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Karyotype and genome size analyses in species of *Helichrysum* (Asteraceae)

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ABSTRACT

Karyotype studies were performed in 18 populations of eight *Helichrysum* species in Iran. Those species showed chromosome numbers of 2n = 2x = 14; 2n = 4x = 24, 28 and 32; 2n = 6x = 36; 2n = 7x = 42; 2n = 8x = 48; 2n = 9x = 54; and 2n = 10x = 60. The chromosome numbers of *H. davisianum*, *H. globiferum*, *H. leucocephalum* and *H. oocephalum* are reported here for the first time. New ploidy levels are reported for *H. oligocephalum* (2n = 4x = 24) and *H. plicatum* (2n = 4x = 32). The chromosomes were metacentric and submetacentric. An ANOVA among *H. globiferum* and *H. leucocephalum* populations showed significant differences for the coefficient of variation for chromosome size, total form percentage and the asymmetry indices, indicating that changes in the chromosome structure of *Helichrysum* species occurred during their diversification. Significant positive correlations among the species and populations studied, in terms of the total chromosome length, lengths of the short arms and lengths of the long arms, indicate that these karyotypic features change simultaneously during speciation events. The genome sizes of *Helichrysum* species are reported here for first time. The 2C DNA content ranged from 8.13 pg (in *H. rubicundum*) to 18.4 pg (in *H. leucocephalum* and *H. davisianum*). We found that C-value correlated significantly with ploidy level, total chromosome length, lengths of the long arms and lengths of the short arms ($p<0.05$), indicating that changes in chromosome structure are accompanied by changes in DNA content.

Key words: C-value, *Helichrysum*, karyotype, polyploidy level

Introduction

The genus *Helichrysum* Mill. (Asteraceae, Gnaphalieae) comprises 500-600 species distributed throughout the African continent and Madagascar (Hilliard 1983; Anderberg 1991; Bayer *et al.* 2007), as well as 41 species distributed across the Mediterranean basin, Macaronesia, central Asia and western Asia (Anderberg 1991). The Mediterranean, European, western Asian and central Asian *Helichrysum* species have been classified into two main groups, which in the latest treatments (Clapham 1976) are recognized at the sectional level: sect. *Helichrysum* and sect. *Virginea* (DC.) Gren. & Godr. The species included in sect. *Helichrysum* are shrubs, subshrubs or herbaceous perennials, with capitula in terminal corymbose synflorescences, bearing phyllaries that are yellow or (in rare instances) white, nearly equaling the florets in length, whereas species in the sect. *Virginea* are caespitose, suffruticose perennials, with capitula solitary or in terminal oligocephalous corymbose synflorescences, with white phyllaries extending beyond the florets (Galbany *et al*. 2006).

According to the Flora Iranica (Rechinger 1980), 19 species of *Helichrysum* occur in Iran and eight of those species are endemic. Most of the species are found in the west and northwest of the country, although four species occur in the central and southern regions.

Helichrysum is a rather variable genus, because a number of modifications strongly affect the morphological aspect of its species, and the taxonomic determination is therefore often uncertain (Giuseppe *et al.* 2002). Although it is a very important genus in the pharmaceutical and cosmetic industries for its medicinal properties, namely its anti-inflammatory, antiallergic, antipsoriatic and diuretic effects (Raimondo & Lentini 1990; Voltalina 1999). Cytological studies of this genus have been restricted to a few studies of chromosome numbers and karyotypes in only 10-12% of the species (Galbany *et al*. 2009). The available chromosome data indicate that polyploidy plays a significant role in the speciation and evolution of the genus. Extensive variation exits in chromosome number and ploidy level within the genus, the latter having been reported as x = 4, 5, 7, 8, 10, 11, 12 and 14 (Galbany *et al*. 2009 & 2008; Carr *et al*. 2005; Castro *et al*. 2006).

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Nuclear DNA content is a specific karyotypic feature that is quite useful for systematic purposes and for evolutionary considerations (Bennet & Leitch 1995). However, to our knowledge, there have been no studies quantifying DNA content in the genus *Helichrysum*.

Little is known about the species of *Helichrysum* that occur in Iran, in terms of karyology and population diversity (ploidy level). The few studies of the genus in the country have mainly focused on reporting chromosome numbers (Ghaffari 1989; Chariat-Panahi *et al.* 1982).

In the present study, we describe, for the first time, the karyotypic features and DNA content (genome size) of *Helichrysum* species in Iran.

Materials and methods

Plant material

Chromosome numbers were determined in 18 populations of eight *Helichrysum* species occurring in Iran (Tab. 1): *H. davisianum* Rech. f.; *H. globiferum* DC.; *H. leucocephalum* Boiss.; *H. oligocephalum* Boiss.; *H. oocephalum* Boiss.; *H. armenium* DC.; *H. plicatum* DC.; and *H .rubicundum* DC. The first five of those species are endemic to Iran. Voucher specimens were deposited in the Herbarium of Shahid Beheshti University (code, HSBU).

Cytological studies

For the karyotypic studies, newly emerged root tips were collected from the seeds of at least ten randomly selected plants of each species, pretreated with 0.002 mmol of 8-hydroxyquinoline for 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 at 60°C in 1N HCl for 10 min. For the cytological studies, we used the squash technique, with 2% aqueous aceto-orcein as the stain. The somatic chromosome number and karyotypic features were studied in at least 10 well-prepared metaphase plates. The chromosomes were photographed by digital camera and measured with ImageTool software, version 3 (Sheidai & Rashid 2007).

The chromosomes were identified as described by Levan *et al.* (1964), and karyotype symmetry was determined as described by Stebbins (1971). We also assessed other karyotypic features (Sheidai & Jalilian 2008), including total form percentage (TF%) and coefficient of variation (CV) of chromosome size, as well as the asymmetry indices (A1 and A2, respectively) devised by Romero-Zacro (1986).

Flow cytometry

We performed flow cytometry, with DAPI (4', 6-Diamidino-2-phenylindole (DAPI) staining, using *Allium cepa* (2C DNA = 33.5 pg) and *Petroselinum crispum* (2C DNA = 4.45 pg, Dolezel *et al.* 2007) as external references. We chopped 0.03 g of plant materials with a sharp razor blade (Otto 1990; Dolezel & Gohde 1995) in 400 µl of nuclei isolation buffer and 1600 µl of DAPI in a Petri dish. The suspension was then filtered through a 50-µm nylon mesh into a labeled sample tube. The relative fluorescence intensity of stained nuclei was measured on a linear scale and at least 5000 nuclei were analyzed for each sample. The total DNA content of a sample was calculated from the G1 peak means (Dolezel *et al.* 2003 & 2007; Dolezel & Bartos 2005) as follows: sample 2C DNA (pg) content = (sample G1 peak mean/standard G1 peak mean) × standard 2C DNA content (pg); and 1 pg of DNA represents 978 Mbp. We estimated nuclear DNA content for three accessions from each species using FloMax software, version 2.4d (Partec, Munich, Germany). The flow cytometry data were analyzed in a completely randomized design, with three replicates.

Statistical analysis

To identify significant differences among the species and populations studied, in terms of karyotype and genome size, we performed ANOVA followed by the least significant difference test (Sheidai & Jalilian 2008). For differences between karyotypic features, we used Pearson's correlation coefficient, whereas we used the Statistical Package for the Social Sciences, version 9.0 (SPSS Inc., Chicago, IL, USA) to compare DNA C-values and certain ecological characters.

In order to group the species studied by similarity in their karyotypic features, we used unweighted pair group method with arithmetic mean (UPGMA) clustering, together with ordination based on principal coordinate analysis (PCoA) and canonical correspondence analysis (CC). For the cluster and PCoA analyses, we used the PAST program, version 5.0 (Hamer *et al.* 2012). We also used the Manhattan distance between the species in the clustering (Sheidai & Jalilian 2008; Podani 2000). The cophenetic correlation coefficient was estimated in order to determine the goodness-of-fit of the clusters to the original data (Sheidai & Jalilian 2008).

Results

Details of the karyotype analyses in *Helichrysum* Mill. species are presented in Tab. 1, as well as in Fig. 1 and 2. The chromosome number was 2n = 10x = 60 for the Sirjan population of *H. leucocephalum*; 2n = 9x = 54 for the Neyriz and Abadeh-Tashk populations of *H. leucocephalum*; 2n = 8x = 48 for the Arsanjan population of *H. leucocephalum*, as well as for the Mavana and Payam populations of *H. globiferum*; 2n = 8x = 48 for the *H. davisianum* population (in Shirkooh); 2n = 7x = 42 for the Tabriz population of *H. globiferum*; 2n = 6x = 36 for the Ardabil population of *H. globiferum*; 2n = 4x = 32 for the *H. plicatum* population (in Solook); 2n = 4x = 28 for the populations of *H. armenium*

Table 1. Karyotypic features of species within the genus *Helichrysum* (Asteraceae) occurring in Iran.

Species	Site	TCL	L	S	L/S	SC	A1	A2	TF%	CV	KF
H. globiferum	Mavana	37.51	0.92	0.65	1.42	2B	0.7	0.32	41.27	32	20m + 4sm
	Payam	29.74	0.69	0.55	1.25	1B	0.8	0.25	44.18	25	24m
	Tabriz	49.88	1.41	0.96	1.47	1B	0.7	0.34	40.53	34	16m + 5sm
	Ardabil	27.55	0.92	0.61	1.51	1B	0.69	0.37	39.77	37	14m + 4sm
	Hamadan	18.64	0.86	0.66	1.30	2B	0.78	0.32	42.77	32	11m + 1sm
	Givi	17.59	0.89	0.65	1.37	1B	0.75	0.32	42.3	32	12m
	Jolfa	16.1	0.8	0.54	1.48	2B	0.69	0.32	40.47	32	9m + 3sm
H. leucocephalum	Aras River	20.96	1.03	0.72	1.43	2B	0.72	0.26	41.33	26	10m + 2sm
	Sirjan	54.36	1.06	0.75	1.41	2B	0.73	0.28	41.47	28	27m + 3sm
	Neyriz	47.56	1.01	0.75	1.35	1C	0.75	0.33	42.65	33	24m + 3sm
	Abadeh-Tashk	46.09	0.99	0.72	1.38	2B	0.73	0.24	41.96	24	24m + 3sm
	Arsanjan	48.56	1.18	0.86	1.37	1B	0.73	0.33	42.40	33	24m
H. armenium	Marmishoo	22.41	0.96	0.64	1.50	1B	0.68	0.25	40.18	25	11m + 3sm
H .davisianum	Shirkooh	42.55	1.04	0.73	1.42	2B	0.72	0.31	41.13	31	21m + 3sm
H. oligocephalum	Touchal	20.34	0.98	0.72	1.36	1C	0.75	0.33	42.18	33	12m
H. oocephalum	Khalaj - kooh	23.18	0.93	0.73	1.27	1B	0.79	0.21	44.13	21	14m
H. plicatum	Solook	29.24	1.04	0.79	1.32	1B	0.77	0.29	43.25	29	16m
H .rubicundum	2 km from Aras River	6.61	0.53	0.42	1.26	1B	0.82	0.29	44.06	29	6m + 1sm

PL – ploidy level; TCL – total chromosome length; L – longest chromosome; S – shortest chromosome; L/S – longest/shortest chromosome ratio; SC – Stebbins class; A1 and A2 – asymmetry index; F% – total form percentage; CV – coefficient of variation; KF – karyotype formula; m – metacentric; sm – submetacentric.

and *H. oocephalum* (in Marmishoo and Khalaj - kooh, respectively); 2n = 4x = 24 for the Jolfa, Hamadan, Givi and Aras River populations of *H. globiferum*, as well as for the *H. oligocephalum* population (in Touchal); and 2n = 2x = 14 for the *H. rubicundum* population (2 km from the Aras River).

The chromosomes were metacentric in all of the species studied, being metacentric and submetacentric in most (Fig. 2). Although four populations of *Helichrysum globiferum* had a chromosome number of 2n = 24, they differed in their karyotype formulae, which indicated that changes in chromosome structure occurred in those populations. Among the various populations of *H. globiferum*, the Aras River, Ardabil, Jolfa, Hamadan, Mavana and Tabriz populations had metacentric and submetacentric chromosomes, whereas the Givi and Pyam populations had only metacentric chromosomes. Among the four populations of *H. leucocephalum*, the Arsanjan population had metacentric chromosomes only, whereas the other three populations had metacentric and submetacentric chromosomes. Similarly, *H. armenium*, *H. davisianum* and *H.Rubicundum* had metacentric and submetacentric chromosomes, whereas *H. oligocephalum*, *H. oocephalum* and *H. plicatum* had only metacentric chromosomes.

Among the populations of *Helichrysum globiferum*, the value for total chromosome length (TCL) was highest (49.88 µm) in the Ardabil population, which is hexaploid with 2n = 36, whereas it was lowest (16.1 µm) in the Jolfa population, which is tetraploid with 2n = 24. The length of the longest chromosome ranged from 1.96 µm (in the Payam population) to 4.56 (in the Tabriz population). Among the populations of *H. leucocephalum*, the Sirjan population, which is decaploid with 2n = 60, had the highest TCL (54.36 µm) and the Abadeh-Tashk population, which is enneaploid with 2n = 54, had the lowest (46.09 µm). The length of the longest chromosome ranged from 2.81 µm (in the Abadeh-Tashk population) to 3.48 µm (in the Arsanjan population). Among all populations and species, the TCL was highest (54.36 µm) in the Sirjan population of *H. leucocephalum* and lowest (6.61 µm) in the *H. rubicundum* population (Aras River), which is diploid with 2n = 14. The size of the longest chromosome ranged from 1.47 µm (in the *H. rubicundum* population) to 4.56 µm (in the Tabriz population of *H. globiferum*, with 2n = 7x = 42).

Among the populations of *Helichrysum globiferum*, the CV for chromosome size was highest (37) in the Ardabil population. Among the populations of *H. leucocephalum*, the Arsanjan and Neyriz populations showed the highest CV value (33). Among all populations and species in this study, total form percentage (TF%) varied from 39.77 (in the Ardabil population of *H. globiferum*) to 44.18 (in the Payam population of the same species).

The *Helichrysum* species and populations studied occupied the 1B, 2B and 1C classes of the Stebbins classification, which are considered the intermediate symmetry classes of this system. The ANOVA of karyotypic features among the populations of *H. globiferum* and *H. leucocephalum*

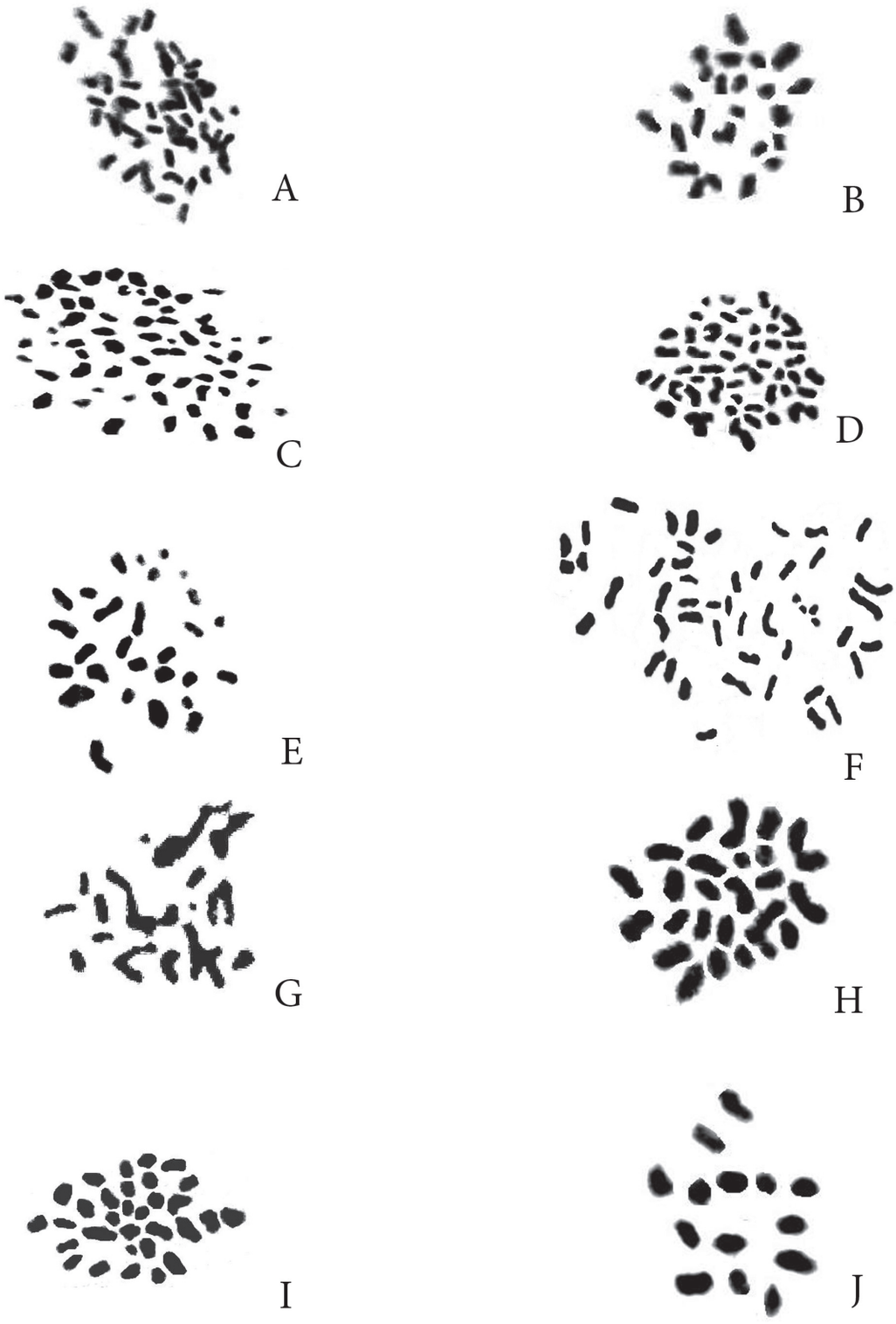

Figure 1. Representative metaphase somatic cells in the studied species of *Helichrysum* (Asteraceae): **A** and **B**) Tabriz and Givi populations of *H. globiferum*, showing 2n = 42 and 2n = 24, respectively; **C** and **D**) Sirjan and Arsanjan populations of *H. leucocephalum*, showing 2n = 60 and 2n = 48, respectively; **E**) *H. armenium*, showing 2n = 28; **F**) *H. davisianum*, showing 2n = 48; **G**) *H. oligocephalum*, showing 2n = 24; **H**) *H. oocephalum*, showing 2n = 28; **I**) *H. plicatum*, showing 2n = 32; and **J**) *H. rubicundum*, showing 2n = 14.

Scale bar = 10 μm.

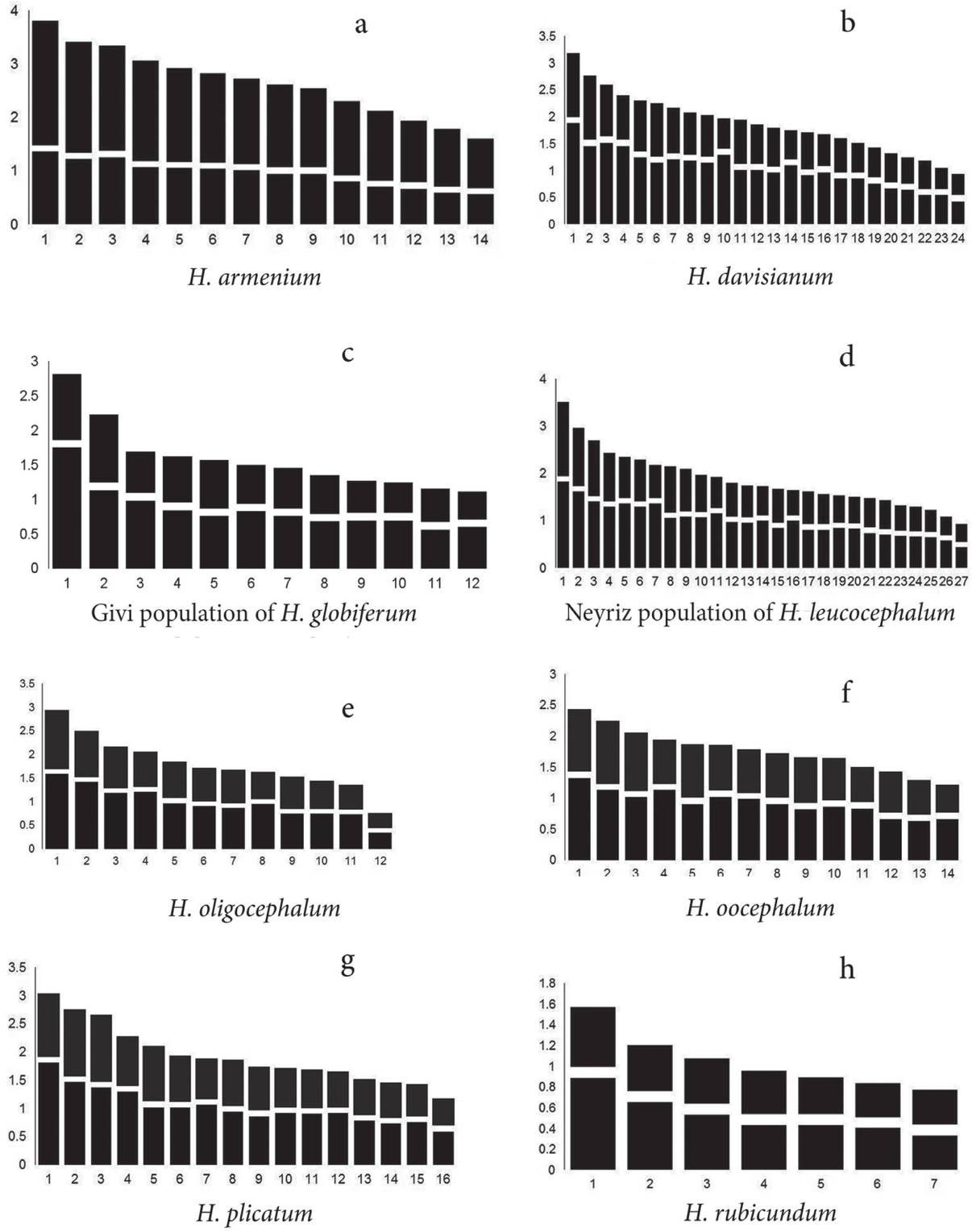

Figure 2. Representative Ideograms of the studied species of *Helichrysum* (Asteraceae): **a**) *H. armenium*; **b**) *H. davisianum*; **c**) Givi population of *H. globiferum*; **d**) Neyriz population of *H. leucocephalum*; **e**) *H. oligocephalum*; **f**) *H. oocephalum*; **g**) *H. plicatum*; and **h**) *H. rubicundum*.

Chromosome size in μm (axis Y).

showed significant difference for CV, TF% and the A2 index. Because these parameters are related to changes in chromosome size and to karyotype symmetry, significant difference in their values indicate structural changes in the chromosomes of the species studied. This is supported by the fact that TF% showed a significant positive correlation with the A1 index.

Pearson's correlation coefficient for karyotype data, DNA content and ecological features showed a significant positive correlation between TCL and ploidy level, as well as between the lengths of the long and short arms of the chromosome (r=0.45, p=0.05). We also found that humidity factor showed a significant positive correlation with TF% and with the A1 index, whereas it showed a significant negative correlation with TCL and with chromosome number. The average annual temperature correlated positively and significantly with ploidy level and with Stebbins class (i.e., with an increase in karyotype asymmetry). However, latitude correlated negatively with the A1 index.

The UPGMA clustering of the species based on karyotype data (Fig. 3) produced two major clusters. The first major cluster comprised two subclusters: one composed of five populations of *Helichrysum globiferum* (G2, G3, G6, G7 and G8), together with the populations of *H. armenium*, *H. oligocephalum*, *H. oocephalum* and *H. plicatum*; and the other composed only of the *H. rubicundum* population. The second major cluster comprised the populations of *H. leucocephalum* and *H. davisianum*, together with three populations of *H. globiferum* (G1, G4, G5), all in close proximity to each other.

According to the CC analysis (Fig. 4), the populations and species of *Helichrysum* were distributed in four groups: the first comprised five of the populations of *H. globiferum* (G2, G3, G6, G7 and G8), together with the population of *H. oligocephalum*; the second group comprised the populations of *H. armenium*, *H. oocephalum* and *H. plicatum*; the third group comprised the populations of *H. leucocephalum* and *H. davisianum*, together with the three remaining populations of *H. globiferum* (G1, G4, G5), all in close proximity; and the fourth group comprised only the population of *H. rubicundum*.

The histograms obtained for analyzing the amount of nuclear DNA in sampled leaves contained two peaks (Fig. 5), one referring to the *Helichrysum* samples and the other referring to the external references. Comparisons of the mean 2C-values among the accessions are shown in Tab. 2. The ANOVA of the flow cytometry data indicated highly significant differences (p<0.05) among the samples studied, the highest value was detected in *Helichrysum leucocephalum*, with 2n = 9x = 54, and *H. davisianum*, with 2n = 6x or 8x = 48 (2C DNA = 18.4 pg for both species), whereas the lowest value was detected in *H. rubicundum*, with 2n = 2x = 14 (2C DNA = 8.13 pg). We found that DNA content showed a significant positive correlation (p<0.05) with ploidy level, TCL and chromosome size (lengths of the long and short arms). These results clearly support our karyotype results, suggesting that, during species diversification, chromosome arm lengths, total chromosome length and genome size increase in parallel with increases in ploidy level. In addition, DNA content showed a significant positive correlation with elevation and a significant negative correlation with humidity factor.

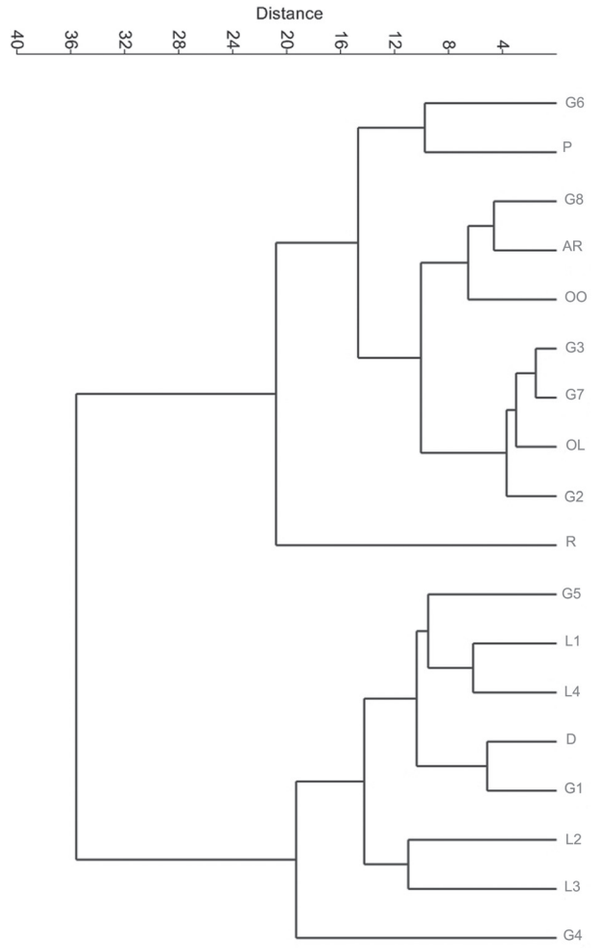

Figure 3. Unweighted pair group method with arithmetic mean clustering of the *Helichrysum* (Asteraceae) populations and species studied, based on karyotype data

G1-G8 – populations of *H. globiferum*: Mavana, Jolfa, Hamadan, Payam, Tabriz, Ardabil, Givi and Aras River, respectively; L1-L4 – populations of *H. leucocephalum*: Arsanjan, Sirjan, Abadeh-Tashk and Neyriz, respectively; D – *H. davisianum*; RU – *H. rubicundum*; OL – *H. oligocephalum*; OO – *H. oocephalum*; AR – *H. armenium*; P – *H. plicatum*.

According to the CC analysis (Fig. 4), ecological features, such as temperature, elevation and humidy factors, as well as ploidy level and C-value, were efficient in separating the species *Helichrysum leucocephalum*, *H. davisianum* and *H. globiferum* from the other species of *Helichrysum*. Rainfall was also a factor that distinguished the species *H. armenium, H. oocephalum* and *H. rubicundum*. The humidity

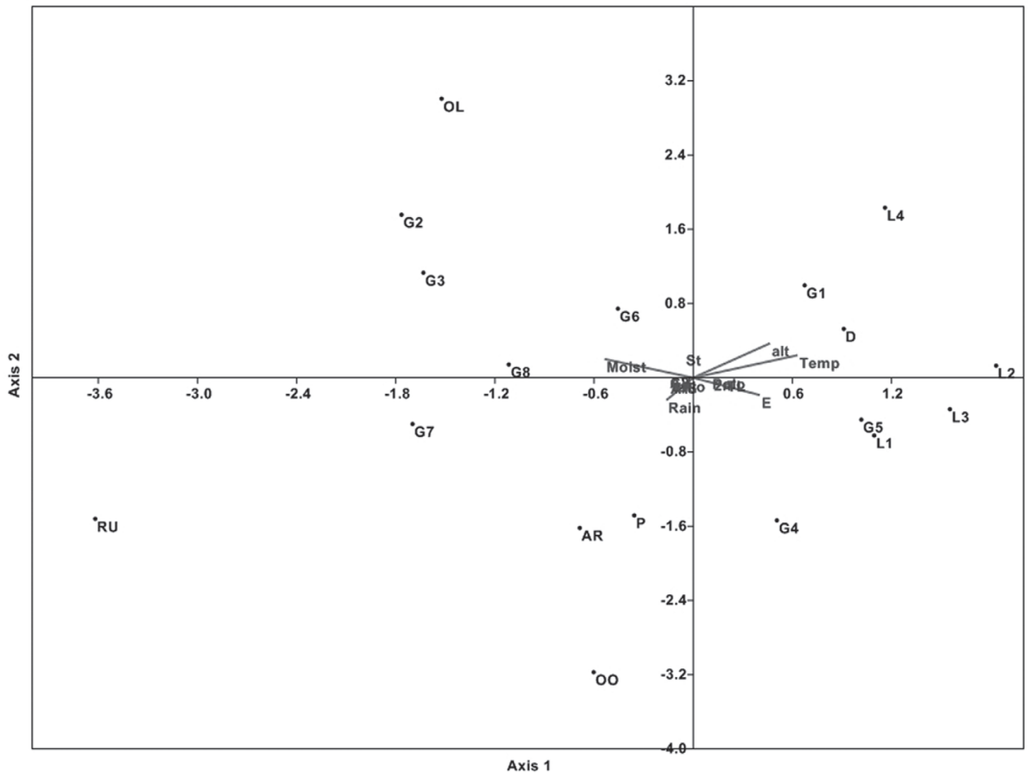

Figure 4. Canonical correspondence analysis ordination of the *Helichrysum* (Asteraceae) populations and species studied.

G1-G8 – populations of *H. globiferum*: Mavana, Jolfa, Hamadan, Payam, Tabriz, Ardabil, Givi and Aras River, respectively; L1-L4 – populations of *H. leucocephalum*: Arsanjan, Sirjan, Abadeh-Tashk and Neyriz, respectively; D – *H. davisianum*; RU – *H. rubicundum*; OL – *H. oligocephalum*; OO – *H. oocephalum*; AR – *H. armenium*; P – *H. plicatum*; Humid – humidity; St – Stebbins class; Elev – elevation; Temp – temperature; Rain – rainfall.

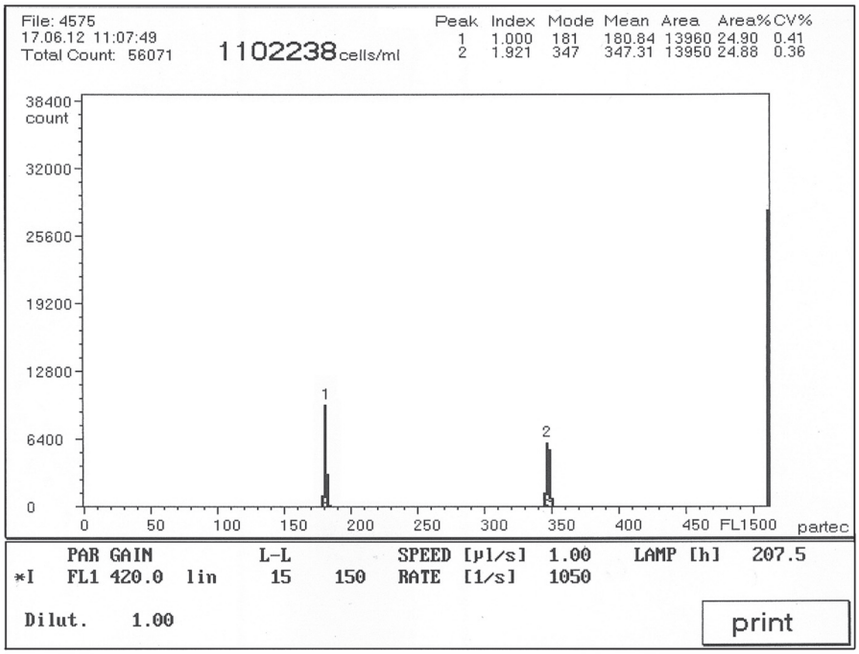

Figure 5. Histogram showing the genome size of *Helichrysum plicatum* (Asteraceae), with *Allium cepa* L. (the second peak) as the reference standard.

Table 2. Nuclear DNA content and genome size in the studied species of *Helichrysum* (Asteraceae).

Species	2C-value	Genome size
H. armenium	12.99	12704.22
H. davisianum	18. 4	17643.12
H. globiferum	17.6	17212.80
H. leucocephalum	18. 4	17643.12
H. oligocephalum	12.48	12205.44
H. oocephalum	8.7	8508.80
H. plicatum	17.5	17115.00
H. rubicundum	8.13	7951.14

parameter was found to play a major role in separating five of the *H. globiferum* populations (G2, G3, G6, G7 and G8) and *H. oligocephalum* from the other *Helichrysum* species.

Discussion

Our results provide additional support for the idea that polyploidy is the main evolutionary trend in the genus *Helichrysum*, as was also suggested by Galbany & Romo (2008). The somatic chromosome numbers reported here are comparable to those reported in earlier studies of *H. armenium* (Chariat-Panahi *et al.* 1982) and *H. rubicundum* (Ghaffari 1989), whereas the chromosome numbers reported here for *H. davisianum*, *H. globiferum*, *H. leucocephalum* and *H. oocephalum* are new to science. In addition, we have reported new chromosome numbers and ploidy levels for *H. oligocephalum* and *H. plicatum*—2n = 4x = 24 and 2n = 4x = 32, respectively—differing from the 2n = 8x = 56 previously reported for both species (Galbany & Romo 2008; Namur & Veraque 1976).

Polyploidy is considered one of the important mechanisms in the evolution of the genus *Helichrysum* (Galbany *et al*. 2006). Exception of *H.rubicundum.* the species studied here showed numerical chromosome polymorphism: within *H. globiferum*, the Jolfa, Hamadan, Givi and Aras River populations were found to be tetraploid (2n = 4x = 24), whereas the Mavana and Payam populations showed octoploidy (2n = 8x = 48) and the Ardabil population showed hexaploidy (2n = 36); among the populations of *H. leucocephalum*, we observed 2n = 8x = 48, 2n = 9x = 54 and 2n = 10x = 60; and the *H. davisianum* population was 2n = 6x or 8x = 28. Different polyploidy levels have also been reported for *H. armenium* and *H. oocephalum* (2n = 4x = 28), *H. oligocephalum* (2n = 4x = 24) and *H. plicatum* (2n = 4x = 32).

According to Galbany *et al*. (2009), the chromosome number most commonly found in *Helichrysum*, mainly in Mediterranean and Asiatic species, is 2n = 28, and two base numbers are found within *Helichrysum*: x = 4, and x = 7. However, in the present study, we observed chromosome numbers of 2n = 14, 24, 28, 32, 36, 42, 48, 54 and 60, as well as base numbers of x = 6, x = 7, and x = 8. Therefore, polyploidy is not only important for the speciation process but also plays a role in the diversification of populations within this genus.

Changes observed in the karyotype formulae of the species and populations indicate the occurrence of changes in chromosome structure within the genus. The fact that the CV obtained for the karyotype in the Ardabil population of *H. globiferum* was higher than that obtained for the karyotype in the Payam population is indicative of the occurrence of changes in chromosome structure within those populations. This also holds true for the Arsanjan and Neyriz populations of *H. leucocephalum* and is further supported by significant differences obtained for TF%, CV, and the A2 index among the *H. globiferum* and *H. leucocephalum* populations. The significant positive correlations among the species and populations studied for the total chromosomes length, lengths of the short arms and lengths of the long arms indicate that these karyotypic features change in concert during speciation events.

The *Helichrysum* species studied here seem to have relatively asymmetrical karyotypes, given that they occupy the 1B, 2B and 1C Stebbins classes. In some of the species and populations, an increase in the ploidy level was accompanied by an increase in karyotype asymmetry. However, that was not observed in any of the *H. globiferum* or *H. leucocephalum* populations studied.

To our knowledge, ours is the first report of the DNA content (genome size) of *Helichrysum* species. The nuclear DNA C-value varies among plant taxa (Yokoya *et al.* 2000), which is considered a means of adaptation. The C-value affects key parameters of plant growth, such as cell cycle, rate of cell division, sensitivity to radiation, ecological behavior in plant communities and life form (Bennett *et al*. 2000). In *Helichrysum* species, the significant correlations between C-value and some karyotypic features, including ploidy level, TCL, length of the short arm and length of the long arm, indicate that changes in chromosome structure have been accompanied by changes in DNA content.

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