New Chromosome Number Reports in *Cousinia* Species (Compositae)

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Summary The cytological study of several populations in 4 species of *Cousinia tenuifolia*, *C. tabrisiana*, *C. urumiensis* and *C. microcephala* showed the occurrence of $2n=2x=26$ chromosome number in all the species studied, information which is new to science. The species formed mainly bivalents and univalents in the metaphase of meiosis I, but in some populations quadrivalents were also formed due to heterozygote translocations. A wide range of meiotic abnormalities were observed including laggard chromosome formation, chromosomes stickiness and tripolar cell formation, leading to the formation of unreduced pollen grains. The occurrence of 0-2 B-chromosomes was observed in most of the species and populations studied. Karyotype analysis showed that the chromosomes are metacentric (m) and submetacentric (sm), varying in size from 0.55 to 1.79 μm . The ANOVA test showed significant difference in total chromosome length, size of the long arm and short arm of the chromosomes as well as arm ratio of the chromosome pair numbers 1–4 and 8, indicating the role of both quantitative and structural changes of chromosome in cytological diversity of *Cousinia* species.

Key words Chromosome pairing, *Cousinia*, Karyotype symmetry, Species diversification.

The genus *Cousinia* (Asteraceae) is of worldwide distribution and comprises about 672 species, of which 235 species occur in Iran. According to Rechinger (1970), the section *Cousinia* contains 10 species in Iran out of which seven are endemic.

The previous studies of the genus *Cousinia* in Iran consider taxonomic (Afzal-Rafii 1980, Attar and Ghahreman 2002) and chromosome number reports (Ghaffari and Djavadi 1998, Ghaffari *et al.* 2000, Djavadi 2005, 2007, Sheidai *et al.* 2006), while the present study considers karyotype and meiotic analyses of 11 populations of the sect. *Cousinia* for the first time.

Materials and methods

Plant material

Meiotic and karyotype studies were performed in 11 populations of 4 *Cousinia* species namely *C. tenuifolia* C. A. Mey., *C. urumiensis* Bornm., *C. tabrisiana* Bunge and *C. microcephala* C. A. Mey. The voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU).

Cytological studies: Meiotic analyses

Meiotic studies were performed on young flower buds collected from at least 10 randomly selected plants from each species and population. Minimum 100 metaphase/diakinesis pollen mother cells (PMCs) and 500 anaphase and telophase cells were analysed for data collection (Sheidai and Rashid 2007). Pollen satiability as a measure of fertility was determined by staining minimum 1000 pollen grains with 2% acetocarmine:50% glycerin (1:1) for about 1/2 h. Round complete pollens

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which were stained were taken as fertile, while incomplete, shrunken pollens with no stain were considered infertile (Sheidai and Rashid 2007). *t*-Test was performed to detect any significant difference in chiasma frequency and chromosome pairing and χ^2 test was performed for meiotic abnormalities among the species studied (Sheidai and Rashid 2007). Neighbor-Joining (NJ) clustering was also performed to show meiotic distinctness of the species from 2 sections studied. Statistical analyses used SPSS ver. 9 (1998) software.

Karyotype analyses

Freshly grown root tips were collected from the seeds of at least ten randomly selected plants in each species, pretreated with 0.002 mol 8-hydroxyquinolin (1–2 h) and fixed in ethanol–acetic acid (3 : 1) for 24 h. The fixed tips were then washed thoroughly in distilled water and macerated in 60°C 1N HCl for about 5 min. Squash technique was used for cytological studies with 2% aqueous aceo-orcein as the stain. The somatic chromosome number and karyotype details were studied in at least 5 well-prepared metaphase plates. The chromosomes were photographed by digital camera and measured by Image Tools3 software.

The chromosomes were identified according to Levan *et al.* (1964), karyotype symmetry was determined according to Stebbins (1971), while other karyotype parameters like total form percentage (TF%), coefficient of variation (CV) of the chromosome size as well as A1 and A2 indices of Romero-Zarco (1986) were determined. The Pearson coefficient of correlation was determined among karyotype features.

Results

All the species studied had $2n=2x=26$ chromosome number, all of which are new to science. Details of chromosome number, chiasma frequency and chromosome pairing in *Cousinia* species are reported in Table 1 and Fig. 1. The species and populations studied showed $2n=2x=13$ chromosome number.

The species and populations studied formed mainly bivalents but a few univalents were also observed. The highest value of ring bivalents (9.18) and lowest values of rod bivalents (3.76) and univalents (0.00) were observed in the Oshnaviyeh population of *C. tenuifolia*, while the lowest value of ring bivalents (3.43) and the highest values of rod bivalents (7.14) and univalents (4.86) occurred in the Romghiosy population of *C. tabrisiana* (Table 1).

Although the species studied are diploid and are expected to form merely bivalents, a few quadrivalents were formed in metaphase of meiosis I in these species. The highest value of quadrivalents occurred in the Romghiosy population of *C. tabrisiana*.

The highest values of total and terminal chiasmata (22.09 and 21.94, respectively) occurred in the Oshnaviyeh population of *C. tenuifolia*, while the lowest value of the same (14 and 13, respectively) occurred in the Romghiosy population of *C. tabrisiana*. The Haris population of *C. urumiensis* showed the highest values of intercalary chiasmata (1.57). An ANOVA test showed no significant difference in chiasma frequency and distribution as well as chromosomes association among *Cousinia* species studied (data not shown).

In order to study the meiotic distinctness of 2 sections of *Cousinia* and *Serratuloideae*, *t*-test and clustering were performed among the species having $2n=26$ chromosome number (Sheidai *et al.* 2005). NJ tree (Fig. 2) clearly separated the species of *C. tabrisiana*, *C. tenuifolia*, *C. urumiensis* and *C. microcephala* of the sect. *Cousinia* from the species of the sect. *Serratuloideae*. This is also supported by *t*-test analysis showing significant difference in meiotic characteristics of the 2 sections (data not shown).

Fig. 1. Representative meiotic and somatic cells in *Cousinia* species. A and B=Meiotic metaphase cells in *C. tenuifolia* and *C. tabrisiana*, respectively. C=Meiotic cell in *C. tabrisiana* showing 1 B-chromosome (arrow). D and E=Tripolar cells in *C. tenuifolia* and *C. microcephala* respectively. F=Potential unreduced pollen grain (bigger size pollen grain) in *C. tenuifolia*. G–J=Somatic metaphase cells in *C. tabrisiana*, *C. urumiensis*, *C. microcephala* and *C. tenuifolia*, respectively. Scale bar=10 μ m.

Table 1. Meiotic characteristics of the *Cousinia* species and populations studied

Species	Locality	2n	ROD	RB	UNI	TEX	IX	TX	IV	Voucher
<i>C. microcephala</i>	Sofian	26	5.26	5.57	4.11	16.57	0.20	16.77	0.06	8600203
<i>C. microcephala</i>	Marand	26	4.58	7.71	1.42	19.84	0.19	20.03	0.00	8600204
<i>C. tabrisiana</i>	Zonoz	26	4.88	7.81	0.63	19.00	1.19	20.00	0.00	8600202
<i>C. tabrisiana</i>	Galle ban	26	5.88	6.79	0.42	19.58	0.06	19.64	0.06	8600205
<i>C. tabrisiana</i>	Rom ghiyosy	26	7.14	3.43	4.86	13.00	1.00	14.00	0.00	8600213
<i>C. tabrisiana</i>	Khoy	26	5.00	6.95	2.10	18.29	0.76	19.05	0.00	8600212
<i>C. urumiensis</i>	Jade ahar	26	5.19	6.77	1.77	19.04	0.04	19.08	0.04	8600206
<i>C. urumiensis</i>	Haris	26	5.04	7.57	0.61	18.00	1.57	19.57	0.04	8600207
<i>C. urumiensis</i>	Khanghah	26	4.96	7.43	1.13	19.70	0.19	19.89	0.02	8600211
<i>C. tenuifolia</i>	Sero	26	5.71	5.38	3.50	16.17	0.50	16.67	0.04	8600208
<i>C. tenuifolia</i>	Oshnaviye	26	3.76	9.18	0.00	21.94	0.15	22.09	0.03	8600209

Abbreviations: ROD=rod bivalents, RB=ring bivalents, UNI=univalents, TX=terminal chiasmata, IX=intercalary chiasmata, TX=total chiasmata, IV=quadrivalents.

Meiotic abnormalities

Meiotic abnormalities observed in *Cousinia* species include stickiness, laggard chromosome and tripolar cell formation (Fig. 1D, E). Laggard chromosomes were observed during anaphases I

Fig. 2. NJ tree of meiotic data in the sections *Cousinia* and *Serratuloideae*.

and II, forming micronuclei in telophases I and II (Fig. 1D). Chromosome stickiness occurred from the early stages of prophase to the late telophase. The number of chromosomes involved in stickiness varied from one to many, forming complete clumps in metaphase.

Larger sized pollen grains (potential unreduced pollen grains) were observed along with the smaller sized pollen grains (normal, reduced pollen grains) in the *Cousinia* species (Fig. 1F). The size of normal (reduced) pollen grains varied from 31.00 to 35.70 μm with a mean value of 42.40 μm . The size of larger (potential unreduced) pollen grains varied from 52.00 to 55.50 μm with the mean value of 52.20 μm .

The presence of giant pollen grains is considered an indication of the production of unreduced ($2n$) pollen grains (Bertagnolle and Thomson, 1995). Unreduced gametes are known to produce individuals with higher ploidy level through a process known as sexual polyploidization, which has been considered the major route to the formation of naturally occurring polyploids (Bertagnolle and Thomson 1995).

B-chromosomes

The occurrence of 0-2 B-chromosomes (Bs) was observed in most of the species and populations studied (Fig. 1C). B-chromosomes were smaller than A-chromosomes and did not pair with them. Bs could arrange themselves in the equator and move to the poles being included in the telophase nucleus.

Karyotype

The somatic chromosome number and details of karyotypes in the *Cousinia* species are presented in Table 2 and Fig. 1. The species studied had $2n=2x=26$ chromosome number, supporting the meiotic analysis. The chromosomes were metacentric (m) and submetacentric (sm) and varied in size from 0.55 to 1.79 μm .

The mean total length of the haploid chromosomes varied from 18.35 μm in the Sero population of *C. tenuifolia* to 38.18 μm in the Jadeh-Ahar population of *C. urumiensis*. The size of the longest chromosome varied from 0.84 μm in the Sero population of *C. tenuifolia* to 1.79 μm in the Jadeh-Ahar population of *C. urumiensis*.

The lowest mean chromosome length occurred in the Oshnaviyeh population of *C. tenuifolia*

Table 2. Karyotype features of the *Cousinia* species and populations studied

Species	Locality	Voucher	TL	L	S	L/S	X	St	A1	A2	TF%	CV	KF
<i>C. microcephala</i>	Marand	8600204	34.46	1.62	1.03	1.58	2.65	2B	0.64	0.26	39	26	13m
<i>C. tabrisiana</i>	Zonoz	8600202	35.36	1.7	1.03	1.68	2.72	2B	0.62	0.32	37.84	32	5m + 8sm
<i>C. tabrisiana</i>	Gale ban	8600205	37	1.69	1.13	1.53	2.85	1B	0.68	0.27	39.77	27	10m+3sm
<i>C. urumiensis</i>	Jadde ahar	8600206	38.18	1.79	1.15	1.6	2.94	1B	0.64	0.24	38.99	24	8m+5sm
<i>C. urumiensis</i>	Heris	8600207	28.4	1.27	0.91	1.42	2.18	1A	0.71	0.18	41.58	18	13m
<i>C. tenuifolia</i>	Sero	8600208	18.35	0.84	0.56	1.51	1.41	1A	0.66	0.26	39.86	26	12m+1sm
<i>C. tenuifolia</i>	Rashkan	8600200	33.6	1.53	1.04	1.52	2.58	1B	0.67	0.25	40.25	25	7m+6sm
<i>C. tenuifolia</i>	Oshnaviyeh	8600209	16.91	0.75	0.55	1.3	1.3	1B	0.72	0.2	42	20	13m

Abbreviations: TL=total chromosome length, L=size of the longest chromosome pair, S=size of the shortest chromosome pair, L/S=ratio of the longest chromosome pair/the shortest chromosome pair, X=the mean chromosome length, St=Stebbins, class, A1 and A2=Romero-Zarco indices, TF%=total form percentage, CV=coefficient of variation, KF=karyotype formulae.

(1.30 μ m), while the highest value of the same occurred in the Jadeh-Ahar population of *C. urumiensis* (2.94 μ m, Table 2).

The highest value of coefficient of variation (CV) (32.00) for the size of chromosomes occurred in the Zonoz population of *C. tabrisiana* indicating the highest degree of size variation among its chromosomes, while the highest value of total form percentage value (TF%) occurred in the Haris population of *C. urumiensis* (41.58) indicating the presence of relatively more symmetrical karyotype in this population.

Pearson correlation determined among the karyotypic features showed a positive significant correlation for total haploid chromosome length, size of the longest and the shortest chromosome pair, as well as the mean chromosome length ($r \geq 0.95$, $p < 0.01$). However, the ratio of the longest/shortest chromosome showed a significant negative correlation ($r = -0.81$) with TF%.

The ANOVA test showed significant difference among the species and populations (data not shown) for total chromosome length, size of the long arm and short arm of the chromosomes as well as arm ratio in the chromosome pair numbers 1–4 and 8. PCA analysis (data not shown) of the karyotype data also identified these characters as the most variable karyotype features among the species studied.

Discussion

Chiasma frequency and distribution are under genetic control and are of adaptive nature (Rees and Dale 1974, Quicke 1993). Quadrivalents occur due to heterozygote translocations which in turn bring about more genetic variation in the next generation. The result of *t*-test and clustering of meiotic data separating *Cousinia* species from 2 sections of *Cousinia* and *Serratuloideae* based on chiasma frequency and distribution as well as chromosomes pairing indicate that the genetic mechanism controlling meiosis in these 2 sections have changed during species diversification.

Meiotic abnormalities observed in *Cousinia* species studied may cause some degree of pollen sterility. Pollen fertility observed in the species studied ranged from 95–100%. Different cytological mechanisms are responsible for the production of 2n gametes (Bertagnolle and Thomson 1995). The detailed cytological study of the *Cousinia* species indicates the possible role of anaphase failure and tripolar formation as well as unreduced meiocyte formation as the main mechanisms for unreduced gamete formation.

B-chromosomes are accessory chromosomes reported in about 1300 species of plants (Camacho *et al.* 2000) and when present in high number may affect negatively the growth and vigor of the plants; but in low number can benefit them. These chromosomes are known to change

the chiasma frequency and genetic recombination in the cells in which they are present. However we did not have enough cells possessing B-chromosomes to analyse statistically the effect of B-chromosomes on chiasma frequency.

Positive significant correlation among total haploid chromosome length, size of the longest and the shortest chromosome pair, as well as the mean chromosome length indicate that during the species' and populations' cytological diversification, an increase in genome size (total chromosome length) is accompanied with an increase in the size of both the long chromosomes and short chromosomes of the complement.

The negative significant negative correlation between the ratio of the longest/shortest chromosomes and TF% indicate that an increase in this ratio (due to structural changes in chromosomes) increases the karyotype asymmetry in the *Cousinia* species studied.

Significant difference was observed for total chromosome length, size of the long arm and short arm of the chromosomes, as well as arm ratio in the chromosome pair numbers 1–4 and 8. Furthermore, PCA analysis reveal that variation in these karyotype features is accompanied with cytological diversity in the species and populations.

Variations in the size of chromosomes, type of chromosomes, TF%, CV and Stebbins class all indicate the role played by structural changes of chromosomes in *Cousinia* species diversification. The ANOVA test showed significant difference in size of chromosomes and chromosome arms among the species studied which indicates the occurrence of significant loss/gain in chromatin material during species diversification. Furthermore, the results of the Pearson correlation show that karyotype asymmetry increases with changes in chromosomes arm ratio.

Therefore it seems that change in the chromosome number, the occurrence of B-chromosomes and unreduced pollen grain formation, chromosomes structural changes along with hybridization, as well as quantitative changes in chromosomes size may have played a role in the *Cousinia* species diversification.

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