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**ARTICLE**

# Species relationship in the genus *Silene* L. Section *Auriculatae* (Caryophyllaceae) based on morphology and RAPD analyses

Masoud Sheidai[1]*, Abbas Gholipour[2], Zahra Noormohammadi[3]

[1]Shahid Beheshti University, GC., Faculty of Biological Sciences, Tehran, Iran, [2]Payame-Noor University, Sari, Iran, [3]Biology Department, School of Basic sciences, Science and Research Branch, Islamic Azad University (SRBIAU), Poonak, Tehran, Iran

**ABSTRACT** Morphological and RAPD studies were performed on *Silene* species of the sect. *Auriculatae* growing in Iran for the first time using phenetic, parsimony and Bayesian analyses. Trees obtained differed in the species groupings although agreed in some parts. Parsimony and Bayesian analyses of morphological characters produced some clades which were not well supported by bootstrap and clade credibility values but UPGMA tree showed a high cophenetic correlation. Grouping based on morphological characters partly support the species affinity given in Flora Iranica. Out of 40 RAPD primers used 15 primer produced reproducible polymorphic bands. In total 347 bands were produced out of which 340 bands were polymorph and 7 bands were monomorph. Among the species studied *S. goniocaula* showed the highest number of RAPD bands (184), while *S. commelinifolia* var. *isophylla* showed the lowest number (123). Some of the species studied showed the presence of specific bands which may be use for species discrimination. NJ and Bayesian trees of RAPD data partly agree with morphological trees obtained.
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**KEY WORDS**

Morphometry
Parsimony
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*Silene*

*Silene* L. is the largest genus of Caryophyllaceae with about 700 species distributed throughout the northern hemisphere; Europe, Asia and northern Africa (Greuter 1995). It includes several important weeds, very beautiful horticultural plants and some medicinal species (Swank 1932). About 110 *Silene* species grow in Iran out of which about 35 species are endemic with very limited geographical distribution (Melzheimer 1988). *Silene* species have been placed in 44 sections (Chowdhuri 1957), but recent molecular studies do not support such sectional classifications particularly for the endemic North American taxa (Oxelman and Lidén 1995; Oxelman and Berglund 1997; Oxelman and al. 2000; Burleigh and Holtsford 2003).

The basic chromosome number of *Silene* is $x = 10$ or 12 (Melzheimer 1978; 1988; Markova and al. 2006; Popp and Oxelman 2007), most of the species are diploid ($2n = 2x = 24$), some are tetraploid ($2n = 4x = 48$) and hexaploid ($2n = 6x = 72$). Few species show higher polyploidy levels for e.g. $2n = c.$ 96, 120 and 192 (Bari 1973), while $2n = 3x = 30$ is reported for *S. fortunei* (Heaslip 1951).

The section *Auriculatae* (Boiss.) Schischkin is the largest section of the genus containing about 35 species in Iran, out of which 21 species are endemic with very restricted distribution in mountainous areas such as Elburz, Zagros and Azarbayejan (Melzheimer 1988). The members of this section are caespitose plants with large flowers placed at the end of short stems. Their inflorescence is unifloral or dichasial. Calyx is cylindrical-clavate, pubescent or glandular-pubescent. The petals have conspicuous auricule at the end of claw.

Different molecular markers have been used in systematic studies. Random Amplified Polymorphic DNA is one of these molecular markers widely used to study genetic polymorphism in plants, identifying hybrid specie and to study the species relationships (Bogani et al. 1994; Sanz-Cortés 2001; Çelebi et al. 2008). For example RAPD markers were used to study the taxonomic status of 42 taxa of species of the genus *Fritillaria* (Çelebi et al. 2008) and based on RAPD, morphological and protein analyses, two species of *F. acmopetala* subsp. acmopetala and *F. sororum* were considered as synonyms. Similarly Badr et al. (2000) showed close affinity of *H. vulgare* to *H. spontaneum* by RAPD analysis. Saitou et al. (2007) studied the hybrid origin of the diploid grass *Calamagrostis longiseta* var. *longe-aristata*, morphometric and genetic analyses of this taxon and its putative parental taxa were performed. The morphometric analyses revealed that, in general, *C. longiseta* var. *longe-aristata* is morphologically intermediate between C. *longiseta* var. *longiseta* and *C. fauriei*. RAPD analyses showed that individuals of *C. longiseta* var. *longe-aristata* were placed in both of the clusters formed by each putative parental taxon.

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*Corresponding author. E-mail: msheidai@yahoo.com
msheidai@sbu.ac.ir

Oxelman (1996) used morphological and molecular markers including Internal Transcribed Spacer DNA (ITS) and RAPD markers to study the species relationships in the genus *Silene* sect. *Sedoides* and concluded that RAPD markers separated the best the species studied compared to either morphological and ITS markers used. Moreover RAPD markers were useful in subsp. delimitation and also confirmed allopolyploid nature of *S. aegaea*.

Biosystematic studies of the genus *Silene* in Iran is confined to few cytological reports only (Sheidai et al. 2008; Gholipour and Sheidai 2010). The present study considers morphometry and RAPD analysis of species relationships in the genus *Silene*, sect. *Auriculatae* of Iran for the first time.

## Materials and Methods

### Plant materials

Morphological and molecular studies were performed in 32 *Silene* species, subspecies and varieties from the sect. *Auriculatae* L. growing in Iran. The species studied are: 1- *S. commelinifolia* Boiss. var. *commelinifolia*, 2- *S. commalinifolia* var. *isophylla* Bornm., 3- *S. commelinifolia* var. *ovatifolia* Melzh., 4- *S. aucheriana* Boiss., 5- *S. nizvana* Melzh., 6- *S. oligophylla* Melzh., 7- *S. meyeri* Fenzl ex Boiss. ssp. *persica*, 8- *S. meyeri* Fenzl ex Boiss. ssp. *meyeri*, 9- *S. rhynchocarpa* Boiss., 10- *S. persica* Boiss., 11- *S. gynodioica* Ghazanfar; 12- *S. lucida* Chowdhuri, 13- *S. erysimifolia* Stapf., 14- *S. hirticalyx* Boiss.& Hausskn., 15- *S. microphylla* Boiss., 16- *S. goniocaula* Boiss., 17- *S. albescens* Boiss., 18- *S. daenensis* Melzh., 19- *S. dschuparensis* Bornm., 20- *S. eriocalycina* Boiss., 21- *S. prilipkoana* Schischk., 22- *S. sojakii* Melzh., 23- *S. sisianica* Boiss. & Buhse, 24- *S. palinotricha* Fenzl. ex Boiss., 25- *S. gertraudiae* Melzh., 26- *S. pseudonurensis* Melzh., 27- *S. elymaitica* Bornm., 28- *S. persepolitana* Melzh., 29- *S. indeprensa* Schischk., 30- *S. crispans* Litw., 31- *S. renzii* Melzh., 32- *S. araratica* Schischk. Moreover, *S. parrowiana* Boiss & Hausskn., from the sect. *Lasiostemones* and *pungens* Boiss., from the sect. *Pinifoliae* were included as out-group species in the RAPD analysis. The voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU).

### Morphometry

In total 40 morphological characters were used for morphometry, including quantitative and qualitative characters taken from published materials on *Silene* (Oxelman 1996), species description given in Flora Iranica (Melzheimer 1980) and personal observations in the field. Quantitative morphological characters were randomly measured in at least 5 plants and the means were used in phenetic analyses. Qualitative characters were coded as binary or multistate characters accordingly.

In phenetic analyses, different clustering method including UPGMA (Unweighted Paired Group using Arithmetic Average) and Neighbor Joining (NJ) clustering as well as ordination plots based on Principal Components Analysis (PCA) and Principal Coordinate Analysis (PCO) were used for grouping of the species studied. Cophenetic correlation and bootstrapping was performed to check the fit of dendrograms obtained (Podani 2000). Factor analysis was used to identify the most variable morphological characters among the species. For clustering, morphological data were standardized (Mean = 0, variance = 1) and used to determine Taxonomic and Euclidean distances (Podani 2000). Similarly unrooted parsimony and Bayesian clustering was performed on morphological data and the results were compared with those of phenetic analyses.

### RAPD analysis

Forty decamer RAPD primers of Operon technology (Alameda, Canada) belonging to OPA, OPH sets were used in molecular study of the wild *Silene*. DNA extraction was done by using the CTAB method (Murry and Tompson 1980) with modification described by De la Rosa et al. (2002). The PCR reaction mixture consisted of 1 ng template DNA, 1 x PCR buffer (10 mM Tris-HCL pH 8.8, 250 mM KCL), 200 µM dNTPs, 0.80 µM 10-base random primers and 1 unit of Taq polymerase, in a total volume of 25 µl. DNA amplification was performed on a palm cycler GP-001 (Corbet, Australia). Template DNA was initially denatured at 92°C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 92°C, primer annealing for 1 min at 36°C and primer extension for 2 min at 72°C. A final incubation for 10 min at 72°C was performed to ensure that the primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 2% agarose gels using 0.5 X TBE buffer (44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0) or 6% polyacrylamide gels. The gels were stained with ethidium bromide and visualized under UV light (Sambrook et al. 2001). A 100 bp DNA ladder (GeneRuler, Fermentas) was used as the molecular standard in order to confirm the appropriate RAPD markers. RAPD markers were named by primer origin, followed with the primer number and the size of amplified products in base pairs.

The reproducible RAPD bands were treated as binary characters and coded accordingly (presence =1, absence = 0). Jaccard similarity as well as Nei·s genetic distance (Nei 1972) were determined among the species studied and used for clustering and ordination based on principal coordinate analysis (PCO; Podani 2000). The fit of dendrograms obtained were checked by cophenetic correlation.

Bayesian clustering using Markova chain Monte Carlo (MCMC) was performed on RAPD data and the results were compared with NJ and UPGMA dendrograms. Bootstrapping was performed by using 10000 replications. NTSYS Ver. 2.02 (1998) was used for clustering and PCO analyses and Bayes-

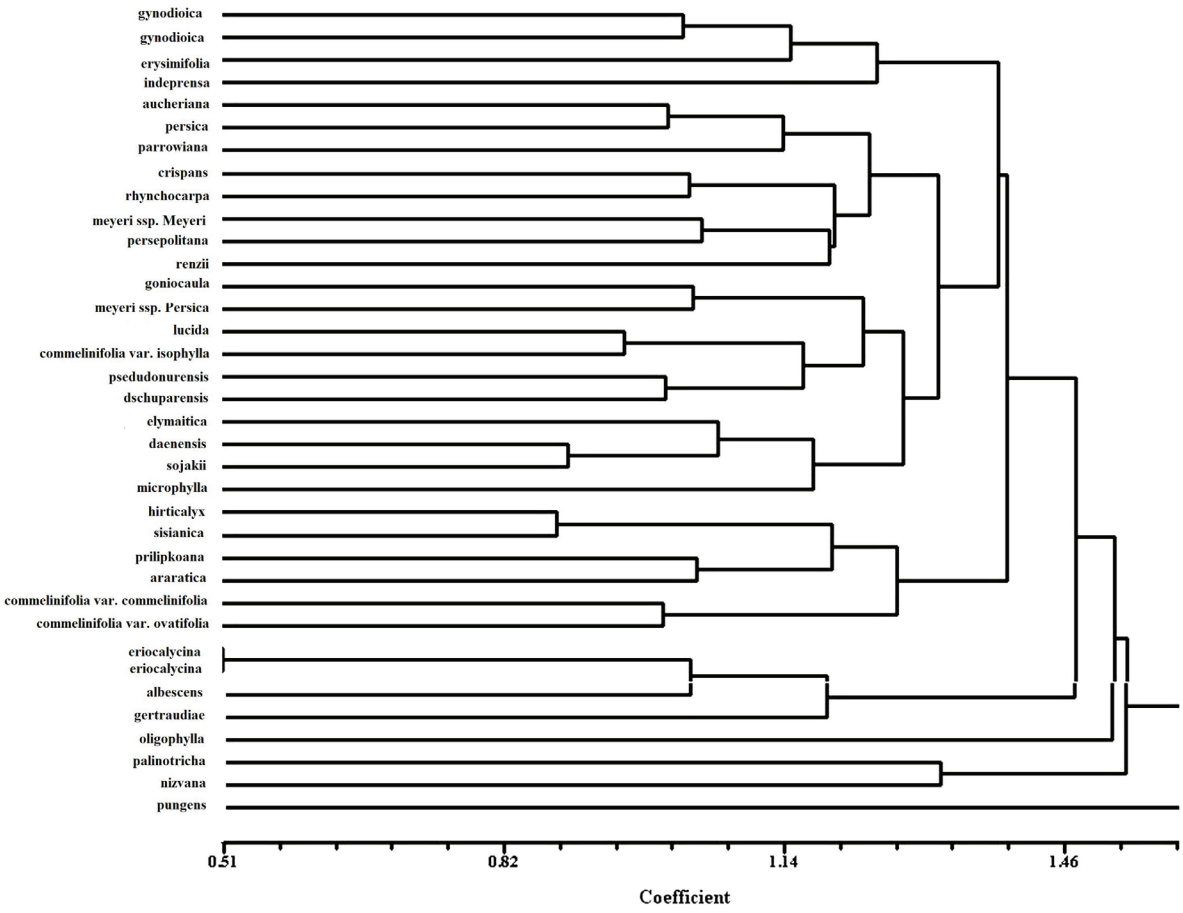


**Figure 1.** UPGMA dendrogram based on morphological characters.

ian clustering was done by Mr. Bayes ver. 3.1 (2005). The trees were obtained by Treeview ver. 1.6. 6 (2001).

## Results and Discussion

### Morphometry

UPGMA and NJ analyses of morphological data produced similar results and due to higher cophenetic correlation value of UPGMA dendrogram (r = 80) it is discussed bellow (Fig. 1). Two species of *S. pungens* and *S. parrowiana* were outgroup taxa used in the analysis, out of which *S. pungens* is separated from the other species but *S. parrowiana* is placed with in-group species studied.

In general 5 major clusters are formed. The first major cluster is comprised of *S. indeprensa*, *S. erysimifolia* and *S. gynodioica*. These species share several morphological characteristics like cespitose-sufferutescent growth form, galandular indumentums, glandular calyx indumentums, absence of calyx inside indumentums, length of coronal scales longer than 1 mm, length of petal limb division longer than ½ limb, point of epipetal filament insertion shorter than 2 mm, capsule situation to calyx included in calyx, lack of testa cell projections and dentate testa cell margin. In Flora Iranica (Melzheimer 1988) these species are placed far from each other which is not supported by morphometric analysis.

The second major cluster is comprised of 2 sub-clusters. Three species of *S. aucheriana*, *S. pseudoaucheriana* and *S. persica* of the first sub-cluster grow in Alpine meadows habitat and share morphological characteristics of cauline leaf length shorter than 25 mm, lateral pedicel length shorter than 1 mm, clavate-cylindric calyx form, reticulate calyx veins, pink-rosea petal color, large conspicuous auricle size, length of alternate filament larger than Epipetal filament, elongate-ovate capsule form, capsule included in calyx, length of antophore 5-10 mm, antophore pubescent in the lower part. These species have been considered close to each other in Flora Iranica too.

The species of *S. crispans*, *S. rynocharpa*, *S. meyeri*, *S. renzi* and *S. persepolitana* also from the same sub-cluster, share morphological characteristics of cauline leaf width shorter than 2.5 mm, elongate-ovate capsule, capsule included in calyx, dentate testa cell margin. In Flora Iranica 3 species

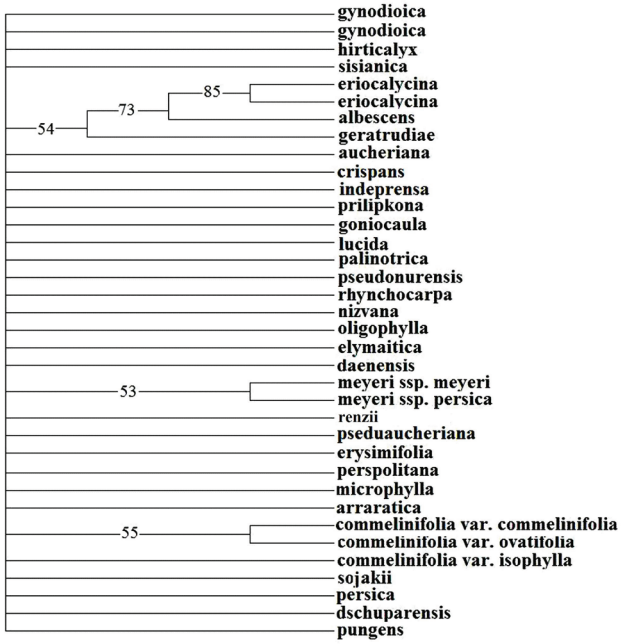


**Figure 2.** Parsimony tree based on morphological characters. (values at the base of clades are bootstrap values).

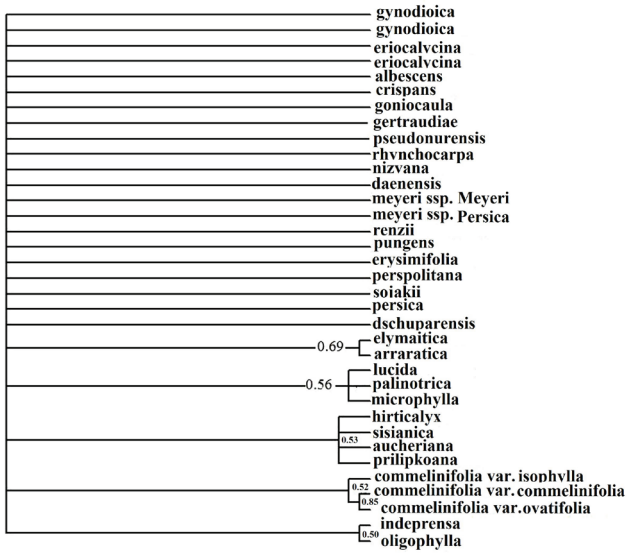


**Figure 3.** Bayesian tree based on morphological characters. (values at the base of clades are clade credibility values).

of *S. meyeri ssp. meyeri*, *S. renzi* and *S. persepolitana* are placed close to each other.

The species of *S. goniocaula*, *S. meyeri* ssp. *Persica*, *S. lucida*, *S. commelinifolia* var. *isophylla*, *S. dschuparensis* and *S. pseudonurensis* from the second sub-cluster share similar morphological characteristics of linear-lanceolate basal leaf form, basal leaf width shorter than 2.5 mm, strigose/lanate calyx indumentums, calyx length 21-32mm, petal limb length shorter than 5 mm, point of epipetal filament insertion shorter than 2 mm, lack of testa cell projections. In Flora Iranica two species of *S. goniocaula* and *S. lucida* have been considered close to each other but other species of this sub-cluster have been placed far from each other.

The species of *S. elymaitica*, *S. daenesis*, *S. sojakii* and *S. microphylla* also from the same sub-cluster share morphological characteristics like cauline leaf length shorter than 25 mm, clavate-cylindric calyx form, pink-rosea petal color, large conspicuous auricle, point of epipetal filament insertion shorter than 2 mm, capsule length longer than 10 mm, capsule included in calyx and dentate testa cell margin. In Flora Iranica two species of *S. elymaitica* and *S. daenesis* are placed close to each other.

The third major cluster is comprised of *S. hirticalyx*, *S. sisanica*, *S. prilipkoana*, *S. araratica*, *S. commelinifolia* var. *commelinifolia* and *S. commelinifolia* var. *ovatifolia* form the third major cluster and share morphological characters like reticulate calyx veins, large conspicuous auricle, claw placed in calyx. In Flora Iranica *S. hirticalyx*, *S. sisanica* and *S. prilipkoana* have been placed close to each other while the other species members of this cluster have been placed far from them. *S. araratica* has also been considered close to varieties of *S. commelinifolia*.

The fourth major cluster is formed by *S. eriocalycina*, *S. albescens* and *S. gertardiae* with the first two species showing more similarity. These species share similar morphological characteristics of cauline leaf length 25-35 mm, compound dichasium inflorescence, alar pedicel length 2-5 mm, lateral pedicel length shorter than 1 mm, clavate-cylindric calyx, internal side of calyx with no indumentums, calyx tooth length shorter than 2.5 mm, large conspicuous auricle, lack of style indumentums, petal claw length shorter than 10 mm, length of petal limb division longer than 1/2 limb, claw placed in calyx, elongate-ovate capsule, capsule length 7-10 mm, antophore with pubescent in the lower part and dentate testa cell margin. In Flora Iranica *S. eriocalycina* and *S. albescens* have been placed close to each other while *S. gertraudiae* is placed far from them.

The species of *S. oligophylla*, *S. palinotricha* and *S. nizvana* are placed far from the other species studied forming the fifth major cluster. These species also differ from each other in morphological characteristics as they join each other with great distance. Two species of *S. oligophylla* and *S. nizvans* have also been placed close to each other in Flora Iranica.

Factor analysis revealed that the first 7 components comprise about 60% of total variance in which, morphological characters like plant height, basal leaf form, basal leaf width, length/width ratio, cauline leaf width, calyx length, calyx tooth length, petal claw length and capsule form showed the highest positive/ negative correlation (>0.60/ <-0.60) and may

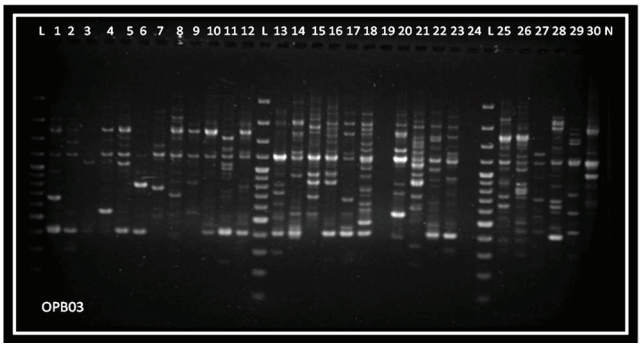


**Figure 4.** RAPD profile of primer OPB-03. Species No. are: 1- *commelinifolia* var. *ovatifolia*, 2- *S. commelinifolia* var. *commelinifolia*, 3- *S. commalinifolia* var. *isophylla*, 4- *S. lucida*, 5- *S. goniocaula*, 6- *S. gynodioica*, 7- *S. albescens*, 8- *S. daenensis*, 9- *S. dschuparensis*, 10- *S. hirticalyx*, 11- *S. eriocalycina*, 12- *S. erysimifolia*, 13- *S. meyeri* ssp. *Meyeri*, 14- *S. persica*, 15- *S. microphylla*, 16- *S. prilipkoana*, 17- *S. sojakii*, 18- *S. sisianica*, 19- *S. palinotricha*, 20- *S. gertraudiae*, 21- *S. nizvana*, 22- *S. rhynchocarpa*, 23- *S. oligophylla*, 24- *S. aucheriana*, 25- *S. pseudonurensis*, 26- *S. elymaitica*, 27- *S. persepolitana*, 28- *S. pungens*, 29- *S. meyeri* ssp. *Persica*, 30- *S. parrowiana*. L = Molecular ladder.

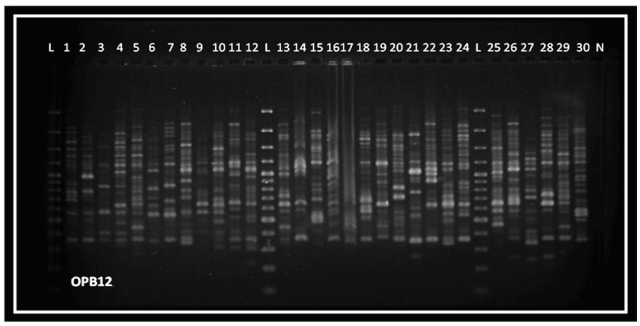


**Figure 5.** RAPD profile of primer OPB-12. Species No. as in Fig. 4.

be considered as the most variable morphological characters among the species studied.

## Parsimony analysis

Cladogram obtained by major parsimony analysis after bootstrapping and using majority rule consensus tree is presented in Figure 2. In general the clades obtained are not well supported by bootstrapping and only one clade containing two species of *S. eriocalycina* and *S. albescens* show >70% bootstrap value and other clades formed, show <60% bootstrap values. The length of tree obtained is 608, with consistency index (CI) = 0.1102, homoplasy index (HI) = 0.8898 and retention index (RI) = 0.0339, indicating the presence of high homoplasy in morphological characters used in taxonomy of *Silene* as also revealed by other studies on other sections of this genus (Oxelman and Lidén 1995; Oxelman 1996; Oxelman and Berglund 1997; Oxelman et al. 2000).

Three distinct clades are present in this cladogram which agrees with our phenetic dendrogram discussed earlier. Three species *S. eriocalycina*, *S. albescens* and *S. gertraudiae* are placed in one clade, *S. meyeri* ssp. *meyeri*, *S. meyeri* ssp. *persica* and S. renzi show close affinity and form a separate clade and two varieties of *S. commelinifolia* var. *commelinifolia* and *S. commelinifolia* var. *ovatifolia* are placed close to each other. The other species studied show polytomy and are not differentiated in separate clades.

## Bayesian analysis

In Bayesian tree (Fig. 3) also only 5 clades are recognized with 0.50-0.85 posterior probability (clade credibility). In the first clade *S. indeprensa* and *S. oligophylla* are placed together which does not agree with UPGMA and parsimony results. Three varieties of *S. commelinifolia* form a separate clade in agreement with parsimony analysis, four species of *S. hirticalyx*, *S. sisanica*, *S. aucheriana* and *S. prilipkoana* comprise a separate clade which partly agrees with UPGMA and parsimony results. Two species of *S. araratica* and *S. elymaitica* show close affinity and form another clade which is not supported by UPGMA and parsimony results.

Therefore it seems that in general morphological studies in *Silene* by different phenetic, cladistic and Bayesian approaches differ from each other and will not lead us to a clear cut grouping unable to show the species interrelationship in the sect. *Auriculatae*. Morphological characters used in the present study are taken from descriptions of *Silene* species provided in Flora Iranica (Melzheimer 1988) and previous taxonomy work on *Silene* (Oxelman 1996). Stevens (1991) states that morphological studies in a genus or among sections usually confront difficulties as clear cut qualitative characteristics separating closely related species are hard to find. Similarly Oxelman (1996) while studying morphological and molecular characteristics of *Silene* species from the sect. *Sedoides* revealed that morphological characters show high level of homoplasy and are not able to differentiate the species studied; however RAPD and ITS molecular characteristics were useful.

## RAPD analysis

Out of 40 RAPD primers used 15 primer produced reproducible polymorphic bands (Table 2., Figs. 4 & 5). In total 347 bands were produced out of which 340 bands were polymorph and 7 bands were monomorph. Among primers used OPB12 produced the highest number of bands (28), while primers OPI12 produced the lowest number of bands (17). In total 11 unique bands were produced with the highest number of unique bands (3) produced by primer OPB05 while some of the primers like OPB12 and OPB20 produced no unique band at all.

Among the species studied *S. goniocaula* showed the highest number of RAPD bands (184), while *S. commelini-*

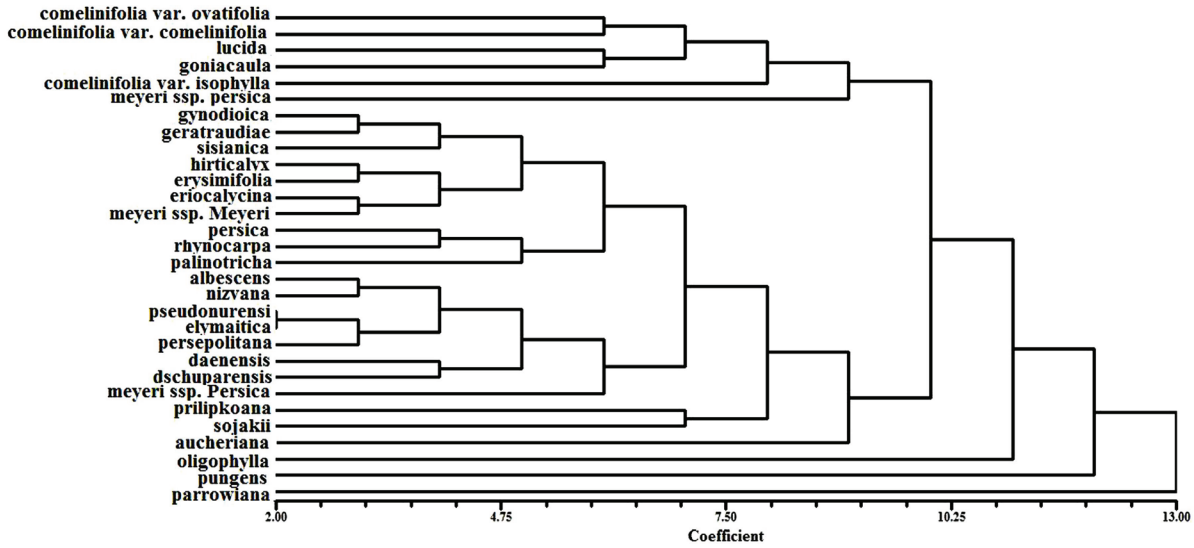


**Figure 6.** NJ dendrogram based on RAPD data.

*folia* var. *isophylla* showed the lowest number (123). Some of the species studied showed the presence of specific bands, for example band no. 14 (400 bp), of the primer OPB-03 was specific for *S. nizvana*, bands No. 2 and 17 (2700 & 470 bp respectively) of the primer OPB-05 was specific for *S. parrowiana* (one of the out-group species used), band No. 18 (450 bp ) of the primer OPB-05 was specific for *S. persepolitana*, band No. 2 (2700 bp ) of the primer OPC-01 was specific for *S. prilipkoana*, band No. 10 (930 bp ) of the primer OPC-03 was specific for *S. pungens* and bands No. 15 and 19 (650 & 450 bp respectively ) of the primer OPI-05 were specific for *S. meyei* ssp. *meyeri*.

NJ and Bayesian dendrograms of RAPD data are presented in Figures 6 & 7. In NJ tree, the out-group species i.e. *S. pungens* is almost placed far from the other species while in Bayesian tree it is not so. In both analyses all three varieties of *S. commelinifolia* var. *commelinifolia*, *S. commalinifolia* var. *isophylla*, *S. commelinifolia* var. *ovatifolia*, are placed close to

**Table 2.** RAPD primers producing bands, their sequences and bands produced.

| Primer | Sequence | No. bands produced | Polymorphic bands | Monomorphic bands | Specific bands |
|---|---|---|---|---|---|
| OPB03 | 5′ CATCCCCCTG 3′ | 28 | 27 | 1 | 1 |
| OPB05 | 5′ TGCGCCCTTC 3′ | 23 | 20 | 3 | 3 |
| OPB07 | 5′ GGTGACGCAG 3′ | 21 | 21 | 0 | 1 |
| OPB12 | 5′ CCTTGACGCA 3′ | 31 | 30 | 1 | 0 |
| OPB20 | 5′ GGACCCTTAC 3′ | 28 | 27 | 1 | 0 |
| OPC01 | 5′ TTCGAGCCAG 3′ | 25 | 25 | 0 | 1 |
| OPC02 | 5′ GTGAGGCGTC 3′ | 18 | 18 | 0 | 0 |
| OPC03 | 5′ GGGGGTCTTT 3′ | 25 | 25 | 0 | 0 |
| OPC04 | 5′ CCGCATCTAC 3′ | 25 | 25 | 0 | 2 |
| OPC06 | 5′ GAACGGACTC 3′ | 24 | 24 | 0 | 0 |
| OPC09 | 5′ CTCACCGTCC 3′ | 20 | 20 | 0 | 0 |
| OPC10 | 5′ TGTCTGGGTG 3′ | 18 | 18 | 0 | 0 |
| OPI05 | 5′ TGTTCCACGG 3′ | 24 | 24 | 0 | 2 |
| OPI12 | 5′ AGAGGGCACA 3′ | 17 | 16 | 01 | 1 |
| OPI16 | 5′ TCTCCGCCCT 3′ | 20 | 20 | 0 | 0 |
| Σ | | 347 | 340 | 7 | 11 |

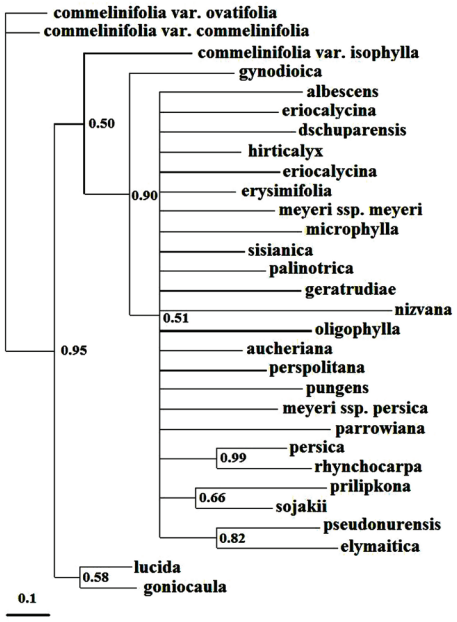


**Figure 7.** Bayesian dendrogram based on RAPD data.

each other along with *S. lucida* and *S. goniocaula* forming a separate cluster. However, two varieties of *S. commelinifolia* var. *commelinifolia* and var. *ovatifolia* show more affinity together and two species of *S. lucida* and *S. goniocaula* also show close relationship. Similarly both trees show close affinity of *S. pseudonurensis* and *S. elymaitica*, *S. prilipkoana* and *S. sojakii*, *S. rhynchocarpa* and *S. persica*. Both trees also show some affinity between *S. hirticalyx, S. erysimifolia*, *S. eriocalycina* and *S. meyeri* ssp. *meyeri* along with *S. gynodioica*, *S. gertraudiae* and *S. sisianica*.

Compairing RAPD tree with UPGMA tree of morphological data shows affinity between *S. lucida* and *S. goniocaula*, between *pseudonurensis* and *S. elymaitica*, between *S. prilipkoana* and *S. sojakii*, between *S. rhynchocarpa* and *S. persica*, and between *S. hirticalyx* and *S. sisianica*. Therefore it seems that both morphological and RAPD analyses are of limited application in showing *Silene* species relationships and further studies using other molecular markers may be performed.

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