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Genetic variability of *Orobanche aegyptiaca* infesting tobacco in Iran by Bayesian analysis

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Abstract: Broomrapes (*Orobanche* L.) are holoparasitic plants, parasitizing roots of a wide range of host plants. In this study, genetic polymorphism among 44 *Orobanche aegyptiaca* Pers. individuals collected from different regions of northwest Iran was investigated using inter-simple sequence repeat (ISSR) markers. Two hundred-sixty one discernible bands were amplified using 20 ISSR primers which 245 (94%) was polymorphic, indicating considerable genetic variation among the examined individuals. The number of polymorphic bands per primer ranged from 4 to 17, averaging 12.25. UPGMA clustering using Jaccard's similarity coefficients revealed six main groups. Genetic similarity coefficients varied from 0.71 (between individuals 23 and 27) to 0.34 (between 13 and 30). A model-based Bayesian approach subdivided 38 out of 44 broomrape genotypes into 2 genetic groups and the remaining ones were categorized as mixed genotypes based on Q values. According to an analysis of molecular variance, 99% of the total variation was partitioned within genetic groups. The results demonstrated the potential usefulness of ISSR markers for determination of genetic variation in *O. aegyptiaca*. Understanding the variability in broomrape is important when attempting to develop resistant host crops against this parasite.

Key words: Broomrapes; genetic polymorphism; inter-simple sequence repeat marker; Iran

Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; ISSR, inter-simple sequence repeat; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeats; UPGMA, unweighted pair group method with arithmetic average.

Introduction

The genus *Orobanche*, commonly known as broomrape, is the largest among the holoparasitic members of Orobanchaceae, and contains over 200 achlorophyllous species which parasitize the roots of various host plants (Parker & Riches 1993; Schneeweiss et al. 2004). This genus is divided into four sections: *Gymnocaulis* Nutt., *Myzorrhiza* (Phil.) Beck, *Trionychon* Wallr., and *Orobanche* (syn. *Osproleon* Wallr.) (Greuter et al. 2000). The most important species (from an agronomic perspective) are found in the sections *Trionychon* and *Orobanche*. Section *Trionychon* includes species such as *O. ramosa* L. and *O. aegyptiaca* (Paran et al. 1997). Individual species of the genus *Orobanche* are difficult to distinguish morphologically, which has led to interest in molecular taxonomic differentiation (Román et al. 2001; Benharrat et al. 2002; Buschmann et al. 2005). Schneeweiss et al. (2004) in view of karyological variability recorded three basic sets of chromosomes, $x = 19$, $x = 12$ and $x = 24$, in the genus *Orobanche*. The first basic set has been found in the members of the section *Orobanche*, the second in the sections *Myzorrhiza* and *Trionychon*, and the third in the section *Gymnocaulis*.

Orobanche spp. grows exclusively in semi-arid and temperate regions of the northern hemisphere such as central and southern Europe, northern Africa and Asia (Uhlich et al. 1955; Buschmann et al. 2005). Species of *Orobanche* have however been introduced by humans into other regions such as southwestern Australia, South and North America and South Africa. (Buschmann et al. 2005). In Iran, 39 species of the *Orobanche* genus have been identified, of which nine taxa are reported to be Iranian endemics (Schiman-Czeika 1964). Most species of Orobanche are found in western and northwestern Iran; some species such as *O. aegyptiaca* and *O. cernua* Loefl. are widely distributed across the country (Saeidi Mehrvarz et al. 2010).

Large-flowered branched-broomrape (*O. aegyptiaca*) is one of the most important parasitic weeds in Iran (Hasannejad et al. 2006; Rumsey & Jury 1991). Flowering period of *O. aegyptiaca* is July to September (Rumsey & Jury 1991). It's flowering stem is 15–20 cm,

usually branched and slender. Stigma lobes are white or bluish (Rumsey & Jury 1991). It is an outcrosser and yet can be a form of facultative autogamy if pollen vectors are absent (Pieterse et al. 1991). It has $2n = 24$ chromosomes (Rumsey & Jury 1991). *O. aegyptiaca* infest crops in the *Solanaceae* and *Cucurbiaceae* families. It is also the most serious holoparasite on tobacco, with yield losses up to 100% having been recorded (Ashrafi et al. 2008). This is of special concern to Northwestern Iran, one of the most favorable regions for oriental tobacco cultivation (Darvishzadeh et al. 2010).

Controlling broomrape by cultural practices or by applying herbicides is extremely difficult, because the holoparasite is directly connected to host plants, is predominantly subterranean, and produces numerous, durable, and small seeds (Buschmann et al. 2005). Given such a scenario, the development of *Orobanche*-resistant varieties is a more efficient way for limiting the effects of this parasitic on crops (Rubiales 2003). *Orobanche* can evolve to overcome host resistance over time. Host-parasite coevolution is reciprocal natural selection on host resistance and parasite infectivity (Thompson 1994). Hence, agronomic breeding programs ought to consider both parasite and host plant genetic diversity (Buschmann et al. 2005).

Earlier studies used biochemical and morphological features to describe and distinguish *Orobanche* populations (Verkleij et al. 1986; Benharrat et al. 2000). However, morphological and biochemical markers are insufficient when applied to holoparasites because variations in these two sets of characteristics are not only reduced compared to most plant species, but also exhibit environmental plasticity. In such a situation, molecular markers are advantageous in assessing genetic diversity over time and space (Duwick 1984). Genetic variation among populations of *Orobanche gracilis* Sm. taxa (var. *gracilis* and var. *deludens* (Beck) A. Pujadas) from northern and southern Spain, infesting different host plant species, was analyzed by RAPD markers (Román et al. 2007). There was a higher level of diversity in the northern populations compared to southern ones (Román et al. 2007). The genetic diversity of *O. cumana* populations from Spain and eastern Europe was investigated by using RAPD markers. They were clearly differentiated by geographic origin and fell into four distinct groups (Pineda-Martos 2009).

ISSR markers have been shown to be particularly useful in genetic fingerprinting and diversity analysis (Godwin et al. 1997; Aghaei et al. 2012). ISSR is an ideal method for fingerprinting and a useful alternative to single-locus or hybridization-based methods because large numbers of DNA fragments are amplified per reaction, representing multiple loci from across the genome (Román et al. 2002; Aghaei et al. 2012). Closely related species can be differentiated, as well as individuals within populations of a single species, with ISSR-PCR (Hristova et al. 2011).

Genetic studies mainly on weedy species of Orobanche, i.e. *O. aegyptiaca* have been limited (Satovic et al. 2009). Detecting genetic differences among *Orobanche* species using ISSR markers demonstrate that ISSR markers can discriminate *O. hederae* Duby from *O. amethystea* Thuill. (Benharrat et al. 2002). In contrast, RFLP and rbcL gene sequence (the nucleotide sequence of plastid genes encoding ribosomal 16S rRNAs applied in phylogenetic studies) were not successful in discriminating *O. hederae* from *O. amethystea* (Benharrat et al. 2000). In another study, there was no variation within *O. ramosa* populations infecting tobacco cultivars in Europe based on ISSR markers (Buschmann et al. 2005). In Bulgaria, Hristova et al. (2011) reported the efficiency of ISSR markers in distinguishing the known sections and genera of *Orobanche*. There is no informative study on the genetic diversity and structure of *O. aegyptiaca* populations infesting tobacco in Northwest Iran, despite the significant damage the parasite inflicts on the region's tobacco crop.

The objective of the present work was to document the genetic variability of *O. aegyptiaca* from northwest of Iran based on ISSR markers using Bayesian analysis.

Material and methods

Plant materials

Forty four individuals of *O. aegyptiaca* naturally infecting cultivated tobacco (*Nicotiana tabaccum* L.) in Northwest Iran were used in this study (Table 1, Fig. 1). DNA was extracted using the CTAB method described by Doyle & Doyle (1987). Fresh inflorescence tissue sampled from each individual (0.2 g) was ground in liquid nitrogen by using a pre-cooled mortar and pestle and transferred to 2.0 mL sterile tubes. Preheated CTAB buffer (800 µL, 2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% (v/v) 2-mercaptoethanol) and 1.3 µL of RNAase (10.0 g L^{-1}) were added to ground tissue in each tube. Tubes were incubated in a water bath at 65 ℃ for 15 min. Subsequently 400 µl of chloroform and isoamylalcohol (24:1 v/v) were added to the tubes. Tubes were mixed well by gentle inversion, incubated on ice for 10 min and centrifuged at 12,000 rpm for 15 min at 4 ℃. The supernatant was transferred to new tubes and DNA was precipitated by adding equal volume of chilled isopropanol plus 0.1 volume ammonium acetate solutions. The tubes with precipitant were placed in a freezer at –20 ℃ for 60 min. After centrifugation at 10,000 rpm for 10 min at 4 ℃, the precipitated pellets were washed with 70% ethanol. Finally the pellets were dried, dissolved in TE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0), and stored at –20 ℃. The concentration of each DNA sample was determined spectrophotometrically at 260 nm (BioPhotometer 6131; Eppendorf, Hamburg, Germany). The quality of the DNA was checked by running 2 µL DNA in 1% (w/v) agarose gels in 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0). Damaged DNA samples were excluded from study.

ISSR analysis

Out of 34 ISSR primers tested (Vancouver, British Columbia, Canada), 20 were found to be polymorphic among individuals and produced clear bands (Table 2). DNA amplification was performed in a final volume of 20 µL containing 30 ng of template DNA, 2 µL 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 µM dNTPs,

Table 1. Description of *Orobanche aegyptiaca* individuals.

[a]No.	Collecting site	Q-matrix S1	Q-matrix S2	[b]Genetic group	Collector (Latitude, Longitude, Altitude)
1	Barbin 1	0.109	0.891	Green	
2	Barbin 2	0.981	0.019	Red	
3	Barbin 3	0.25	0.75	Mix	
4	Barbin 4	0.117	0.883	Green	
5	Barbin 5	0.994	0.006	Red	
6	Urmia A0	0.995	0.005	Red	
7	Urmia A1	0.736	0.264	Mix	
8	Urmia A4	0.985	0.015	Red	
9	Urmia A5	0.051	0.949	Green	
10	Urmia A6	0.017	0.983	Green	Urmia Tobacco Research Center (37°33′09″ N, 45°04′33″ E, 1362m)
11	Diylakh-Marangalou	0.016	0.984	Green	
12	Gharagozlou	0.076	0.924	Green	
13	Tappeh Torkaman	0.006	0.994	Green	
14	Marangalou	0.975	0.025	Red	
15	Shortulu	0.993	0.007	Red	
16	Nayyerlou	0.088	0.912	Green	
17	Torkaman	0.229	0.771	Mix	
18	Salim Abad	0.985	0.015	Red	
19	Sari-Baglou	0.004	0.996	Green	
20	Khoy 1-2	0.207	0.793	Mix	Khoy Jahad-e-Keshavarzi Organization (38°33′01″ N, 44°57′07″ E, 1139m)
21	Khoy 38-3	0.021	0.979	Green	
22	Abdollah-Hachi23	0.988	0.012	Red	
23	Abdollah-Hachi 26	0.987	0.013	Red	
24	Abdollah-Hachi 29	0.993	0.007	Red	
25	Abdollah-Hachi 30	0.99	0.01	Red	Bukan Jahad-e-Keshavarzi Organization (36°31′15″ N, 46°12′32″ E, 1372m)
26	Abdollah-Hachi 34	0.994	0.006	Red	
27	Abdollah-Hachi38	0.988	0.012	Red	
28	Ghazanasar	0.847	0.153	Red	
29	Oshnaviyeh3	0.97	0.03	Red	Oshnaviyeh Jahad-e-Keshavarzi Organization (37°02′22″ N, 45°05′53″ E, 1450m)
30	Piranshahr2-2	0.968	0.032	Red	Piranshahr Jahad-e-Keshavarzi Organization (36°42′03″ N, 45°08′28″ E, 1572m)
31	Marivan2	0.846	0.154	Red	
32	Marivan3	0.951	0.049	Red	
33	Marivan 4	0.017	0.983	Green	
34	Marivan 5	0.919	0.081	Red	
35	Marivan 6	0.4	0.6	Mix	
36	Marivan 9	0.989	0.011	Red	
37	Marivan 13	0.11	0.89	Green	Marivan Jahad-e-Keshavarzi Organization (35°31′37″ N, 46°10′34″ E, 1495m)
38	Marivan 14	0.068	0.932	Green	
39	Kalbala 26	0.087	0.913	Green	
40	Kalbala 29	0.016	0.984	Green	
41	Kalpain 7	0.643	0.357	Mix	
42	Kalpain 15	0.056	0.944	Green	
43	Kalpain 17	0.081	0.919	Green	
44	Balak	0.991	0.009	Red	

[a]Individuals number which used in Fig. 3; [b]Two distinguished genetic groups including Green and Red that illustrated in Fig. 3

10 pM primer, 0.5 Unit of Taq DNA polymerase and appropriate volume of double-distilled water. PCR reactions was carried out in a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany) programmed for an initial step of 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 45 s at 40–60 °C (depending on primers sequence) and 2 min at 72 °C, and 10 min at 72 °C as a final step. PCR products were mixed with an equal volume of formamide dyes (98% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and resolved in a 1.6% (w/v) agarose gel in 0.5× TBE buffer, stained with ethidium bromide (1.0 μg mL^{-1}) and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA). DNA amplification by the "A12" primer was repeated two times.

Data analysis

A binary data matrix was constructed by scoring amplified bands as present (1) or absent (0) for each sampled *O. aegyptiaca* individual. Jaccard (Jaccard 1908), Dice (Nei & Li 1979) and Simple Matching (Sneath & Sokal 1973) similarity coefficients calculated on the binary data matrix were input to agglomerative clustering algorithms for constructing dendrograms. The efficiency of clustering algorithms and their goodness of fit were measured using cophenetic correlation coefficient. Moreover, principal coordinate analysis (PCoA; Kovach 1999) was used to confirm the results of the cluster analysis. Data analyses were implemented in NTSYS-pc version 2.02e software (Rohlf 1998).

In order to reveal genetically homogeneous groups in studied *O. aegyptica*, Bayesian approach implemented in

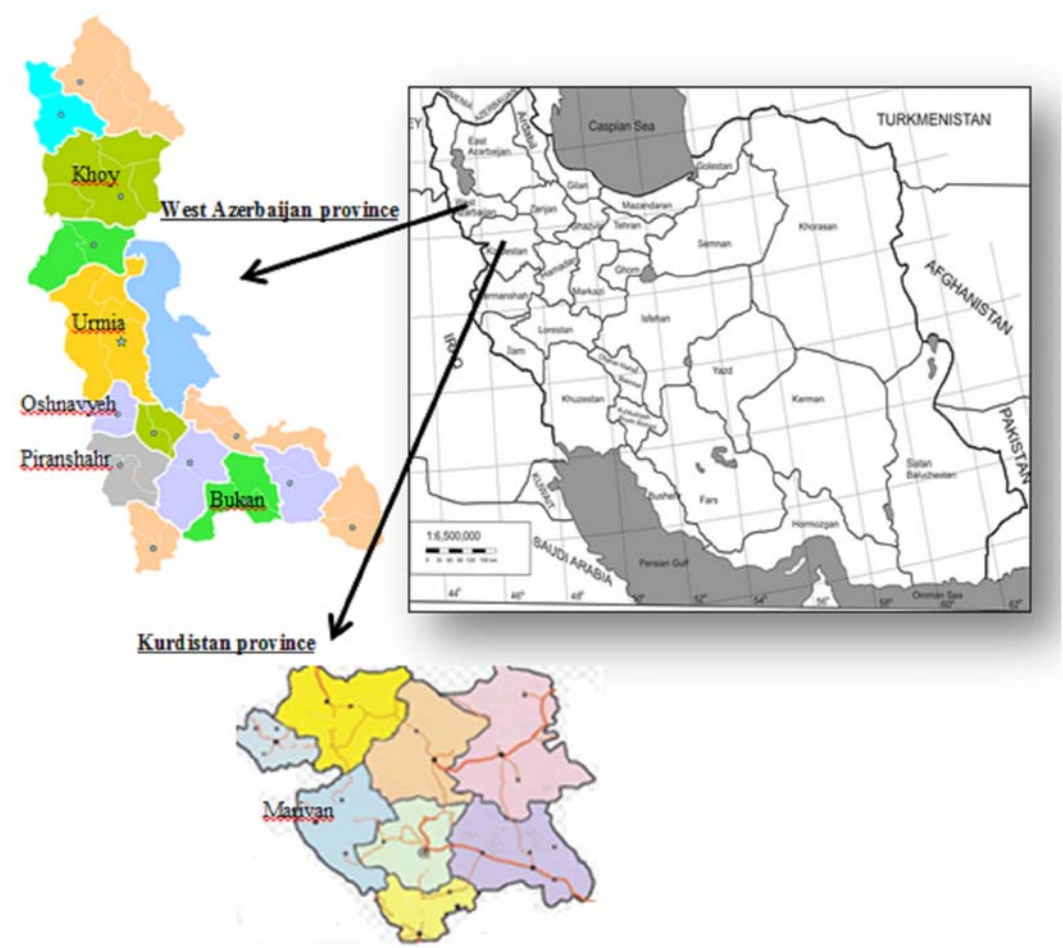

Fig. 1. Collecting regions of *Orobanche aegyptiaca* individuals from northwest Iran.

Structure 2.3.4 (Pritchard et al. 2000) was employed. This clustering method is based on the allocation of individual genotypes to K clusters in such a way that Hardy–Weinberg equilibrium and linkage equilibrium are valid within clusters, whereas these kinds of equilibrium are absent between clusters. Five independent runs were performed, setting the number of sub populations (K) from 1 to 10, burn in time and Markov Chain Monte Carlo (MCMC) replication number both to 100,000 and a model for admixture and correlated allele frequencies. The appropriate number of K was identified based on the posterior probability of the data for a given k (Evanno et al. 2005). Inferred ancestry estimates of individuals (Q-matrix) were derived for the selected genetic groups (Pritchard et al. 2000). An individual was discretely assigned to a subpopulation when more than 80% of its genome composition came from that subpopulation (Courtois et al. 2013). AMOVA (Excoffier et al. 1992) was performed using GenAlEx 6.4 software (Peakall & Smouse 2006) to estimate the variance within and among genetic subpopulations. Admixed individuals were divided to groups based on their composition. If for an individual the majority of its composition was red it was assigned to group red.

Results and discussion

Quantifying genetic diversity

Genetic diversity of 44 *O. aegyptiaca* individuals was investigated by using 20 ISSR primers (Table 2). The primers generated 261 clear bands, of which 245 (94%) were polymorphic. DNA amplification was repeated with the "A12" primer twice on all individuals and good reproducibility (higher than 95%) was observed in the produced banding patterns. This was consistent with what Buschmann et al. (2005) and Hristova et al. (2011) observed concerning the reproducibility of ISSR markers. The total number of bands per ISSR primer ranged from 5 [primer (AG)8T] to 19 [primer (AC)8G], with an average of 13.1 (Table 2). The number of polymorphic bands per primer ranged from 4 to 17, averaging 12.25 (Table 2). Amplified fragments ranged in size from 200 to 1500 bp.

A model-based Bayesian approach assigned 38 out of 44 *O. aegyptiaca* genotypes into two genetic groups and the remaining ones were categorized as mixed based on their Q values (Table 1, Figs 2 and 3). Genotypes from Bukan were totally allocated to Red genetic group (Table 1, Fig. 3). Marivan and Urmia genotypes were allocated to Green, Red and Mixed genetic groups (Table 1, Fig. 3). Hence, it was not possible to distinguish exact origin-related of identified genetic groups. However since this report is concerned to parasites growing on crops, in addition to their ability to disperse pollen and/or seeds, these results may arise from human acts that are involved on its distribution through the transport of commercial seeds (Román et al. 2007).

Table 2. Primers, amplification conditions and polymorph ISSR markers used on 44 *Orobanche aegyptiaca* individuals.

Primer name	Sequence(5'→3')	Annealing temperature(°C)	Total number of bands	Polymorphic bands	Percentage of polymorphic bands
UBC807	$(AG)_8T$	44	5	4	80
UBC808	$(AG)_8C$	46	14	11	78.6
UBC810	$(GA)_8T$	43	12	10	83.3
UBC811	(GA)8C	51	14	14	100
UBC812	$(GA)_8A$	49	15	13	86.7
UBC816	$(CA)_8T$	50	14	14	100
UBC818	$(CA)_8G$	45	12	10	83.3
UBC825	$(AC)_8T$	56	11	10	90.9
UBC827	(AC)8G	47	19	17	89.4
UBC834	(AG)8YT	40	16	16	100
UBC854	(TC)8RG	35	9	9	100
UBC857	$(AC)_8YG$	49	13	13	41.7
UBC864	(ATG)6	51	14	14	100
UBC880	(G(GA)2)3	42	16	16	100
A7	$(AG)_{10}T$	49	11	10	90.9
A12	(GA)6CC	30	9	9	100
A13	$(GT)_6CC$	52	14	12	85.7
CA&AC	(CA)6AC	41	14	14	100
CA6Rg	(CA)6RG	48	16	16	100
CAg5	$(CAG)_5$	60	13	13	100
Total	–	–	261	245	93.9
Mean	–	–	13.1	12.3	91.1

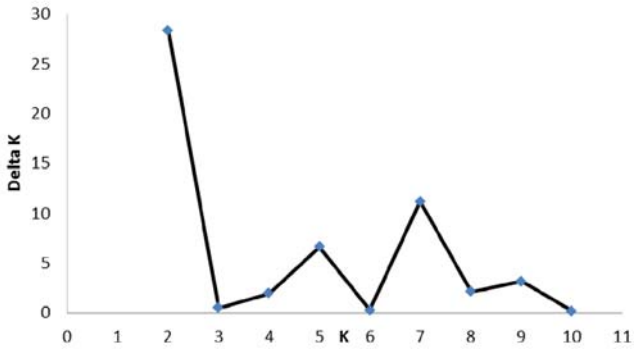

Fig. 2. Bilateral charts to determine the optimal number of K.

Table 3. Comparison of different methods for constructing similarity matrices and dendrograms.

Similarity matrices	Algorithm	Co-phenetic coefficient[a]
Jaccard	UPGMA	$r = 0.714$
	Complete linkage	$r = 0.513$
	Single linkage	$r = 0.631$
Dice	UPGMA	$r = 0.694$
	Complete linkage	$r = 0.612$
	Single linkage	$r = 0.485$
Simple Matching	UPGMA	$r = 0.669$
	Complete linkage	$r = 0.597$
	Single linkage	$r = 0.488$

[a]A measure of how successful cluster analysis has been in partitioning the data. Dic (Nei; Li, 1979); Jaccard (Jaccard, 1908); Simple Matching (Sneath and Sokal, 1973). UPGMA: Unweighted pair-group method using arithmetic average.

The AMOVA analyses revealed that 1% of the total variation was attributed to the variability among the genetic groups whereas 99% was accumulated within the genetic groups. Studies on population genetics in broomrape species have clearly revealed that out-crossing species i.e. *O. aegyptiaca* (Pieterse et al. 1991; Brault et al. 2007) tend to have higher within-population genetic diversity than between populations (Satovic et al. 2009). According to Satovic et al. (2009), one can infer the mating system by using AMOVA on dominant marker data. The level of differentiation among genetic groups (1%) suggests an allogamous mating system. Our results are similar to those of Román et al. (2002) on six *O. crenata* populations using ISSR markers, showing an among-population diversity of only 5%. The out-crossing behavior of *O. crenata* was verified by analyses carried out by Verkleij et al. (1991), using isozymes. *O. gracilis* var. *gracilis* populations in northern Spain show a high level of genetic diversity (approximately 80%) within populations (Román et al. 2007). Vaz-Patto et al. (2008) studied *O. foetida* populations with AFLP markers and reported that 13.7% of the genetic variability was among populations and 86.3% of the genetic variability was within populations. Variability and gene flow in *Orobanche* species attacking crops is probably related not only to their mating systems, but also reflect human intervention in distributing species from one region to another (Román et al. 2007).

Quantifying genetic relationships

Among the different similarity indices and clustering methods tested, the highest co-phenetic correlation coefficient ($r = 0.714$) was achieved with the UPGMA method based on Jaccard's similarity coefficient (Table 3). Therefore, Jaccard's similarity coefficient was used as the basis for depicting the genetic diversity of

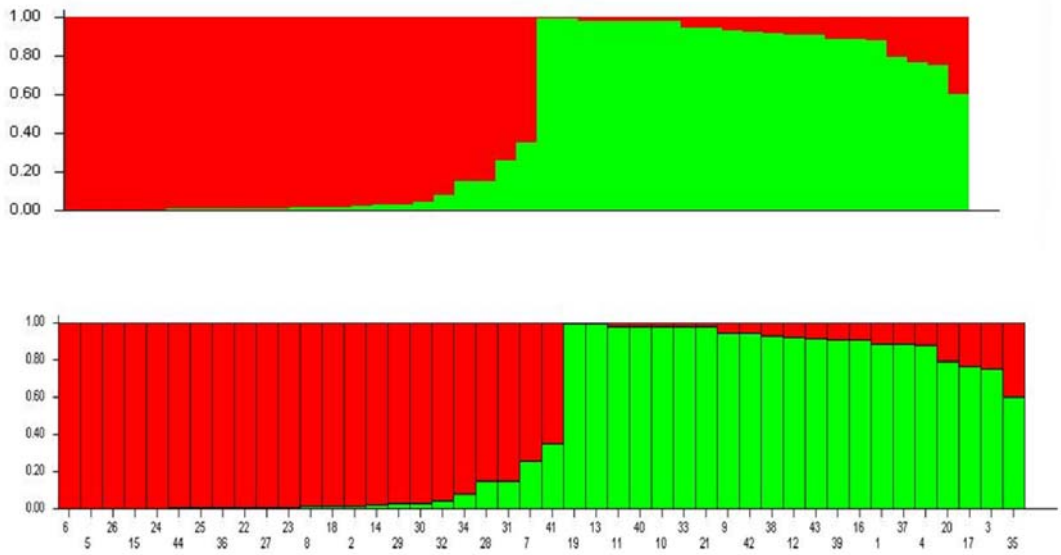

Fig. 3. Genetic relatedness of 44 genotypes of *Orobanche aegyptiaca* analysed by *Structure* program. Numbers on the y-axis indicate the membership coefficient and on the x-axis indicate the genotypes code. Genotypes with the same color belong to the same genetic groups.

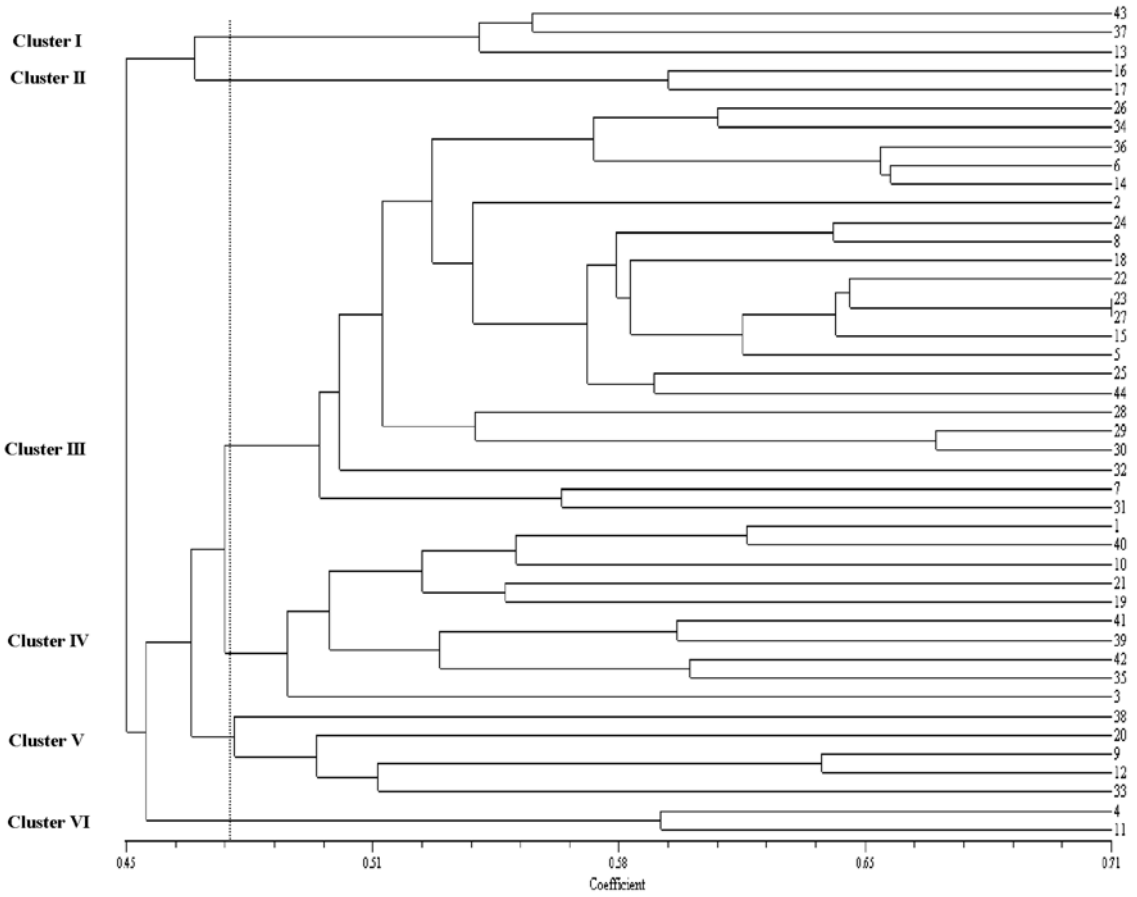

Fig. 4. UPGMA cluster analysis of 44 *Orobanche aegyptiaca* individuals based on Jaccard's similarity coefficient.

the *O. aegyptiaca* individuals. Genetic similarity coefficients varied from 0.71 between individuals 23 and 27 (both from genetic group Red) to 0.34 between individuals 13 (from genetic group Green) and 30 (a mixed individual). Individuals were placed in 6 main clusters (Fig. 4). In each cluster, there were genotypes from differed genetic groups including Green and Red (Figs 3 and 4). The UPGMA dendrogram of individuals and structure analysis did not exactly divide the individuals into distinct groups resembling the three geographically-defined regions (north and south of West Azerbaijan province and Kurdistan province).

Generally, genotypes were evenly distributed along the dendrogram and subgroups, revealing high intrapopulation genetic diversity. For instance, individuals 2 and 4 collected from the same region were categorized in different groups. Also they were allocated into two different subgroups (Fig. 3). Therefore, two geographically proximal individuals can exhibit different PCR patterns. These results demonstrate the potential usefulness of ISSR method in discriminating genetically distinct Orobanche individuals or clones which are not isolated spatially (Benharrat et al. 2002). However, Román et al. (2007) observed the separation of individuals according to their taxonomical and the geographical origins. However, the high level of genetic variation within *Orobanche* populations identified in the present study has to be considered in future crop breeding programs (Pieterse et al. 1991) if selection programs are involved with specific parasite races at different geographical regions (Satovic et al. 2009).

In conclusion, ISSR markers are suitable for investigating genetic diversity among *Orobanche aegyptiaca* genetic groups and are able to discriminate between individuals. Related genotypes were not clustered spatially. The high level of genetic diversity within *O. aegyptiaca* populations in tobacco growing regions in Iran is important to consider when developing resistant host crops against this parasite.

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