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REGULAR PAPER

Iranian *Onobrychis carduchorum* (Fabaceae) populations: morphology, ecology and phylogeography

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Aims – Iran is one of the main centres of diversity for the genus *Onobrychis* Miller. This study includes 55 specimens from eleven representative wild populations of *Onobrychis carduchorum* C.C.Towns. originating from their natural habitats in Iran. The aims of this study are to provide a detailed taxonomical, morphological, genetic and ecogeographical characterization of *O. carduchorum* in Iran.
Methods – The specimens which represent all Iranian populations were biometrically assessed using 45 quantitative and fifteen qualitative morphological characters. The phenotypic variation among the populations depends on various environmental factors; thus at each sample site we recorded data regarding longitude, latitude, altitude, slope inclination, slope orientation, substrate, minimum and maximum annual temperatures, number of rainy days, annual precipitation as well as soil traits including texture, electrical conductivity, organic carbon, total nitrogen, available phosphorus, available potassium, total neutralizing value, pH, and saturation percentage. The floristic composition of each population habitat was examined as an indicator of environmental conditions. To assess genetic variation, we obtained nucleotide sequence data from the internal transcribed spacer of the nuclear ribosomal DNA (ITS) and carried out genomic fingerprints using inter-simple sequence repeat (ISSR) analysis.
Results – Cluster analysis of morphological characters showed that the eleven populations could be divided into two major groups including five subgroups. Principal component analysis (PCA) of floristic data confirmed the two major morphological groups suggesting habitudinal segregation among the groups and the indicative value of floristic composition of study sites in assessing intraspecific variation in the target species. Furthermore, canonical correspondence analysis (CCA) of ecogeographic data showed correlations between morphological variations and ecogeographic factors. Longitude, latitude, substrate, available potassium, clay%, total nitrogen, organic carbon, slope orientation, sand%, texture, altitude and rainy days are apparently the main environmental variables associated with morphological groups of *O. carduchorum*. Both ITS and ISSR data indicate that *O. carduchorum* is a young species with a recent divergence of its populations.

Key words – ecology, ISSR genomic fingerprints, ITS sequence data, morphology, *Onobrychis carduchorum*.

INTRODUCTION

The genus *Onobrychis* Miller (Papilionoideae, Fabaceae) comprises about 130 annual or perennial, mostly caulescent herbs species (Mabberley 2008) and constitutes a major group within the tribe Hedysareae DC (Polhill 1981, Lock 2005). Its distribution ranges from the Mediterranean region to Caucasia, the Zagros Mountains in Iran and Central Asia. Most species are concentrated in south-western Asia, especially Iran and Anatolia, making this area the main centre of genetic diversity of the genus (Yildiz et al. 1999). Rechinger (1984) treated 77 species under nine sections. *Onobrychis* sect. *Onobrychis* – with nearly fifteen species in Iran – is one of the most important sections of the genus.

Onobrychis carduchorum C.C.Towns., which belongs to this section, is a perennial herb with particularly high levels of morphological variation among populations (based on own observations in the field and herbarium specimens) rendering it especially interesting for studying the underlying factors determining this variation. In conjunction with other

taxonomic studies on *Onobrychis* spp. in Iran (e.g. Ranjbar et al. 2004, 2007, 2009a, 2009b, 2010a, 2010b, 2010c, 2010d, 2010e, Toluei et al. 2010, 2012), the present investigation was carried out to provide for the first time a detailed taxonomic, morphological, genetic and ecogeographical characterization of *O. carduchorum* in Iran and to test whether different populations of *O. carduchorum* are differentiated with respect to the corresponding environmental conditions. Because of lack of information about the Iranian range of this species, we here present a description of this species in Iran and compare it to information derived from occurrences of the species in other countries.

Onobrychis carduchorum is a species ranging from Iran and Iraq to Syria and Turkey (fig. 1A) (Rechinger 1984, Davis et al. 1988, Townsend et al. 1984). The most important morphological character for distinguishing *O. carduchorum* from the other species of *Onobrychis* sect. *Onobrychis* is having keels that are conspicuously shorter than the standards. The other species of *Onobrychis* sect. *Onobrychis* in Iran have keels approximately as long as or rarely longer than standards (Rechinger 1984, Davis et al. 1988). As the other species of this section, *O. carduchorum* is an important perennial herb that can be used for high-protein fodder for ruminants and equines, for increasing the nutritive value of drought-resistant pastures due to its nitrogen fixation, and for soil conservation (Abou-El-Enain 2002, Elena 2006). The species is however not cultivated in Iran, so that the risk of introgression from a cultivated gene pool of this species can be considered to be very low.

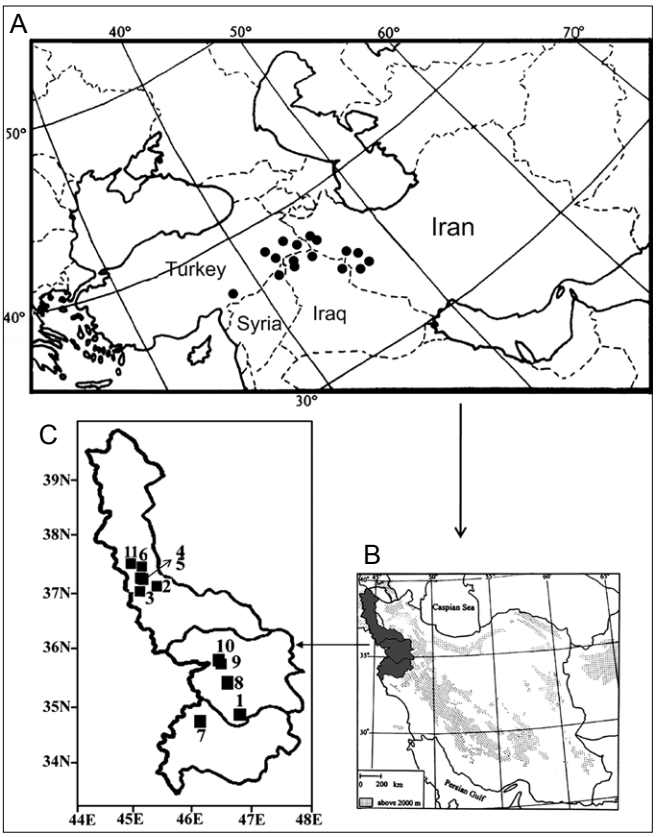

Figure 1 – Distribution map of *O. carduchorum* in Iran, Iraq, Turkey and Syria (A); map of Iran representing estimated range limit of *O. carduchorum* (B) and the location of investigated *O. carduchorum* populations and their numbers (C).

The effect of environmental conditions on morphological variation in plant traits has been studied before (e.g. Ojeda et al. 1998, Fujita et al. 2002, Ellison et al. 2004, Gómez-González et al. 2004, Christensen 2005, Cheng et al. 2010). The cited publications illustrate that morphological variations are frequently associated with environmental conditions and geography. Therefore, it appears that geographically structured morphological variation reflects phenotypic responses to environmental gradients and the evolutionary history of populations. Morphological differentiation may represent an initial stage of the evolutionary process of adapting to environmental conditions. Therefore, elevated morphological variation within and among populations of one species may indicate ecological adaptation. In this paper the effect of environmental conditions on morphological variation of all Iranian populations of *O. carduchorum* was analysed, and compared with the floristic composition of populations and their genetic differentiation.

In the present study the vegetation of particular habitats was examined as indicative of environmental conditions and intraspecific variation. Just as environmental conditions might influence species-specific morphological traits, they may determine composition of the vegetation. Floristic composition in plant communities is highly sensitive to environmental conditions (Braun-Blanquet 1964, Kent & Coker 1992); and thus, in ecological studies, plants are frequently used as environmental indicators (Odland et al. 2006). The knowledge of the floristic composition of an area is a perquisite for any ecological and phytogeographical study and for conservation management activities. In studying any particular piece of vegetation, from an ecological point of view, our first step must be to determine the facts as they exist on the ground: facts regarding the vegetation, on the one hand; facts regarding the habitat, on the other (Nichols 1930).

Any kind of changing floristic composition in different habitats is indicative of different ecological factors, that lead to inter- and intraspecific variation. Floristic composition has only rarely been considered as indicative of different ecogeographical factors (e.g. Fakhre-Tababaei et al. 2000, Fujita et al. 2002, Akhani et al. 2003, Kalvandi et al. 2004, Atri et al. 2009, Naghavi et al. 2009, Toluei et al. 2010, Yavari & Shahgolzari 2012). The results of these investigations suggest that floristic composition may represent a proxy for intraspecific variation.

In this study, the genetic variation of different Iranian populations of *O. carduchorum* was investigated for the first time by means of nucleotide sequence data from the internal transcribed spacer of the nuclear ribosomal DNA (ITS) and by genomic fingerprints established via inter-simple sequence repeat PCR (ISSR). Among the nuclear markers, ITS regions have been especially useful at lower taxonomic levels in many angiosperm groups, including Fabaceae (Wojciechowski et al. 1993, Käss & Wink 1997a, 1997b, Aïnouche & Bayer 1999, Davis et al. 2002). Also ISSR analysis has become a powerful tool to assess genetic diversity among closely related species and to detect similarities between and

Table 1 – ***Onobrychis carduchorum* populations**.
Population numbers, their localities, coordinates, voucher numbers and population size (extension, number of plants) are indicated.

Population number	Province	Locality	Coordinates	Voucher number	Extension (m^2)	Number of plants
1	Kurdistan	Kamyaran to Ravansar	34°47.423'N 46°49.374'E	BASU 20014	12	20
2	Azerbaijan Gharbi	Naqadeh to Oshnavieh, past Jaldian, Jaldian turn, before Oshnavieh and Piranshahr bifurcate	37°03.131'N 45°19.527'E	BASU 20015	4	8
3	Azerbaijan Gharbi	Oshnavieh	37°00.188'N 45°07.029'E	BASU 20016	6	10
4	Azerbaijan Gharbi	Oshnavieh to Orumieh, first turn before Sekani village	37°07.044'N 45°08.872'E	BASU 20017	6	5
5	Azerbaijan Gharbi	Oshnavieh to Orumieh, first turn before Sekani village	37°07.036'N 45°08.904'E	BASU 20018	9	15
6	Azerbaijan Gharbi	Oshnavieh to Orumieh, 3 km to Sekani village	37°12.014'N 45°07.826'E	BASU 20019	9	15
7	Kermanshah	Javanrud to Tazehabad, 20 km to Tazehabad	34°44.868'N 46°16.268'E	BASU 20020	8	12
8	Kurdistan	Sanandaj to Marivan, Shovisheh	35°21.564'N 46°40.891'E	BASU 20021	30	20
9	Kurdistan	Marivan to Saqqez, 65 km past Marivan, between Aqjeh and Qamjian	35°46.943'N 46°25.711'E	BASU 20022	12	15
10	Kurdistan	Marivan to Saqqez, 65 km past Marivan, between Aqjeh and Qamjian	35°46.939'N 46°25.733'E	BASU 20023	15	15
11	Azerbaijan Gharbi	Oshnavieh to Orumieh, 2–3 km to Jarabad village	37°13.423'N 45°01.533'E	BASU 20024	8	10

within species (Zietkiewics et al. 1994, Paäakinskiene et al. 2000, Ghariani et al. 2003, Treutlein et al. 2003, Chennaoui-Kourda et al. 2007).

Specifically, four main objectives are addressed in this paper: (i) to detect the geographic pattern of the population distribution of *O. carduchorum* in Iran and its relationship to environmental gradients defined by several ecogeographic variables; (ii) to study the relationship between variation in plant morphological traits and environmental variables; (iii) to test whether floristic data can be used as a proxy for intraspecific variation and (iv) to assess the genetic variation of different populations and their correlation with other data sets.

MATERIALS AND METHODS

Sample collection

Fifty-five specimens of *O. carduchorum* were collected from eleven populations across Iran during 2008 and 2009 (April to July). These eleven populations represent the only known *O. carduchorum* populations in Iran (based on over ten years of extensive studies of the genus *Onobrychis* in Iran: e.g. Karamian et al. 2010a, 2010b, Ranjbar et al. 2004, 2007, 2009a, 2009b, 2010a, 2010b, 2010c, 2010d, 2010e, 2011, Toluei et al. 2010, 2012). The specimens were prepared according to established herbarium techniques and voucher specimens were deposited in the herbarium of Bu-Ali-Sina University (BASU). Information on collection sites is listed in table 1. Identification of the specimens was performed using the Flora Iranica and other related floras (Rechinger 1984, Davis et al. 1988, Townsend et al. 1984) as well as a revision monograph (Širjaev 1926).

Floristic data

The vegetation composition of the eleven sampled populations was recorded as an indicator of environmental conditions and intraspecific variation. Floristic data were collected using the phytosociological method after Braun-Blanquet (1964). Within each population a representative vegetation patch was chosen; this area was homogenous from the floris-

Table 2 – List of characters and related numerical codes used in morphological studies.
The qualitative characters were encoded according to the multi-state method, and the related means were considered for quantitative characters. The third column is indicative of abbreviations that have been used for the characters in electronic appendix 2.

No.	Characters	Character abbreviation	Unit of measurement
1	Plant height	a	cm
2	Stem length	b	cm
3	Length of stem hair	v1	mm
4	Leaf length	e	cm
5	Petiole length	f	cm
6	Number of leaflets	g	In no.
7	Leaflet length	h	mm
8	Leaflet width	i	mm
9	Hair length of adaxial surface of leaflet	b2	mm
10	Hair length of abaxial surface of leaflet	c2	mm
11	Stipule length	c	mm
12	Stipule width	d	mm
13	Length of stipule hair	y1	mm
14	Inflorescence length	l	cm
15	Peduncle length	m	cm
16	Length of peduncle hair	l2	mm
17	Number of flowers per raceme	n	In no.
18	Bract length	o	mm
19	Bract width	p	mm
20	Length of bract hair	f2	mm
21	Pedicel length	r	mm
22	Length of pedicel hair	g2	mm
23	Calyx length	s	mm
24	Calyx width	u	mm
25	Length of calyx tube	v	mm
26	Length of calyx teeth	t	mm
27	Length of calyx hair	j2	mm
28	Standard length	y	mm
29	Standard width	z	mm
30	Keel length	a1	mm
31	Keel width	b1	mm
32	Keel claw length	c1	mm
33	Wing length	e1	mm
34	Wing width	f1	mm
35	Wing claw length	g1	mm
36	Wing auricle length	h1	mm
37	Pod length	j1	mm
38	Pod width	k1	mm
39	Number of crest spines	q1	mm
40	Spines length	o1	mm
41	Spines width	p1	mm
42	Number of areoles per pod	n1	In no.
43	Length of pod hair	m1	mm
44	Seed length	r1	mm
45	Seed width	s1	mm

No.	Characters	Character abbreviation	Numerical code
46	Leaflet shape	j	1- oblong-elliptic 2- oblanceolate 3- linear-oblong
47	Hair state of adaxial surface of leaflet	z1	1- glabrous 2- sparse 3- loose 4- dense
48	Hair state of abaxial surface of leaflet	a2	1- sparse 2- loose 3- dense
49	Standard shape	w	1- elliptic 2- obovate
50	Standard apex	x	1- rounded 2- acute 3- truncate 4- emarginated 5- retuse
51	State of stem hair (density)	t1	1- sparse 2- loose 3- dense
52	State of stem hair	u1	1- appressed 2- half-upright 3- upright
53	State of peduncle hair	m2	1- glabrous 2- sparse 3- loose 4- dense
54	Stipule shape	w1	1- lanceolate 2- triangular
55	State of stipule hair	x1	1- glabrous 2- ciliate 3- sparse 4- loose
56	State of bract hair	e2	1- glabrous 2- ciliate 3- sparse 4- loose
57	Bracteole shape	i2	1- lanceolate 2- linear 3- lanceolate-linear
58	State of pedicel hair	n2	1- dense 2- loose
59	State of calyx hair	j2	1- tube is sparse and dentate is dense 2- both tube and dentate are sparse
60	State of pod hairs	l1	1- dense 2- loose

tic-ecologic view-point and included individuals of *O. carduchorum*. For these patches both floristic and ecogeographic data were recorded. For statistical analysis, we encoded the floristic composition of each representative patch according to the presence or absence of each species (electronic appendix 1). Floristic data were analyzed with MVSP Vers. 3.2 (Kovach 1985–2002) by the principal component analysis (PCA) method. Distinct floristic composition and ecogeographical factors of *O. carduchorum* habitats were used to define separate populations. Thus the size of each population is based on presence of *O. carduchorum* individuals and stability of floristic composition and ecogeographical variation.

Morphological data

The studied specimens (five in each population) were assessed by biometric data: 45 quantitative and fifteen qualitative morphological characters were chosen and evaluated (table 2). For statistical analysis, we initially encoded the qualitative characters as multi-state data, and for the quantitative characters standardized means were used. The phenetic analysis was carried out with five individuals (a, b, c, d, e) per population using the unweighted pair-group method with arithmetic averages (UPGMA) (Sneath & Sokal 1973) and Percent Similarity coefficients in MVSP Vers. 3.2 (Kovach 1985–2002). The results are illustrated in a phenogram. Morphological variations among and within populations were analyzed using one-way ANOVA. A post hoc follow-up test was applied to determine which means differ from each other; the Tukey (HSD) and Bonferroni follow-up tests were used. The distribution of the morphological diagnostic characters per population is graphically represented via box plots produced with SPSS Ver. 9 software. The box plots provide a simple graphical representation of the distribution of the morphological data and are used to identify the morphological traits characteristic for each identified group. Using the bivariate correlation option in SPSS, Pearson's correlation coefficients (Snedecor & Cohran 1968, Turna 2004) between pairs of morphological characters were computed, and two-tailed tests of significance were applied.

Molecular data

Sequence data of the internal transcribed spacer of nuclear ribosomal DNA (ITS) and ISSR genomic fingerprints were obtained to estimate the genetic variation of different populations. Total genomic DNA was isolated from dried leaf material using the standard CTAB (hexadecyltrimethylammonium bromide) extraction method (Doyle & Doyle 1987) and purified through QIAquick silica columns (Qiagen Inc., Hilden, Germany). In a pilot study, we used five individuals for each random population to analyse both ITS sequences and ISSR fingerprints. ITS sequences were exactly or approximately (double peaks in a few positions) the same in all individuals of the same population. Also in ISSR fingerprints using different ISSR primers intrapopulation variation was negligible. Similar results were obtained for other species of sect. *Onobrychis*. In the final analysis, one or two individuals from each population were analyzed because there was no considerable intrapopulation genetic variation in this species.

Nucleotide sequence data (ITS) – The ITS region was amplified using the ITS4 and ITS5 primers (White et al. 1990). PCR amplifications were performed in 50 µl reaction volumes containing: 0.5–1 µg DNA, 10 pmol of each primer, 1.5 µl dNTPs (10 mM), 5 µl Taq polymerase buffer, 10 mg/ml bovine serum albumin (BSA) and 0.8 units Taq DNA polymerase (Pharmacia Biotech, Freiburg, Germany). PCR amplifications were carried out on a thermal cycler (Biometra, Göttingen, Germany) using the following parameters: initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 90 s and a final extension at 72°C for 5 min. PCR products were precipitated with 4 mol/l NH4Ac and ethanol (1:1:6) followed by centrifugation for 15 min. For each sample the ITS region was sequenced using the ITS4 primer. Sequencing was carried out on an ABI 3730 automated capillary sequencer (Applied Biosystems) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 by STARSEQ GmbH (Mainz, Germany). ITS sequences were aligned with Bioedit Ver. 7.0.5 (Hall 1999) and adjusted manually. Trees were reconstructed with the Neighbor Joining method using MEGA5 (Tamura et al. 2011) and parsimony optimality criterion using PAUP* version 4.0b10 (Swofford 2002). For reconstructing a maximum parsimony tree using PAUP*, the heuristic search option was selected using 100 replications of simple addition sequence and TBR branch-swapping with MulTrees on and steepest descent off. As the aligned ITS matrix included IUPAC nucleotides ambiguity codes for representing heterozygous sites, we inferred haplotype phases using the PHASE algorithm (Stephens et al. 2001, Stephens & Donelly 2003) in DnaSP v 5 (Librado & Rozas 2009). The two inferred sequences for each individual were used for further analysis. A phylogenetic tree was drawn using Tree View (Page 1996). Confidence of each node of the trees was tested by bootstrapping (Felsenstein 1985) with 1000 replicates. The ITS sequences of *Onobrychis viciifolia* (BASU 23172; GenBank JX290360), *Onobrychis bungei* (BASU 23116; GenBank JX290363), *Onobrychis transcaspica* (BASU 19171; GenBank JX290361), *Onobrychis persica* (BASU 23139; GenBank JX290362), *Onobrychis sosnovskyi* (BASU 23156; GenBank JQ780469), *Onobrychis gontsharovii* (BASU 23119; GenBank JQ780471), *and Onobrychis verae* (BASU 23164; GenBank JQ780474) (our unpublished data) were included in the analyses as outgroups.

ISSR fingerprinting – Genomic fingerprints by ISSR-PCR were used to analyze the genetic variation in *O. carduchorum*. PCR was performed in a final volume of 25 µl containing 30–60 ng of genomic DNA, 20 pmol of the primer $(GACA)_4$, 0.1 mmol/l each of dGTP, dCTP and dTTP, 0.045 mmol/l dATP, 0.1 µl (α-^{33}P)-dATP (Amersham Biosciences), 0.6 units of Taq DNA polymerase (Pharmacia Biotech) and 2.5 µl of 10 × amplification buffer (100 mmol Tris-HCl pH 8.5, 500 mmol KCl and 15 mmol $MgCl_2$, 5% Triton × 100). PCR amplifications were performed in a DNA thermal cycler (Biometra, Göttingen, Germany). Initial denaturation was for 5 min at 94°C; followed by 38 cycles of 45 s at 94°C, 60 s at 48°C, 120 s at 72°C, and 10 min at 72°C for final elongation. PCR products were separated by high-resolution vertical polyacrylamide gel electrophoresis for

Table 3 – Ecogeographical variables of *O. carduchorum* populations.
These data were included in canonical correspondence analysis (CCA). The qualitative characters were converted to numerical values.

Ecogeographical factors	Population numbers										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Altitude (m)	1539	1520	1412	1686	1699	1681	1395	1523	1791	1801	1828
Slope inclination	20%	5%	45%	40%	40%	5%	45%	50%	30%	35%	35%
Slope orientation	S	SW	NW	W	W	SW	SW- S	NE- N	NW	NW	W-NW
Maximum temperature (°C)	21.5	17.9	17.6	17.6	17.6	17.6	21.5	21.4	20.6	20.6	17.6
Minimum temperature (°C)	8.1	6.2	5.4	5.4	5.4	5.4	8.1	5.5	5	5	5.4
Rainy days (In no)	79	86.5	90	90	90	90	79	85.7	96	96	90
Annual precipitation (mm)	524.2	672.7	341	341	341	341	524.2	458.4	991.2	991.2	341
Substrate	Soil	Soil	Soil	Soil	Soil and gravel	Soil	Stony	Soil and gravel	Soil and gravel	Soil and gravel	Soil
Texture	SCL	CL	SL	SCL	SL	SCL	C	SCL	SCL	SL	SCL
Clay%	29	34	8	29	16	25	42	23	23	12	26
Sand%	54	36	73	47	68	58	34	51	53	71	49
Silt%	17	30	19	24	16	17	24	26	24	17	25
EC ($\times 10^3$ mmhos/cm)	0.32	0.38	0.261	0.322	0.229	0.307	0.43	0.362	0.291	0.275	0.332
OC%	0.56	2.24	1.13	0.64	1.15	1.48	1.07	2	2.32	0.68	3.2
pH	7.46	7.4	7.29	7.44	7.49	7.46	7.35	7.61	7.49	7.53	7.31
av. K (ppm)	352.7	708.8	303.2	431.8	293.3	590.1	362.6	332.9	342.8	214.1	471.4
av. P (ppm)	5.2	17.4	16.8	14	3.8	20.6	10.2	6.2	18	14.6	14
Total N%	0.05	0.22	0.11	0.06	0.11	0.14	0.1	0.2	0.23	0.06	0.32
SP	39.3	37.9	16.1	38.4	17.1	35	42.3	33.4	34.5	17	36.5
TNV%	8.5	23.5	0.5	4.5	6.5	5	39	15	5.5	0.5	4

3 h at 65 W using a Base Acer Sequencer (Stratagene, La Jolla, San Diego, CA, USA). After drying, the denaturating gels were exposed for 24 h to X-ray film (BioMax MR Film, Kodak, Taufkirchen, Germany). For an estimation of the reproducibility of the ISSR data, DNA from four individuals (randomly selected from different populations) was extracted twice, and the replicated samples were scored independently. Each ISSR band was considered as a character and the presence or absence of the band was scored in binary code (present = 1, absent = 0). A data matrix was assembled and analyzed using PAUP* Ver. 4.0b10 (Swofford 2002) and a pair-wise distance matrix was generated based on total character differences. The genetic relationships among the populations were analyzed by Neighbor Joining method based on distance measures of total character differences. Confidence of each node of the tree was tested by bootstrapping (Felsenstein 1985) with 1000 replicates.

Ecogeographic data

Data obtained from each site included: altitude, longitude, latitude, slope inclination, slope orientation, substrate, min. and max. annual temperature, rainy days per year, mean annual precipitation and soil traits including texture, sand%, clay%, silt%, electrical conductivity (EC), organic carbon (OC%), total nitrogen (Total N%), available phosphorus (av. P%), available potassium (av. K%), total neutralizing value (TNV%), pH and saturation percentage (SP). Soil samples were collected from 0–30 cm depth and measured in the soil laboratory with routine methods of soil analysis. Climatic information for each collecting site was obtained from the Islamic Republic of Iran Meteorological Organization (IRIMO), see table 3. These data were converted to numerical values for canonical correspondence analysis (CCA), a technique for direct gradient analysis. Variables constraining the scores of populations are represented by arrows, the correlations of these variables to CCA axes being proportional to length, direction, and angle of arrows to axis. Ecogeographic data were also analyzed by the PCA method for grouping of different populations based on overall similarity in ecogeographic data. Correlation between pairs of ecogeographical variables was evaluated using Pearson's correlation coefficient.

RESULTS

The geographic and habitat range of *O. carduchorum*

Our extensive survey has shown that within Iran, *O. carduchorum* is only distributed in western Iran, in the Azerbaijan Gharbi, Kurdistan and Kermanshah provinces from 34° to 38° northern latitudes and 45° to 47° eastern longitudes (fig. 1B). Analysing the floristic composition of the collection sites by PCA resulted in two major groups (fig. 2) which are,

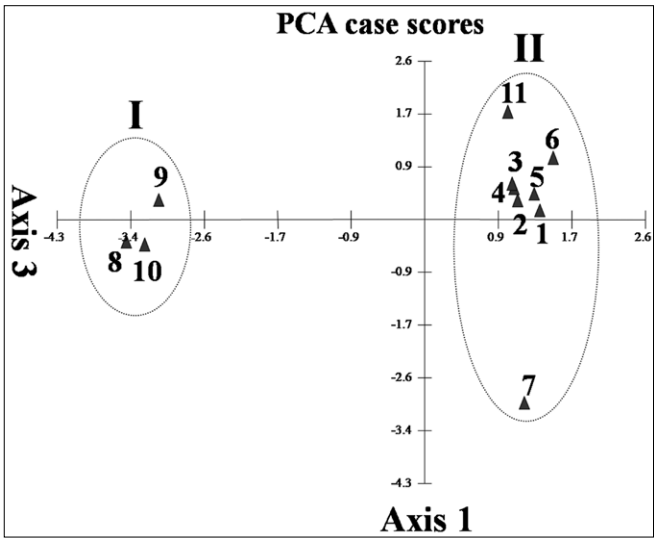

Figure 2 – PCA of floristic data from eleven collection sites of *O. carduchorum* in Iran. Populations are summarized into two groups (I, II) based on overall distance in the diagram.

to some extent, congruent with the geographic position of populations (fig. 1C): Group I consisted of populations 8, 9 and 10 from the centre of the Iranian distribution while group II consisted of populations 2, 3, 4, 5, 6 and 11 from the north-west and populations 1 and 7 from the south. It should be mentioned that population 7 is geographically isolated from all other populations in the south-west of the study area. Although it clusters with group II, its floristic composition is distinct (fig. 2). The floristic composition of this collection site includes *Bunium caroides* (Boiss.) Hausskn. ex Bornm., *Coronilla scorpioides* (L.) W.D.J.Koch, *Hippocrepis unisiliquosa* L., *Lamium amplexicaule* L., and *Trigonella turkmena* Popov as characteristic species that were not found in other collection sites (electronic appendix 1). Populations 8, 9 and 10 are separated from the others because their unique floristic composition includes *Astragalus curvirostris* Boiss., *Astragalus daghdaghabadensis* Maassoumi, *Cerastium dichotomum* L., *Eremopoa persica* (Trin.) Roshev., *Phlomis olivieri* Benth., *Ziziphora capitata* L. and *Zoegea leptaurea* L. as characteristic species (electronic appendix 1).

Morphological variation in *O. carduchorum*

The results of the morphometric analysis based on five individuals (a, b, c, d, e) from each population is represented in an UPGMA phenogram with percent similarity coefficients (fig. 3). All individuals deriving from the same population cluster together. Two groups of populations were detected: populations 8, 9 and 10 forming group I and the other populations group II. There is a further subdivision of group II into five subgroups with similarity level 94.2%: populations 1, 2 and 3 clustered individually (groups C, D and E), populations 8, 9 and 10 formed a separate group (A) and populations 4, 5, 6, 7 and 11 formed another group (B). Note that the two major morphological groups (fig. 3) are in concord-

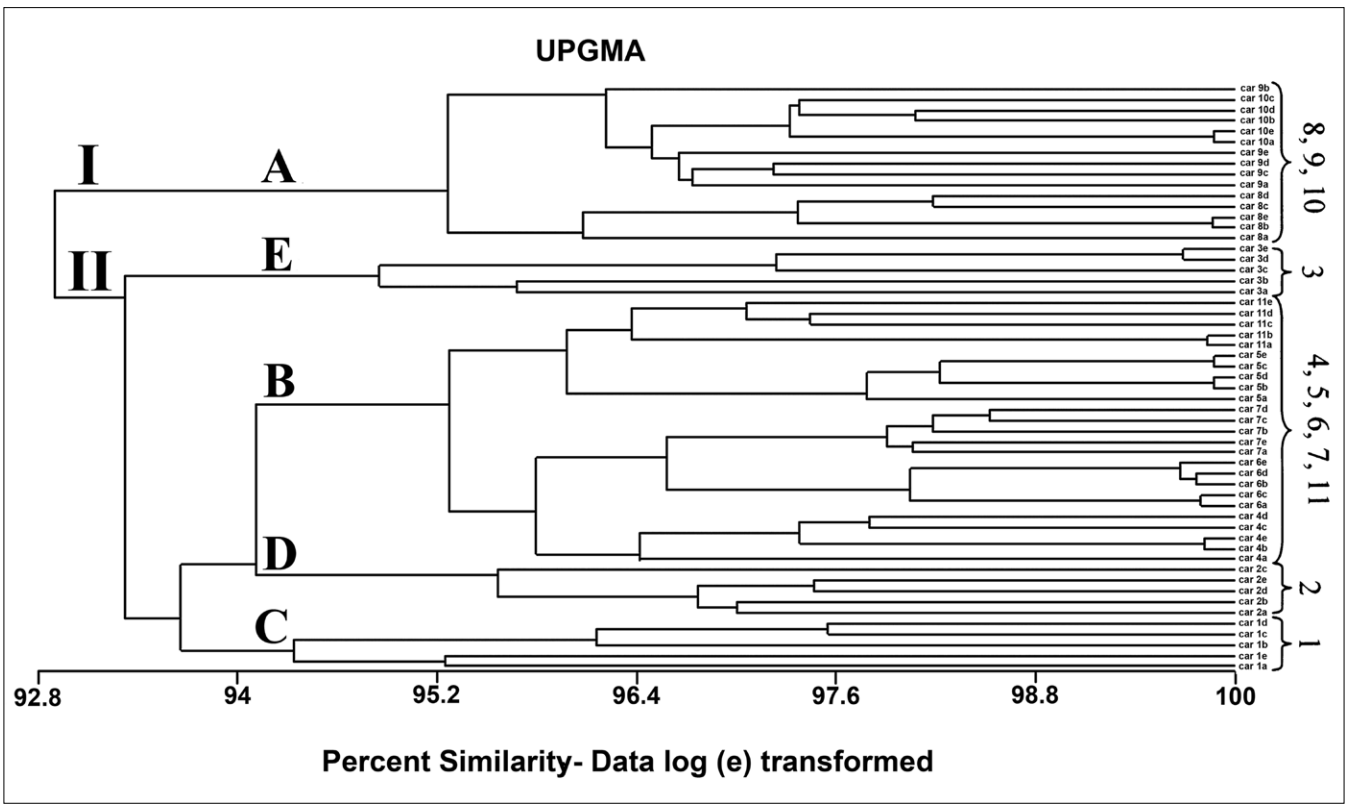

Figure 3 – The UPGMA phenogram based on the analysis of morphological data in five individuals (a, b, c, d, e) per population (1–11) of *O. carduchorum* collected from different ecogeographic conditions (habitats) in Iran. Two main groups (I and II) and five subgroups (A, B, C, D and E) can be distinguished. Individuals from the same population always cluster together.

ance with the subdivision of populations based on floristic data (fig. 2).

Most morphological characters showed significant differences at $\alpha = 0.05$ among populations using one-way ANOVA; the only non-significant characters were wing length, bract width and hair length of adaxial surface of leaflet (electronic appendix 2). Population 1 (Group C) was separated from the other groups by minimum length of wing auricle, with glabrous, ciliate or sparse stipules and bracts; population 2 (Group D) by maximum length and width of pod, maximum number of crest spines; population 3 (Group E) by minimum mean of plant height, minimum mean stem length, minimum mean leaflet length and width, minimum length of inflorescence and peduncle; populations 8, 9 and 10 (Group A) by maximum height of plant, maximum length of stem (electronic appendix 3).

Correlation analyses using Pearson's correlation coefficients revealed that most morphological characters are significantly correlated with probabilities of P = 0.05 or P = 0.01 (electronic appendix 3). Tukey (HSD) and Bonferroni tests after ANOVA showed that five morphological groups (A, B, C, D, E) are significantly different at P = 0.05 for the mentioned diagnostic characters (see electronic appendix 2 for statistical analyses).

Genetic variation in *O. carduchorum*

The aligned ITS data matrix (GenBank accession numbers: JX290341-JX290359, appendix 4) consisted of 482 characters of which 479 characters are constant and three are variable and parsimony-informative. All variable sites presented double peaks (coded with IUPAC ambiguity codes) in some sequences. Neighbor Joining analyses of ITS sequences including ambiguity codes (fig. 4A) showed that all populations of *O. carduchorum* clustered separately from the other *Onobrychis* species (used as outgroups) with high bootstrap support (97%). There is not much genetic structure within *O.*

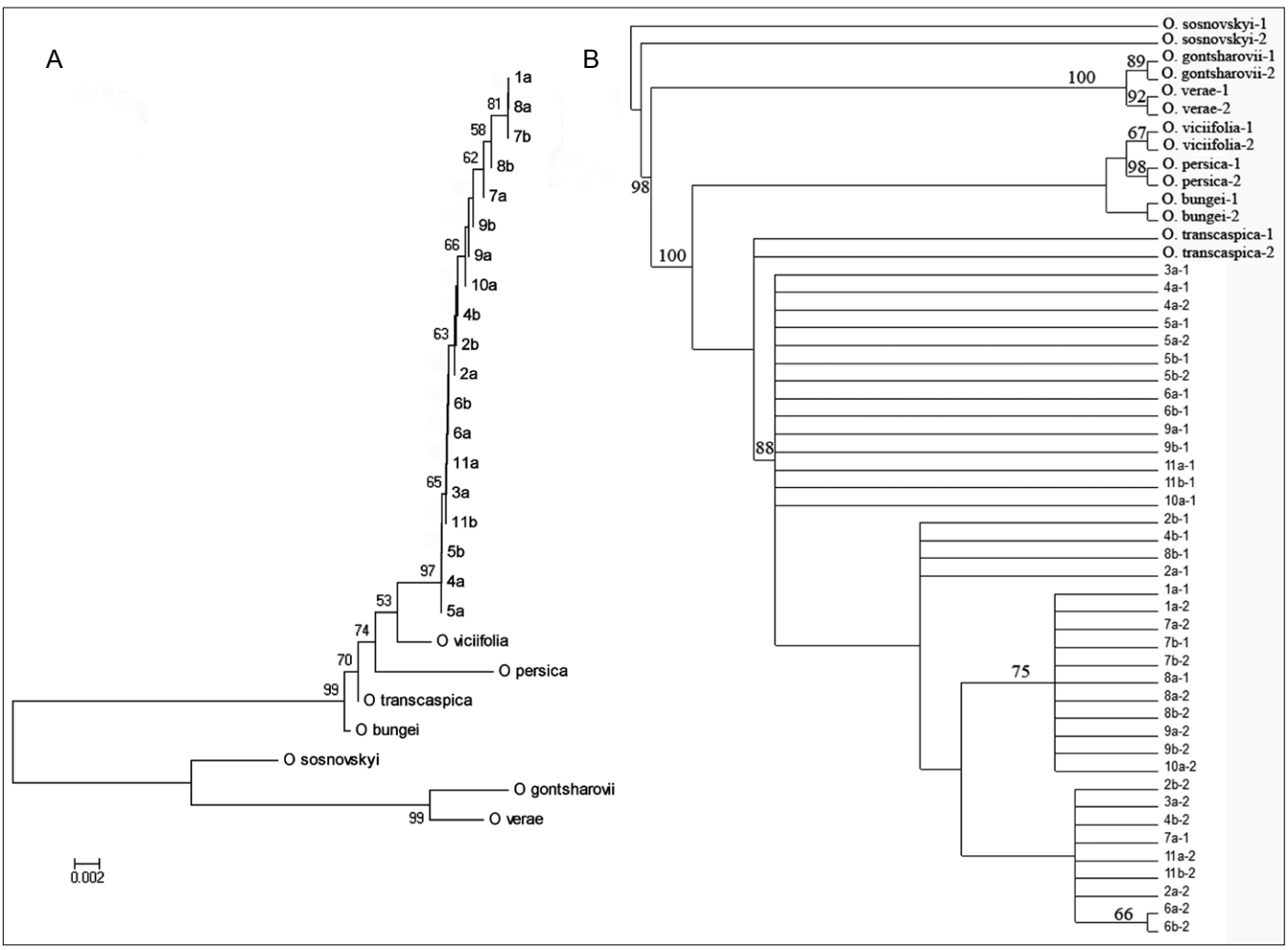

Figure 4 – NJ phylogram (A) and strict consensus tree with parsimony optimality criterion (B) based on ITS sequences data of *O. carduchorum* populations (maximum two individuals a and b from each population). The phased data set (two sequences for each individual) was used for parsimony analysis. The ITS sequences of *O. viciifolia*, *O. bungei*, *O. transcaspica*, *O. persica*, *O. sosnovskyi*, *O. gontsharovii* and *O. verae* were included in the analyses as outgroups. All populations of *O. carduchorum* clustered separately from the other *Onobrychis* species with high bootstrap support value. Populations of *O. carduchorum* are closely related as the trees are poorly resolved. Only populations 1, 7, 8, 9 and 10 (southern populations) form a clade with bootstrap support value of 75. Numbers refer to bootstrap values greater than 50% (from 1000 replications).

carduchorum except that populations 1, 7, 8, 9 and 10 in the south form a separate clade. The strict consensus tree generated by maximum parsimony on phased data (fig. 4B) is in concordance with the NJ tree and presents a bootstrap support value of 75 for the clade including populations 1, 7, 8, 9 and 10 and a bootstrap value of 88 for separating of *O. carduchorum* populations from other species.

The ISSR amplification generated a total of forty distinguishable bands ranging from 247 to 1700 bp in size across the 20 analysed individuals. Within populations the ISSR profiles were identical. Differences were detected between populations: A Neighbour-joining tree (fig. 5) based on distance measures of total character differences shows two main groups, the first one including populations 1, 2, 7, 8, 9, 10, and 11, and the second populations 3, 4 and 5. However, the bootstrap values for supporting these two clades are weak. Population 6 clustered separately with a high bootstrap support of 100%.

Correlation of morphological, floristic and genetic variation

The two major morphological groups (fig. 3) are in concordance with the two groups of populations based on floristic data (fig. 2). However, genetic variation between populations based on both ITS and ISSR data differs from morphological groups: The comparison of the two morphological groups with the two groups based on ITS data (fig. 4A) shows that populations cluster together in both data sets (with exception of populations 1 and 7). The results of ISSR data is indicative of a separation of population 6 from the other populations; this result differs from floristic, morphologic and ITS data. Comparing the clustering of populations across all four data sets (floristic, morphological, ITS, ISSR), only populations 8, 9 and 10 on the one hand and 3, 4 and 5 on the other hand consistently cluster together.

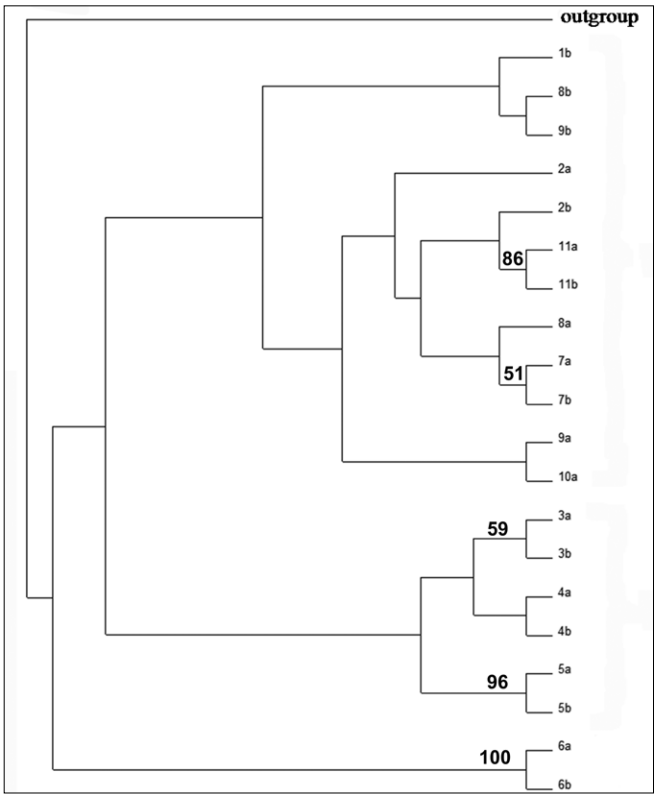

Figure 5 – Neighbour-joining tree generated from ISSR data of *O. carduchorum* populations representing two major groups with weak bootstrap support value (maximum two individuals a and b from each population). This tree is indicative of separation of population 6 from the others. Bootstrap values greater than 50% were shown above the branches (from 1000 replications).

Correlation of morphological and genetic variation with ecogeographic variables

Ecogeographical data – Ecogeographic direct gradient analysis, as shown by CCA (fig. 6) illustrates the relative importance of each environmental variable in the distribution of the populations. Correlation analysis using Pearson's correlation coefficient identified significant correlation between many pairs among the twenty examined ecogeographical variables (electronic appendix 5). CCA axis 1 is positively correlated with av. K, SP and slope orientation and negatively correlated with substrate and slope inclination. CCA axis 2 is negatively correlated with number of rainy days, sand and texture but positively correlated with TNV, EC, clay, substrate, SP, minimum and maximum temperature (electronic appendix 6). Figure 6 shows that TNV, substrate, EC, clay and sand are the most determinant factors for population clustering followed by texture, rainy days, av. K, slope orientation, SP, minimum and maximum temperature being the second-most important factors. Altitude, pH, OC, precipitation and total N are the lowest effective factors for population clustering. The PCA analysis of the populations based on overall similarity of ecogeographic data (fig. 7) showed two groups.

Morphological and ecogeographic data – UPGMA analysis on morphological and PCA analysis on ecogeographical data sets identified each two groups of populations with the same population composition (compare figs 3 & 7). Ecogeographic direct gradient analysis, as shown by CCA, can establish correlations between five morphological subgroups (so between their diagnostic morphological characters) and certain ecogeographic factors: based on fig. 6, total N and OC seemed to be the important effective factor for separation of population 1. Also based on table 1, population 1 occurs at maximum longitude compared to the other populations so longitude can be the most effective factor for separation of population 1. The av. K and slope orientation seems to be the most critical factor for separation of population 2. The minimum percentage of clay, the maximum percentage of sand, texture, altitude and rainy days are the most effective factors for population 3. Substrate appears to be a prominent determining ecogeographic factor for populations 8, 9 and 10. Also these populations exist at similar latitudes (35°N).

Genetic data and ecogeographical data – Furthermore, two main groups resulting from ITS sequence data are also geographically separated but we could not find significant relationship between ISSR data and ecogeographical variables used in this study; only available phosphorus can be detected as the most effective factor for separation of population 6.

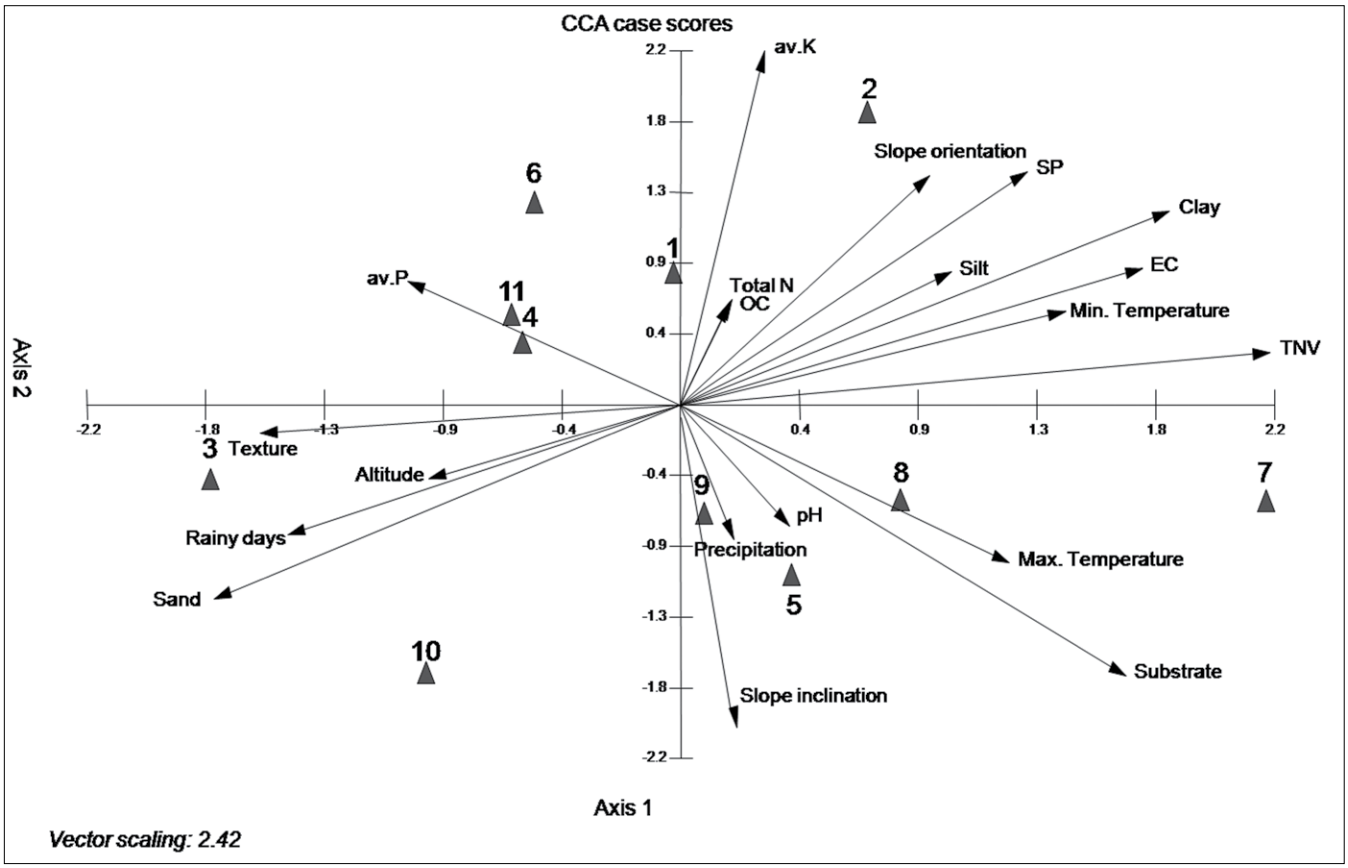

Figure 6 – Canonical correspondence analyses (CCA) for collection sites of *O. carduchorum* populations in Iran based on twenty ecogeographic variables. Information on populations (triangles, numbers 1–11) can be found in table 1. Abbreviations are explained in Materials and methods section.

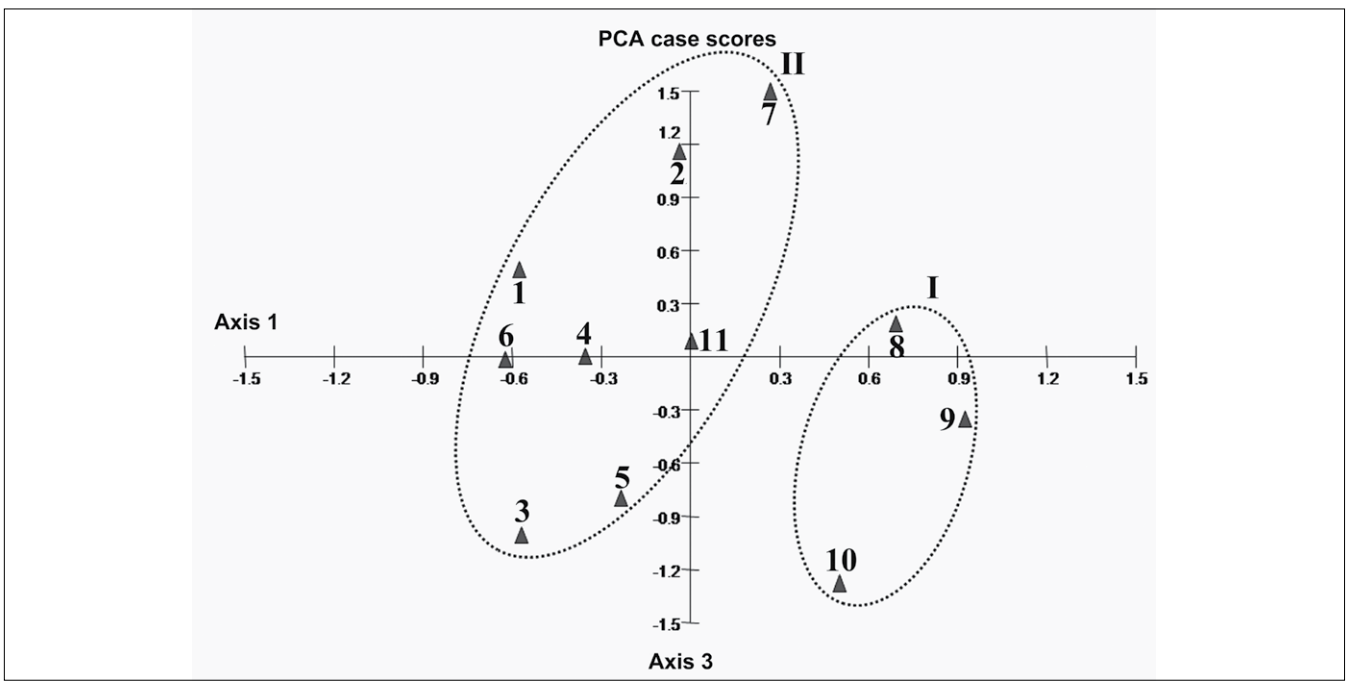

Figure 7 – PCA of eleven *O. carduchorum* populations in Iran based on the twenty ecogeographic data. Populations are summarized into two groups (I, II) based on overall similarity of ecogeographic data.

Table 4 – **Comparison of Iranian *O. carduchorum* populations with other populations.**
The most important differences of morphological characters of Iranian *O. carduchorum* populations with other populations from Iraq, Syria and Turkey. In Flora Iranica, *O. carduchorum* has been recorded for Iraq, Turkey and Syria.

No.	characters	Iranian populations	Flora of Iraq	Flora of Turkey	Flora Iranica
1	Plant height (cm)	20–80	20–40(–50)	30–50	20–40(–50)
2	Stipule length (mm)	1.5–13	4–6	-	4–6
3	Number of leaflets (in pairs)	1–8	4–6	4–6	4–6
4	Leaflet length (mm)	4–43	10 –25	-	10 –25
5	Leaflet width (mm)	1–11	2–7	-	2–7
6	Maximum length of lower petioles (cm)	23	15	-	-
7	Peduncle length (cm)	3–18	7–12	-	-
8	Bract length (mm)	1–3	c. 2	-	2
9	Calyx length (mm)	5–8	5–7	5–7	5–7
10	Standard length (mm)	8–10.5	7–10	7.5–10	7–10
11	Standard width (mm)	5–8.5	5	-	5
12	Keel length (mm)	6–8.5	5–7	5–7	5–7
13	Wing length (mm)	2.2–4	c. 3	2	3
14	Pod length (mm)	6–11	7–9	7–10	7–9
15	Number of crest spines	6–12	c. 8	-	c. 8
16	Spine length (mm)	0.5–4	-	2(–3)	-
17	Seed length (mm)	2.5–6	5–6	-	5–6
18	Seed width (mm)	1.5–4	3.5	-	3.5

Figure 6 shows that population 7 is separated from the others by maximum percent of clay, TNV, maximum temperature, SP and EC, stony substrate and minimum percent of sand. But we did not see any separation of this population in morphological and genetic trees.

PCA and CCA analysis based on floristic and ecogeographical data give the eigenvalues shown in appendix 7.

Ecogeographic circumscription of *O. carduchorum* in Iran – The present study shows that *O. carduchorum* populations exist under the following ecogeographic conditions: soil textures being clay loam (CL), sandy loam (SL), sandy clay loam (SCL) and clay (C), ranging from pH 7.29–7.61, EC 0.229–0.43, OC 0.56–3.2, clay 8–42%, sand 34–73%, silt 16–30%, av. K 214.1–708.8, av. P 3.8–20.6, Total N 0.05–0.32%, SP 16.1–42.3 and TNV 0.5–39%; substrate is soil, stone and a mixture of soil and gravel; slope orientations are 5–50% inclination; lowest altitude 1395 m a.s.l., highest altitude 1828 m a.s.l.; max. annual temperature ranging from 17.6–21.5°C, min. annual temperature from 5–8.1°C; number of rainy days/year 79–96; annual precipitation 341–991.2 mm.

DISCUSSION

Morphological circumscription of the Iranian *O. carduchorum* populations

Onobrychis carduchorum is an Irano-Turanian element. The geographical distribution range of *O. carduchorum* in Iran is limited and the species occurs only in western Iran. Also the north and south geographic range of this species are not connected (fig. 1B & C). There is no record on *O. carduchorum* in Flora Iranica (Rechinger 1984) but it has been recorded from Iran in Flora of Iraq (Townsend et al. 1984) and Flora of Turkey (Davis et al. 1988). There are special descriptions for *O. carduchorum* in Iraq and Turkey. Because a description of Iranian *O. carduchorum* does not exist, we present here a relevant morphological circumscription. This description differs in some morphological characters from populations in Turkey, Syria and Iraq (table 4).

Onobrychis carduchorum C.C.Towns. – Kew Bulletin 21: 446 (Townsend 1968) – Perennial with procumbent or erect stems, 20–80 cm high, many-stemmed; stems green with raised pale lines, ascendance, semi-appressed or appressed-hairy. Stipules lanceolate- or ovate-acuminate, 1.5–13 mm, white, scarious, membranous with brownish midribs, sparingly pilose, free. Leaves 2–38 cm, imparipinnate with 1–8 pairs of leaflets, lower leaves longer than upper, lower petioles to c. 23 cm, the upper leaves with fewer leaflets, petiolate or subsessile; leaflets oblong-elliptic, linear-oblong to oblanceolate, mucronulate, 4–43 × 1–11 mm, those of the basal leaves usually densely silky with silvery apressed hairs on both surfaces at least when young, those of the upper leaves more frequently moderately appressed-hairy on both surfaces or subglabrous above. Inflorescence racemose, many-flowered (10–42), rather dense (± lax), somewhat elongating in fruit, 4–28 cm long; peduncles 3–18 cm, with sparse or dense hairs, exceeding the leaves; bracts membranous, subulate, 1–3 mm, with ciliate and sparse hairs or glabrous. Pedicel 1–2.5 mm long. Calyx 5–8 mm, campanulate, teeth decreasing from the upper pair to the lower, whole calyx especially the teeth and sinuses densely flexuous-hairy, upper teeth c. 1.5–2.5 times as long as the tube; bracteoles filiform to lanceolate, membranous minute. Corolla pale pink, with darker veins, 1.5 times as long as the calyx; stand-

ard broadly elliptic or obovate, 8–10.5 × 5–8.5 mm, glabrous, claw very short; wings very small, 2.2–4 mm, lamina broadly deltoid-oblong, shortly clawed with broad, blunt auricles; keel conspicuously shorter than the standard, 6–8.5 mm, deltoid-oblong, truncate, shortly clawed. Ovary uniovulate. Pod suborbicular, 6–11 mm, ± densely and shortly appressed-hairy, upper suture almost straight, convex lower suture with 6–12 subulate, simple spines; spinose on disk and crest, spines of the latter up to 0.5–4 mm long, often curved; disk foveolate, with 7–12 alveoles. Seeds reniform, 2.5–6 × 1.5–4 mm, brown, smooth, somewhat compressed.

Based on morphological variations, five groups were identified with the Iranian populations (see fig. 3); here we have presented the description of each group based on important and distinguishing features:

Group A (populations 8, 9 and 10): plant height 43–80 cm, stem length 17–61 cm, inflorescence 7.5–28 cm long, peduncle 4–18 cm long, length of leaflet 6–43 mm, width of leaflet 1.5–11 mm, wing auricle 0.4–0.7 mm, without glabrous stipules and bracts, pods 7–9 × 5–7 mm, number of spines 5–10.

Group B (populations 4, 5, 6, 7 and 11): plant height 25–52 cm, stem length 6–43 cm, inflorescence 6–23 cm long, peduncle 3–13.5 cm long, length of leaflet 5–24 mm, width of leaflet 1.5–7 mm, wing auricle 0.5–0.7 mm, without glabrous stipules and bracts, pods 6–9 × 4.5–7.5 mm, number of spines 6–10.

Group C (population 1): plant height 40–57 cm, stem length 14–42 cm, inflorescence 5.5–23 cm long, peduncle 3–15 cm long, length of leaflet 7–21 mm, width of leaflet 2–7 mm, wing auricle 0.3–0.4 mm, with glabrous, ciliate or sparse stipules and bracts, pods 6–9 × 4.5–6.5 mm, number of spines 8–10.

Group D (population 2): plant height 23–60 cm, stem length 11–48 cm, inflorescence 6–22 cm long, peduncle 4–13 cm long, length of leaflet 5–23 mm, width of leaflet 1.5–5 mm, wing auricle 0.5 mm, without glabrous stipules and bracts, pods 8–11 × 6.5–8 mm, number of spines 6–12.

Group E (population 3): plant height 22–42 cm, stem length 6–29 cm, inflorescence 4–13 cm long, peduncle 3–7 cm long, length of leaflet 4–14 mm, width of leaflet 1.2–4.5 mm, wing auricle 0.7–0.9 mm, without glabrous stipules and bracts, pods 6–8 × 4.5–7 mm, number of spines 6–8.

Phylogeography and genetic variation

Chennaoui-Kourda et al. (2007) have investigated genetic diversity of two legume genera *Sulla* and *Hedysarum* which are closely related to *Onobrychis* and in the same tribe (Hedysareae), using ISSR markers. ISSR analysis of Sulla and *Hedysarum* revealed a high level of variability in Mediterranean species suggesting that the ISSR technology is a powerful and efficient approach in genetic diversity analysis of legumes. Moreover, with regard to species discrimination, ISSR markers appeared to be more powerful than ITS sequences. Whereas we found no or very little variation within a population, forty different genotypes were detected between populations as compared to three variable sites in the ITS data set. The clades arising from the ISSR analysis of *O. carduchorum* are not in concordance with morphologic groups nor with any of the ecogeographical variables. ISSR data indicate that population 6 is genetically different but there is no evidence for a separation of this population from morphological data.

Considering that species with restricted ranges and small population sizes are more likely to be self-compatible, as proved for two restricted *Astragalus* species (Karron 1989), and self-compatibility and self-pollination are common in many species of Fabaceae (Arroyo 1981, Gomes da Silva et al. 2011), the near-absence of ISSR variation within *O. carduchorum* populations may suggest that plants reproduce by self-fertilization (autogamy). If so, individuals within populations are genetically closely related and variation is expected to lie essentially among, and not within populations. Restricted gene (pollen) flow between populations can contribute to enhancing morphological differentiation.

Both ITS and ISSR data analysis showed that the populations of *O. carduchorum* are genetically closely related because the genetic distances between clades are very small. The low gene diversity across various populations may be the result of a common gene pool. Hence, based on our results, the observed morphological variation within *O. carduchorum* is probably linked to environmental variables or alternatively, the morphological characters have already fixed but that the populations are genetically very young.

Environmental correlations

Furthermore our results showed that two main groups based on overall similarity of ecogeographic data (fig. 7) are in concordance with two floristic and morphological groups. The floristic composition of each environment is in close correlation with a combination of ecological factors, representing the best criteria for the diagnosis of its ecological factors (Guinochet 1973). Thus, in ecological studies, floristic compositions can be used as environmental indicators and indicative of intraspecific variation. Our study confirms prominent ecogeographical patterns in the different variations of wild populations and provides continuing evidence that floristic classification is still a useful and efficient tool for distinguishing inherent variations among natural populations. Additionally, other taxonomic markers (morphological, molecular, chemical etc.) and complementary techniques are necessary for identifying and evaluating different levels of variation, as indicated by various other studies (e.g. Naghavi et al. 2009, Karamian et al. 2010a, Toluei et al. 2010). CCA analysis was used for determining the effect of different ecogeographic data on population distribution. TNV, substrate, EC, clay and sand were the main factors associated with the distribution of *O. carduchorum*, while morphological groups are determined mostly by longitude, latitude, substrate, av. K, clay, total N, OC, slope orientation, sand, texture, altitude and rainy days.

Morphological variation may not only mirror plastic responses to a varying environment but also entail some degree of adaptive genetic differentiation among populations. Based on ITS data, a divergence of *O. carduchorum* from its

sister species is apparent. Regarding the intraspecific variation between different populations of *O. carduchorum* ITS sequence data showed two main groups (fig. 4A) – these two groups were also geographically separated (fig. 1C). Thus two apparent ecotypes for this species can be distinguished: a high-latitude ecotype (populations 2, 3, 4, 5, 6 and 11) and a low-latitude ecotype (populations 1, 7, 8, 9 and 10). But reciprocal transplantation is necessary to determine if local adaptation is present. Since the morphological differences between the two geographically isolated groups are not sufficiently distinct to be unequivocally recognized in the field or in the herbarium the two ecotypes are not considered as distinct subspecies.

As already mentioned, the risk of introgression from cultivated *O. carduchorum* can be considered to be low. Another concern could be the effect of grazing on population variation. Herbivores can affect the structure of plant communities in space and in time. As a consequence plant can respond to herbivory in varied ways. Heavy grazing by domestic livestock reduces the quality and the quantity of both food and cover. Consequently, plant populations and communities are affected, in both the ecological and evolutionary sense (Milchunas et al. 1988, Perevolotsky & Seligman 1998). However, none of the populations included in this study were influenced by grazing herbivores.

Finally, intra-specific variation in plants is often regarded as crucial to adaptive mechanisms in response to different environments (Mal & Doust 2005). *Onobrychis carduchorum* has a wide distribution range in western Iran and populations differ in their morphological traits. Based on different studies (e.g. Richards et al. 2005, Cheng et al. 2010) species with a greater morphological variation are believed to be better adapted to the environment than species that have less morphological variation.

As other studies have also pointed out (e.g. Yang 1991, Odland et al. 2006), studies of plant populations in different geographic areas, which involve sampling of floristic, morphologic, genetic and environmental data, may give valuable information about the ecological demands of the actual species, its responses to different environmental conditions, and can help us to understand the determining factors of plant adaptation and evolution.

SUPPLEMENTARY DATA

Supplementary data are available at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data),and consists of the following: (1) floristic composition of *O. carduchorum* collection sites (pdf format); (2) statistical analyses of morphological data including the results of correlation analyses using Pearson's correlation coefficients, one-way ANOVA and Tukey (HSD) and Bonferroni tests after ANOVA (text format); (3) box plots of diagnostic morphological characters of *O. carduchorum* populations that resulted from analysis with SPSS software (pdf format); (4) aligned ITS data matrix including GenBank accession numbers (fas format); (5) Correlation analysis of ecogeographical variables using Pearson's correlation coefficient (text format); (6) interset correlations for twenty ecogeographical variables of collection sites of *O. carduchorum* populations accounted for the first two axes of the canonical correspondence analysis (CCA) (pdf format); (7) eigenvalues, percentage and cumulative percentage variance of populations data accounted for by the first three axes of the principal components analysis (PCA) based on floristic data (1 = floristic), the canonical correspondence analysis (CCA) of population distribution and twenty environmental variables (2 = ecogeographic) and the PCA based on twenty environmental variables (3 = ecogeographic) for the *O. carduchorum* populations in Iran (pdf format).

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