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Molecular taxonomy and phylogeny of *Silene* species (Caryophyllaceae) using DNA-based markers

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Abstract

DNA markers have provided valuable tools in various analyses ranging from phylogenetic analysis to the positional cloning of genes. *Silene* is a genus of flowering plant in the family Caryophyllaceae. This study compared the phylogenetic relationship within and between 16 *Silene* sections, including 24 species (Silenoideae: Caryophyllaceae) using some molecular markers such as Random Amplification of Polymorphic DNA (RAPD), Microsatellite Primed PCR (MP-PCR), Anchored Microsatellite Primed PCR (AMP-PCR) andSequence-Related Amplified Polymorphism (SRAP), Phylogenetic analysis was carried out by parsimony, and neighbour joining model. Finally, our molecular analysis did not confirm morphological viewpoints completely.

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Introduction

Silene L. is the largest genus in the family Caryophyllaceae with nearly 700 species world-wide (Melzheimer, 1988) is mainly distributed in temperate regions of the northern hemisphere and has its principle centre of diversity in the Mediterranean region and the Middle East. The genus is mostly hermaphrodite although a few species are dioecious or gynodioecious (Bari, 1973; Greuter, 1995). The circumscription of the genus has long been controversial, with an expanding tendency during the past decades (Greuter, 1995; Chowdhuri, 1957; McNeill, 1978; Greuter *et al.*,1984; Šourková, 1971). The genus *Silene* includes several important weedy species, some very beautiful horticultural plants and some medicinal plants (Swank, 1932; Vestal, 1952; Oxelman *et al.*, 1995). The most important characters that separate sections are morphology and venation of the calyx, length and pubescence of the anthophore, shape and size of the corolla and coronal scales, capsule and seed.

The genus *Silene* includes 98 species in Iran with 35 endemic to the Iranian plateau (26 species for Iran). The Iranian species are placed in 21 sections (Melzheimer, 1988). Major areas of species for the Iranian sections occur in west of the country (Edalatiyan*et al.*, 2010).

Genetic marker is any trait representing genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as signs or flags (Angaji, 2011). There are three major types of markers: morphological markers, which themselves are phenotypic traits or characters; biochemical markers, which include allelic variants of proteins and enzymes; and DNA (molecular) markers, which reveal sites of variation in DNA (Barh, 2013).

Morphological variations, unfortunately, are not sufficient to differentiate between a large number of individuals, or between groups or subgroups within species, simply because the level of polymorphism is low. Besides, morphological characters or phenotypic expressions are affected by environmental changes, so stability of expression is low and sometimes uncertain. Certain disadvantages of morphological markers can be overcome by biochemical markers (Pratik, 2007).

Biochemical markers are usually enzyme variants with the same functional role but structurally different. These are highly influenced by environment and relatively few biochemical assays are available (Angaji, 2011).

DNA –based markers offer several advantages over other markers. They are less affected by environmental factors. There are potentially an unlimited number of molecular markers available. Finally, they are much more polymorphic than other markers (Angaji, 2011; Singh *et al.*, 2013).

DNA markers may be broadly divided into four classes based on the method of their detection: 1) hybridization-based e.g. Restriction Fragment Length Polymorphism (RFLP), 2) PCR-based e.g. RAPD, MP-PCR, AMP-PCR, SRAP 3) molecular markers based on PCR followed by hybridization, and 4) DNA sequence based-markers (Angaji, 2011; Fu *et al.*, 2008).

PCR-based molecular markers offer the potential to reduce time, effort and expense required for molecular technology and these markers can be generally divided into single primer PCR methods and PCR methods using a pair of primers. This research was carried out by single primer PCR methods, such as MP-PCR and AMP-PCR, since no prior knowledge of DNA sequence is required for them as well as pair of primers method, for instance Sequence-related amplified polymorphism (SRAP), due to more stringency compared to the former ones (Angaji, 2011; Li *et al.*, 2001).

Tandem arrays are widely distributed throughout plant and animal genomes that display high levels of

genetic variation based on differences in the number of tandemly repeating units at a locus. In MP-PCR and AMP-PCR, detected polymorphisms are not due to microsatellite length variation in most cases, but are often caused by small insertion/deletion (indel) in intervening sequences (Angaji. 2011; Dávila*et al.*, 1999; Sharma *et al.*, 1995).

The rapidly growing amount of gene in databases opened the door to the development of DNA profiling strategies that are directed at specific coding DNA regions of interest. SRAP is a new marker system based on PCR reaction and more reproducible, stable and less complex compared with other molecular marker techniques. Two primers are used each of which consists of the following three elements: 1) an arbitrary filler sequence of 10 to 11 bases at 5'-end, 2) the sequence motifs CCGG and AATT in the forward and reverse primer, respectively, and 3) three selective bases at the 3'-end.

The rationale behind the primer architectures is that exon sequences are known to be more GC rich than other regions of the genome. In contrast, the core sequencesof the second primer (AATT) is designed to bind to AT-rich sequences, which are preferentially found in non-coding regions(Angaji, 2011; Fu *et al.*, 2008; Ferriol *et al.*, 2003; Budak *et al.*, 2004; Budak *et al.*, 2004; Espósito *et al.*, 2007; Fu *et al.*, 2008).

Material and method

Plant material and DNA extraction:

Herbarium specimens were provided from Herbarium of Kharazmi University (FAR), and Central Herbarium of Tehran University (TUH). The plant samples were used from the below specimens (Table1).

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Numerous protocols for DNA extraction from plants have been published (Doyle *et al.*, 1990; Scott *et al.*, 1996; Csaikl *et al.*, 1998; Sharma *et al.*, 2000; Li *et al.*, 2001; Pirttilä *et al.*, 2001; Drabkova *et al.*, 2002; Shepherd *et al.*, 2000; Mogg *et al.*, 2003; Haymes *et al.*, 2004). Total DNAs were isolated from leaf tissue by the CTAB method (Saghai-Maroof *et al.*, 1984) which is modified by Doyle *et al.* in1987. Fifty primers were used for amplification, including MP-PCR, AMP-PCR, SRAP and RAPD. Among them, 36 markers were polymorphic (Table 2, 3).

Single primer PCR methods

In this study 28 RAPD, MP-PCR and AMP-PCR primers were polymorphic.

Pair of Primers method

In this research, SRAP markers were applied as functional markers. Eight polymorphic SRAP were found.

PCR amplification and separation for scorning

Each PCR amplification reaction micro tube (50µL) contained 5µL of DNA template, 4 µL primer, 28 µL water, and 13 µL Taq DNA polymerase Master MIX Red which cosists of dNTPs, Tris-HCL pH 8.5, $(NH_4)_2SO_4$, $MgCl_2$, 0.2% Tween 20 and Taq polymerase. The PCR amplification was carried out in a Peltier thermal cycler in 35 cycles, denaturation at 95°C for1min, primer annealing at 42°C for 1 min and extension at 72°C for 2 min. Amplification products were analyzed by electrophoresis through 8% (w/v) agarose gel and the gel was cast and **run** using TAE at 76 V for 30 min and stained by ethidium bromide. Gels were visualized under UV light and photographed and then PCR amplification products were scored as present (1) or absent (0). The data were analyzed by PAUP version 0.4 and shown as Neighbour-joining tree.

Result

Cladistics

Following cladogram is according to result, which obtained from the molecular researches on 24 *Silene* species. This cladogram has shown an interesting shape that caused species placed at different locations.

Table 1. List of Specimens were used.

Species	Author	Collector	Herbariums numbers	Herbarium
S.apetala	Willd.	Atayi	3650	[FAR]
S.astro–iranica	Rech.f., Aell. & Esfand	Veiskarami	23960	[TUH]
S.aucheriana	Boiss.	Bidarlord	2246	[FAR]
S.cappadocica	Boiss. & Heldr	Bidar	2249	[FAR]
S.chlorifolia	SM.	Bidar	2248	[FAR]
S.commelinifolia	Boiss.	without collector	23250	[FAR]
S.compacta	Fisch.	Ghahreman *et al.*	35576	[TUH]
S.conoidea	L.	without collector	8995	[FAR]
S.dichotoma	Ehrh	Ghahreman&Mozaffarian	17431	[TUH]
S.italic	(L.) Pers	without collector	4182	[FAR]
S.latifolia	Poir	Moradi & Siadati	40178	[TUH]
S.linearis	Decne	Ghahreman & Mozaffarian	5612	[TUH]
S.macrowizicii	Schischk.	Eslami	29572	[TUH]
S.marschallii	C.A.Mey.	Ghahreman & Sheikholeslami	6271	[TUH]
S.multifida	(Adams) Rohrb.	Ghahreman *et al.*	17527	[TUH]
S.nana	Kar. & Kir.	Ghahreman *et al.*	28488	[TUH]
S.noctiflora	L.	Rezvanian	23098	[TUH]
S.odontopetala	Fenzl	Bidar Lord	2266	[FAR]
S.persica	Boiss.	without collector	023254	[FAR]
S.schafta	J.G.Gmel. ex Hohen	Attar & Dadjou	14689	[TUH]
S.spergulifolia	(Willd.) M.B.	Bidar	2259	[FAR]
S.swertiifolia	Boiss.	Gilani	8221	[FAR]
S.tenella	C.A.Mey.	Bidar	2261	[FAR]
S.vulgaris	(Moench) Garcke	Eslami	29566	[TUH]

Discussion

In this study, the results of molecular analysis of 24 species of genus *Silene* were compared with morphological classification, and similarities and differences were reported.

The molecular phylogenetic analysis showed that *Silene* is divided into two major clades with strong support (Fig. 1) and not correspond to previous informal grouping (Melzheimer, 1988). One group contains *S.italica* and *S.linearis.* Polytomic relationship has been shown; despite they have many differences in morphological characters. The poor resolution within *Silene* may be attributed to rapid radiation, recombination among homoeologues, homoplasy, or any combination of these factors.

Another major group divided into three subgroups. One subgroup has two species: *S.apetala* and *S.vulgaris*. They are near in our cladgram, but they are not at the same section. *S.apetala* belongs to Sect. *Lasiocalycinae* with these characters: annual or biennial, calyx cylindric-ovate or campanulate, petal limb entire or emarginate; claw glabrous; anthophore glabrous (Edalatiyan *et al.*, 2010). But *S.vulgaris* belongs to Sect. *Inflatae.* It has morphological characters such as perennial, calyx oblong to campanulate, petal limb bifid; claw glabrous or

pubescent; anthophore puberulent rarely glabrous (Edalatiyan *et al.*, 2010).

Table 2. List of polymorphic RAPD, AMP- PCR and MP-PCR primers.

NO	Primer	No of bp
1	5'- CTG TTG CTA C-3'	10
2	5'-CAG TGC TGT G-3'	10
3	5'-ACA CAC GCT G-3'	10
4	5'-TCT CTC TCT C-3'	10
5	5'-ACA CAC ACA C-3'	10
6	5'-GTG TGT GTG T-3'	10
7	5'-GGC GGCGGC GGC-3'	12
8	5'-GAA GAA GAA GAA-3'	12
9	5'-GGT GGT GGT AA-3'	11
10	5'- ATA TAT ATA T-3'	10
11	5'-AGA GAG AG-3'	8
12	5'-TGT GTG TGT G-3'	10
13	5'- CAC ACA CAC A-3'	10
14	5'- CCG CCGCCG CCG-3'	12
15	5'-CTT CTT CTT CTT-3'	12
16	5'- CAC ACA CAC A-3’	10
17	5'- CAC ACA CAC A-3’	10
18	5'- ATA TAT ATA T-3’	10
19	5'-GAG AGA GAG A-3’	10
20	5'-AGA GAG AGA GT-3’	10
21	5'-ACA CAC ACA C-3’	10
22	5'-GAA GAA GAA GAA-3’	12
23	5'-GTG TGT GTG T-3’	10
24	5'-TAT ATA TAT A-3’	10
25	5'-CTC TCT CTC T-3’	10
26	5'-CTA TCT ATC T-3’	10
27	5'-TGT GTG TGT G-3’	10
28	5'- CTA TCT ATC T-3’	10

In one subgroup, nine species from different sections show polytomy, so it is not possible to identify their relationship from molecular data.

One monophyletic group has been shown, which contains 11 species from different sections. *S.asteroiranica* and *S.dichotoma* are sister group. They are in one clade. *S.asteroiranica* is from Sect. *Rigidulae* which has the following characters: annual; inflorescence regularly dichasial; calyx cylindric-clavate, umblicate at the base, glandular-pubescent rarely glabrous; petal limb bifid; Anthophore pubescent (Edalatiyan *et al.*, 2010). *S.dichotoma* is in Sect. *Lasiocalycinae* with these morphological characters: Annual or biennial, inflorescence dichasial, with long monochasial branches rarely monochasium, Calyx cylindric-ovate or campanula, Petal limb entire or emarginated, Anthophore glabrous.

Table 3. List polymorphic SRAP primers.

1	5'-GAC TGC GTA CGA ATT AAT-3' 5'-GAC TGC GTA CGA ATT GAC-3'
2	5'-GAC TGC GTA CGA ATT AAT-3' 5'-TGA GTC CAA ACC GGA TA-3'
3	5'-GAC TGC GTA CGA ATT AAT-3' 5'-TGA GTC CAA ACC GC-3'
4	5'-GAC TGC GTA CGA ATT AAT-3' 5'-TGA GTC CAA ACC GGA AT-3'
5	5'-GAC TGC GTA CGA ATT AAT-3' 5'-GAC TGC GTA CGA ATT GAC-3'
6	5'-GAC TGC GTA CGA ATT AAT-3' 5'-TGA GTC CAA ACC GGA TA-3'
7	5'-GAC TGC GTA CGA ATT AAT-3' 5'-TGA GTC CAA ACC GGA GC-3'
8	5'-GAC TGC GTA CGA ATT AAT-3' 5'-TGA GTC CAA ACC GGA AT-3'

The two above-mentioned species have shown close relationship with species *S. Spergulifolia*; despite it is in Sect. *Spergulifoliae* with following morphological characters: perennial, claw pubescent, not auriculate; anthophore pubescent or glabrous; staminal filaments glabrous (Edalatiyan *et al.*, 2010). *S.macrowizicii* from Sect. *Lasiostemones* that has these characters such as, perennial, claw often auriculate; anthophore pubescent; staminal filaments pubescent (Edalatiyan *et al.*, 2010) is sister group with *S.conoidea* from Sect. *Conoimorpha* with these morphological characters: annual; claw auriculate; anthophore hairy (Edalatiyan *et al.*, 2010), that two above species described before is sister group with *S.compacta* is in sect. *Compactae*. This section has these morphological characters: biennial to annual; inflorescence compact at tip of stems; calyx cylindric-clavate, glabrous; petal limb emarginate or entire; claw auriculate (Edalatiyan *et al.*, 2010). According to our study, *S.nana* has close relationship to all above

species. This species is in Sect. *Saponariodeae,* it is annual, glabrous; inflorescence dichasial; calyx campanula-ovate, not umblicate at the base, glabrous; claw auriculate, (Edalatiyan *et al.*, 2010).

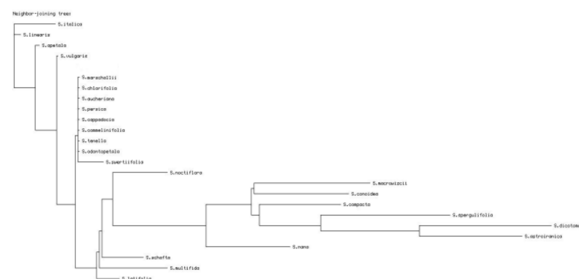

Fig. 1. Phylogenetic neighbour-joining tree cladogram based molecular markers data on species of *Silene.*

S.latifolia and *S.noctiflora* sorted in Sect. *Melandriformes are* not too close. They have some morphological characters such as perennial or annual, inflorescence dichasial; flowers hermaphrodite; calyx oblong to campanula, glandular-eglandular pubescent; petal limb bifid; claw rarely auriculate, coronal scales usually present; anthophore pubescent rarely glabrous; staminal filaments pubescent; styles 3 or 5 (Edalatiyan *et al.*, 2010). But all the other sections have three styles.
According to our cladogram*S.schafta* and *S.multifida* and *S.latifolia* have close relationship, but they are not in the same section.*S.schafta* belongs to Sect. *Schaftae* with these morphological characters: paniculate inflorescence; calyx cylindric-clavate, glandular-eglandular pubescent; petal limb bifid; coronal scales present (Edalatiyan *et al.*, 2010). *S.schafta* has different calyx shape from two others. *S.multifida* from Sect. *Fimbriatae* has these morphological characters: inflorescence dichasial; flowers hermaphrodite; calyx campanula, glandular-pubescent; petal limb divided into many parts; coronal scales absent rarely present (Edalatiyan *et al.*, 2010).

The result of our study partially confirmed the morphological affinities between sections. According to our study, Sect. *Inflate* shows the close relationship with four other sections, while from the morphological study it did not show any affinity with these four sections.

In our cladogram, the species *S.macrowizcii* of Sect. *Lasiostemones* and species *S.spergulifolia* of Sect.*Spergulifoliae*, and *S.vulgaris* of Sect. *Inflate which* before had shown close relatives with morphological study, are not shown these relatives by molecular tests. About species *S.apetela* and *S.dicotoma* which based on morphological characters were located in the same section and *S.linearis* and *S.astroiranica* were classified in the same section, and also *S.noctiflora* and *S.latifolia* which were in the one section, are far from each other in the cladogram by using molecular markers such as MP-PCR AND AMP-PCR and SRAP.

Our analysis strongly suggests that morphological data do not possess sufficient phylogenetic signal.
All characters that have been considered to be of major taxonomic value in *Silene* were found to be homoplastic in our analysis, *i.e.*, having evolved several times independently like life form and venation of calyx. As our molecular analysis did not confirm morphological viewpoints completely, so section classification is artificial (Melzheimer, 1988).

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