Genetic Investigation of Wild Species of Subfamily Malvoideae in Saudi Arabia as Inferred From SCoT and ISSR Markers

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Original article

Keywords: Phylogenetic, Taxonomy, molecular marker, Malvaceae

Posted Date: December 30th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-135572/v1>

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**Genetic investigation of wild species of subfamily Malvoideae in Saudi Arabia as
inferred from SCoT and ISSR markers**

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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), the Inter Simple Sequence Repeats DNA (ISSR), and united ISSR and SCoT markers. Ten SCoT and five ISSR primers created 149 polymorphic enlarged fragments, which jagged to a comparatively high level of genetic difference in Malvoideae. SCoT markers exposed a higher level of polymorphism (89 bands) than ISSR (60 bands). Gathering of genotypes within groups was greatly similar when SCoT and ISSR consequent dendrograms were compared. Five clusters and clades can be documented within Malvoideae, which regularly verify, but also partially deny, traditional groupings. Taxonomic and phylogenetic consequences are debated in contrast with the existing morphological and phylogenetic data. The results of this study offer useful data for evaluating the taxonomy of Malvoideae at both tribes, infrageneric, and subgeneric levels. In general, the results reported here are largely consistent with the phylogenetic findings that *Abutilon*, *Hibiscus*, and *Malva* are polyphyletic. Also, species of sections *Bombicella* and *Malva* are highly heterogeneous. The most exciting result of this analysis was identifying *Senra incana* with unique characters and telling that it should be preserved as a separate tribe. In the similar way, distinctive variances between the closely related genera *Fioria* and *Althaea* were also noted suggesting that they should be placed in different tribes. Furthermore, the present results indicate some level of parallel among the species of *Pavonia* and support the monophyly of this genus.

Keywords: Phylogenetic, Taxonomy, molecular marker, Malvaceae

Introduction	٣٥
Malvaceae is a universal family of herbs, shrubs, and small trees with a primary focus	٣٦
of genera in the tropical regions and including about 244 genera with some 4225	٣٧
species (Christenhusz & Byng, 2016). It is a natural family, simply distinguished by	٣٨
its monadelphous stamens, monothechal anthers, simple entire mostly palmate leaves,	٣٩
stellate hairy indumentum, and large echinate pollen grains. It is closely related to	٤٠
Tiliaceae, Bombacaceae, and Sterculiaceae but differs in the possession of one-celled	٤١
anthers and monadelphous stamens (Hutchinson, 1967; Fryxell, 1997; Heywood,	٤٢
1993; La Duke & Doeby, 1995; Mabberley, 1997). Bayer (1999), Bayer & Kubitzki	٤٣
(2003) ordered Malvaceae into Nine subfamilies: Bombacoideae Brownlowioideae,	٤٤
Byttnerioideae, Dombeyoideae, Grewioideae, Helicteroideae, Malvoideae,	٤٥
Sterculioideae, and Tilioideae based on morphological and molecular data. The	٤٦
systematic of Malvaceae mostly at the subfamilies, tribal, and generic level, is often	٤٧
unsure (Bentham & Hooker, 1862; Schumann, 1889; Bates 1968). Kearney (1951)	٤٨
divided the family Malvaceae into four tribes viz. Malpeae, Ureneae, Hibisceae, and	٤٩
Malveae, which are divided into four subtribes viz. Abutilinae, Malvinae, Siodinae,	٥٠
and Ureneae. Furthermore, Hutchinson (1967) divided Malvaceae into 5 tribes viz.	٥١
Malopeae, Hibisceae, Malveae, Abutilae, and Ureneae based on the characters of	٥٢
fruits, while Schultze-Motel (1964) recognized three tribes. Besides, La Duke&	٥٣
Doebley (1995) and Krebs (1994) separated into five or six tribes: Malopeae,	٥٤
Malveae, Hibiscieae, Abutilieae, Ureneae, and Decaschistieae. Recently, Bayer &	٥٥
Kubitzhi (2003) and Takhtajan (2009) classified subfamily Malvoideae (formerly	٥٦
Malvaceae), into four main tribes viz. Kydieae, Gyosspieae, Hibisceae including	٥٧
(<i>Fioria</i> , <i>Hibiscus</i> , <i>Pavonia</i> , and <i>Senra</i>) and Malveae including <i>Abutilon</i> , <i>Althaea</i> , and	٥٨
<i>Malva</i>) based on morphological and molecular data. In the flora of Saudi Arabia,	٥٩
Collenette (1999) recognized 11 genera comprising 38 species. However, Chaudhary	٦٠
(1999) reported 13 genera and 54 species of Malvaceae includes cultivated species.	٦١
Molecular markers have excessive standing in detecting different parental genotypes	٦٢
over the estimation of genetic difference, which appreciated in cultivar identification	٦٣
(Abdel Khalik et al., 2014). The use of molecular markers for the finding and	٦٤
corruption of DNA polymorphism is one of the most significant progress in the field	٦٥
of molecular genetics. Among the diverse molecular markers, Start Codon Targetted	٦٦
DNA (SCoT) and Inter Simple Sequence Repeat (ISSR) is the simple, rapid, highly	٦٧
effective, and searching procedures. These markers have been used to classify and	٦٨

define the genetic range in many plants (Zietkiewicz et al., 1994; Bornet &	٦٩
Branchard, 2001; Collard & Mackill, 2009; Celka et al., 2010; Hamidi et al., 2014;	٧٠
Ibrahim et al., 2016). In the works analysis, we could not find any phylogenetic	٧١
investigation (molecular markers, ISSR, and SCOT) of Malvaceae species. The	٧٢
present study aims to assess the interspecific genetic diversity among some species of	٧٣
<i>Malvoideae</i> from Saudi Arabia by the means of the SCOT and ISSR methods to	٧٤
obtain shared results. To verify whether these results match with the systematics of	٧٥
the genera as proposed by most traditional and new classification systems for	٧٦
<i>Malvoideae</i> tribes and to report the taxonomic difficulties of the subfamily.	٧٧
Material and Methods	٧٨
Plant materials	٧٩
The leaf samples of the studied taxa are collected from wild inhabitants and some	٨٠
herbarium samples. The voucher specimens of the studied species are placed in the	٨١
herbarium of the Department of Biology of Umm Al-Qura University (UQU)	٨٢
(TABLE 1).	٨٣
Plant genomic DNA extraction	٨٤
Total genomic DNA was extracted from leaf samples. The leaves were first crushed	٨٥
into a fine powder in liquid nitrogen using a pestle and mortar following the CTAB	٨٦
protocol (Porebski et al., 1997; Hussien et al., 2003).	٨٧
Estimation for the DNA concentration:	٨٨
Run 2 µl of the parent's DNA samples on 1% agarose gel in assessment to 10 µl of a	٨٩
DNA size marker (100bp DNA Ladder). To estimate DNA concentration, link the	٩٠
degree of fluorescence of the DNA sample with the different bands in the DNA size	٩١
marker.	٩٢
ISSR and SCoT- PCR reactions	٩٣
A set of 5 primers ISSR and SCoT (TABLE 2) was used in the finding of	٩٤
polymorphism. The amplification reaction was carried out in 25 µl reaction volume	٩٥
containing 1X PCR buffer, 1.5 mM MgCl ₂ , 0.2 mM dNTPs, 1 µM primer, 1 U Taq	٩٦
DNA polymerase, and 30 ng template DNA.	٩٧
Thermocycling Profile	٩٨
PCR amplifications were achieved in a Perkin-Elmer/GeneAmp® PCR System 9700	٩٩
(PE Applied Biosystems) planned to fulfill 35 cycles after an initial denaturation cycle	١٠٠
for 5 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an	١٠١

hardening step at 50°C for 1 min, and an elongation step at 72°C for 1.5 min. The	۱۰۲
primer extension segment was extended to 7 min at 72°C in the final cycle.	۱۰۳
Detection of the ISSR and SCoT Products	۱۰۴
The amplification products were fixed by electrophoresis in a 1.5% agarose gel	۱۰۵
comprising ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. A 1kb DNA	۱۰۶
ladder was used as a molecular size standard. PCR products were imagined on UV	۱۰۷
light and photographed using a Gel Documentation System (BIO-RAD 2000)	۱۰۸
(FIGUREs 1 & 2).	۱۰۹
Data Analysis	۱۱۰
The banding patterns created by ISSR and SCoT-PCR marker analyses were allied to	۱۱۱
determine the genetic resemblance of the samples under study (TABLE 3). The	۱۱۲
pairwise similarity among the genotypes characterized in the different lanes can	۱۱۳
be enumerated using indexes of similarity was estimated according to the Dice	۱۱۴
coefficient (Sneath & Sokal, 1973). For phylogenetic investigation, each amplified	۱۱۵
band was preserved as a unit character, forgetting its concentration, and was scored	۱۱۶
in terms of a binary code based on the presence (1) and absence (0) in the gel.	۱۱۷
Only pure and reproducible bands were measured for counting. Three datasets	۱۱۸
were used, SCoT, ISSR, and a combined dataset of SCoT and ISSR. The numerical	۱۱۹
method working only the presence or absence of a band as its difference feature. The	۱۲۰
binary qualitative data matrices were then used to build similarity matrices based	۱۲۱
on similarity coefficients. The similarity matrices were then used to generate	۱۲۲
dendrograms by using Unweighted Pair Group Method with Arithmetic Average	۱۲۳
(UPGMA)(Sneath&Sokal,1973).	۱۲۴
Results	۱۲۵
SCoT analysis:	۱۲۶
Ten primers were used for the ScoT analysis to examine the outline of genetic	۱۲۷
differences among the measured species of the family Malvaceae growing in Saudi	۱۲۸
Arabia. Among the primers verified, only five exposed a polymorphism. Each primer	۱۲۹
was verified on all samples and was selected for genotype analysis if its patterns were	۱۳۰
reproducible and constant. Polymorphic bands were selected for recognizing the	۱۳۱
genetic resemblance for the group of species. A whole of 89 reproducible	۱۳۲
polymorphic bands were formed by using 5 ScoT-PCR primers. The average	۱۳۳
similarity coefficient extended from 0.55 to 1.00. The maximum number of	۱۳۴
polymorphic amplification DNA fragments gotten per primer was 20 bands for the	۱۳۵

primers SCoT-18 and SCoT-35. The relationships between the studied taxa are existing with the help of a dendrogram constructed based on similarity factors. For ease of calculation, the 100 bands were booked together and the number of bands for each size of DNA fragments (bp) was counted for every species. One branches and four clusters with about 0.65 similarities were found (FIGURE 3): (i) a branch including *Senra incana*; (ii) a cluster comprising Eight species of *Abutilon* and *Althaea ludwigii* with about 0.65 genetic similarities; (iii) a cluster includes *Fioria vitifolia* and *Hibiscus deflersii* with about 0.75 genetic similarities; (iv) a cluster with of *Hibiscus purpureus*, *Hibiscus micranthus*, *Sida alba*, and *Malva parviflora*; (v) a cluster includes *Malva sylvestris*, *Malva neglecta*, *Pavonia arabica* and *Pavonia kotschy* with about 0.67 genetic similarities.

ISSR analysis:

In whole five primers for ISSR were used to examine the designs of genetic variations among the species of Malvaceae growing in wild habitat in Saudi Arabia. In total, 60 (49 are polymorphic and 11 are monomorphic) reproducible bands were caused by the five ISSR primers; and these bands were used for studying the genetic similarity among the species. The average similarity coefficient ranged from 0.60 to 1. The results indicated that all primers were polymorphic. The highest number of polymorphic amplification DNA fragments gotten per primer was 12 for the primer ISSR- 4. 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 (FIGURE 4): (i) a branch including *Fioria vitifolia*; (ii) a branch including *Hibiscus deflersii*; (iii) a cluster *Althaea ludwigii* and Eight species of *Abutilon* with 0.75 genetic similarities; (iv) a cluster including *Hibiscus purpureus* and *Hibiscus micranthus* with about 0.75 genetic similarities; (v) a cluster including a subgroup of *Sida alba*, *Malva parviflora* and *Malva verticillata*, *Malva sylvestris*, *Malva neglecta*, *Pavonia arabica* with a similarity score of 0.75, and the subgroup of including *Pavonia kotschy* and *Senra incana* with a similarity score of 0.88.

Combined SCoT and ISSR analysis:

The UPGMA dendrogram gotten from the cluster analysis of SCoT and ISSR shared data existing a near similar clustering pattern, with similarity coefficient ranging 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 (FIGURE 5): (i) a branch including *Senra incana*; (ii) a branch *Fioria vitifolia*; (iii) a cluster comprising *Hibiscus deflersii*, *Malva sylvestris* and *Malva neglecta*, *Sida alba*, *Malva parviflora* and *Malva verticillata* with a similarity score of 0.67; (iv) a cluster includes three groups; one group includes *Pavonia arabica* and *Pavonia kotschyi* and the second one contains Eight species of *Abutilon*, the third group includes *Althaea ludwigii* with a similarity score of 0.73; (v) a cluster consist of *Hibiscus purpureus* and *Hibiscus micranthus* with about 0.85 genetic similarities.

Discussion

Several authors have tried to offer an accepTABLE system to classify 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 are based on one or two traits such as life forms, carpel morphology, number and position of ovules in each carpel, fruits, and seeds. Morphological characters in plants could be affected by environmental conditions. Thus, the use of morphological characters for classification may effect in divergences.

SCot and ISSR molecular markers are broadly used for population genetic analysis of different plants creating more dependable and reproducible bands (Nagaoka & Ogiyara, 1997; Collard & Mackill, 2009; Zhang et al., 2015; Sadek & Ibrahim, 2018). The present study, however, has established that both SCoT and ISSR techniques along with the right statistical tools could be definitely useful to evaluate the genetic diversity and phylogenetic analysis in subfamily Malvoideae. Although SCoT and ISSR markers showed significant differences in detecting polymorphism and discriminating capacity, they showed nearly similar topology in dendrograms generated based on similarity matrices. A extremely significant link between these two dendrograms suggested that both markers were similarly efficient for measuring phylogenetic relationships among the investigated taxa. Moreover, the genotype scattering on the consensus tree based on the shared banding patterns of SCoT and ISSR may significantly vary because each technique may magnify different parts of the genome. The SCoT markers are very reproducible due to the use of longer primers, and ISSR amplifies the region between two microsatellites. Hence, the polymorphisms reveal the variety of these regions of the genome. It is, therefore, better to use the mixture of banding patterns of the two techniques to use more parts

sites of the genome that will increase the strength of the consensus tree. General, our	٢٠٢
results obtained from the SCoT and ISSR analyses proposed groups and partially	٢٠٣
established the tribes, subtribes, and sections classification of Malvoideae by the most	٢٠٤
recent traditional (Mattei, 1915; Kearney, 1951; Schultze-Motel, 1964; Hutchinson,	٢٠٥
1967; Bates, 1968; La Duke & Doebley, 1995) and a recent phylogenetic	٢٠٦
classification based on molecular data (Fryxell, 2002; Bayer & Kubitzki, 2003; Tate	٢٠٧
et al., 2005; Reveal's, 2012).	٢٠٨
<i>Abutilon</i> group (G2 B)	٢٠٩
The taxonomy of <i>Abutilon</i> is very difficult mostly due to a high degree of plasticity	٢١٠
and so adaptations to climatic conditions. According to Hutchinson (1967), the tribe	٢١١
Abutilae comprises two subtribes: Abutilinae (include <i>Abutilon</i>) and Sidinae (include	٢١٢
<i>Sida</i> and <i>Malvastrum</i>). The tribe Abutilae however, was comprised within Malveae	٢١٣
in the system of Takhtajan (2009). Based on molecular data Reveal (2012) separated	٢١٤
both subtribes Sidinae and Abutilinae from Malveae under tribe Sidieae. The	٢١٥
infrageneric classification of <i>Abutilon</i> is unwell understood. The genus has been	٢١٦
ordered into sections and subsections by some workers, but only for quite limited	٢١٧
geographical areas such as Schumann (1891) for Brazilian species, Mattei (1915) for	٢١٨
East African species (using the ranks stirps and substirps), and Fryxell (1988) for	٢١٩
Mexican species. Mattei (1915) grouped the East African <i>Abutilon</i> into three natural	٢٢٠
stirps (Capsulati, Cephalocarpi, and Monospermi) and seven substirps (Fruticosi,	٢٢١
Cuspidati, Indici, Graveolenti, Mericarpi, Blepharocarpi, and Mutici) based on the	٢٢٢
carpels, seeds, and leaves morphology.	٢٢٣
The systematic revision on <i>Abutilon</i> (Mattei, 1915) including all species distributed in	٢٢٤
Saudi Arabia, allows us to compare our results with him. He treated <i>Abutilon</i>	٢٢٥
<i>fruticosum</i> under stirps Capsulati substirps Fruticosei; <i>A. ramosum</i> in stirps Capsulati	٢٢٦
substirps Cuspidati; <i>A. figarianum</i> in stirps Cephalocarpi substirps Graveolenti;	٢٢٧
<i>Abutilon bidentatum</i> , <i>A. hirtum</i> , and <i>A. grandifloium</i> in stirps Cephalocarpi substirps	٢٢٨
Mierocarpi; <i>A. muticum</i> and <i>A. pannosum</i> in stirps Cephalocarpi substirps Mutici.	٢٢٩
Moreover, Fryxell (2002) presented an eventual study and nomenclature of more than	٢٣٠
500 names at the specific rank and 25 names in the infrageneric rank of <i>Abutilon</i> . He	٢٣١
treated <i>Abutilon bidentatum</i> and <i>A. grandifloium</i> in section Beloere; <i>A. muticum</i> , <i>A.</i>	٢٣٢
<i>pannosum</i> , and <i>A. figarianum</i> in section Muticum; <i>A. fruticosum</i> in section	٢٣٣
Oligocaroe. Also, Fuertes Aguilar et al. (2003) studied phylogenetic relations	٢٣٤
between members of the subtribe Abutilinae and mostly the taxa in the <i>Sida</i> generic	٢٣٥

alliance based on the internal transcribed spacers of nuclear ribosomal DNA (ITS) ۲۳۶
 from 58 species of Malvaceae were sequenced. The ITS data show that *Abutilon* and ۲۳۷
Sida were not monophyletic. Similarly, Tate et al. (2005), studied the phylogenetic ۲۳۸
 relations within tribe Malveae based on sequence data from (ITS) regions of the 18– ۲۳۹
 26S nuclear ribosomal repeat and accepted two main clades: one comprising *Abutilon* ۲۴۰
 and *Sida* (Abutilon alliance) and a second covering the rest of taxa, and they revealed ۲۴۱
 that *Abutilon*, *Sida*, and *Tetrasida* are not monophyletic. Furthermore, Taia (2009) ۲۴۲
 investigated the systematic revision of Five species of *Abutilon* from Saudi Arabia ۲۴۳
 based on morphology and provided that close relation among species and classified ۲۴۴
 them into two groups. ۲۴۵

According to the combined SCoT and ISSR tree (group 2B), the studied taxa of ۲۴۶
Abutilon were grouped in one cluster. This cluster is splitting off into two groups. One ۲۴۷
 group includes *Abutilon bidentatum*, *A. hirtum*, *A. grandifloium*, and *A. muticum* ۲۴۸
 (Stirps Cephalocarpi), and *A. ramosum* (stirps Capsulati) with 0.82 genetic ۲۴۹
 similarities. These species are morphologically distinguished by the absence of ۲۵۰
 epicalyx, mericarps that lack wings, dorsally dehiscent fruit, and by leaves that are ۲۵۱
 sub-entire to serrate margins. The second group includes *A. pannosum* and *A.* ۲۵۲
figarianum (Stirps Cephalocarpi) and *A. fruticosum* (Stirps Capsulati) with 0.80 ۲۵۳
 genetic similarities. These species are actual comparable, as the three have largely ۲۵۴
 ovate or rounded leaf blades, with either rounded or acute apices. Meanwhile our ۲۵۵
 results are inherited data, and we propose that species of *Abutilon* form a polyphyletic ۲۵۶
 group. Our data support the previous work of treating tribe Abutileae (Sidieae sensu ۲۵۷
 Reveal 2012) with its two subtribes as distinct from Malveae. Therefore, these results ۲۵۸
 partially agree with Mattei (1915) and Fryxell (2002), and congruent with those of ۲۵۹
 Fuertes Aguilar et al. (2003), Tate et al. (2005), and Reveal (2012). ۲۶۰

***Pavonia* group (G 2 A)** ۲۶۱

This group comprises only *Pavonia arabica* and *P. kotschyi*. It has been documented ۲۶۲
 as a distinct clade with 0.80 genetic similarities. These species can be clearly defined ۲۶۳
 based on various features: the perennial, presence of epicalyx, leaves not lobed, ۲۶۴
 mericarps 5, and seed 1 in each mericarp. A Fryxell (1997) investigated *Pavonia* of ۲۶۵
 the new world and show that all species of the genus have the same base chromosome ۲۶۶
 number $x=7$. *Pavonia* placed in a separate tribe Ureneae by most authors (Bentham & ۲۶۷
 Hooker, 1862; Schultze-Motel, 1964, Hutchinson, 1967 & Cheek 2007). While ۲۶۸
 Takhtajan (2009) and Reveal (2012) placed the genus with *Hibiscus* in the tribe ۲۶۹

Hibiscieae. Our data support the previous work of treating <i>Pavonia</i> in a separate tribe	270
Ureneae and agree with those authors.	271
<i>Althaea</i> clade (G 2 C)	272
Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012) treated this genus	273
in tribe Malveae. However, Jun Qiana et al. (2020) investigated phylogenetic analysis	274
of <i>Althaea rosea</i> based on whole cp genome sequences and show that a close	275
relationship of <i>Althaea</i> with <i>Gossypium</i> and <i>Hibiscus</i> in Malvaceae. Rendering to the	276
combined SCoT and ISSR tree, the results do not support the placement of <i>Althaea</i>	277
<i>ludwigii</i> in tribes Malveae or Hibiscieae. This is due to the assignment of <i>Althaea</i>	278
<i>ludwigii</i> within a distinct branch with high genetic similarities. This species is well	279
known morphologically from the other species by having an annual life form, 3	280
segments epicalyx, indehiscent fruit, many mericarps, each one includes one seed.	281
This result disagrees with those of Bentham & Hooker (1862), Hutchinson (1967),	282
and Reveal (2012) Jun Qiana et al. (2020) for treating it as tribe Malveae or	283
Hibiscieae. More data are necessary for deciding this assumption.	284
<i>Senra</i> clade (G 3)	285
This branch includes only <i>Senra incana</i> . It has been known as a separate clade with	286
high genetic similarities and can be clearly distinguished based on several features:	287
spheroidal pollen shape, seed hairy, style 5-branched, capsule with 1-locule, and 1-	288
seed, calyx without oil glands.	289
Bentham & Hooker (1862), Hutchinson (1967), treated this genus in the tribe	290
Hibiscieae. However, Reveal (2012) placed <i>Senra</i> in tribe Gossypieae, based on	291
molecular data. Our data support the previous work of treating <i>Senra</i> in tribe	292
Gossypieae (sensu Reveal, 2012).	293
<i>Fioria</i> clade (G 5)	294
According to our results, <i>Fioria vitifolia</i> shows the largest distance from all other	295
groups, and is distinct from the others by the characters of the persistent calyx with 5-	296
toothed, calyx without oil glands, fruit 5-broadly winged, and seed reniform,	297
tuberculate. Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012)	298
treated this genus in the tribe Hibiscieae. Our data support the previous work of	299
treating <i>Fioria</i> in a tribe Hibiscieae and congruence with those authors.	300
<i>Hibiscus</i> groups (G 1 & G 4)	301
Concerning the cluster and clade of <i>Hibiscus purpureus</i> , <i>H. micranthus</i> , and <i>H.</i>	302
<i>deflersii</i> , our results do not support the monophyly of the non-natural section	303

Bombycella. This is due to the situation of <i>Hibiscus purpureus</i> , <i>H. micranthus</i> , and <i>H. deflersii</i> within two separate clusters and clade with 0.82 genetic similarities.	۳۰۴
	۳۰۵
Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012) treated this genus	۳۰۶
in the tribe Hibisceae. Hochreutiner (1900) revised the genus <i>Hibiscus</i> and classified	۳۰۷
it into 12 sections and he concluded that <i>Hibiscus</i> is very heterogeneous and needs	۳۰۸
additional attention. Ulbrich in Engler (1921) divided section Bombycella into three	۳۰۹
subsections: Syriaca, Eubombycella, and Africana. Moreover, Cufodontis (1948)	۳۱۰
overview <i>Hibiscus</i> species in Africa and treated <i>Hibiscus purpureus</i> , <i>H. micranthus</i> ,	۳۱۱
and <i>H. deflersii</i> in section Bombycella. Furthermore, Fryxell (1980) concluded that	۳۱۲
section <i>Bombicella</i> is the second most diverse section of <i>Hibiscus</i> , after	۳۱۳
section <i>Furcaria</i> , and is a paraphyletic section. Also, they cited that the chromosome	۳۱۴
number extremely variable, because the chromosome base number of American	۳۱۵
species $x=11$ diploids, and African species $x=16$ diploids or tetraploids, while	۳۱۶
Australian species $2n= 54$ allotetraploids. Since our results are inherited data, and we	۳۱۷
propose that species of section Bombicella subsection Eubombycella (sensu Engler,	۳۱۸
1921; Cufodontis, 1948) form a paraphyletic group. Therefore, these data agree with	۳۱۹
those of Hochreutiner (1900) and Fryxell (1980) for treating section Bombycella as a	۳۲۰
heterogeneous section.	۳۲۱
Malva-Sida group (G 1)	۳۲۲
Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012) treated <i>Malva</i> in	۳۲۳
the tribe Malveae. However, they preserved <i>Sida</i> in the tribes Malveae, Abutilaeae, and	۳۲۴
Sidaeae respectively. Based on flower structure characteristics, Baker (1890) divided	۳۲۵
the genus <i>Malva</i> into three sections: Bismalva, Bibracteolata, and Fusciculata, which	۳۲۶
includes (<i>Malva neglecta</i> , <i>M. parviflora</i> , <i>M. sylvestris</i> , and <i>M. verticillata</i>). However,	۳۲۷
Dalby (1968) separated the European species of the genus <i>Malva</i> into two sections:	۳۲۸
Bismalva and Malva. The section Bismalva includes species with single flower in the	۳۲۹
leaf axils or a congested, terminal raceme, while the species of the section Malva have	۳۳۰
two or more flowers in each leaf axil. Bates (1968) suggested that <i>Malva</i> might be	۳۳۱
polyphyletic because it has chromosome number in the range $2n=14$ should be	۳۳۲
considered diploids, and $2n=40-44$ species are hexaploids and this number is related	۳۳۳
to <i>Sida</i> and other taxa. Furthermore, Luque & Devesa (1986) reported that	۳۳۴
chromosome base number hexaploids $2n= 42$ in <i>M. neglecta</i> , <i>M. parviflora</i> , <i>M.</i>	۳۳۵
<i>sylvestris</i> , and dodecaploids with a chromosome count of (76, 84, 112) in <i>M.</i>	۳۳۶
<i>verticillata</i> . Besides, Fryxell (1997) reported that <i>Sida</i> has the same base chromosome	۳۳۷

number $x=7$ or 8 ($2n=14, 28; 2n=16, 32$), and is heterogeneous. Ray (1995) based on an ITS sequence analysis, differentiated two groups: Malvoid and Lavateroid, and he placed all species of *Malva* in the malvoid group. Similarly, Garcia et al. (2009) used five molecular markers (ITS, matK + trnK, ndhF, trnL-trnF, psbA-trnH), to investigate a phylogenetic hypothesis of *Malva* alliance (Malvaceae), and they show that *Althaea*, *Lavatera*, and *Malva* are highly polyphyletic. Also, they treated *Malva neglecta* and *M. sylvestris* in a subgroup with 0.96 genetic similarities, and *M. parviflora* and *M. verticillata* in another subgroup with 0.82 genetic similarities based on total plastid markers. Furthermore, Celka et al., (2010) used ISSR and ISJ markers to determine genetic relationships among eight *Malva* taxa. The species are classified into two groups consistent to the sections *Bismalva* (*M. alcea*, *M. excisa*, and *M. moschata*) and *Malva* (*M. neglecta*, *M. pusilla*, *M. sylvestris*, and *M. verticillata*). According to the combined SCoT and ISSR tree, the results do support the placement of *M. neglecta*, *M. parviflora*, *M. sylvestris*, and *M. verticillata* in section *Malva*, but is not monophyletic. This is due to the placement of *M. parviflora*, *M. verticillata*, and *Sida alba* within a separate sub-cluster, and *M. neglecta*, *M. sylvestris*, and *Hibiscus deflersii* in another sub-cluster with 0.71 genetic similarities. This cluster is distinguished morphologically from the other species by having schizocarp fruit, leaves unlobed, many mericarps, base chromosome number $x=7$.

In general, there is some congruence between morphological results and those of phylogenetic studies. Our results reported here 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, along with the division into sections based on morphology suggested by Baker (1890), Dalby (1968).

Conclusions

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), the Inter Simple Sequence Repeats DNA (ISSR), and combined ISSR and SCoT markers. Five clusters and clades can be accepted within Malvoideae, which mostly verify, but also partially reverse, traditional groupings. The results of this study offer valuable data for judging the taxonomy of Malvoideae both at tribes, infrageneric, and subgeneric levels. In general, our results reported here are largely consistent with the phylogenetic findings that *Abutilon*, *Hibiscus*, and *Malva* are polyphyletic. Besides, species of sections *Bombicella* and *Malva* are highly

heterogeneous. A remarkable result from this study was to identify *Senra incana* with distinctive characters and telling that it should be preserved as a separate tribe. In the same way, distinguishing differences between the closely related genera *Fioria* and *Althaea* were also noted suggesting that they should be placed in different tribes. Additional support comes from the molecular data of RAPD and ISSR which indicate that some level of similarity among the species of *Pavonia* and support the monophyly of this genus. Nevertheless, we believe that molecular and morphological tactics should be joint to arrive at a generally accepted phylogenetic reconstruction of Malvoideae. Moreover, a broad study covering extra species from different genera would be necessary to make a more exhaustive classification.

Acknowledgments

The authors would like to thank the Research and Development Grants Program for National Research Institutes and Centers (GRANTS), Graduate Research Program, King Abdulaziz City for Science and Technology (KACST), Kingdom of Saudi Arabia, grant number (Project ID: 1-18-01-007-0001) for the financial funding. Also, we are thankful to the herbarium director and curator of the King Saud University and Agriculture and Water Research Center, Riyadh, (KSU and RAWRC).

Authors' contributions

The authors contributed to the work done in the manuscript as follows: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; Kadry and Suad, conducted the laboratory experiments. Kadry and Suad presented the data in table and graphs and prepared the preliminary version of the manuscript. Kadry and Suad revised the experimental design, guided the data analyses, interpretation, and manuscript revision. Kadry, guided the laboratory experiments, helped in the preparation of the first version of the manuscript and analysis of the experimental data. All authors read and approved the final manuscript.

Funding

This work was funded by Research and Development Grants Program for National Research Institutions and Centers (GRANTS), Graduate Research-Program, King Abdulaziz City for Science and Technology (KACST), Riyadh, Kingdom of Saudi Arabia, grant no: 1-18-01-007-0001.

Availability of data and materials

All relevant data are within the manuscript.

Ethics approval and consent to participate	٤٠٦
Not applicable.	٤٠٧
Consent for publication	٤٠٨
Not applicable.	٤٠٩
Competing interests	٤١٠
The authors declare that they have no competing interests.	٤١١
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FIGUREs captions

FIGURE 1. DNA polymorphism created by five ISSRs primers from the genomic DNA of the examined species of Malvoideae. Species names are settled and numbered as in TABLE 1.

FIGURE 2. DNA polymorphism generated by five SCoT primers from the genomic DNA of the investigated species of Malvaceae. Species names are settled and numbered as in TABLE 1.

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

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

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

TABLEs captions

TABLE 1. List of taxa used in the study (Chaudary, 1999), a comparison of traditional (Bentham & Hooker, 1862; Hutchinson, 1967) and phylogenetic classifications (Bayer & Kubitzki, 2003; Jennifer et al., 2005; Reveal's system, 2012).

TABLE 2. The characteristics of ISSR and SCoT primers sequencing and amplification products generated by the studied taxa.

TABLE 3: Genetic similarity matrix among the studied taxa as FIGURED according to the Dice coefficient from a combination of SCoT and ISSR primers. Species names from 1-21 as in TABLE 1.

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Figure 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

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

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

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

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

Table 1. List of taxa used in the study (Chaudary, 1999), a comparison of t traditional (Bentham & Hooker, 1862; Hutchinson, 1967) and phylogenetic classifications (Bayer & Kubitzhi, 2003; Jennifer et al., 2005; Reveal's system, 2012)

No	taxa	Source and voucher	Bentham & Hooker (1862)	Hutchinson (1967)	Bayer & Kubitzhi, 2003; Jennifer et al. 2005	Reveal's system (2012).	Present study SCoT & ISSR
1	<i>Abutilon bidentatum</i> Hochst.	Wadii Thalolah near AL-Baha, Suad Al-Ruzayza, 11 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe:Sideae Subtribe: Abutilinae	G 2 B
2	<i>Abutilon hirtum</i> (Lam.) Sweet	Mahil Aseir, Suad Al-Ruzayza, 8 15 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
3	<i>Abutilon figarianum</i> Webb	Wadii Qusai, Suad Al-Ruzayza, 5 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
4	<i>Abutilon fruticosum</i> Guill.	Near Al-Howtah, Alfarhan and J. Thomas, 22274 (KSU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
5	<i>Abutilon pannosum</i> (G.Forst.) Schltdl.	Al-Edabi, Suad Al-Ruzayza, 17 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
6	<i>Abutilon ramosum</i> (Cav.) Guill. & Perr.	Shada mountiain, Suad Al-Ruzayza,2 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
7	<i>Abutilon grandifolium</i> (Willd.) Sweet	Fifa mountain, Suad Al-Ruzayza,15 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
6	<i>Abutilon muticum</i> Sweet	Alardah, Suad Al-Ruzayza,6 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
7	<i>Abutilon pannosum</i> (G.Forst.) Schltdl.	Al-Edabi, Suad Al-Ruzayza, 17 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae. Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B
8	<i>Abutilon ramosum</i> (Cav.) Guill.	Shada mountiain, Suad Al-Ruzayza,2 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Abutilinae	G 2 B

9	<i>Althaea ludwigii</i> L.	Qassim Road, Suad Al-Ruzayza, 22 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	<i>Malva</i> alliance	Tribe :Malveae Subtribe: Malvinae	G 2 C
10	<i>Fioria vitifolia</i> (L.) Mattei	Al Darb, near Abha, Suad Al-Ruzayza, 21 (UQU)	Tribe: Hibiscieae	Tribe: Hibiscieae	-	Tribe: Hibiscieae	G 5
11	<i>Hibiscus deflersii</i> Schweinf.	Taif, Suad Al-Ruzayza , 37 (UQU)	Tribe: Hibiscieae	Tribe: Hibiscieae	-	Tribe: Hibiscieae	G 1
12	<i>Hibiscus purpureus</i> L.	Fifa mountain, Alfarhan and J. Thomas, 999 (KSU)	Tribe: Hibiscieae	Tribe: Hibiscieae	-	Tribe: Hibiscieae	G 4
13	<i>Hibiscus micranthus</i> L.f.	Raidah, Suad Al-Ruzayza, 34 (UQU)	Tribe: Hibiscieae	Tribe: Hibiscieae	-	Tribe: Hibiscieae	G 4
14	<i>Sida alba</i> L.,	Fifa mountain, J. Thomas and R. Basahi, 21876 (KSU)	Tribe: Malveae Subtribe: Sidinae	Tribe: Abutilaeae. Subtribe: Sidinae	<i>Abutilon</i> alliance	Tribe :Sideae Subtribe: Sidinae	G 1
15	<i>Malva parviflora</i> L.,	Alkarj, Suad Al-Ruzayza, 29 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	<i>Malva</i> alliance	Tribe :Malveae Subtribe: Malvinae	G 1
16	<i>Malva verticillata</i> L.,	AL-ahsaa, Suad Al-Ruzayza, 32 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	<i>Malva</i> alliance	Tribe :Malveae Subtribe: Malvinae	G 1
17	<i>Malva sylvestris</i> L.	Al-Qasim, Buradah, Suad Al-Ruzayza, 46 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	<i>Malva</i> alliance	Tribe :Malveae Subtribe: Malvinae	G 1
18	<i>Malva neglecta</i> Wallr.	Syll., Ushaqur, Suad Al-Ruzayza, 2 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	<i>Malva</i> alliance	Tribe :Malveae Subtribe: Malvinae	G 1
19	<i>Pavonia arabica</i> Hochst.	Wadi Bani Zaher, S. Chaudhary, 7087 (RAWRC)	Tribe: Ureneae	Tribe: Ureneae	-	Tribe: Hibiscieae	G 2A
20	<i>Pavonia kotschy</i> Hochst.	Jeddah, Suad Al-Ruzayza, 40 (UQU)	Tribe: Ureneae	Tribe: Ureneae		Tribe: Hibiscieae	G 2A
21	<i>Senra incana</i> Cav.	Harrat Al-Shara, Suad Al-Ruzayza, 41(UQU)	Tribe: Hibiscieae	Tribe: Hibiscieae		Tribe: Gossypieae	G 3

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Table 2. The characteristics of ISSR and SCoT 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
ISSR	ISSR- 1	5'-AGAGAGAGAGAGAGAGGYC-3'	11	1	10	91 %
	ISSR- 2	5'-AGAGAGAGAGAGAGAGGYG-3'	11	2	9	82 %
	ISSR- 3	5'-ACACACACACACACACYT-3'	12	2	10	83 %
	ISSR- 4	5'-ACACACACACACACACYG-3'	14	2	12	86 %
	ISSR- 5	5'-GTGTGTGTGTGTGTGTGYG-3'	12	4	8	67 %
SCoT	SCoT-7	ACAATGGCTACCACTGAC	15	1	14	93 %
	SCoT-16	CCATGGCTACCACCGGCA	21	2	19	90 %
	SCoT-18	CCATGGCTACCACTAGCA	22	2	20	91 %
	SCoT-35	AACCATGGCTACCACCAC	23	3	20	87 %
	SCoT-36	CACCATGGCTACCACCAT	19	3	16	84 %
Total number			160	22	138	

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

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	0.61	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

Figures

Figure 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

Figure 2

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

Figure 3

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

Figure 4

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

Figure 5

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

Supplementary Files

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