Species relationship and diversification in the genus *Arenaria* L. (*Caryophyllaceae*)

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Abstract. Twenty-two *Arenaria* taxa and one outgroup species, *Minuartia glandulosa,* were used in the morphological and molecular studies. UPGMA and parsimony trees of morphological characters grouped some of the species in the subgenus *Arenaria* together, but other species were grouped with those of the subgenus *Eremogone*, possibly due to the high level of homoplasy in morphological characters. Nineteen out of the 30 used RAPD primers produced 414 reproducible polymorphic bands. A total of 32 unique bands were obtained in different species, which may be used in the species delimitation. NJ and UPGMA trees of RAPD data grouped the species similarly to the morphological trees. The obtained results, along with our earlier cytological data, indicate how difficult is to treat the genus *Arenaria* taxonomically, as well as the fact that a combination of morphological evolution (including characters change/reversal), cytological evolution (including aneuploidy occurrence, structural changes of the chromosomes, duplication and deletion in the chromosomes content, unreduced gamete formation, and B-chromosomes occurrence) and change in the DNA content (insertion/deletions) have been involved in diversification of the *Arenaria* species.

Key words: *Arenaria*, morphometry, parsimony, phenetic, RAPD.

Introduction

The family *Caryophyllaceae* Juss., the pink or carnation family, is cosmopolitan and contains about 3000 species and 86 genera (Harbaugh & al. 2010). This family is primarily Holarctic in distribution, manifesting its species diversity mainly in the Mediterranean and Irano-Turanian regions (Bittrich 1993).

Determination of relationships within the *Caryophyllaceae* has been difficult, owing to the fact that many genera are not well defined morphologically and are difficult to distinguish (Bittrich 1993), and that it is difficult to find phylogenetically useful morphological characters due to convergence of characters used in taxonomic classifications (Harbaugh & al. 2010).

Molecular studies of the *Caryophyllaceae* have revealed the monophyly of the family (Rettig & al. 1992; Downie & Palmer 1994; Downie & al. 1997; Fior & al. 2006), but Smissen & al. (2002), on the basis of chloroplast ndhF gene, and Fior & al. (2006), using a combination of chloroplast (matK) and nuclear ribosomal DNA (nrDNA; ITS) data, have shown that the traditional subfamilies of *Alsinoideae*, *Caryophylloideae*, and *Paronychioideae* are not monophyletic.

The *Arenaria* complex belongs to subfamily *Alsinoideae* – a group that traditionally includes the large genera *Arenaria* and *Minuartia* and some nine smaller genera (McNeill 1962; Wagner & al. 1999). The genus *Arenaria* L. consists of about 306 species, mainly distributed across Eurasia, America and north Africa, and 17 annual and perennial species in Iran (Melzheimer 1980).

In *Flora Iranica* (Melzheimer 1980), the genus *Arenaria* contains two subgenera: *Arenaria* and *Eremogone* (Fenzl) Fenzl. The first subgenus comprises three sections: 1- *Parviflorae* McNeill, 2 – *Rotundiflorae* McNeill and 3 – *Arenaria*, while subgenus *Eremogone* contains five sections: 1 – *Eremogone* (Fenzl) Fenzl, 2 – *Glomeriflorae* Fenzl ex Williams, 3 – *Rigidae* McNeill, 4 – *Scariosae* McNeill, and 5 – *Sclerophyllae* (Boiss.) McNeill,

Cytological studies into the *Arenaria* indicate the occurrence of $2n$ = 14, 16, 20, 22, 24, 30, 34, and 36 in the genus (Celebioglu & Favarger 1989; 1993; Runemark 1996; Chambers & al. 1998; Nieto Feliner 2000; Castro & Rossello 2005; Fadaei & al. 2010). Molecular study specifically of the genus is confined to the findings of Fior & Karis (2007), using nuclear (*ITS*) and chloroplast (*matK*) sequence data.

Biosystematic study of the genus *Arenaria* in Iran is also limitted to a preliminary cytological report (Fadaei & al. 2010). That is why, for the first time the present study has considered Random Amplified Polymorphic DNA (RAPD) and morphometric analyses of 22 *Arenaria* species growing in Iran (Table 1), so as to present starting data for further molecular and phylogenetic studies of the relationship between these species.

RAPD markers were used to study the taxonomic status, as well as the species relationship in different plant groups, including *Fritillaria* (Çelebi & al. 2008), *Hordeum* (Badr & al. 2000; Sheidai & al. 2008), wild olive (*Olea cuspidata*, Sheidai & al. 2010), *Silene* (Sheidai & al. 2010), etc. They have been also used to show the hybrid nature of the plant taxa (Saitou & al. 2007).

Table 1. Studied Arenaria species.

No.	Species	Section	Subgenus
1	*A. minutissima* Rech.f. & Esfand.	*Parviflorae*	*Arenaria*
2	*A. bulica* Stapf ex F.N.Williams	*Parviflorae*	*Arenaria*
3	*A. rotundifolia* M.Bieb. .	*Rotundifoliae*	*Arenaria*
4	*A. balansae* Boiss.	*Rotundifoliae*	*Arenaria*
5	*A. serpyllifolia* L.	*Arenaria*	*Arenaria*
6	*A. leptoclados* (Rchb.) Guss.	*Arenaria*	*Arenaria*
7	*A. graminea* C.A.Mey.	*Eremogone*	*Eremogone*
8	*A. kandovanensis* Fadaie, Sheidai & Assadi	*Capillares*	*Eremogone*
9	*A. dianthoides* Sm.	*Glomeriflorae*	*Eremogone*
10	*A. cucubaloides* Sm.	*Glomeriflorae*	*Eremogone*
11	*A. gypsophiloides* L. var. *gypsophiloides*	*Glomeriflorae*	*Eremogone*
12	*A. gypsophiloides* L. var. *glabra* Fenzl	*Glomeriflorae*	*Eremogone*
13	*A. holostea* M.Bieb.	*Glomeriflorae*	*Eremogone*
14	*A. szowitsii* Boiss.	*Rigidae*	*Eremogone*
15	*A. polycnemifolia* Boiss.	*Scariosae*	*Eremogone*
16	*A. zargariana* Parsa	*Scariosae*	*Eremogone*
17	*A. tetrasticha* Boiss.	*Sclerophyllae*	*Eremogone*
18	*A. persica* Boiss.	*Sclerophyllae*	*Eremogone*
19	*A. insignis* Litv.	*Sclerophyllae*	*Eremogone*
20	sp2	---------	---------
21	sp3	---------	---------
22	sp4	---------	---------
23	*Minuartia glandulosa* (Boiss. & A.Huet) Bornm.		

Material and methods

Plant material

Morphological and molecular studies into 22 *Arenaria* species/varieties have been carried out (Table 1). Details of localities and voucher numbers are given in Appendix 1. Voucher specimens are deposited in the Herbarium of Shahid Beheshti University (HSBU).

Appendix 1. Studied species, their localities and voucher No.

A. leptoclados: Mazandaran, between Ramsar airport and Kalar-abad, coastal lands, Alt: 0–10 m, collected: *Gharib and Zare*, No. 5930.
A. graminea: West Azerbaijan, Euroumiyeh, Ghasemloo valley, Natural Research Protected area, collected: Larti, *Ghasempoor and Alizadeh*, N. 1701.
A. holostea: West Azerbaijan, Khoy, Gheris village, Jahanam-Darre valley, 2000 m, collected: *Heidari, Shanaki and Siyamaki*, No. 936.
A. szowitsii: East Azerbaijan, Urmiyah to Salmas, Ghooshchi pass, 1700–1900 m, collected: *Fadaie and Nasiri*, No. 1047F
A. insigni: Semnan, Shahmirzad, Chashm elevation, 2660 m, collected: *Fadaie and Nasiri*, No. 1029F.
A. zargariana: Tehran, Haraz road, hills of S.E. Abesard, 1930 m, collected: *Fadaie and Nasiri*, No. 1071F.
A. minutissima: Chaharmahalobakhtiyari, S. Sibak, N. slope of Kallar mountain, 3235 m, collected: Fadaie, *Nasiri and Shahrokhi*, No. 1064F.
A. tetrasticha: Esfahan, Semirom, Hana, Gavtappeh mountains, 2400 m, collected: *Shams*, No. 13028.
A. rotundifolia: Mazandaran, 50 km S.W. Chaloos, above Delir village, 2800 m, collected: *Assadi and Maasoumi*, No. 51632.
A. persica: Hamedan, Ganjnameh to Shahrestanak, Ski station, 2800 m, *Fadaie and Safikhani*, No. 1096F.
A. gypsophiloides var. *gypsophiloides*: West Azerbaijan, Maraghe, Sahand mountain, Research station, 2505 m, collected: *Fadaie and Nasiri*, No. 1081F.
A. gypsophiloides var. *glabra*: East Azerbaijan, Tabriz, Heris, Hiyagh, northern slopes of Akouzdaghi elevations, collected: *Fadaie and Nasiri*, No. 1086F.
A. bulica: Fars, Eghlid, Bell mountain, collected: *Gholipoor*, No. 1100.
A. balansae: Chahar mahalobakhtiyari, South Sibak, northern slope of Kallar mountain, 2930 m, collected: *Fadaie, Nasiri and Shahrokhi*, No. 1062F.
A. serpyllifolia: Hamedan, Simineabaroo village, Alvand mountain, left side of the Dirt Road, collected: *Safikhani and Kalvandi*, No. 3337.

Appendix 1. Continuation.

A. dianthoides: East Azerbaijan, Tabriz, Yam, northern slope of Mishoudagh mountain, 2060 m, collected: *Fadaie and Nasiri*, No. 1082F.
A. kandovanensis: Tehran, 48 km off Chaloos to Tehran, Kandovan elevations, 1112 m, collected: *Fadaie and Nasiri*, No. 1092F.
A. polycnemifolia: Gorgan, 4 km off Chaharbagh to Shahkouh, northern slopes of Shahkouh, 2050 m, collected: *Fadaie and Nasiri*, No. 1056F.
sp2: Hamadan: Famenin, Ghorveh Karafs, mountains of W. E. of Karafs, 2000–2600 m, collected: *Mozaffariyan*, No. 64541.
A. cucubaloides: Urmiyah, Sero, 1800m, collected: *Foroughi*, No. 1621.
sp3: West Azerbaijan, Salmas to Urmiyah road, Ghooshchi pass, 1750 m, collected: *Runemark and Foroughi*, No. 19588.
sp4: Kerman, 70 km N.W. of Ravar near Kuhbahan, Davedan mountain, 2400–3200 m, collected: *Assadi and Bazgosha*, No. 56164 .

Morphometry

A total of 30 morphological characters were used for morphometry (Table 2), including quantitative and qualitative characters taken from floras and personal observations in the field. Quantitative morphological characters were randomly measured in at least five plants and the means were used in the analyses. Qualitative characters were coded as binary or multistate characters accordingly (Table 2).

Unweighted Paired Group using Arithmetic Average (UPGMA), Neighbor Joining (NJ), as well as ordination plots based on Principal Components Analysis (PCA) and Principal Coordinate Analysis (PCO) were used for grouping of the studied species. Cophenetic correlation and bootstrapping were performed

Table 2. Morphological characters and their coding.

No.	Characters	States and codes
1	Life cycle	annual (0), perennial (1)
2	Habit type	dense caespitose (0), lax caespitose (1), non caespitose (2)
3	Stem direction	erect (0), ascending(1), decumbent (2)
4	Stem indumentum	pubescent in end of stem(0), pubescent in inflorescent axis (1), glabrous or pubescent in end of stem (2), pubescent (3), glandular pubescent (4), glandular (5), glabrous (6)
5	Plant height	shorter than 4cm (0), 4–10 cm (1), longer than 10–30 cm (2), longer than 30–60 cm (3)
6	Sterile leaf length	absent (0), 0.2–2 cm (1), longer than 2–5.5 cm (2), longer than 5.5–17.5 cm (3)
7	Basal leaf shape	absent (0), elliptic (1), linear acerose (2), lorate linear (3), obovate (4), oblanceolate-obovate (5), lanceolate to linear- lanceolate (6)
8	Basal leaf length	absent (0), 0.1–0.15 cm (1), longer than 0.15–0.3 cm (2), longer than 0.3- shorter than 3cm (3), 3–12.5 cm (4)
9	Basal leaf width	absent (0), 0.3–1 cm (1), longer than 1–2.5 cm (2)
10	Basal leaf margin	absent (0), entire (1), serrulate (2), ciliolate (3)
11	Median leaf shape	absent (0), acerose (1), linear (2), linear-acerose (3), lorate-linear (4), ovate-lanceolate (5), lanceolate-ovate (6), oblanceolate-obovate (7), elliptic-obovate(8), lanceolate-linear lanceolate (9), oval-circular cordate (10)
12	Median leaf margin	absent (0), thin membranous ciliolate (1), serrulate (2), entire (3)
13	Indumentum of median leaf	leaf absent (0), pubescent(1), glabrous (2)
14	Terminal leaf shape	absent (0), lorate (1), long deltoid-lanceolate to sub acerose (2), linear-aserose (3), linear-lanceolate (4), subulate to long subulate (5), deltoid (6), long obovate (7), ovate-lanceolate (8), obovate-oblanceolate (9), acerose (10)
15	Terminal leaf margin	absent (0), membranous serrulate (1), membranous (2), entire (3), membranous ciliolate (4)
16	Terminal leaf length	absent (0), 2mm to shorter than 4.5 mm (1), 4.5 mm to shorter than 12 mm (2), 12–40 mm (3)
17	Terminal leaf width	absent (0), 0.5–2 mm (1), longer than 2–4.5 mm (2), longer than 4.5–6.0 mm (3)
18	Median leaf length	absent (0), 0.2 cm to shorter than 1cm (1), 1 cm to shorter than 3cm (2), 3–5 cm (3), longer than 5–17 cm (4)
19	Median leaf width	absent (0), shorter than 0.5 mm (1), 0.5–1 mm (2) longer than 1–3.5 mm (3), longer than 3.5–6.5 mm (4)
20	Cauline leaf sheath length	cauline leaf absent (0), 0.5 mm (1), longer than 0.5–1mm (2), longer than 1–8 mm (3)
21	Internode length	absent (0), 0.2–0.5 mm (1), longer than 0.5–1.5 mm (2), longer than 1.5–7 mm (3)
22	Inflorescent position	apical and lateral (0), apical (1)
23	Inflorescent type	capitate (0), cyme (1), cymose panicle (2)
24	Inflorescent density	lax (0), compact (1)
25	Lowest bract length	absent (0), 1.5–7 mm (1), longer than 7–21 mm (2), longer than 21–38 mm (3)
26	Flower bract length	1.5–4.5 mm (0), longer than 4.5–8 mm (1)
27	Pedicle length	0.2–1cm (0), longer than 1–5cm (1)
28	Pedicle indumentum	glabrous (0), pubescent (1), glabrous to pubescent (2), glandular pubescent (3)
29	Sepal shape	lanceolate (0), oval carinate (1), lanceolate to long lanceolate (2), ovate (3), ovate-lanceolate (4), caudate lanceolate (5), lanceolate to subulate (6)

Table 2. Continuation.

No	Characters	States and codes
30	Sepal length	2–3 mm (0), longer than 3–5.5 mm (1), longer than 5.5–10 mm (2)
31	Sepal width	0.5–1 mm (0), longer than 1–3 mm (1), longer than 3–4.6 mm (2)
32	Sepal length/width	
33	Sepal margin	thin membranous (0), membranous (1), developed membranous (2)
34	Sepal tip	acute (0), obtuse (1), acute to membranous obtuse (2), acute to caudate (3), obtuse to membranous rounded (4)
35	Sepal color	green (0), green and in upper part dark (1), often entirely and sometimes in upper part dark (2)
36	Sepal indumentum	glabrous (0), pubescent (1), glandular pubescent (2), glabrous to glandular pubescent in base (3)
37	Sepal nerves	3 (0), 3–5 (1), 3–7 (2), 3–9 (3), 3–11 (4), 5–9 (5)
38	Petal shape	oblong to linear oblong (0), obovate to oblong (1), oblong – elliptic (2), oval (3), obovate to long spathulate (4), sub rhomboid (5), lanceolate (6), long obovate to linear oblong (7)
39	Petal length	1–2.5 mm (0), longer than 2.5 mm to shorter than 4 mm (1), 4–6 mm (2), longer than 6 mm to shorter than 11mm (3), longer than 11–16 mm (4)
40	Petal width	0.5 mm (0), longer than 0.5 mm to-1.1 mm (1), 1.2–2 mm (2), longer than 2 mm to 5.5 mm (3)
41	Petal length / width	
42	Capsule shape	urceolate to long urceolate (0), urceolate (1), spherical (2), ovate-urceolate (3), cup-shaped (4), suboblong
43	Capsule length	2–4 mm (0), longer than 4 mm to 6.5 mm (1), longer than 6.5 mm to9 mm (2)
44	Capsule width	1.2–2.5mm (0) longer than 2.5 mm to 3 mm (1), longer than 3 mm to 5.5 mm (2)
45	Capsule length/width	
46	Sepal/capsule length	
47	Filament length	1–3 mm (0), longer than 3 mm to 6.5 mm (1), longer than 6.5 mm to 9.5 mm (2)
48	Staminal glands	absent (0), present (1)
49	Seed shape	oblong-elliptic (0), reniform (1), orbicular ovate to dropped ovate (2),comma-shaped (3)
50	Seed length	0.3–1mm (0), more than 1 mm to less than 2.5 mm (1), 2.5 mm to 3.2mm (2)
51	Seed width	0.3–0.8mm (0), more than 0.8 mm to 1.5 mm (1), 2–2.3 mm (2)
52	Seed ornamentation	round (0), triangular (1), sinuate (2), spheroid (3), subfunnel-shaped (4), septum-shaped (5), irregular pyramidical (6), suboblong (7), tuberculate (8), truncate(9)
53	Seed indumentum	Glabrous (0), pubescent (1)

to check the fit of obtained dendrograms (Podani 2000). For clustering, morphological data were standardized (mean = 0, variance = 1) and used to determine the taxonomic and Euclidean distances (Podani 2000). Parsimony and Bayesian clustering was applied on morphological data and the results were compared with those of phenetic analysis.

RAPD analysis

Thirty-five decamer RAPD primers of Operon technology (Alameda, Canada) belonging to OPA, OPH sets, were used in the molecular study of wild olives. DNA extraction was implemented by using the CTAB method (Murry & Tompson 1980), with a modification after De la Rosa & al. (2002). The PCR reaction mixture consisted of 1 ng template DNA, 1 × 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. Final incubation for 10 min at 72 °C was carried out 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 & al. 2001). A 100 bp DNA ladder (GeneRuler, Fermentas) was used as molecular standard, in order to confirm the appropriate RAPD markers. RAPD markers were named according to primer origin, followed by 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 and Nei's genetic distance (Nei 1972) were determined among the studied

species and used for clustering and ordination based on the principal coordinate analysis (PCO) (Podani 2000). The fit of obtained dendrograms was checked by cophenetic correlation.

Bayesian clustering using Markov Chain Monte Carlo (MCMC) was applied to RAPD data and the results were compared with NJ and UPGMA dendrograms. Bootstrapping was carried out by use of 10 000 replications. NTSYS Ver. 2.02 (1998) was applied for clustering and PCO analysis and Bayesian clustering was achieved by Mr. Bayes ver. 3.1 (2005). The trees were obtained by Tree View ver. 1.6. 6 (2001).

Results and discussion

Morphometry

A taxonomic study of the collected species has helped us identify three new species labeled sp2–4 and included in a further morphomtery analysis. UPGMA and NJ analyses of morphological data produced similar results and, owing to the higher cophenetic correlation value of UPGMA dendrogram (r = 80), are discussed bellow.

In general, four major clusters were formed (Fig. 1), the first of which comprised *A. insignis*, sp4, *A. kandovanensis*, sp2 and *A. persica*. These species shared such morphological characters like length, width and shape of median cauline leaf; length, width, shape and indumentum of sepals, type and density of inflorescence and absence of staminal glands.

The second major cluster was formed by *A. szowitsii* and sp3, which have been placed close to each other, *A. gypsophiloides* var. *gypsophiloides*, *A. gypsophiloides* var. *glabra* and *A. graminea* which have been placed close to each other, *A. dianthoides* and *A. holostea*, *A. cucubaloides* var. *cucubaloides,* and *A. cucubaloides* var. *glabra*. *A. zargariana* joined these species at some distance (Fig. 1).

A. szowitsii and sp3 shared morphologically such characters like absence of basal leaf; margin of cauline leaf and indumentum of sepals, and absence of staminal glands. Two varieties of *A. gypsophiloides* var. *gypsophiloides* and *A. gypsophiloides* var. *glabra* had several similar morphological characters, but differred in indumentum of sepals, stem and pedicle; shape of sepal tip and shape of capsule and, along with *A. graminea,* shared such morphological features like direction of stem, length, width, shape and margin of median and terminal cauline leaves, length and margin of sepals, and absence of staminal glands.

The third major cluster contained *A. minutissima*, *A. tetrasticha*, *A. polycnemifolia*, *A. leptoclados*, *A. serpyllifolia* var. *serpyllifolia,* and *A. serpyllifolia* var. *macrocepala*.

A. leptoclados and two varieties of *A. serpyllifolia* var. *serpyllifolia* and *A. serpyllifolia* var. *macrosepala* were members of sect. *Arenaria* from subgen. *Arenaria* and showed similar morphological characters in the direction of stem; indumentum of stem; absence

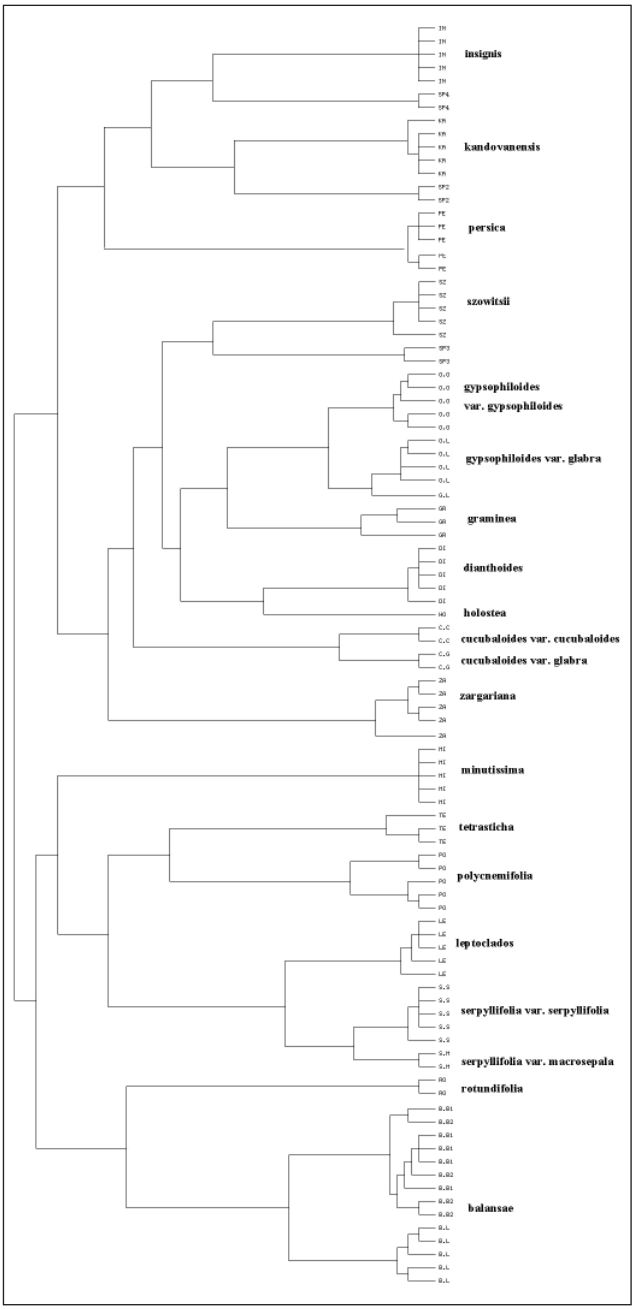

Fig. 1. UPGMA dendrogram based on morphological characters.

of sterile leafy shoot and basal leaf, shape and margin of terminal cauline leaf; length and margin of median cauline leaf, number of nerves in sepals, length and shape of petals, shape of seed and absence of staminal glands.

Two species of *A. rotundifolia* and *A. balansae* from sect. *Rotundifoliae* comprised the fourth major cluster. In general, the grouping obtained by UPGMA did not agree with sectional positions of the studied species.

Parsimony analysis produced 400 best trees, which after bootstrapping produced a final tree of 452 steps, with a Consistence Index (CI) value of 0.35, Homoplacy Index (HI) of 0.64 and Retention Index (RI) of 0.85. The parsimony tree supported the UPGMA dendrogram by a 70 % bootstrap value, indicated distinctness of *A. insignis,* and recognized two sp2 and sp4 as separate entities.

The first major clade (Fig. 2) in the parsimony tree grouped the species of the sections in subgen. *Eremogone*, with a good bootstrap value (70 %). the same was true for the third major clade containing the other species of this subgenus. However, two species, *A. serpyllifolia* var. *serpyllifolia* and *serpyllifolia* var. *macrosepala,* from subgen. *Arenaria* sect. *Arenaria* were placed close to this clade. The parsimony tree also showed close affinity between *A. cucubaloides* var. *cucubaloides* and *A. cucubaloides* var. *glabra* and *A. holostea* subsp. *macrantha* of the sect. *Glomeriflorae*.

Although some species of the subgenus *Arenaria* were grouped together, others have been placed close to the species of subgenus *Eremogone*. On the basis of morphological characters we recognized three possible new species, coded as sp2, sp3 and sp4. Specimens of sp4 showed close affinity to *A. graminea* subgen. *Eremogone* of the sect. *Eremogone*, while sp2 and sp3 specimens were separated from the other studied species by 100 % bootstrap values.

Fior & Karis (2007) have stated that morphological characters in *Arenaria* and *Moehringia* are affected by a high level of homoplasy, and valuable information was provided when they were analyzed in combination with the molecular data. These combined data have shown that *Moehringia* is paraphyletic to *Arenaria,* while the Iberian taxa belonging to *Moehringia* sect. *Pseudomoehringia* McNeill are more closely related to *Arenaria*.

The Consistency index (CI), Homoplasy index (HI) and Retention index (RI) values of the obtained parsimony tree indicated homoplasy in the morphological characters used in the taxonomy of *Arenaria*. This is a known problem in *Caryophllaceae* and, as Harbaugh & al. (2010) have stated, there is difficulty in determining phylogenetically the useful characters

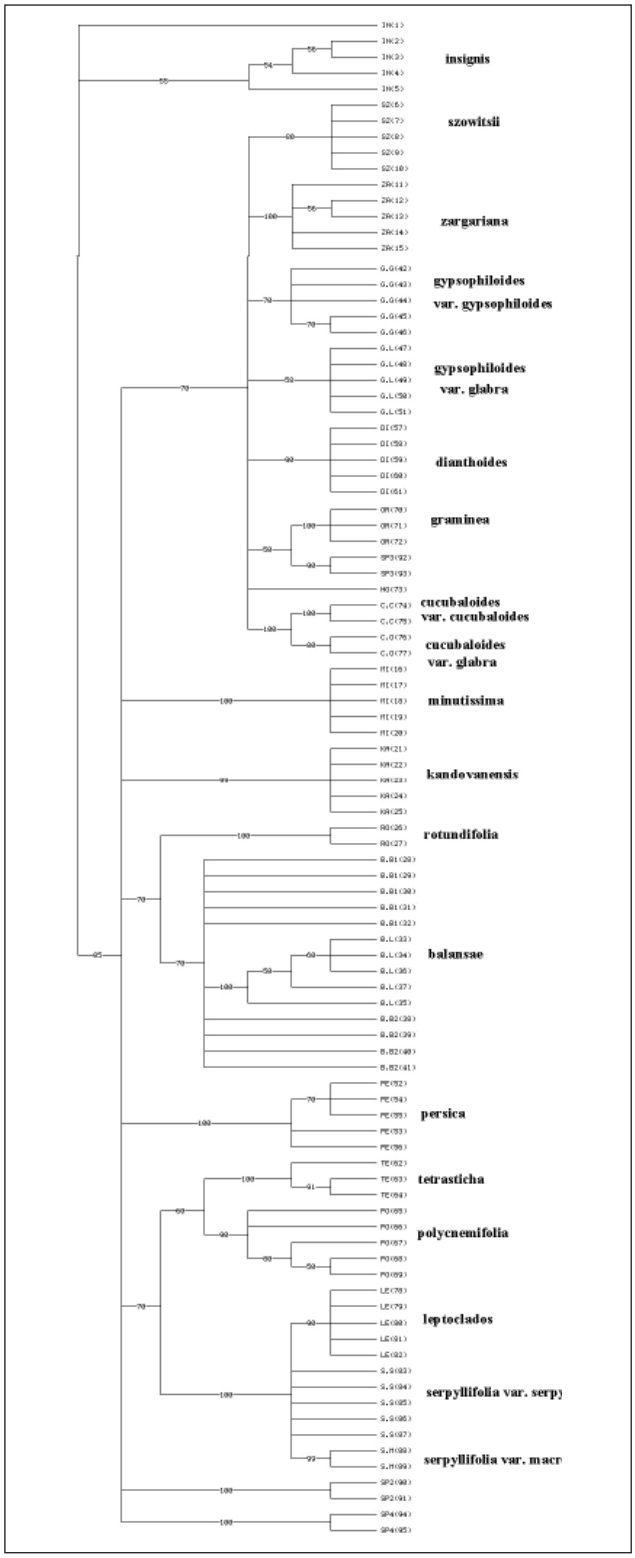

Fig. 2. Parsimony tree of morphological characters.

and the possibility of convergence of characters used in taxonomic classifications (Harbaugh & al. 2010). The Bayesian tree (not shown) of the morphological characters did not produce any clear species relationships.

Stevens (1991) has stated that since it is hard to find clear-cut qualitative characteristics separating the closely related species, morphological studies among the sections and inside a genus usually encounter difficulties. Furthermore, Bittrich (1993) has stated that understanding the relationships within the *Caryophyllaceae* is difficult, partly because many of the genera are not well defined morphologically and are difficult to distinguish.

RAPD analysis

Nineteen of the RAPD primers produced 414 reproducible polymorphic bands (Fig. 3). Primers OPC-05 and OPR-08 produced the highest number of bands (33 and 32 respectively), while primers OPC-08 and OPI-18 produced the lowest number of bands (12 and 13 respectively). The species *A. leptoclados* and *A. zargariana* showed the highest number of bands (163 and 162 respectively), while sp2 showed the lowest number of bands (59 bands). A total of 32 unique bands were obtained, with primer OPC-12 having the highest number of unique bands (Table 3). *A. kandovanensi* and *A. polycnemifolia* had four unique bands while, *A. zargariyana* had three unique bands. Such unique bands may be used in taxonomic delimitation of the species.

NJ and UPGMA dendrograms (Fig. 4) of the RAPD analysis grouped together *A. szowitsii*, *A. zargariana* and *A. insignis* of subgen. *Eremogone,* supporting the morphological analysis (Figs 1 and 2). Similarly, *A. gypsophiloides* var. *gypsophiloides* and *A. gypsophiloides* var. *glabra* of sect. *Glomeriflorae*, subgen. *Eremogone* showed affinity in RAPD markers, similarly to morphological characteristics. *A. rotundifolia* of subgen. *Arenaria* and *A. persica* of subgen. *Eremogone* joined at some distance in both RAPD and morphological trees.

Arenaria serpyllifolia var. *serpyllifolia* and *A. leptoclados* of subgen. *Arenaria* showed affinity in both RAPD and morphological analyses. sp4 also joined these two species at some distance in both analyses.

Three species, *A. cucubaloides* var. *cucubaloides*, A. *graminea* and *A. holostea* subsp. *Macrantha* from subgen. *Eremogone,* also showed close relationship in

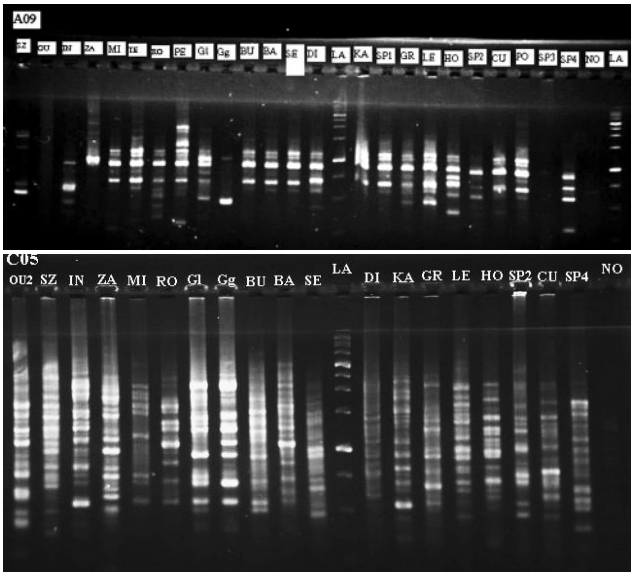

Fig. 3. RAPD profile of OPR-A09 (top) and OPR-C05 in the *Arenaria* species (bottom).

Abbreviations: SZ = *A. szowitsii*, **OU** = *Minuartia glandulosa*, **IN** = *A. insigis*, **ZA** = *A. zargariana*, **MI** = *A. minutissima*, **TE** = *A. tetrasticha*, **RO** = *A. rotundifolia*, **PE** = *A. persica*, **GL** = *A. gypsophiloides* var. *glabra*, **GG** = *A. gypsophiloides* var. *gypsophiloides*, **BU** = *A. bulica*, **BA** = *A. balansae*, **SE** = *A. serpyllifolia*, **DI** = *A. dianthoides*, **LE** = *A. leptoclados*, **GR** = *A. graminea*, **KA** = *A. kandovanensis*, **CU** = *A. cucubaloides*, **HO** = *A. holostea*, **PO** = *A. polycnemifolia*, **LA** = molecular ladder, and **NO** = No. DNA.

Table 3. RAPD primers used and the bands obtained in *Arenaria* species.

No.	Primer	Primer sequences	Total No. bands	Polymorphic bands	Unique bands
1	OPA02	5' TGCCGAGCTG3'	23	23	4
2	OPA04	5' AATCGGGCTG 3'	22	22	4
3	OPA05	5' AGGGGTCTTG3'	21	21	0
4	OPA09	5' GGGTAACGCC3'	21	21	2
5	OPA10	5' GTGATCGCAG3'	26	26	1
6	OPA11	5' CAATCGCCGT 3'	22	22	1
7	OPA12	5' TCGGCGATAG3'	21	21	3
8	OPA13	5' CAGCACCCAC 3'	25	25	1
9	OPB12	5' CCTTGACGCA 3'	18	18	1
10	OPC04	5' CCGCATCTAC 3'	21	21	0
11	OPC05	5' GATGACCGCC 3'	33	33	2
12	OPC06	5' GAACGGACTC 3'	23	23	1
13	OPC08	5' TGGACCGGTG 3'	12	12	0
14	OPC12	5' TGTCATCCCC3'	24	24	5
15	OPH19	5' CTGACCAGCC 3'	20	20	2
16	OPI18	5' AATGCGGGAG 3'	13	13	0
17	OPM19	5' CCTTCAGGCA 3'	17	17	1
18	OPR02	5' CACAGCTGCC 3'	20	20	0
19	OPR08	5' CCCGTTGCCT3'	32	32	3
		Total	**414**	**414**	**31**

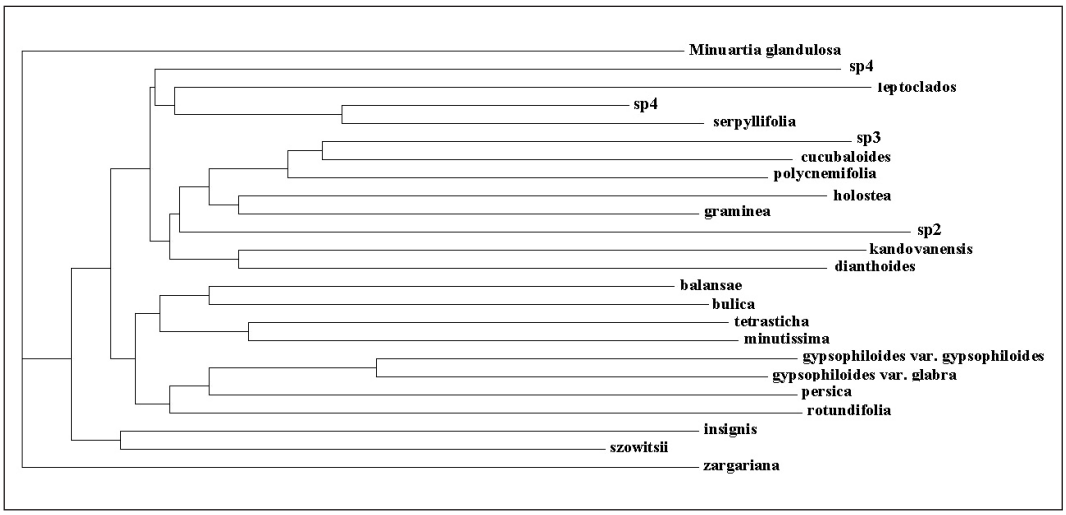

Fig. 4. NJ dendrogram of RAPD data.

both RAPD and morphological trees. Parsimony and Bayesian trees could not group the studied species in RAPD data.

After reviewing the molecular studies of *Caryophyllaceae* and performing a phylogenetic analysis of three chloroplast gene sequences (matK, trnL-F, and rps16), Harbaugh & al. (2010) have stated that (1) the currently delimited subfamilies within *Caryophyllaceae* are not natural groups and should be abandoned, (2) the genera *Arenaria* and *Minuartia* and several other genera are not natural groups and further studies should be carried out to clarify their phylogenetic relationships, while the genus *Arenaria* itself and the subgen. *Arenaria* within it are polyphyletic.

Cytological evolution in *Arenaria* includes occurrence of polyploidy/aneuploidy (chromosome numbers ranging from 2n = 14, 16, 20, 22, 24, 30, 34 and 36, Celebioglu & Favarger 1989; 1993; Runemark 1996; Chambers & al. 1998; Nieto Feliner 2000; Castro & Rossello 2005; Fadaei & al. 2010). However, our earlier karyotype and meiotic analyses of 16 populations of seven *Arenaria* species growing in Iran (Fadaei & al. 2010) have shown the occurrence of only diploid chromosome numbers, i.e. $2n = 2x = 22$ in *A. persica*, *A. insignis*, *A. gypsophiloides*, A. *polycnemifolia*, *A. zargariana*, *A. szowitsii,* and *A. minutissima*. These species differed significantly in total size of the chromosomes, size of the short arms and long arms, indicating the role of quantitative genomic changes in the *Arenaria* species diversification. They also differed in their karyotype formulae, indicating the occurrence of structural changes in their chromosomes, which is also supported by RAPD diversity explained in this paper. The observed different RAPD loci indicate variations in DNA nucleotide contents of the studied *Arenaria* species.

Meiotic analysis of these species also revealed the occurrence of heterozygote translocation between two pairs of chromosomes, which in turn may increase the amount of genetic variability in the next generation.

The process of species diversification in the genus *Arenaria* seems to be complicated, as we have reported recently the formation of unreduced pollen grains in *A. gypsophiloides* var. *glabra,* and the occurrence of B-chromosomes in *A. insignis* and *A. polycnemifolia* (Fadaei & al. 2010).

Unreduced pollen grain formation in the diploid species may be considered as a future plan for higher polyploidy production, an adaptive/genomic strategy to encounter new environmental conditions, also reported in other plant species, including *Bromus*, *Stipa*, *Festuca* (Sheidai 2008), *Achillea* (Sheidai & al. 2009a), *Silene* (Sheidai & al. 2009b) and *Oryzopsis* (Sheidai & al. 2010).

The occurrence of B-chromosomes may be also beneficial for the plants that have them by increasing the amount of genetic recombination as already reported in many other plant species (Camacho & al. 2000), including *Brassica napus* (Sheidai & al. 2006), *Punica granatum* (Sheidai 2007) and *Aegilops* (Sheidai 2008). Therefore, a combination of morphological evolution (including characters change/reversal), cytological evolution (including aneuploidy occurrence, structural changes of the chromosomes, duplication and deletion in the chromosomes content, unreduced gamete formation and B-chromosomes occurrence) and change in the DNA content (insertion/deletions) have been involved in the *Arenaria* species diversification.

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