DOI: http://dx.doi.org/10.22092/cbj.2012.100412

Cytological evaluation of annual species of the *Onobrychis* genus in Iran

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Received: October 2011

ABSTRACT

F. Ghanavati, N. Nematpajooh, M. Khosrow Chahli, and S. Safaei Chaeikar. 2012. Cytological evaluation of annual species of the *Onobrychis* genus in Iran**. Crop Breeding Journal 2(1):17-24.**

The genus *Onobrychis* is an important forage crop consisting of approximately 130 annual and perennial species. In this study, 13 populations of five *Onobrychis* species were analyzed. The basic chromosome number varied from x=7 to x=8. According to Stebbins' classification, populations such as *O. aucheri* subsp. *psammophila*, *O. crista-galli* (1) and *O. crista-galli* (2) were classified in symmetric class B, while the others were classified in A. Based on interchromosomal symmetry, *O. aucheri* subsp. *tehranica* and *O. crista-galli* (1) had the most asymmetrical and evolutionary karyotype, and *O. crista-galli* (6) had the most symmetrical karyotype. Based on intrachromosomal symmetry, *O. crista-galli* (6) had the most asymmetrical karyotype. Populations were divided into three classes by cutting dendrogram resulting from cluster analysis (Ward) using six parameters (SA, LA, TL, AR, r-value, CI). The greatest distance observed was between *O. crista-galli* (3) and *O. crista-galli* (5), while the smallest distance was between *O. crista-galli* (2) and *O. crista-galli* (6). Populations were separated into three classes using two indices (A_1 and A_2). The greatest distance observed was between *O. crista-galli* (6) and *O. aucheri* subsp. *tehranica,* while the smallest distance was between *O. crista-galli* (5) and *O. crista-galli* (3).

Key words: number of chromosomes, interchromosomal symmetry, intrachromosomal asymmetry, asymmetrical karyotype

INTRODUCTION

Sainfoin (*Onobrychis* Miller), with more than 130 annual and perennial species, can be found from the Mediterranean region to central Asia. Most of these species are restricted to northwest Asia, especially Iran and Anatolia, making this area the main center of genetic diversity for the genus (Yildiz *et al.*, 1999). Many species are grown to produce high protein fodder for many animals and play an important role in soil environment by increasing the nutritive value of drought-resistant pasture (Abou-El-Enain, 2002). According to flora Iranica, this genus is subdivided into two subgenera, namely, *Onobrychis*, with four sections, and *Sisyrosema*, with five sections. They can be distinguished by their different karyotypes, morphological features and geographical origins (Rechinger, 1984). Thirty-one endemic species have been found in Iran, which has 69 species of the *Onobrychis* genus, including 13 annual and 56 perennial species.

A study of the karyotypes of four species of sainfoin has shown that *O. aucheri* subsp. *tehranica*, *O. scrobiculata*, *O. melanotricha* and *O. oxyptera*, all with 16 chromosomes, are diploid (Ansari *et al.*, 2000). Abou-El-Enain (2002) showed that the basic chromosome number in *Onobrychis* varied from 7, 8 and 9, and that their chromosomal type was metacentric and sub-metacentric. Morphological features and chromosomal characteristics of two subspecies, *O. aucheri* subsp. *tehranica* and *O. aucheri* subsp. *psammophila*, showed that the two subspecies differ in terms of morphology and chromosomes, and therefore cannot be considered as two subspecies of one species (Hatami and Nasirzadeh, 2006). Ghanavati *et al.* (2010) examined nine species of section *Heliobrychis* and showed that the basic chromosome number was x=8 and x=9, and that *O. heliocarpa* and *O. lunata* were in Stebbins class A, while *O. oxyptera* was in class 3B.

The present study conducted a mitosis analysis of 13 populations of *Onobrychis*, some of which are endemic to Iran, and tried to identify the chromosome number, shape, size and karyotypic evolution and species relationship by using multivariate statistical analysis methods.

MATERIALS AND METHODS

Seed of 13 populations of *O. caput-galli*, *O. pulchella*, *O. aucheri*, *O. micrantha* and *O. crista-galli* collected from six locations of Iran were germinated on wet filter paper in Petri dishes and kept at 22°C temperature for three days. Root tip meristems obtained from seedlings were pre-treated in 8-hydroxyl-quinalin (2 mM) at 4°C for 5 h, fixed in a 1:1 (v/v) solution of 10% formalin and 1% chromic acid for 24 h at 4°C. The root tips were then rinsed for 3 h in distilled water and stored in 70% ethanol at 4°C. For hydrolyzing, the root tips were treated with 1N NaOH for 10 min at 60°C and stained with aceto-iron-hematoxilin solution for 4 h at 30°C. After each step, root tips were washed briefly in distilled water. A segment of the meristematic region 1 mm in length was excised and macerated in cytase enzyme at room temperature for 1 h. Squash preparations on slides were made using 45% acetic acid (Aghayev, 1998).

Chromosome measurements including long arm (LA), short arm (SA), total length of chromosome set (TL) [L+S], arm ratio (AR) [L/S] and Centromic Index (CI)[S/L+S] were taken from 15 and 10 enlarged well-spread metaphases. They were used for performing a karyotype analysis of each population, using Micromeasure software developed by the Biology Department of Colorado State University, USA, available at http://www.Colostate.edu/Depts/Biology/Micromeasure. Karyotype asymmetry was estimated by three different methods, namely, total form percentage (TF %) [(∑S/∑TL) ×100] (Huziwara, 1962); difference of relative length (DRL) [$Max_{RL\%}$–$Min_{RL\%}$]; intrachromosomal asymmetry index (A1) [1-Σ($\bar{S}/\bar{L}$)/n] and interchromosomal asymmetry index (A2) [Sd/x] (Romero Zarco, 1986). Both indices (A_1 and A_2) are independent of chromosome number and size.

Karyotypic evolution was determined using the symmetry classes of Stebbins (Stebbins, 1971). Karyotype formula was determined by chromosome morphology based on centromere position according to the classification of Levan (Levan *et al.*, 1964). For each population, karyograms were drawn based on length of chromosome (arranged from larger to smaller).

To determine the variation between populations, one-way unbalanced ANOVA was performed on normal data, and parameter means were compared using Duncan's multiple range test. Principal components analysis (PCA) was performed to evaluate the contribution of each karyotypic parameter to the ordination of species. Clustering was performed using the Ward method to examine karyotypic similarity among populations. Numerical analyses were performed using SAS version 6.1, SPSS version 18 and StatistiXL version 1.7.

RESULTS

Results showed that two basic chromosome numbers (x=7 and x=8) and ploidy levels (2n=2x=14, 2n=2x=16 and 2n=4x=32) are present in the *Onobrychis* genus. One ploidy level was observed for *O. caput-galli* (2n=2x=14) and *O. pulchella* (2n=2x=16) and two ploidy levels for *O. aucheri* (2n=2x=16, 2n=4x=32) and *O. crista-galli* (2n=2x=16, 2n=4x=32) (Table 1). The karyotypes of diploid and tetraploid populations are illustrated in Fig. 1.

The long-arm mean value varied from 4.39 µm in *O. caput-galli* (1) to 20.73 µm in *O. crista-galli* (3). The short-arm average varied from 2.30 µm in *O. caput-galli* (1) to 15.24 µm in *O. crista-galli* (3). The mean value for total chromosome length varied from 6.69 µm in *O. caput-galli* (1) to 35.97 µm in *O. crista-galli* (3). Finally, the mean value of arm ratio ranged from 11.62 in *O. aucheri* subsp. *tehranic*a to 33.73 in *O. micrantha* (Table 2). The chromosomes of these populations were mostly metacentric or sub-metacentric; the chromosomes of *O. crista-galli* (6) were sub-metacentric, while those of other populations were a combination of metacentric and sub-metacentric.

Symmetry types of Stebbins (Stebbins, 1971) are given in Table 1. In terms of the Stebbins system (Stebbins, 1971), the karyotypes of populations, mostly of class 2A, are considered primitive classes in this system. One population was classified in group 1A, eight populations in class 2A, one population in group 1B, and three populations — namely, *O. aucheri* subsp. *psammophila*, *O. crista-galli* (1) and *O. crista-galli* (2)— from tetraploid populations were classified in group 2B (Table 1).

The intrachromosomal asymmetry index (A_1) expresses the arm ratio of each pair of homologous chromosomes (Romero Zarco, 1986). The interchromosomal asymmetry index (A_2) corresponds to Pearson's coefficient of dispersion and gives an idea of the asymmetry caused by the different lengths of the chromosomes. Using Romero-Zarco (Romero Zarco, 1986) asymmetry indices A_1 and A_2, we could identify the more asymmetric karyotypes among populations that had similar Stebbins classes of symmetry. For example, in class 2A populations, *O. crista-galli* (6) possessed the highest A_1 value (0.50) and its DRL value was 4.10; therefore, it has a more asymmetric karyotype. *O. crista-galli* (3) possessed the lowest A_1 value (0.24) and, hence, a more symmetric karyotype.

Table 1. Karyotypic characteristics of 13 populations of *Onobrychis*.

Taxon	Section	Origin sites	2n	x	DRL	%TF	%S	A
***O. caput-galli* 1**	***Lophobrychis***	**Kermanshah: Eslamabad**	**14**	**7**	**8.54**	**34.42**	**54.73**	**0.4**
***O. caput-galli* 2**	***Lophobrychis***	**Lorestan, Khoram Abad**	**14**	**7**	**8.17**	**34.83**	**58.15**	**0.4**
***O. pulchella* 1**	***Lophobrychis***	**Khorasan:Mashhad, Sade Torogh**	**16**	**8**	**8.35**	**40.14**	**52.83**	**0.3**
***O. pulchella* 2**	***Lophobrychis***	**Khorasan: Kalat Naderi**	**16**	**8**	**6.58**	**36.57**	**57.71**	**0.3**
O. aucheri* subsp. *psammophila	***Heliobrychis***	**Kashmar**	**32**	**8**	**4.73**	**33.19**	**45.46**	**0.4**
O. aucheri* subsp. *tehranica	***Heliobrychis***	**Tehran: Chitgar park**	**16**	**8**	**8.56**	**42.20**	**49.88**	**0.2**
O. micrantha	***Lophobrychis***	**Khorasan: Mashhad, Sade Torogh back**	**16**	**8**	**7.85**	**36.86**	**53.26**	**0.4**
***O. crista-galli* 1**	***Lophobrychis***	**Kermanshah: Sarepolezahab**	**32**	**8**	**5.74**	**41.60**	**41.16**	**0.2**
***O. crista-galli* 2**	***Lophobrychis***	**Kermanshah: Salase Babajani**	**32**	**8**	**5.51**	**38.60**	**44.83**	**0.3**
***O. crista-galli* 3**	***Lophobrychis***	**Fars: Firuzabad**	**32**	**8**	**3.97**	**42.38**	**54.83**	**0.2**
***O. crista-galli* 4**	***Lophobrychis***	**Lorestan: Koohdasht**	**32**	**8**	**3.78**	**35.60**	**54.65**	**0.4**
***O. crista-galli* 5**	***Lophobrychis***	**Lorestan: Koohdasht**	**16**	**8**	**5.47**	**36.53**	**64.32**	**0.4**
***O. crista-galli* 6**	***Lophobrychis***	**Kermanshah: Gilane Gharb**	**16**	**8**	**4.10**	**32.71**	**72.05**	**0.5**

2n: Somatic chromosome number; x: Basic chromosome number; DRL: Difference of relative length; %TF: Total form percentage; % S: Relative A_1: Intrachromosome asymmetry index; A_2: Interchromosome asymmetry index; SC: Stebbins' symmetry classes; Sat: satellites; KF: Karyotype f sm: submetacentric; st-subtelocentric.

Table 2. Means of *Onobrychis* populations resulting from chromosome analysis.

Taxon	LA	SA	TL	AR	r-value	CI
***O. caput-galli* 1**	**4.39^{a*}**	**2.30^{a}**	**6.69^{a}**	**13.93^{a}**	**3.86^{a}**	**2.44^{a}**
***O. caput-galli* 2**	**4.50^{a}**	**2.46^{a}**	**7.08^{a}**	**13.54^{a}**	**4.09^{a}**	**2.48^{a}**
***O. pulchella* 1**	**5.10^{a}**	**3.42^{ab}**	**8.53^{a}**	**12.47^{a}**	**5.69^{b}**	**3.26^{bc}**
***O. pulchella* 2**	**6.34^{ab}**	**3.65^{ab}**	**9.99^{a}**	**15.16^{a}**	**4.95^{ab}**	**2.97^{abc}**
O. aucheri* subsp. *psammophila	**6.14^{ab}**	**3.58^{ab}**	**9.72^{a}**	**15.16^{a}**	**4.82^{ab}**	**2.93^{abc}**
O. aucheri* subsp. *tehranica	**6.19^{ab}**	**4.52^{bc}**	**10.72^{a}**	**11.62^{a}**	**5.84^{b}**	**3.34^{c}**
O. micrantha	**10.80^{c}**	**5.37^{cd}**	**16.18^{b}**	**33.73^{e}**	**8.39^{c}**	**5.39^{d}**
***O. crista-galli* 1**	**9.57^{bc}**	**6.84^{de}**	**16.41^{b}**	**24.36^{cd}**	**11.91^{de}**	**7.19^{e}**
***O. crista-galli* 2**	**18.88^{de}**	**11.87^{f}**	**30.76^{d}**	**28.48^{de}**	**10.76^{d}**	**6.73^{e}**
***O. crista-galli* 3**	**20.73^{e}**	**15.24^{g}**	**35.97^{e}**	**22.00^{bc}**	**12.33^{e}**	**6.87^{e}**
***O. crista-galli* 4**	**19.80^{de}**	**10.94^{f}**	**30.74^{d}**	**29.80^{de}**	**9.33^{c}**	**5.78^{d}**
***O. crista-galli* 5**	**6.19^{ab}**	**3.46^{ab}**	**9.65^{a}**	**14.35^{a}**	**4.67^{ab}**	**2.92^{abc}**
***O. crista-galli* 6**	**17.02^{d}**	**8.28^{e}**	**25.31c**	**16.86^{bc}**	**4.04^{a}**	**2.64^{ab}**

LA: long arm; SA: short arm; TL: total length; AR: arm ratio; r-value: ratio of short arm to long arm CI: centromere index.

*** Means in each column followed by similar letter(s) are not significantly different at the 5 probability level-using Duncan's Multiple Range Test.**

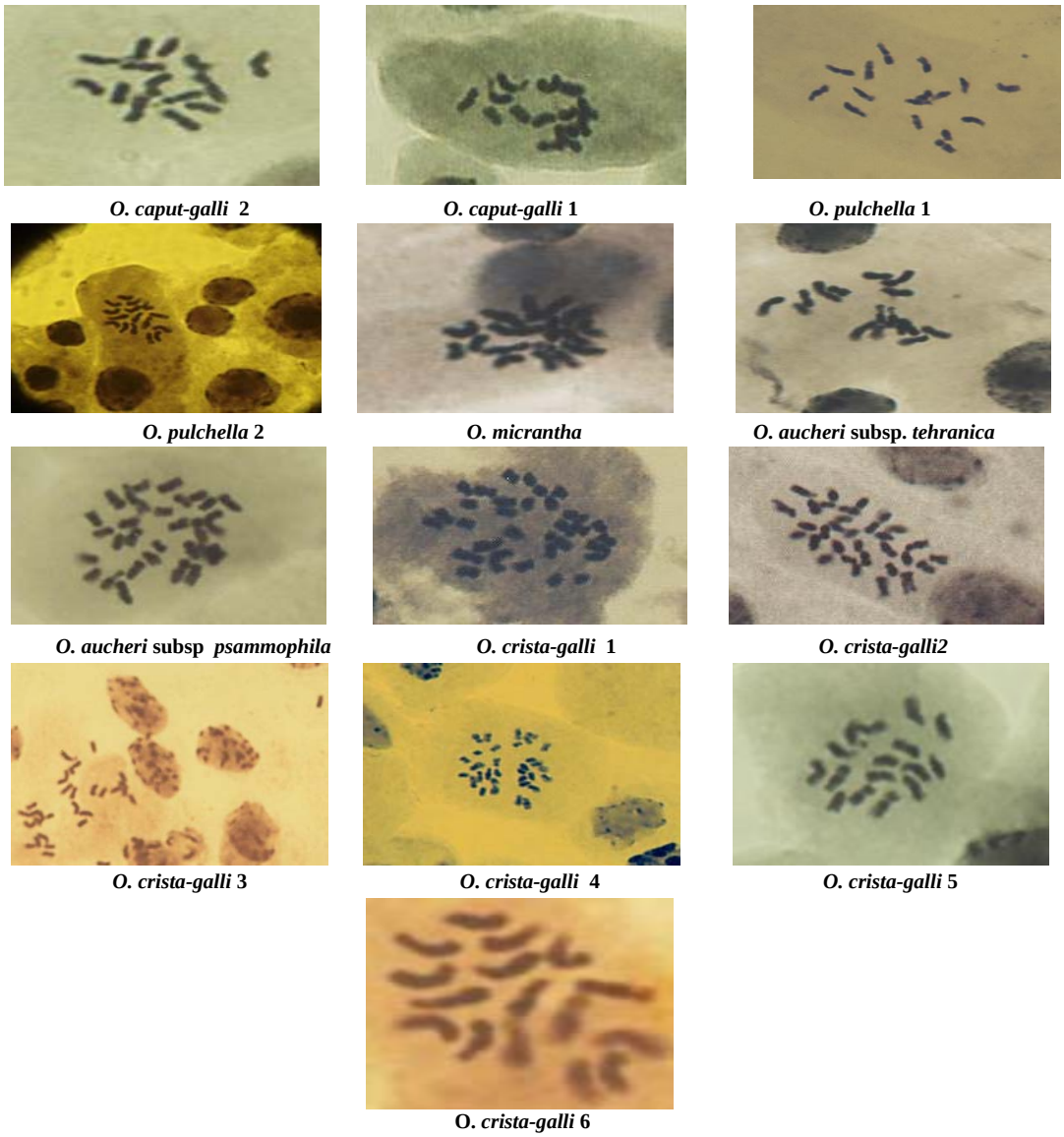


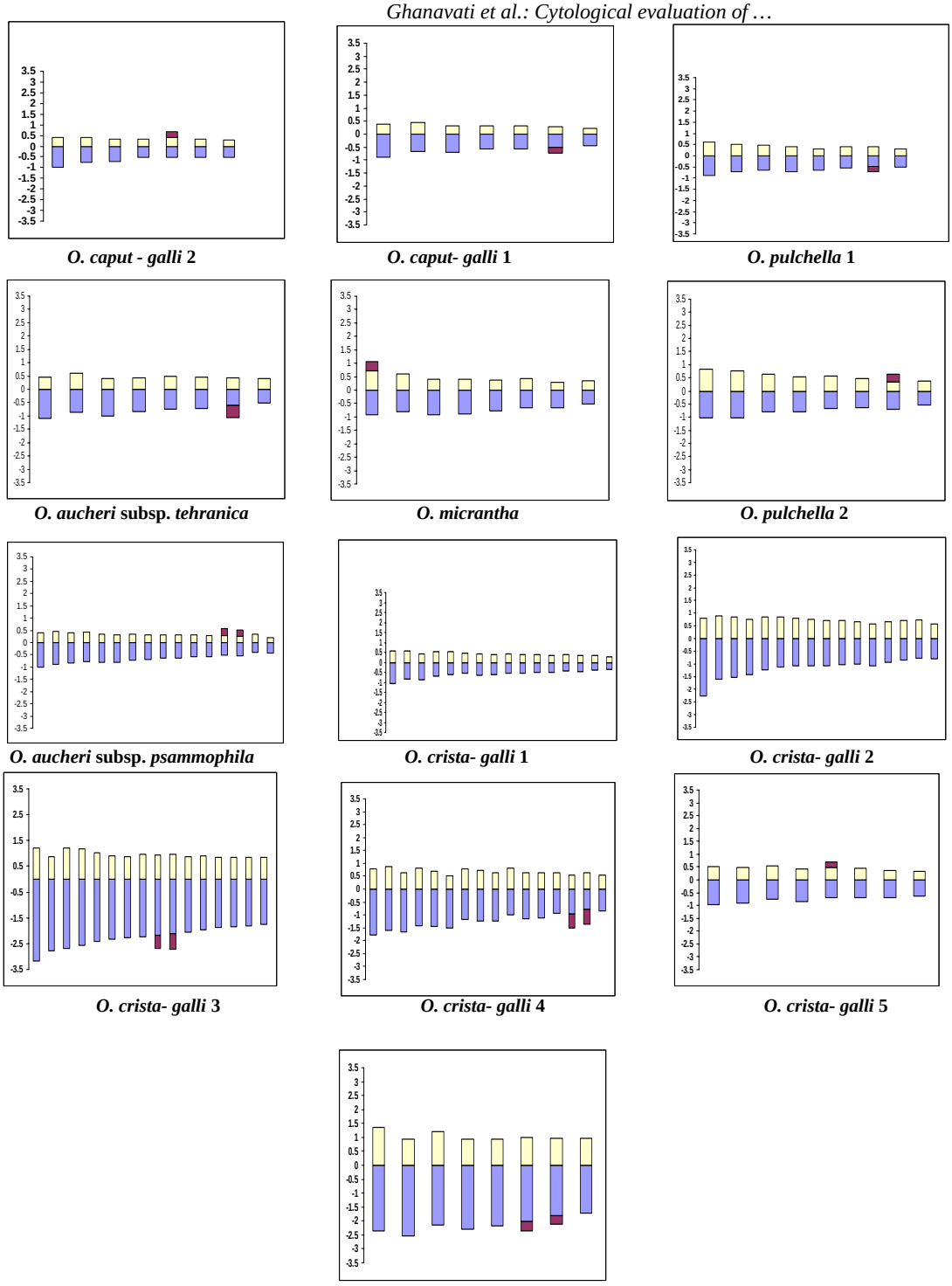
Fig. 1. Karyotypes of 13 diploid and tetraploid *Onobrychis* populations.

Similarly, in class 2B populations, *O. aucheri* subsp. *psammophila* possessed the highest value for A_1 (0.49) and the highest asymmetric karyotype in this group, and *O. crista-galli* (1) had the lowest value for A_1 (0.26) and the lowest symmetric karyotype in this group (Table 1).

Populations classified in group 2A also showed the lowest A_2 values (ranging from 0.10 to 0.18), and the highest %TF (from 32.71 to 42.38) (Table 1).

In general, based on intrachromosomal asymmetry (A_1 and %TF), *O. crista-galli* (6) had the most asymmetrical and evolutionary karyotype, while *O. crista-galli* (3) had the most symmetrical karyotype of all the populations. According to interchromosomal asymmetry (A_2 and DRL), *O. aucheri* subsp. *tehranica* had the most asymmetrical karyotype of all the populations (Table 1). The asymmetry index %TF ranged from 32.71 to 42.38, the intrachromosomal asymmetry index (A_1) varied from 0.24 to 0.50, and the interchromosomal asymmetry index (A_2) ranged from 0.10 to 0.23 (Table 1). Most populations had two or four pairs of small visible satellites connected to the short or long arms of the chromosomes (Fig. 2; Table 1).

The analysis of variance based on an unbalanced completely randomized design demonstrated that there were significant differences (P<0.01) among the populations for all the measured traits (Table 4). The principal component analysis (PCA) of the karyotypic parameters showed that the first two principal components accounted for 93.64% of total variances. Component one (81.63%) consisted of total chromosome length, long-arm length, short-arm length, long arm to short arm ratio, short arm to long



***O. caput - galli* 2** | ***O. caput- galli* 1** | ***O. pulchella* 1**

O. aucheri* subsp. *tehranica | ***O. micrantha*** | ***O. pulchella* 2**

O. aucheri* subsp. *psammophila | ***O. crista- galli* 1** | ***O. crista- galli* 2**

***O. crista- galli* 3** | ***O. crista- galli* 4** | ***O. crista- galli* 5**

***O. crista- galli* 6**

Fig. 2. Idiogram of 13 diploid and tetraploid *Onobrychis* populations.

arm ratio, and the centromere index, which had the highest eigenvalue (Table 4).

Grouping of the populations was studied based on their relative karyotypic as well as mitotic characteristics (Table 2; Fig. 4). Cutting the dendrogram resulted from cluster analysis by the Ward method; based on two indices (A_1 and A_2), the populations were classified into three groups. The greatest distance was between *O. crista-galli* (6) and *O. aucheri* subsp. *tehranica*, and the smallest distance was between *O. crista-galli* (3) and *O. crista-galli* (5). However, population karyotypic parameters of the populations (Fig. 3). The greatest distance was between *O. crista-galli* (3) and *O. caput-galli* (5), and the smallest distance was between *O. caput-galli* (6) and *O. caput-galli* (2).

DISCUSSION

In this study, the basic chromosome numbers were x=7 and x=8 for diploid populations, and only

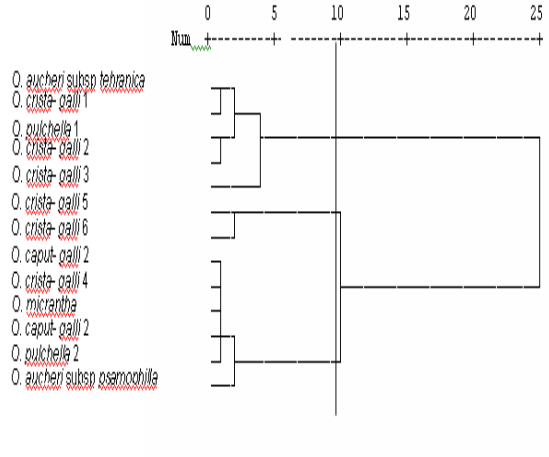


Fig. 3. Cluster analysis (Ward) dendrogram based on two parameters (A_1 and A_2) of 13 populations.

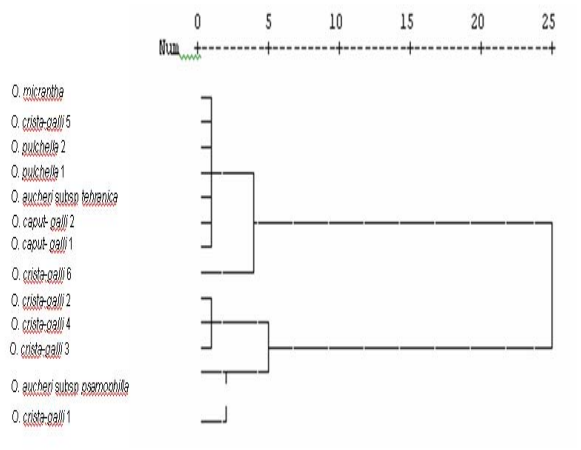


Fig 4. Dendrogram for 13 populations of *Onobrychis* produced by analyzing six karyotypic parameters using Ward's cluster analysis method.

x=8 for tetraploid populations. Goldblatt (1981) suggested x=14 as the basic number for the Faboideae subfamily, x = 8 for the Hedysareae tribe, and x = 8 or 7 for the *Onobrychis* genus. Numerous reports (Semerenko and Shvets, 1989; Baltisberger, 1991; Magulaev, 1995; Slavik *et al.*, 1993; Mohamed, 1997; Oberprieler and Vogt, 1996; Abou-El-Enain, 2002; Hesamzadeh Hejazi and Ziaei Nasab, 2009a, b; Ghanavati *et al.*, 2010) have shown that the most frequent basic chromosome numbers for the *Onobrychis* genus are were also classified into three groups based on x=7 and x=8, while ploidy levels vary. Analysis of karyotype formulae showed that generally in all diploid and tetraploid species, the number of "m" chromosomes was higher than the number of "sm" chromosomes, except for *O. caput-galli* (1) and (2), *O. aucheri* subsp. *psammophila*, and *O. crista-galli* (4), (5) and (6). This finding is in agreement with the conclusions reached by Hesamzadeh Hejazi and Ziaei Nasab (2009a) and Ghanavati *et al.* (2010) regarding other species in this genus. In 13 populations some chromosome pairs carried secondary constructions on their short or long arms (Fig. 2; Table 1). As a result, species also could be differentiated by the number, type and position of satellites.

Onobrychis crista-galli (6) had the highest A_1 value (0.50) and exhibited the most asymmetrical and intrachromosomally derived karyotypes, while *O. crista-galli* (3) was identified as having the most symmetrical karyotypes (Table 1).

As a matter of fact, lower DRL values illustrated more symmetry of karyotype; *O. aucheri* subsp. *tehranica* and *O. crista-galli* (4) with DRL 8.56 and 3.78 values, respectively, had the most asymmetrical and symmetrical karyotypes. Similarly, high DRL values lead to more variation in chromosome construction.

Different populations of several *Onobrychis* species show numerical chromosome polymorphism. For example; Darlington and Wylie (1995), Goldblatt and Johnson (1993), and Hesamzadeh Hejazi and Ziaei Nasab (2009b) reported a diploid chromosome number (2n=14; 2n=16 and 2n=16) for *O crista-galli* species, while Goldblatt and Johnson (1998) and Ansari Asl *et al.* (2001) reported a tetraploid chromosome number (2n=32) for *O. crista-galli* species. Ansari Asl *et al.* (2001) declared a diploid (2n=16) chromosome number for *O. aucheri* subsp. *tehranica* and a tetraploid (2n=32) for *O. aucheri* subsp. *psammophila*. However, in this study, 2n=2x=16 and 2n=4x=32 were found in different populations of *O. crista-galli* in Iran.
Analysis of variance showed significant differences (P<0.01) in the size of chromosomes as well as in the long arm to short arm ratio among diploid and tetraploid populations. These results indicate there is significant variation in chromatin in *Onobrychis* species (Table 4).

Considering the changes in the interchromosomal asymmetry index (A_2) among diploid and tetraploid species, the lowest value was observed in the diploid species with x=8 (*O. crista-galli* (6)) and the highest value was also observed in diploid and tetraploid species with x=8 (*O. aucher* subsp. *tehranica* and *O. crista-galli* (1)) (Table 1). Cluster analysis based on cytological data identified populations with the lowest metric distance that could be used in breeding programs to incorporate genetic variation (Fig. 4).

Table 3. Means of *Onobrychis* populations resulting from chromosome analysis.

CI	r-value	AR	TL	SA	LA	Taxon
2.44[a]	3.86[a]	13.93[a]	6.69[a]	2.30[a]	4.39[a*]	*O. caput-galli* 1
2.48[a]	4.09[a]	13.54[a]	7.08[a]	2.46[a]	4.50[a]	*O. caput-galli* 2
3.26[bc]	5.69[b]	12.47[a]	8.53[a]	3.42[ab]	5.10[a]	*O. pulchella* 1
2.97[abc]	4.95[ab]	15.16[a]	9.99[a]	3.65[ab]	6.34[ab]	*O. pulchella* 2
2.93[abc]	4.82[ab]	15.16[a]	9.72[a]	3.58[ab]	6.14[ab]	*O. aucheri* subsp. *psammophila*
3.34[c]	5.84[b]	11.62[a]	10.72[a]	4.52[bc]	6.19[ab]	*O. aucheri* subsp. *tehranica*
5.39[d]	8.39[c]	33.73[e]	16.18[b]	5.37[cd]	10.80[c]	*O. micrantha*
7.19[e]	11.91[de]	24.36[cd]	16.41[b]	6.84[de]	9.57[bc]	*O. crista-galli* 1
6.73[e]	10.76[d]	28.48[de]	30.76[d]	11.87[f]	18.88[de]	*O. crista-galli* 2
6.87[e]	12.33[e]	22.00[bc]	35.97[e]	15.24[g]	20.73[e]	*O. crista-galli* 3
5.78[d]	9.33[c]	29.80[de]	30.74[d]	10.94[f]	19.80[de]	*O. crista-galli* 4
2.92[abc]	4.67[ab]	14.35[a]	9.65[a]	3.46[ab]	6.19[ab]	*O. crista-galli* 5
2.64[ab]	4.04[a]	16.86[bc]	25.31c	8.28[e]	17.02[d]	*O. crista-galli* 6

LA: long arm; SA: short arm; TL: total length; AR: arm ratio; r-value: ratio of short arm to long arm; CI: centromere index.

*** Means in each column followed by similar letter(s) are not significantly different at the 5% probability level-using Duncan's multiple range test.**

Table 4. Analysis of variance of karyotypic parameters.

Centromere index	r-value	Arm ratio	Total length	Long arm	Short arm	Degrees of freedom	Source of variation
10.31[**]	29.63[**]	167.02[**]	319.86[**]	120.18[**]	50.58[**]	12	Genotype
0.06	0.33	6.35	4.01	2.16	0.45	26	Error
19.19	8.88	9.21	12.37	12.98	10.62		CV%

r-value: ratio of short arm to long arm.

Table 5. Eigenvectors from the first 2 principal components (PC1 and PC2) of 6 karyotypic parameters to classify 13 populations of *Onobrychis*.

PC 2	PC 1	Parameters
-0.34	0.94	Total length
-0.37	0.92	Long arm
-0.28	0.94	Short arm
0.28	0.81	Arm ratio
0.37	0.89	r-value
0.72	4.89	Eigenvalue
12.01	81.63	Percentage of variance
93.64	81.63	Cum. percentage of variance

r-value: ratio of short arm to long arm.

Grouping based on karyotypic parameters indicated *O. crista-galli* (3) was located far from *O. crista-galli* (5), and grouping using A_1 and A_2 indices showed that *O. crista-galli* (6) was far from *O. aucheri* subsp. *tehranica* (Fig. 3).

This study showed that variation in chromosomal traits is one of the mechanisms of inter- and intraspecies diversification in the *Onobrychis* genus. Differences in karyotypic formulae and asymmetric indices found among the species suggest that structural variation of chromosomes may contribute to the diversification of the genus. These genomic differences could be used for breeding purposes.

CONCLUSION

The basic chromosome number of *Onobrychis* species varied between x=7 and x=8, but their chromosomal variation was very high. Based on interchromosomal symmetry, *O. aucheri* subsp. *tehranica* and *O. crista-galli* (1) had the most asymmetrical and evolutionary karyotype, while *O. crista-galli* (6) had the most symmetrical karyotype. However, intrachromosomal symmetry information showed that *O. crista-galli* (6) had the most asymmetrical karyotype.

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