

The evolution of *Dianthus polylepis* complex (Caryophyllaceae) inferred from morphological and nuclear DNA sequence data: one or two species?

Mohammad Farsi · Maryam Behroozian ·
Jamil Vaezi · Mohammad Reza Joharchi ·
Farshid Memariani

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Abstract *Dianthus polylepis* complex consists of two already known endemic species, *Dianthus polylepis* and *D. binaludensis*, in Khorassan-Kopetdagh floristic province. The taxonomic position of these species has long been debated. The aim of the present study is to shed light on the evolutionary relationships of the members of the complex using morphological and molecular data. In morphological study, firstly, 56 vegetative and floral characters were measured on 33 specimens of the both species. Multivariate analyses were performed on 25 (out of 56) significantly discriminating morphological traits. In molecular study, we sequenced alleles obtained from a region between 2nd and 6th exons of the gene coding for the enzyme dihydroflavonol 4-reductase copy1 (DFR1). Morphological results show that most of a priori identified accessions were not grouped in a posteriori classification. It is difficult to discriminate *D. polylepis* from *D. binaludensis* in morphological continuum among the accessions. Results obtained from the molecular data indicated no monophyly for the members of the *D. polylepis* complex. Consistency between the morphological and molecular results shows that *D. polylepis* and *D. binaludensis* were not morphologically and molecularly well differentiated. Therefore, we propose a new combination as *D. polylepis* subsp. *binaludensis*.

Keywords Caryophyllaceae · *Dianthus polylepis* complex · Morphology · Molecular phylogeny · Iran

Introduction

One of the fundamental problems in plant taxonomy concerns with the plant identification, especially for distinguishing closely related or recently evolved species (Rieseberg et al. 2006; Fazekas et al. 2009; Yan et al. 2011).

Dianthus L. (Caryophyllaceae) with over 300 species worldwide is characterized by its extensive morphological variability at both inter- and intraspecific levels (Erhardt 1990, 1991; Friedman et al. 2001; Bloch et al. 2006). Identification of some of the species in the genus is problematic due to their morphological plasticity (Sultan 1987; West-Eberhard 1989; Crespi et al. 2004). In reality, this variability seems to be due to variations occurred in sexual organs resulting from cross pollination (Collin and Shykoff 2003).

Linder (2008) compiled several examples of rapid radiations in plants. This process appears to be common in plants (Kadereit et al. 2004; Linder 2008; Bittkau and Comes 2009). Many phylogenetic studies revealed that the evolutionary radiations have been occurred in *Dianthus* (Balao et al. 2010; Valente et al. 2010). A consequence of radiations, however, is that the rates of morphological diversity could be raised making species delimitation difficult (Linder 2008; Balao et al. 2010). On the other hand, polyploidy, hybridization, and genome duplication, common evolutionary forces in plants, act as potential drivers of plant radiations (Ramsey and Schemske 1998; Otto 2007; Paun et al. 2009; Soltis and Soltis 2009; Wood et al. 2009). These processes are among phenomena occurring in

M. Farsi · M. Behroozian · M. R. Joharchi · F. Memariani
Research Centre for Plant Sciences,
Ferdowsi University of Mashhad, Mashhad, Iran

J. Vaezi (✉)
Department of Biology, Faculty of Sciences,
Shahid Chamran University of Ahvaz, Ahvaz, Iran
e-mail: J.vaezi@Scu.ac.ir

the complexes and complicate the study of taxonomy and evolution in certain plant groups. However, in addition to morphological studies, including data from ecology (Mandakova and Munzbergova 2006), cytology (Balao et al. 2009; Al-Saghir 2010), and molecular methods (Oxelman et al. 1997; Linder et al. 2006; Pillon et al. 2007) could assist in resolving taxonomic problems of species complexes.

The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA is highly employed in phylogenetic publications (Baldwin et al. 1995; Volkov et al. 2007). The markers like rDNA nrITS and chloroplast DNA evolve slowly compared to speciation in recently radiated taxa and thus, may not provide enough information to resolve relationships (Baldwin et al. 1998; Sang 2002; Small et al. 2004; Whittall et al. 2006). Lu et al. (2002) showed that analyses of ITS sequences provided low resolution among *Dianthus* species distributed in China. This homogeneity of the accessions seems to be due to the radiation phenomenon where there has not been possibly enough time to differentiate the ribotypes in this recently evolved taxon.

Low-copy nuclear genes may provide more information to resolve relationships and therefore, could overcome the problem of low resolution within recent radiations (reviewed in Sang 2002; Small et al. 2004). Thus, numerous investigations have attempted to evaluate the use of low-copy nuclear genes to reconstruct phylogenies and to estimate their evolutionary impact (Fan et al. 2004; Syring et al. 2005; Janssens et al. 2007; Tu et al. 2008; Duarte et al. 2010; Naumann et al. 2011).

Our current study is focused on *Dianthus polylepis* Bien. ex Boiss. as a species complex in Khorassan-Kopetdagh floristic province, northeast of Iran and southern Turkmenistan. This species has a large range of variation in morphological traits. For this reason, taxonomists did not reach the same conclusion to identify specimens of the species in different geographical areas. Rechinger (1983) described *D. binaludensis* Rech.f. as a new species from Binalud Mountains. He considered *D. binaludensis* and *D. polylepis* to be distinct, independent species (in “Flora Iranica”, Rechinger 1988), while Assadi (1985) declared these two species as synonyms and nominated it as *D. polylepis*. In Flora Iranica (Rechinger 1988) *D. polylepis* is distinguished from *D. binaludensis* by bract numbers, length of calyx, branches of stem, length of fimbria, width of calyx, and length of calyx dents. However, these traits have never been assessed in a phylogenetic context, and thus whether they can be used as discriminating variables to define the monophyly of *D. polylepis* and *D. binaludensis* remained ambiguous. *Dianthus polylepis* is widely distributed in northeast of Iran and partly in southern Turkmenistan, on Hezar-Masjed and Kopetdagh mountain

ranges and also on southern and southeastern extensions of Khorassan-Kopetdagh ranges, while distribution range of *D. binaludensis* is locally restricted to Binalud Mountains (Fig. 1). Jafari and Behroozian (2010) reported the same ploidy level ($2n = 2x = 30$) for the both species.

The problematic taxonomic position of *D. polylepis* compared with *D. binaludensis* (hereafter the *D. polylepis* complex) is still uncertain. Our main objective is to unravel the evolutionary history of this complex. To address this goal, we used morphological data and sequence data from the gene coding for the enzyme dihydroflavonol 4-reductase (DFR). The enzyme dihydroflavonol 4-reductase functions most obviously in the reduction of three dihydroflavonols (dihydrokaempferol, dihydroquercetin, and dihydromyricetin) to leucoanthocyanidins (Martens et al. 2003). This gene is presented as a small, tandemly arrayed three-gene family (DFR-A, DFR-B, and DFR-C; Inagaki et al. 1999) in the genus *Ipomoea* or with two functional copies (DFR1 and DFR2; Xie et al. 2004) in *Medicago*. However, we used the dihydroflavonol 4-reductase copy1 (DFR1) copy in the present phylogenetic study.

Materials and methods

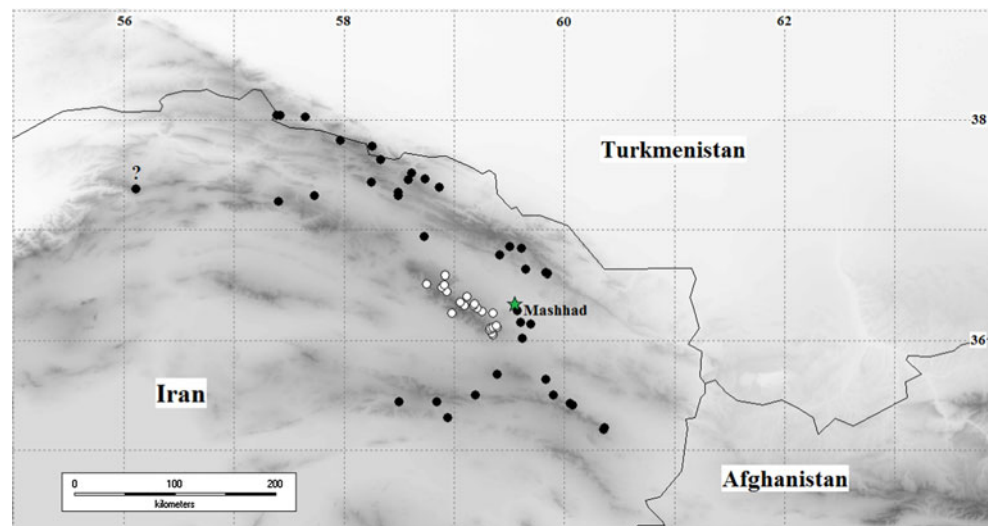
Plant materials

Morphological analyses were carried out with 33 herbarium [Ferdowsi University of Mashhad Herbarium (FUMH)] and field-collected *Dianthus* specimens including 18 and 15 individuals of *D. polylepis* and *D. binaludensis*, respectively. Of these, 3 and 10 specimens (*D. binaludensis* and *D. polylepis*, respectively) were included in the molecular analysis (Table 1). Accessions were chosen to represent the extensive morphological diversity that can be found particularly in distribution range of *D. polylepis*. One individual of *Dianthus crinitus* subsp. *tetralapis* was included as outgroup in the molecular study (Table 1). The specimens of each species included in this work are classified a priori using Flora Iranica (Rechinger 1988). For the molecular analysis, the leaf materials of collected specimens were dried in silica gel and stored at room temperature. Voucher specimens of the samples collected were deposited in FUMH.

Morphological character analysis

Fifty-six morphological characters (including quantitative and qualitative) were measured and/or scored (Table 2). All quantitative measures were made using a ruler with the precision of 1 mm. All qualitative traits were numerically codified as binary or multi-status criteria (from 0 to 9,

Fig. 1 Geographical distribution of *Dianthus polylepis* (*black circles*) and *D. binaludensis* (*white circles*) in Khorassan-Kopetdagh mountain ranges. The localities are based on herbarium records (FUMH) and distribution data in the “Flora Iranica” (Rechinger 1988). The recorded specimen by Rechinger (1988) from Almehr in Golestan National Park (*the question mark*) has not been confirmed by Akhani (1998)



depending on the states of each trait). Univariate analyses were used to determine which characters most effectively discriminated the species. First, quantitative data were tested for normality using the Kolmogorov–Smirnov test. Transformation methods were applied to normalize variables that were not normally distributed. Then, the Mann–Whitney *U* test was used to compare differences between two species. All univariate analyses were implemented using SPSS release 18.0.0 (SPSS Inc., Chicago, USA).

Multivariate analyses were performed on the 25 significantly discriminating characters of the raw matrix. Characters were standardized by dividing the centered traits by their standard deviation. Inter-variable correlations were applied in the analysis due to the presence of two different units of measurements (Legendre and Legendre 1998). Principal component analysis (PCA) was used to ordinate the specimens on the reduced space without a priori knowledge of species identity. The PCA was performed using CANOCO ver. 4.5 (Ter Braak and Smilauer 2002). Canonical discriminant analysis (CDA) was also performed on the morphological data set to determine the percentage of specimens correctly assigned to their a priori species. This analysis was implemented in SPSS release 18.0.0 (SPSS Inc., Chicago, USA).

Molecular study

DNA extraction, primer designing, PCR amplification, sequencing and cloning

Total genomic DNA was extracted following the Doyle and Doyle (1987) CTAB protocol or with a modification (an additional RNAase step) of the Dellaporta method (1983).

For the DFR1 gene, we designed a primer pair by blasting partial mRNA sequences of *Dianthus caryophyllus* L. (GenBank Accession No. AB071787), *D. plumarius*

L. (GenBank Accession No. AF267172), *D. gratianopolitanus* Vill. (GenBank Accession No. AF291097) with similar sequences of the order Caryophyllales followed by alignment with a complete DFR1 sequence of *Arabidopsis thaliana* (L.) Heynh. (GenBank Accession No. NM123645) using BioEdit sequence Alignment Editor (Hall 1999). The alignment was refined manually. Subsequently, two conserved 5′ → 3′ regions between the 2nd and 6th exons were selected as a primer pair: DFR1F (5′CATATC GCCACACCTATGGACTTTG3′) and DFR1R (5′CGTCA GTTCTTCGACGAATTG3′). The primers were designed using the Amplifx program version 1.5.4. (Jullien 2008).

Amplification of the DFR1 region was done in 25- μ l reactions containing 2.5 μ l 10 \times PCR buffer (Fermentas, Lithuania), 1.25 μ l MgCl₂ (25 mM, Fermentas, Lithuania), 0.2 mM of each dNTP, 2 U of Taq polymerase, 100 μ mol/L of each primer, and ca. 200 ng genomic DNA. An initial 5-min denaturation step at 95 °C was followed by 35–37 cycles of denaturation (1 min at 94 °C), annealing at 60 °C for 1 min, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. PCR products were purified according to PEG purification (Joly et al. 2006). Direct sequencing was conducted using Macrogen’s sequencing service (Macrogen Inc., Korea). Sequences were edited using Sequencher (version 4.1, Gene Codes Inc., Ann Arbor, Michigan).

Direct sequences with two or more single nucleotide polymorphisms (SNPs) were cloned using the pGEM-T vector (Promega Corporation, WI, USA) following the manufacturer’s protocol and transformed into competent *Escherichia coli* at 42 °C. The transformed bacteria were screened on a selective and solid LB petri dish media containing suitable antibiotics, X-gal, and IPTG (isopropyl β -D-1-thiogalactopyranoside) at 37 °C overnight. Five to ten positive colonies were arbitrarily chosen and directly amplified via colony PCR using the universal M13 primers

Table 1 Voucher specimens included in the morphological (Mor.) and molecular (Mol.) study of *Dianthus polylepis* complex followed by GenBank accessions of alleles sequenced in the current study

Species	Locality	Latitude, longitude	Voucher no.	Mor.	Mol.	DFR1 GenBank Acc.
<i>D. binaludensis</i>	W. Mashhad, Pivezhan	36.100N, 59.317E	43070	✓	–	–
<i>D. binaludensis</i>	NW. Neyshabur, Bar waterfall	36.515N, 58.751E	34885	✓	–	–
<i>D. binaludensis</i>	S. Chenaran, Fereizi to Binalud summit, Gurghi	36.507N, 58.906E	24116	✓	–	–
<i>D. binaludensis</i>	S. Chenaran, Fereizi, Dahane-Jaji	36.45N, 58.933E	24075	✓	–	–
<i>D. binaludensis</i>	S. Chenaran, between Fereizi and Abghad	36.483N, 58.934E	36481	✓	–	–
<i>D. binaludensis</i>	S. Chenaran, Fereizi, Heyte-Shaban	36.508N, 58.915E	23353	✓	–	–
<i>D. binaludensis</i>	W. Mashhad, Kalate-Zabetian	36.249N, 59.352E	10473	✓	–	–
<i>D. binaludensis</i>	S. Chenaran, Golmakan, Cheshme-Sabz	36.345N, 59.056E	22586	✓	–	–
<i>D. binaludensis</i>	W. Mashhad, kang mountains	36.296N, 59.203E	27624	✓	B1	KC595328/KC595329/KC595330
<i>D. binaludensis</i>	W. Mashhad, Zoshk, Cheshme gholgholi	36.316N, 59.176E	44456	✓	–	–
<i>D. binaludensis</i>	W. Mashhad, Binalud mountain, Kordine	36.267N, 59.255E	36532	✓	–	–
<i>D. binaludensis</i>	S. Mashhad, Moghan mountains	36.107N, 59.341E	33675	✓	B2	KC595324/KC595325/KC595331/KC595332
<i>D. binaludensis</i>	E. Neyshabur, Buzhan mountains	–	3397	✓	–	–
<i>D. binaludensis</i>	S. Chenaran, Mountains of after Dolatabad dam	–	3409	✓	–	–
<i>D. binaludensis</i>	S. Mashhad, Moghan mountains	–	3396	✓	–	–
<i>D. binaludensis</i>	W. Mashhad, Pivezhan	36.100N, 59.317E	43069	–	B3	KC595312/KC595333/KC595334/KC595335
<i>D. polylepis</i>	NW. Kashmar, Ghale-Jugh mountains	35.301N, 58.942E	28888	✓	P8	KC595350/KC595353/KC595354/KC595355
<i>D. polylepis</i>	Ghuchan, Faruj	37.435N, 58.249E	29326	✓	P9	KC595314/KC595315/KC595318/KC595319
<i>D. polylepis</i>	Dargaz, mountains before Chelmir	–	3413	✓	P10	KC595320/KC595321
<i>D. polylepis</i>	Fariman, hills of around Fariman dam	–	3398	✓	–	–
<i>D. polylepis</i>	Dargaz, Tivan	–	3420	✓	P4	KC595313/KC595345/KC595346
<i>D. polylepis</i>	N. Mashhad, mountains of Karde village	–	3410	✓	–	–
<i>D. polylepis</i>	Dargaz, Tandooreh national park	–	44585	–	P5	KC595316/KC595347/KC595348/KC595349
<i>D. polylepis</i>	W. Mashhad, Deh Gheibi	36.148N, 59.691E	44218	✓	P3	KC595317/KC595322/KC595323/KC595342/KC595343/KC595344
<i>D. polylepis</i>	W. Mashhad, Khaje Morad	36.169N, 59.600E	44217	✓	P11	KC595351/KC595356
<i>D. polylepis</i>	N. Mashhad, Dorbadam strait	37.4685N, 58.734E	44428	✓	P12	KC595352/KC595357/KC595358/KC595359
<i>D. polylepis</i>	SE. Ghuchan, between Borselan and Golkharan	36.9393N, 58.724E	44419	✓	–	–

Table 1 continued

Species	Locality	Latitude, longitude	Voucher no.	Mor.	Mol.	DFR1 GenBank Acc.
<i>D. polylepis</i>	N. Mashhad, Balghur	36.837N, 59.613E	27535	✓	P2	KC595336/KC595337/KC595338
<i>D. polylepis</i>	N. Mashhad, Karim Abad strait	36.848N, 59.500E	44210	✓	–	–
<i>D. polylepis</i>	Torbate Jaam, Bardo forest	35.424N, 60.049E	34805	✓	–	–
<i>D. polylepis</i>	Kashmar to Neyshabur	35.442N, 58.496E	20733	✓	–	–
<i>D. polylepis</i>	Fariman, Chartakab	35.500N, 59.897E	11298	✓	–	–
<i>D. polylepis</i>	N. Torbate Heidarie, Khomari pass., gypsy hills	35.501N, 59.189E	39170	✓	P1	KC595339/KC595340/KC595341
<i>D. polylepis</i>	N. Mashhad, Khwor mountains	–	3411	✓	–	–
<i>D. polylepis</i>	W. Torbate Heidarie, S. Roodmajan	–	13572	✓	–	–
<i>D. crinitus</i> subsp. <i>tetralapis</i>	Torbate Jaam; Saleh Abad, Zaloo mountain	35.893N, 60.745E	34589	–	C6	KC595326/KC595327/KC595360/ KC595361/KC595362/KC595363

All voucher specimens are deposited in FUMH

under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. The PCR products were directly purified and sequenced using MacroGen's sequencing service (MacroGen Inc., Korea).

Molecular data analyses

The DFR1 sequences were aligned using Clustal W (Thompson et al. 1994) as implemented in BioEdit sequence Alignment Editor (Hall 1999). To remove redundant intra-individual alleles, the aligned sequences were collapsed using the program COLLAPSE version 1.2 (Posada 2004). We identified two different sets of alleles within the DFR1 aligned sequences. In order to avoid affecting our phylogenetic study by the gene tree/species tree problem probably due to gene duplication (Page and Charleston 1997) and likewise to discriminate between orthology and paralogy, we used split-based methods (e.g., Bryant and Moulton 2002). These methods compute networks based on splits to provide visual representation of reticulate events such as recombination and gene duplication (Huson and Bryant 2006). In addition, we used bootstrap value as a sampling method to confidentially inference duplication event (Zmasek and Eddy 2001). However, the network was constructed based on the NeighbourNet algorithm (Bryant and Moulton 2004) on all DFR1 sequences joined with the bootstrapping splits. This analysis implemented in the SplitsTree 4 program (Huson and Bryant 2006). The network detected two distinct

groups (hereafter DFR1-1 and DFR1-2, Fig. 2) with a bootstrap value of 64 %. The groups are recognized by an indel (insertion and deletion) of seven base pairs as well as three SNPs within 3rd intron of the gene. Therefore, in final phylogenetic inference we analyzed two groups as two separate data sets.

Indel matrix was calculated for both data sets (DFR1-1 and DFR1-2) separately, using the "simple indel coding" approach (Simmons and Ochoterena 2000). The indel matrices were generated automatically by the indel coding tool of Seqstate (Müller 2005).

To determine which substitution models for Bayesian inference best fitted the sequence data, we used MrModel test 2.2 (Nlander 2004) with executable MrModelblock file in PAUP* version 4.10 b (Swofford 2002). For the both data sets, the HKY + I + G substitution model was assigned as the best fitting model considering the Akaike information criterion (AIC). Bayesian MCMC inference was performed for one million and 6,500,000 generations for DFR1-1 and DFR1-2, respectively, with MrBayes version 3.1.2 (Huelssenbeck and Ronquist 2001) using the substitution model mentioned above. We confirmed convergence and burn-in phases by comparing the posterior probabilities of different splits between pairs of identical runs using TRACER version 1.3 (Rambaut and Drummond 2003). Convergence occurred after one million and 6,500,000 generations for DFR1-1 and DFR1-2, respectively. After excluding the 2,500 and 25,000 trees for DFR1-1 and DFR1-2 data sets, respectively, of the burn-in phases, the 50 % majority rule consensus trees were computed. Trees were visualized using TreeView version 1.6.6 (Page 2001).

Table 2 Description and results of univariate analyses of the morphological characters used in the current study

No.	Character	Abbreviation	Mean		Mann–Whitney test (<i>P</i> value)
			<i>D. polylepis</i>	<i>D. binaludensis</i>	
1	Plant height (mm)	PLHT	328.06	272.67	0.027
2	Stem length (mm)	STLT*	283.33	246.67	0.062
3	Stem diameter (mm)	WOST	0.972	1.17	0.033
4	Lower internode length (mm)	LINL	39.86	34.33	0.044
5	Upper internode length (mm)	UINL	39.58	52.17	0.006
6	Length of lower leaf (mm)	LOLL*	17.72	16.07	0.63
7	Width of lower leaf (mm)	WOLL	0.9	1.08	0.036
8	Length of middle leaf (mm)	LOML*	14.72	14.33	0.79
9	Width of middle leaf (mm)	WOML*	0.83	0.88	0.53
10	Length of upper leaf (mm)	LOUL*	8.09	6.18	0.97
11	Width of upper leaf (mm)	WOUL	0.77	1.13	<0.0001
12	Length of lower sheath (mm)	LSLL*	2.76	2.7	0.96
13	Length of middle sheath (mm)	LSML*	1.73	1.8	0.36
14	Length of upper sheath (mm)	LSUL*	1.14	0.98	0.073
15	Bract number (count)	BRNU	7.11	4.33	0.002
16	Length of outermost bract (mm)	LOLB	6.89	5.37	<0.0001
17	Width of outermost bract (mm)	WOLB	1.99	2.41	0.03
18	Length of innermost bract (mm)	LIUB	10.06	6.9	<0.0001
19	Width of innermost bract (mm)	WIUB*	3.25	3.25	0.38
20	Margin thickness of outermost bract (mm)	TOBS	0.27	0.69	<0.0001
21	Inner bract to calyx length ratio (ratio)	LBCR	0.48	0.32	<0.0001
22	Length of calyx (mm)	LTCA*	21.75	19.9	0.086
23	Width of calyx dent (mm)	WDCA*	3.01	3.29	0.057
24	Length of calyx dent (mm)	LCAD*	8.86	8.17	0.126
25	Width of calyx dent (mm)	WCAD*	1.36	1.48	0.215
26	Length of petal without fimbria (mm)	LOPE	2.5	3.3	0.018
27	Width of petal without fimbria (mm)	WOPE*	1.81	1.9	0.442
28	Length of claw (mm)	LOAU*	20.33	19.13	0.259
29	Width of claw (mm)	WOAU*	1.27	1.23	0.708
30	Length of fimbria (mm)	LOFI*	1.63	1.87	0.307
31	Width of fimbria (mm)	WOFI	0.25	0.42	<0.0001
32	Protruded petal from calyx (mm)	PPFC	3.42	5.03	0.005
33	Length of seed (mm)	LOSD*	3.49	3.32	0.441
34	Width of seed (mm)	WOSD	1.29	1.48	0.009
35	Length of anther (mm)	LOAN*	1.42	1.37	0.873
36	Width of anther (mm)	WOAN*	0.55	0.65	0.145
37	Length of stigma (mm)	LOST*	15.89	16.87	0.135
38	Length of ovary (mm)	LOOV	6.65	7.98	0.003
39	Width of ovary (mm)	WOOV	2.04	2.37	0.044
40	Branches of stem (1: single; 2: branched; 3: both)	BOST*	2.67	2.73	0.605
41	Generative branch (1: stem with a flower; 2: stem with two or several flowers)	GEBR*	1.17	1	0.421
42	Inflorescent type (1: single; 2: several)	IFTY*	1.17	1	0.421
43	Thickness of middle leaf veins (1: non-specified; 2: trinerves; 3: both)	TMLV	1.94	1.47	0.018
44	Thickness of middle sheath veins (1: without nerve; 2: trinerves; 3: both)	TMSV*	1.83	1.53	0.145
45	Thickness of lower leaf veins (1: non-specified; 2: trinerves; 3: both)	TLLV*	1.94	1.6	0.093

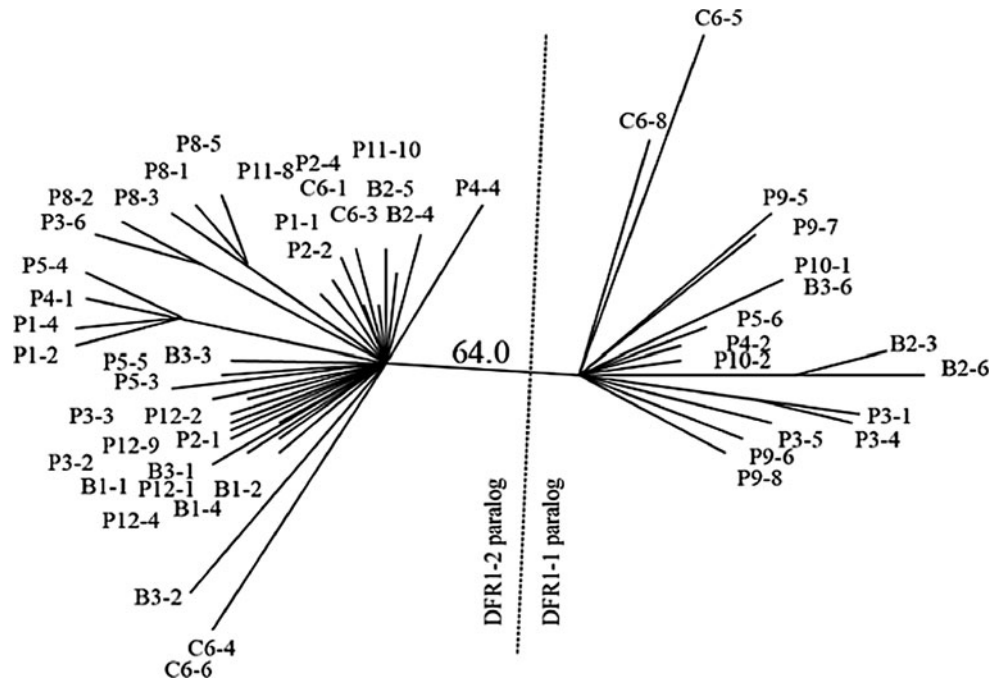
Table 2 continued

No.	Character	Abbreviation	Mean		Mann–Whitney test (<i>P</i> value)
			<i>D. polylepis</i>	<i>D. binaludensis</i>	
46	Thickness of lower sheath veins (1: without nerve; 2: trinerves; 3: both)	TLSV*	1.28	1	0.178
47	Type of sheath of middle leaf (1: membranous-squamous; 2: membranous-herbaceous; 3: both)	TSML*	1.83	2	0.421
48	Type of sheath of lower leaf (1: membranous-squamous; 2: membranous-herbaceous; 3: both)	TSLL*	1	1.13	0.532
49	Shape of upper bract tip (1: mucronate; 2: acuminate; 3: cuspidate; 4: coudate)	SUBT	2	3	<0.0001
50	Shape of lower bract tip (1: acute; 2: acuminate; 3: cuspidate; 4: mucronate)	SLBT	1.61	3	<0.0001
51	Thickness of lines and grooves of bract (1: non-specified; 2: specified in tip; 3: specified along the bract)	TLBG*	1.78	2	0.29
52	Thickness of lines and grooves of calyx (1: non-specified; 2: specified in tip; 3: specified along the bract)	TLCG	2.33	2.93	0.003
53	Shape of dent tip (1: mucronate; 2: acute)	SODT	1.33	2	0.001
54	Fimbria division (1: regular; 2: irregular)	FIDI	1	2	<0.0001
55	Tip shape of petal fimbria (1: acute; 2: rotundate)	TSPF	2	1.2	<0.0001
56	Petal trichomes (1: imberbous; 2: glabrous)	PETR*	1.67	2	0.108

Measured units are specified in the parenthesis. Non-significantly differentiating characters between two species *D. polylepis* and *D. binaludensis* are marked as asterisk

P < 0.05 is used to reject the null hypothesis of non-significantly differentiating characters

Fig. 2 Bootstrapping NeighbourNet network of dihydroflavonol 4-reductase (DFR1) alleles derived from the two species of the present study. Two different types of DFR1 gene indicated by paralog 1 and 2 are separated by an indel of seven base pairs and three single nucleotide polymorphisms. The accessions are the same as those indicated in Table 1. The arrow shows the bootstrap support for splits resulting from separation of the paralogs



Results

Univariate analyses

Results of normality of the quantitative characters using the Kolmogorov–Smirnov test showed that 15 characters were

not normally distributed. Distribution of some of these variables was not normalized following the transformation methods; therefore, the non-parametric Mann–Whitney test was implemented. Results of the statistical Mann–Whitney test showed that 14 of 21 (67 %) vegetative and 17 of 35 (49 %) floral characters cannot significantly discriminate

the individuals included in the current study (Table 2). As a result, these characters were excluded from subsequent analyses. The remaining 25 (44.6 %) characters significantly differentiated the specimens of the two species, *D. polylepis* and *D. binaludensis*. Interestingly, the characters length of calyx (LTCA), branches of stem (BOST), length of fimbria (LOFI), width of calyx (WDCA), and length of calyx dents (LCAD) which are used as discriminating traits between the two species based on Flora Iranica (Rechinger 1988), are not significantly differentiated *D. polylepis* from *D. binaludensis* based on the univariate results (Table 2). Thus, these traits are excluded from subsequent analyses.

Multivariate analyses

The first and second principal component axes (PC1 and PC2) account for 98.7 % of the total variation. The PC1 is the most effective axis in discriminating between the individuals of the species with 95.2 % variation (Fig. 3). The PC1 has the highest loading for plant height (PLHT), bract number (BRNU), width of outermost bract (WOLB), and fimbria division (FIBI), and the PC2 has the highest loading for upper internode length (UINL), length of inner

bract to calyx length ratio (LBCR), and apex shape of upper bract (SUBT). According to the first axis, there is not a boundary to delineate among the individuals of the two species. Moreover, intraspecific variations are greater than the interspecific ones; 9 of 15 accessions of *D. binaludensis* are grouped within the accessions of *D. polylepis*. On the other hand, 9 of 18 accessions of *D. polylepis* are grouped within the accessions of *D. binaludensis*.

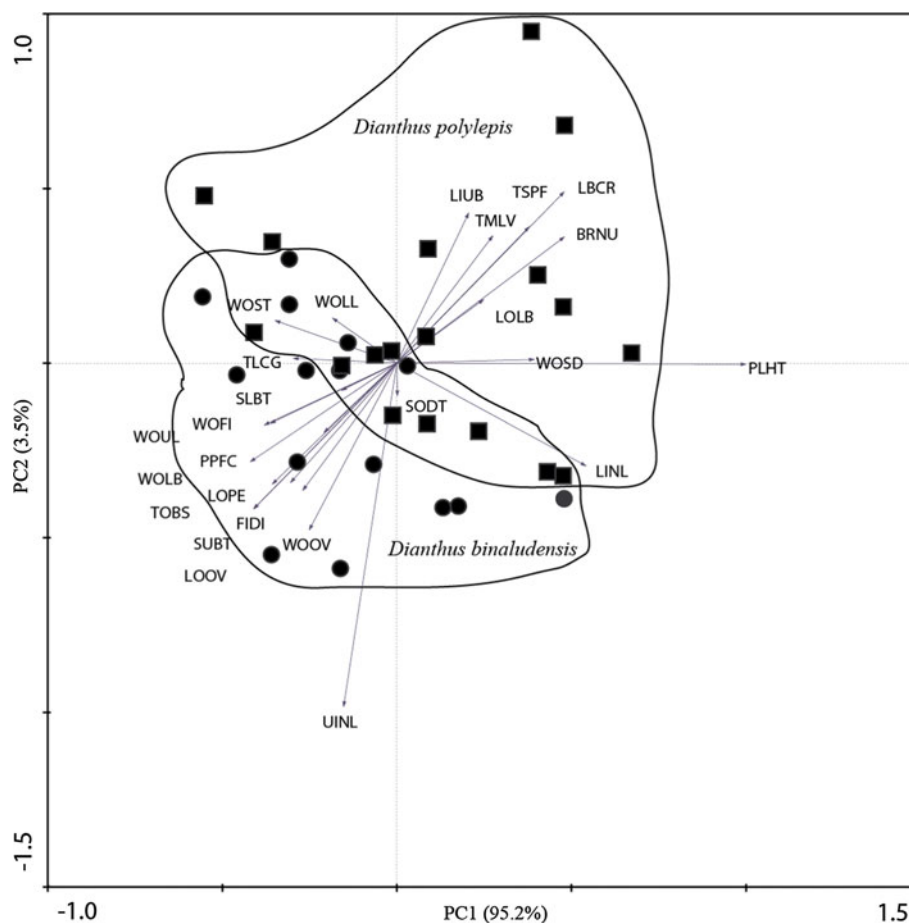
Result of the CDA (Table 3) is consistent with that obtained from the PCA. It shows that for *D. binaludensis*, 6 of 15 (40 %) of the specimens are correctly classified [9 of 15 (60 %) accessions are grouped with *D. polylepis*]. For *D. polylepis*, 9 of 18 (50 %) of individuals are correctly categorized [9 of 18 (50 %) accessions are classified with *D. binaludensis*].

Molecular analyses

DFR1-1 analysis

The DFR1-1 data compiled for our analysis consisted of 16 alleles, after removing seven repeated intra-individual sequences. A total length of 965 nucleotides (including gap characters) of aligned alleles was analyzed. Of the total

Fig. 3 Principal component analysis (PCA) of 25 morphological data comprising 15 and 18 accessions of *D. binaludensis* (circles) and *D. polylepis* (squares), respectively. The arrows represent the character vector, which were scaled to 1 in this analysis. Abbreviations used for each character vector are explained in Table 2



characters in the aligned matrix, statistically, 57 sites were variable (5.9 %), of which 43 (4.5 %) were parsimony informative. After collapsing the sequences, no shared alleles were found among the species. The 50 % majority rule consensus tree (Fig. 4) resulting from the Bayesian analysis show that the assemblage of both species, *D. polylepis* and *D. binaludensis*, form a monophyletic group with a strong support [posterior probability (PP) = 0.96] with respect to the outgroup. All clones from the same species for this orthologous copy (DFR1-1) do not form monophyletic groups. Furthermore, three alleles of *D. binaludensis* are scattered within the clade I (Fig. 4) where the majority of the alleles of *D. polylepis* are present. This clade is a strongly supported sister group (PP = 0.96) to two clades (clades II and III, Fig. 4) comprising two accessions of *D. polylepis*. Relationships between the latter clades, however, are polytomous.

DFR1-2 analysis

The primary alignment of the orthologous DFR1-2 data set comprises 53 sequences with 952 characters excluding coded gaps. In total, 900 (94.5 %) aligned characters were constant, 8 (0.8 %) were variable but parsimony uninformative, and 44 (4.6 %) were parsimony informative. Result

of sequence collapsing showed that five clones of the species *D. binaludensis* were identical and 12 repeated sequences were found within the pooled alleles of *D. polylepis*. The recurrent sequences were removed from the DFR1-2 data matrix. In addition, no identical alleles were found among the species included in the current study. The remaining 36 sequences were used for subsequent analysis. The phylogenetic tree generated using the Bayesian inference is presented in Fig. 5. Two of four alleles of the outgroup nested within the ingroup clade (Fig. 5). The phylogenetic tree (Fig. 5) confirms the results obtained from the DFR1-1 genic region. However, the phylogenetic analysis suggested that neither *D. binaludensis* nor *D. polylepis* is monophyletic. It showed that the species are two closely related lineages.

Discussion

Morphological evidence

Many researchers showed that morphometric method is a powerful tool for assessing morphological relationships amongst closely related taxa (reviewed by Rieseberg and Ellstrand 1993; Otieno et al. 2006; Owen et al. 2006; Seppä

Table 3 Results of an a posteriori classification using canonical discriminant analysis based on 33 accessions and 25 morphological characters

A priori group	N	A posteriori group		Percentage correctly classified
		<i>D. polylepis</i>	<i>D. binaludensis</i>	
<i>D. polylepis</i>	18	9	9	50
<i>D. binaludensis</i>	15	9	6	40
Total	33	18	15	45

Fig. 4 Phylogenetic consensus tree of the DFR1-1 data set resulting from Bayesian analysis using the two species under study and one outgroup species. The number on the branch represents Bayesian posterior probabilities. The Roman numbers (I–III) on the branches are explained in the text. The letters indicated in the parenthesis correspond to the accessions represented in the Table 1

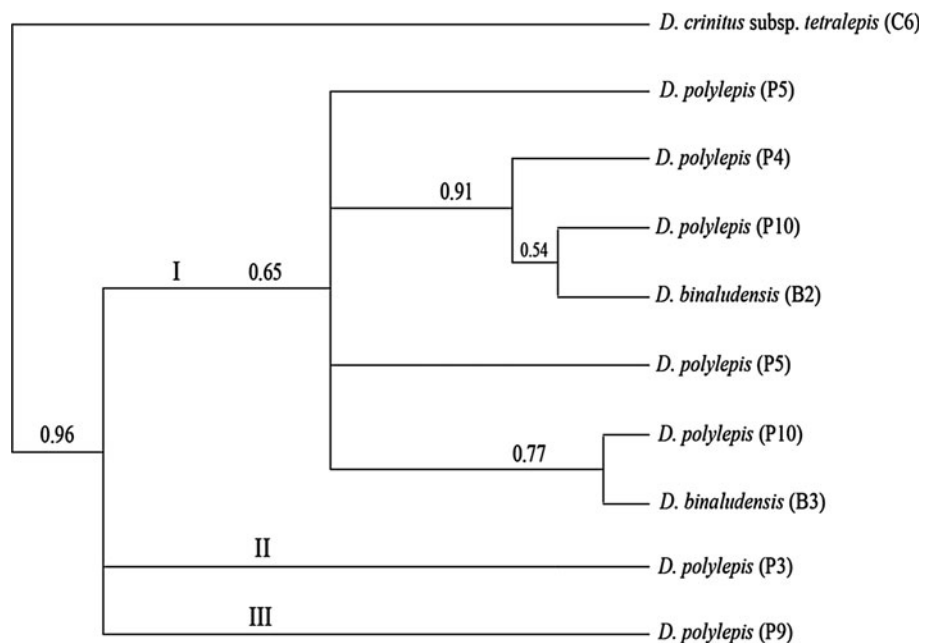
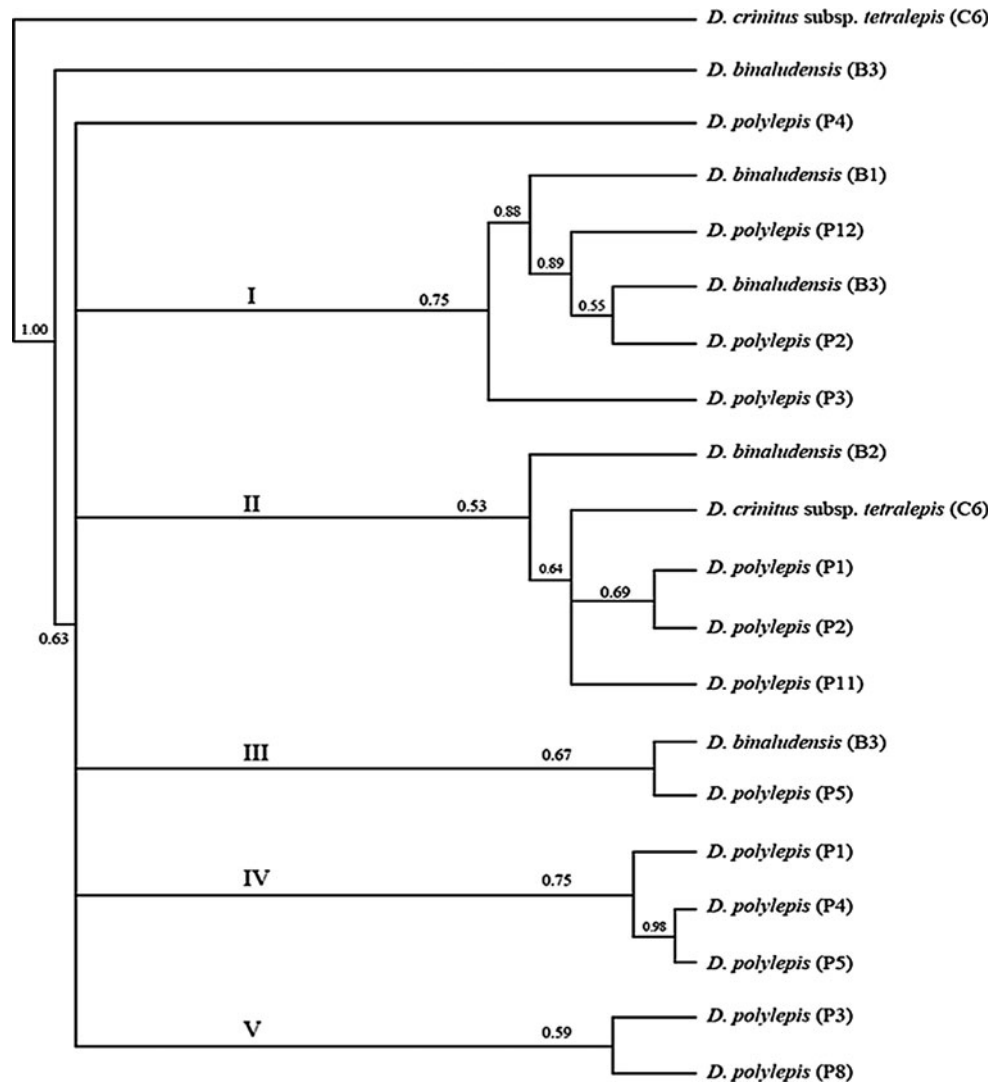


Fig. 5 Majority rule consensus tree resulting from the Bayesian analysis based on DFR1-2 sequence data using the two species under study and one outgroup species. The number on the branch represents Bayesian posterior probabilities. The Roman numbers (I–V) on the branches are explained in the text. The letters indicated in the parenthesis correspond to the accessions represented in the Table 1



et al. 2011). The Mann–Whitney test results reveal that 7 of 21 (33 %) vegetative characters significantly discriminate the two species, *D. polylepis* and *D. binaludensis*, in contrast to 18 of 35 (51 %) generative variables (Table 2). In general, 25 of 56 (44.6 %) both vegetative and floral traits are effective in discriminating the species under study. In contrast to Flora Iranica (Rechinger 1988) which used the characters including length of calyx (LTCA), branches of stem (BOST), length of fimbria (LOFI), width of calyx (WDCA), and length of calyx dents (LCAD) in discriminating the two species, our univariate statistical analysis (Table 2) suggests that these variables do not significantly differentiate the two species. These traits have a continuous morphological variation in the *D. polylepis* complex and it seems that they have no taxonomic significance in separating *D. polylepis* from *D. binaludensis*. Moreover, in Flora Iranica (Rechinger 1988) the corolla color is also used as a character in differentiating the members of the *D. polylepis* complex. According to this flora, the corolla color

is yellow-white in *D. binaludensis* while it is pale in *D. polylepis*. In addition, during our taxon sampling for the present study, we identified various colors even within a population of both species. For this reason, the authors decided not to include this character in the morphometric study.

The multivariate analyses of morphological variation in the *D. polylepis* complex did not support segregation of the complex into two separate species as described in Flora Iranica (Rechinger 1988). Based on the first axis (first principal component) where the greatest amount of variance (95.2 %) exists (Fig. 3), there is no obvious boundary separating *D. polylepis* and *D. binaludensis*. In addition, almost half of the individuals of the *D. polylepis* complex placed in the ordination area overlapping the two species (Fig. 3). Likewise, this evidence is confirmed using the CDA method. Based on this analysis, a posteriori classification indicated that only 40 and 50 % of a priori identified specimens (according to Flora Iranica Rechinger 1988) of

D. binaludensis and *D. polylepis*, respectively, were correctly classified (Table 3). It appears that interspecific boundary is disturbed probably due to increasing morphological variation following a broad taxon sampling in the present work.

The geographic distribution range of *D. binaludensis* is restricted to Binalud Mountains, whereas *D. polylepis* is widely distributed in Khorassan-Kopetdagh mountain ranges outside Binalud range (Fig. 1). Binalud Mountains consist of relatively thick successions of sedimentary, metamorphic, and igneous rock (Sheikholeslami and Kolehpeyma 2012), while Hezar-Masjed and Kopetdagh Mountains are mostly comprised of sedimentary rocks of limestone (Nowrouzi et al. 2007). It seems that *D. polylepis* has been highly successful species and it covers a large area at present. Probably, during expanding its geographical range a few populations of *D. polylepis* with regards to their own ecological preferences may have become geographically and ecologically separated in the Binalud Mountains. Gradually, some morphological features of these radiating populations have been changed resulting in the recognition of *D. binaludensis* (sensu Flora Iranica Rechner 1988). These populations may still be interfertile with the main populations and clearly represent only variants of one widespread species, *D. polylepis*.

Molecular evidence

The DFR1-1-based phylogenetic tree (Fig. 4) confirms the monophyly of the assemblage of the two species under study with a strong support (PP = 0.96) with respect to *Dianthus crinitus* subsp. *tetralepis* as outgroup. Contrastingly, the phylogenetic tree obtained from the DFR1-2 marker showed that two alleles of the outgroup are grouped within the clade II (Fig. 5) where some alleles of the ingroup are present. Clearly, haplotypes shared by recently differentiated species such as *Dianthus* species (Balao et al. 2010; Valente et al. 2010) could occur from the evolutionary processes including interspecific hybridization, incomplete lineage sorting or ancestral polymorphisms that had been present prior to the divergence of a group of species (Olsen and Schaal 1999). Although interspecific hybridization may be possible within the genus *Dianthus* with its known cross pollination (Collin and Shykoff 2003) it seems, however, that the incomplete lineage sorting and/or ancestral polymorphism processes have played prominent roles in the evolutionary history of the recently evolved and rapidly radiating genus (Balao et al. 2010; Valente et al. 2010).

The results obtained from the both phylogenetic trees (Figs. 4, 5) are generally consistent with the patterns of morphological variation. The phylogenetic trees obtained

from the both DFR1 data sets suggested that neither *D. polylepis* nor *D. binaludensis* is monophyletic (Figs. 4, 5). A group of alleles from the both species form a clade (clade I, Fig. 4) in the phylogenetic tree resulted from the DFR1-1 data. Similarly, two clades of the DFR1-2 phylogenetic tree (clades II and III, Fig. 5) show that the alleles of the both species are also grouped together.

From both the morphological and the DNA data, we suggest that *D. polylepis* and *D. binaludensis* have not yet fully diverged. Currently, they grow in different locations and diverse habitats. *Dianthus polylepis* is more common in its distribution ranges from the southern areas of Turkmenistan to the northeast of Iran, but all *D. binaludensis* populations are endemic to the Binalud Mountains in close proximity to *D. polylepis* populations (Fig. 1). We propose that during the evolutionary history of *D. polylepis*, some members of its northern populations (probably Hezar-Masjed populations) extended their range southwards along Binalud Mountains. This scenario is consistent with the somewhat greater haplotype diversity found in *D. polylepis* (Fig. 4, clades II and III; Fig. 5, clades IV and V; independently evolved haplotypes within their populations) and the nesting of *D. binaludensis* haplotypes within a larger clade of *D. polylepis* haplotypes (Figs. 4, 5, clade I). It is likely that the migrated populations of *D. polylepis* have followed diversification of some morphological characters such as bract numbers, length of petals, and width of fimbria after the populations have established and adapted to growing on the igneous rocks of Binalud Mountains.

Conclusion

The results of the current study suggest that *Dianthus binaludensis*, which is distributed on Binalud Mountain, northeast of Iran, displays local morphological divergence. Nevertheless, phenotypic divergence may be associated with substrate specificities and may traditionally be interpreted as a result of environmental variation (Shaw et al. 1994; Bijlsma et al. 2000). The morphometric analysis, however, indicated that no strong discontinuities existed between the two species under study. The morphological and molecular data and analyses obtained from the present study evoked serious concerns about the taxonomic status of *D. polylepis* and *D. binaludensis* as two independent species. In summary, we do not believe that a number of morphological traits (Table 2) are sufficient for taxonomic splitting of *D. binaludensis* from widely distributed *D. polylepis*. Therefore, our taxonomic interpretation of the *D. polylepis* complex is almost in agreement with the taxonomic treatment proposed by Assadi (1985). However, based on our results from the both morphometric and

molecular analyses, the *D. polylepis* complex is interpreted as one species, with two subspecific taxa.

Key to the subspecies of *Dianthus polylepis*

1. Bracts 6–12 (14), lanceolate, acuminate or acute, from 1/3 up to total calyx length. Calyx indistinct costate–striate *Dianthus polylepis* Bienert ex Boiss. subsp. *polylepis*

2. Bracts 4 (6), ovate, cuspidate, up to 1/4 calyx length. Calyx distinct costate – striate *Dianthus polylepis* Bienert ex Boiss. subsp. *binaludensis* (Rech. f.) Vaezi & Behroozian **comb. nov.**

Syn. *D. binaludensis* Rech. f., Pl. Syst. Evol. 142: 242 (1983).

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References

Akhani H (1998) Plant biodiversity of Golestan National Park. *Stapfia* 53:1–411

Al-Saghir MG (2010) Perspective on Chromosome Numbers in the Genus *Pistacia* L. (Anacardiaceae). *Int J Plant Breed Genet* 4:153–157

Assadi M (1985) The genus *Dianthus* L. (Caryophyllaceae) in Iran. *Iran J Bot* 3:9–54

Balao F, Casimiro-Soriguer R, Talavera M, Herrera J, Talavera S (2009) Distribution and diversity of cytotypes in *Dianthus broteri* as evidenced by genome size variations. *Ann Bot* 104:965–973

Balao F, Valente LM, Vargas P, Herrera J, Talavera S (2010) Radiative evolution of polyploid races of the Iberian carnation *Dianthus broteri* (Caryophyllaceae). *New Phytol* 187:542–551

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann Missouri Bot Gard* 82:247–277

Baldwin BG, Cawford DJ, Francisco-Ortega J, Kim S, Sang T, Stuessy T (1998) Molecular phylogenetic insights into the origin and evolution of island plants. In: Soltis DE, Soltis PS, Doyle JJ (eds) *Molecular systematics of plants II*. Kluwer, Boston, pp 410–441

Bijlsma R, Van der Velde M, Van de Zande L, Boerema AC, Van Zanten BO (2000) Molecular markers reveal cryptic species within *Polytrichum commune* (common hair-cap moss). *Plant Biol* 2:408–414

Bittkau C, Comes HP (2009) Molecular inference of a Late Pleistocene diversification shift in *Nigella s. lat.* (Ranunculaceae) resulting from increased speciation in the Aegean archipelago. *J Biogeogr* 36:1346–1360

Bloch D, Werdenberg N, Erhardt A (2006) Pollination crisis in the butterfly-pollinated wild carnation *Dianthus carthusianorum*? *New Phytol* 169:699–706

Bryant D, Moulton V (2002). NeighborNet: an agglomerative method for the construction of planar phylogenetic networks. Algorithms in bioinformatics. In: Guigo R, Gusfield D (eds) *Second international workshop, WABI, Rome, Italy. Lecture Notes in Computer Science*, vol 2452, pp 375–391

Bryant D, Moulton V (2004) Neighbour-Net: an agglomerative method for the construction of phylogenetic networks. *Mol Biol Evol* 21:255–265

Collin CL, Shykoff JA (2003) Outcrossing rates in the Gynomonocious-Gynodioecious species *Dianthus sylvestris* (Caryophyllaceae). *Amer J Bot* 90:579–585

Crespi AL, Bernardos S, Paiva J, Amich F, Fernandes CP, Castro A (2004) An approach to phenotypic analysis and environmental variability. The examples of the genera *Dianthus* L. and *Lotus* L. in the north of Portugal. *Acta Bot Croat* 63:35–48

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version 2. *Plant Mol Biol Rep* 1:19–21

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19:11–15

Duarte JM, Wall PK, Edger PP, Landherr LL, Ma H, Pires JC, Leebens-Mack J, dePamphilis CW (2010) Identification of shared single copy nuclear genes in *Arabidopsis*, *Populus*, *Vitis* and *Oryza* and their phylogenetic utility across various taxonomic levels. *BMC Evol Biol* 10:61

Erhardt A (1990) Pollination of *Dianthus gratianopolitanus* Vill. *Plant Syst Evol* 170:125–132

Erhardt A (1991) Pollination of *Dianthus superbus* L. *Flora* 185: 99–106

Fan C, Purugganan MD, Thomas DT, Wiegmann BM, Xiang QY (2004) Heterogeneous evolution of the *Myc-like* anthocyanin regulatory gene and its phylogenetic utility in *Cornus* L. (Cornaceae). *Mol Phylog Evol* 33:580–594

Fazekas AJ, Kesanakurti PR, Burgess KS, Percy DM, Graham SW, Barrett SCH, Newmaster SG, Hajibabaei M, Husband BC (2009) Are plant species inherently harder to discriminate than animal species using DNA barcoding markers? *Mol Ecol Resour* 9:130–139

Friedman H, Hagiladi A, Resnick N, Barak A, Umiel N (2001) Ethylene-insensitive related phenotypes exist naturally in a genetically variable population of *Dianthus barbatus*. *Theor Appl Genet* 103:282–287

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98

Huelsenbeck JP, Ronquist F (2001) MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17:754–755

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 23:254–267

Inagaki Y, Johzuka-Hisatomi Y, Mori T, Takahashi S, Hayakawa Y, Peyachoknagul S, Ozeki Y, Iida S (1999) Genomic organization of the genes encoding dihydroflavonol 4-reductase for flower pigmentation in the Japanese and common morning glories. *Gene* 226:181–188

Jafari A, Behroozian M (2010) A cytotoxic study on *Dianthus* L. species in north eastern Iran. *Asian J Plant Sci* 9:58–62

Janssens BS, Geuten K, Viaene T, Yuan YM, Song Y, Smets E (2007) Phylogenetic utility of the *AP3/DEF* K-domain and its molecular evolution in *Impatiens* (Balsaminaceae). *Mol Phylog Evol* 43:225–239

Joly S, Starr JR, Lewis WH, Bruneau A (2006) Polyploid and hybrid evolution in roses east of the Rocky Mountains. *Am J Bot* 93:412–425

Jullien N (2008). Amplifx. A tool for seeking and designing new primers. Ver. 1.5.4. <http://www.softpedia.com/get/Other/Home-Education/AmplifX.shtml>

Kadereit J, Griebeler E, Comes H (2004) Quaternary diversification in European alpine plants: pattern and process. *Proc R Soc Lond B* 359:265–274

Legendre P, Legendre L (1998) *Numerical ecology*. Elsevier Press, Amsterdam

Linder H (2008) Plant species radiations: where, when, why? *Proc R Soc B* 363:3097–3105

- Linder HP, Dlamini T, Henning J, Verboom GA (2006) The evolutionary history of *Melianthus* (Melianthaceae). *Amer J Bot* 93:1052–1064
- Lu Z, Cai YM, Qiang Z, Yu ZH, Ren HM (2002) Sequence of the ITS region of nuclear ribosomal DNA (nrDNA) in Xinjiang wild *Dianthus* and its phylogenetic relationship [J]. *Acta Genet Sinica* 29:549–554
- Mandakova T, Munzbergova Z (2006) Distribution and ecology of cytotypes of the *Aster amellus* aggregates in the Czech Republic. *Ann Bot* 98:845–856
- Martens S, Knott J, Seitz CA, Janvari L, Yu SN (2003) Impact of biochemical pre-studies on specific metabolic engineering strategies of flavonoid biosynthesis in plant tissues. *Biochem Eng J* 14:227–235
- Müller K (2005) SeqState: primer design and sequence statistics for phylogenetic DNA data sets. *Appl Bioinformatics* 4:65–69
- Naumann J, Symmank L, Samain MS, Müller KF, Neinhuis C, dePamphilis CW, Wanke S (2011) Chasing the hare—evaluating the phylogenetic utility of a nuclear single copy gene region at and below species level within the species rich group *Peperomia* (Piperaceae). *BMC Evol Biol* 11:357
- Nlander JAA (2004). MrModeltest v2. Program distributed by the author, Evolutionary Biology Centre, Uppsala University. <http://www.abc.se/~nylander/>
- Nowrouzi G, Priestley KF, Ghafory-Ashtiani M, Javan Doloei G, Rham DJ (2007) Crustal velocity structure in Iranian Kopehdagh, from analysis of P-waveform receiver functions. *J Sustain Energy Environ* 8:187–194
- Olsen KO, Schaal BA (1999) Evidence on the origin of cassava: phylogeography of *Manihot esculenta*. *Proc Natl Acad Sci USA* 96:5586–5591
- Otieno DF, Balkwill K, Paton AJ (2006) A multivariate analysis of morphological variation in the *Hemizygia bracteosa* complex (Lamiaceae, Ocimeae). *Plant Syst Evol* 26:19–38
- Otto SP (2007) The evolutionary consequences of polyploidy. *Cell* 131:452–462
- Owen E, Semple JC, Baum BR (2006) A multivariate morphometric analysis of the *Symphytotrichum boreale*—*S. nahanniense*—*S. welshii* complex (Asteraceae: Astereae). *Canad J Bot* 84:1282–1297
- Oxelman B, Liden M, Berglund D (1997) Chloroplast rps16 intron phylogeny of the tribe Sileneae (Caryophyllaceae). *Plant Syst Evol* 206:393–410
- Page DM (2001) TreeView (Win32) Version 1.6.6. <http://taxonomy.zoology.gla.ac.uk/rod/rod.html>
- Page DM, Charleston MA (1997) From gene to organismal phylogeny: reconciled trees and the gene tree/species tree problem. *Mol Phyl Evol* 7:231–240
- Paun O, Forest F, Fay MF, Chase MW (2009) Hybrid speciation in angiosperms: parental divergence drives ploidy. *New Phytol* 182:507–518
- Pillon Y, Fay MF, Hedrén M, Batean RM, Devvey DS, Shipunov AB, Bank MVD, Chase MW (2007) Evolution and temporal diversification of western European polyploid species complexes in *Dactylorhiza* (Orchidaceae). *Taxon* 56:1185–1208
- Posada D (2004) Collapse: describing haplotypes from sequence alignments, Ver.1.2. <http://darwin.uvigo.es/software/collapse.htm>
- Rambaut A, Drummond AJ (2003) Tracer v 1.3. <http://evolve.zoo.ox.ac.uk>
- Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploidy formation in flowering plants. *Annu Rev Ecol Syst* 29:467–501
- Rechinger KH (1983) Acht neue Arten der Gattung *Dianthus* (Caryophyllaceae) aus dem Gebiet der Flora Iranica. *Plant Syst Evol* 142:239–246
- Rechinger KH (1988) Flora Iranica, No. 163. Caryophyllaceae II. Akademische Druck- u. Verlagsanstalt, Graz
- Rieseberg LH, Ellstrand NC (1993) What can morphological and molecular markers tell us about plant hybridization? *Crit Rev Plant Sci* 12:213–241
- Rieseberg LH, Wood TE, Baack EJ (2006) The nature of plant species. *Nature* 440:524–527
- Sang T (2002) Utility of low-copy nuclear gene sequences in plant phylogenetics. *Crit Rev Biochem Mol Biol* 37:121–147
- Seppä P, Helanterä H, Trontti K, Punttila P, Chernenko A, Martin SJ, Sundström L (2011) The many ways to delimit species: hairs, genes and surface chemistry. *Myrmecol News* 15:31–41
- Shaw AJ, Gutkin MS, Bernstein BR (1994) Systematics of the tree mosses (*Climacium*, Musci); genetic and morphological evidence. *Syst Bot* 19:263–272
- Sheikholeslami MR, Kouhpeyma M (2012) Structural analysis and tectonic evolution of the eastern Binalud Mountains, NE Iran. *J Geodyn* 61:23–46
- Simmons MP, Ochoterena H (2000) Gaps as characters in sequence-based phylogenetic analyses. *Syst Biol* 49:369–381
- Small RL, Cronn RC, Wendel JF (2004) Use of nuclear genes for phylogeny reconstruction in plants. *Aust Syst Bot* 17:145–170
- Soltis PS, Soltis DE (2009) The role of hybridization in plant speciation. *Annu Rev Plant Biol* 60:561–588
- Sultan SE (1987) Evolutionary implications of phenotypic plasticity in plants. *Evol Biol* 21:127–178
- Swofford DL (2002). PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer Press, Sunderland, MA
- Syring J, Willyard A, Cronn R, Liston A (2005) Evolutionary relationships among *Pinus* (Pinaceae) subsections inferred from multiple low-copy nuclear loci. *Amer J Bot* 92:2086–2100
- Ter Braak CJF, Smlauer P (2002) CANOCO Reference manual and user's guide to Canoco for Windows: Software for Canonical Community Ordination (version 4). Microcomputer Power, Ithaca
- Thompson JD, Higgins DG, Gibson TJ (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Tu T, Dillon MO, Sun H, Wen J (2008) Phylogeny of *Nolana* (Solanaceae) of the Atacama and Peruvian deserts inferred from sequences of four plastid markers and the nuclear *LEAFY* second intron. *Mol Phylog Evol* 49:561–573
- Valente LM, Savolainen V, Vargas P (2010) Unparalleled rates of species diversification in Europe. *Proc R Soc B* 277:1489–1497
- Volkov RA, Komarova NY, Hemleben V (2007) Ribosomal DNA in plant hybrids: inheritance, rearrangement, expression. *Syst Biodiv* 5:261–276
- West-Eberhard MJ (1989) Phenotypic plasticity and the origins of diversity. *Ann Rev Ecol Syst* 20:249–278
- Whittall JB, Medina-Marino A, Zimmer EA, Hodges SA (2006) Generating single copy nuclear gene data for a recent adaptive radiation. *Mol Phylog Evol* 39:124–134
- Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH (2009) The frequency of polyploid speciation in vascular plants. *Proc Natl Acad Sci USA* 106:13875–13879
- Xie DY, Jackson LA, Cooper JD, Ferreira D, Paiva NL (2004) Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol 4-reductase from *Medicago truncatula*. *Plant Physiol* 134:979–994
- Yan HF, Hao G, Hu CM, Ge XJ (2011) DNA barcoding in closely related species: A case study of *Primula* L. sect. *Proliferae* Pax (Primulaceae) in China. *J Syst Evol* 49:225–236
- Zmasek CE, Eddy SR (2001) ATV: display and manipulation of annotated phylogenetic trees. *Bioinformatics* 17:383–384