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Flow cytometric estimation of the nuclear genome size of 22 Echinops (Asteraceae) taxa from Turkey

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Abstract: Taxonomic classification of the genus *Echinops* (Asteraceae) is still unclear, mostly because of the small morphological differences between the species. Estimation of genome size is helpful in species identification and in establishing a relationship between them; however, nuclear DNA content has been established for only 25% of known *Echinops* species. In the present study, in addition to the chromosome number, the DNA content of species in 22 taxa belonging to 3 sections (*Echinops*, *Oligolepis*, and *Ritropsis*) was estimated using flow cytometry; 9 of the species are endemic to Turkey. For 16 of the species this is the first report on their genome size. The chromosome numbers of the studied species were $2n$ = 28, 30, 32, or 34; the 2C DNA content ranged from 5.55 to 13.96 pg, and the mean DNA content per chromosome from 0.19 to 0.45 pg. The possible chromosome rearrangements during evolution of the genus are discussed. It is suggested that the ancestral section of the genus *Echinops* is *Oligolepis*, and the most modern one *Echinops*. The results allowed for the verification of the taxonomic position of some *Echinops* species, which previously were classified based only on morphological characteristics.

Key words: 2C DNA content, chromosome number, endemic species, flow cytometry, taxonomy

1. Introduction

The genus *Echinops* L. (Asteraceae, Cardueae) consists of approximately 120 species, distributed mostly in tropical Africa, the Mediterranean basin, and temperate regions of Eurasia (Jäger, 1987; Bobrov, 1997). In Turkey, the genus is composed of 25 species belonging to three sections, *Echinops*, *Oligolepis*, and *Ritropsis*; most of these are endemic and/or endangered (Hedge, 1975; Gemici and Leblebici, 1992; Vural et al., 2010; Vural, 2012; Vural and Şapcı, 2012). Because of the strong morphological uniformity of the *Echinops* species their taxonomic classification is very difficult to determine. In most cases, the diagnostic characters used for infrageneric delimitation are limited to the bracts of the uniflowered capitula, or their number or degree of connation of the inner bracts (Hedge, 1975; Kožuharov, 1976; Rechinger, 1979; Bobrov, 1997). The diversity of the fillaries is related to the fact that the one-seeded capitulum is the unit of dispersal, and therefore has an adaptive value (Davis, 1956; Sánchez-Jiménez et al., 2010). In addition to morphological studies, molecular techniques have been used to address the taxonomic problems of this genus (Garnatje et al., 2005; Sánchez-Jiménez et al., 2010). All studied *Echinops* species are diploids (or rather diploidized ancient polyploids); however, their basic chromosome number varies (x = 14, 15, 16, or 17; Sheidai et al., 2000; Garnatje et al., 2004a, 2004b), which, to some extent, can be used to verify the systematic position of a species. This variation indicates that there was a centric fusion/fission during species diversification, although it is not known which chromosome number is the primitive one (Sheidai et al., 2000).

Since genome size is an important karyological feature characteristic of an organism, estimation of nuclear DNA content has been used to resolve taxonomic and evolutionary problems (Godelle et al., 1993; Bennett and Leitch, 1995; Bennett, 1998; Zoldos et al., 1998; Naganowska et al., 2006; Klos et al., 2009; Chramiec-Głąbik et al., 2012). In angiosperms, evolution seems often to be accompanied by genome size changes (Garnatje et al., 2004a). Flow cytometry is a fast, simple, and accurate method to measure DNA content, and is most commonly

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used for such analyses (Doležel and Bartoš, 2005; Sliwinska et al., 2005; Doležel et al., 2007). However, 2C DNA content of *Echinops* has been reported for only about 30 species (Garnatje et al., 2004a; Sánchez-Jiménez et al., 2012; GSAD, 2014), about 25% of the total number in this genus.

The aim of the present study was to determine 2C DNA content in 26 populations of *Echinops* species belonging to three sections, *Echinops*, *Oligolepis*, and *Ritropsis*, growing in Turkey and relate it to chromosome number. Genome sizes are recorded for 22 species/subspecies, 16 of which have not been measured before, including 2C-values for 2 newly discovered species, *E. antalyensis* and *E. borae* (Vural, 2012). The results provide new information that can be used to verify the taxonomic status of *Echinops* species.

2. Materials and methods

2.1. Plant material

Because dried-preserved material of *Echinops* is unreliable (Sánchez-Jiménez et al., 2012), the fresh leaves of seedlings were used for genome size estimation. Achenes of 22 *Echinops* taxa (26 populations) belonging to 3 sections, *Echinops*, *Oligolepis*, and *Ritropsis*, were collected from wild populations in Turkey (Table 1) in 2007–2010 and identified according to Vural et al. (2010), Vural (2012), Vural and Şapcı (2012), and Vural and Dadandı (unpublished). Seedlings were grown in a growth chamber under a 16-h photoperiod at 22 ± 2 °C. Only *E. emiliae* is a biennial; all other species are perennial.

Zea mays L. CE-777 (5.43 pg/2C; Lysák and Doležel, 1998) and *Secale cereale* L. cv. Dankovske (16.19 pg/2C;

Table 1. Provenance of the studied populations of *Echinops* species from which seeds were collected.

Section	Taxon	Location	Latitude (N)	Longitude (E)	Altitude (m)
Echinops L.	*E. emiliae* P.H.Davis*	Antalya	36°32′473″	30°25′456″	2020
	E. heterophyllus P.H.Davis	Hakkari	37°40′0795″	43°44′814″	2749
	E. mersinensis Gemici & Leblebici*	Mersin	37°03′602″	34°47′133″	326
	E. microcephalus Sm.	Eskişehir	39°49′593″	30°22′615″	850
	E. onopordum P.H. Davis*	Antalya	36°38′025″	30°26′665″	970
	E. ossicus K.Koch	Amasya	40°45′000″	35°49′653″	1047
	E. pungens Trautv. subsp. *adenoclados* Hedge*	Van	38°22′722″	43°23′602″	2340
	E. pungens Trautv. subsp. *polyacanthus* (Iljin) Hedge	Erzurum	39°53′831″	42°20′786″	1713
	E. pungens Trautv. subsp. *pungens*	Kayseri	38°40′043″	35°32′358″	1750
	E. pungens Trautv. subsp. *transcaucasicus* (Iljin) Hedge	Erzurum	30°53′833″	42°20′786″	1940
	E. ritro L.	Konya	37°47′611″	33°41′304″	991
	E. sphaerocephalus L. subsp. *albidus* (Boiss. & Spruner) Kožuharov	Kırklareli	41°38′750″	27°29′664″	194
	E. sphaerocephalus L. subsp. *sphaerocephalus*	Kayseri	38°40′541″	35°32′741″	1150
	E. vaginatus Boiss. & Hausskn.	Malatya	37°59′362″	38°00′395″	1337
Oligolepis Bunge	*E. dumanii* C.Vural*	Adana	36°46′371″	35°45′420″	2
	E. melitenensis Hedge & Hub-Mor.*	Malatya	38°29′546″	38°12′937″	746
	E. phaeocephalus Hand.-Mazz. *	Şırnak	42°10′857″	37°23′404″	404
Ritropsis Greuter & Rech. f.	*E. antalyensis* C.Vural*	Antalya	36°52′768″	30°39′671″	20
	E. borae C.Vural*	Mersin	36°28′643″	34°10′294″	15
	E. orientalis Trautv. *CV 3739*	Kırşehir	39°08′373″	34°10′813″	1042
	E. orientalis CV 4413	Sivas	40°14′890″	38°06′757″	757
	E. orientalis CV 4452	Iğdır	40°06′750″	43°33′093″	1630
	E. spinosissimus Turra subsp. *bithynicus* (Boiss.) Greuter *CV 4398*	Tokat	40°06′245″	35°36′620″	767
	E. spinosissimus subsp. *bithynicus CV 4468*	Elazığ	38°49′550″	39°58′573″	1116
	E. spinosissimus subsp. *bithynicus CV 4580*	Mardin	37°05′256″	41°18′165″	460
	E. spinosissimus Turra subsp. *spinosissimus*	Antalya	36°44′619″	30°35′693″	5

*species endemic to Turkey

Doležel et al., 1998) were used as internal standards for flow cytometry.

2.2. Chromosome counting

Chromosome counting was performed for all species except *E. heterophyllus*. The chromosome number of this, for the same seed sample as used in the present study, was published previously by Vural et al. (2012). Root tips from germinated seeds were incubated in a saturated solution of α-bromonaphthalene for 16 h at 4 °C and fixed for 2 h in Carnoy's solution. The root tips were hydrolyzed in 1 N HCl for 10–15 min at 60 °C, stained with Feulgen solution, and squashed in 45% (v/v) glacial acetic acid. Cells with a good spread of chromosomes were evaluated using an Olympus BH2 microscope.

2.3. Flow cytometry

Samples of young leaves of *Echinops* and an internal standard (Table 2) were co-chopped with a sharp razor blade in a plastic petri dish in 1 mL of Galbraith's buffer (Galbraith et al., 1983), supplemented with propidium iodide (PI; 50 µg/mL) and ribonuclease A (50 µg/mL). The

Table 2. Number of chromosomes (2*n*) and nuclear DNA content (2C) of *Echinops* species in Turkey.

Section	Taxon	2*n*	DNA content 2C ± SD (pg)	2C/2*n* (pg)	Internal standard**
Echinops	*E. emiliae*	34	12.816 ± 0.074 d*	0.377 f	S
	E. heterophyllus	30	12.534 ± 0.087 e	0.418 c	Z
	E. mersinensis	30	13.565 ± 0.086 b	0.452 a	Z
	E. microcephalus	30	9.376 ± 0.062 g	0.313 g	Z
	E. onopordum	32	13.962 ± 0.101 a	0.436 b	Z
	E. ossicus	30	7.880 ± 0.165 j	0.263 j	Z
	E. pungens subsp. *adenoclados*	32	13.043 ± 0.058 c	0.408 d	Z
	E. pungens subsp. *polyacanthus*	32	12.598 ± 0.038 e	0.394 e	Z
	E. pungens subsp. *pungens*	32	13.465 ± 0.035 b	0.421 c	Z
	E. pungens subsp. *transcaucasicus*	32	12.585 ± 0.100 e	0.393 e	Z
	E. ritro	30	9.166 ± 0.075 h	0.305 h	S
	E. sphaerocephalus subsp. *albidus*	32	6.166 ± 0.119 p	0.193 p	S
	E. sphaerocephalus subsp. *sphaerocephalus*	30	8.415 ± 0.051 i	0.280 i	Z
	E. vaginatus	30	11.974 ± 0.107 f	0.400 e	Z
Mean for the section			**11.253**	**0.361**	
Oligolepis	*E. dumanii*	28	6.595 ± 0.074 mn	0.235 n	S
	E. melitenensis	30	5.549 ± 0.019 r	0.185 r	S
	E. phaeocephalus	28	6.814 ± 0.087 l	0.245 lm	S
Mean for the section			**6.319**	**0.222**	
Ritropsis	*E. antalyensis*	30	7.632 ± 0.082 k	0.254 k	Z
	E. borae	28	5.559 ± 0.095 r	0.199 p	S
	E. orientalis CV 3739	28	6.392 ± 0.101 no	0.228 o	S
	E. orientalis CV 4413	28	6.328 ± 0.034 op	0.226 o	S
	E. orientalis CV 4452	28	6.285 ± 0.078 op	0.224 o	S
	E. spinosissimus subsp. *bithynicus CV 4398*	28	6.793 ± 0.080 lm	0.243 lm	S
	E. spinosissimus subsp. *bithynicus CV 4468*	28	6.958 ± 0.071 l	0.249 kl	S
	E. spinosissimus subsp. *bithynicus CV 4580*	28	6.753 ± 0.090 lm	0.241 mn	S
	E. spinosissimus subsp. *spinosissimus*	28	6.364 ± 0.096 o	0.227 o	S
Mean for the section			**6.563**	**0.232**	

*Values in columns followed by different letters are significantly different at P = 0.05 (Tukey's test).
**Z, *Zea mays*; S, *Secale cereale*

suspension of nuclei was passed through a 50-µm mesh nylon filter and analyzed directly after preparation using a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer, equipped with a high-grade solid-state laser with green light emission at 532 nm, long-pass filter RG 590 E, DM 560 A, as well as with side (SSC) and forward (FSC) scatters. For each sample, nuclear DNA content in 7000–10,000 nuclei was measured, using linear amplification. Analyses were performed on 5 individuals per population. Histograms were collected as FCS files and evaluated manually by FloMax software (Partec GmbH, Münster, Germany), using gating. The coefficient of variation (CV) of the G_0/G_1 peak of *Echinops* species ranged between 1.99% and 5.72%. Nuclear DNA content was calculated according to the following equation (Galbraith et al., 1997):

sample 2C DNA content (pg/2C DNA) = (sample G_1 peak mean/standard G_1 peak mean) × standard 2C DNA content

Additionally, mean DNA content per chromosome (2C/2*n*) was calculated.

2.4. Statistics

The results were statistically evaluated using one-way analysis of variance and Tukey's test (P = 0.05). The Spearman's coefficient of rank correlation was calculated to quantify the relationship between the chromosome number, DNA content per chromosome, genome size, and altitude.

3. Results and discussion

Genome size (2C-value) and chromosome number (2*n*) are crucial parameters for establishing lineage diversification within the genus *Echinops* (Sánchez-Jiménez et al., 2012). Using these characteristics, Sánchez-Jiménez et al. (2012) created a phylogenetic tree and were able to deduce some cytogenetic changes taking place in its evolutionary history. However, since phylogenetic karyological changes within *Echinops* are still not clear, further studies on a wider range of species were suggested. The present study reports both the 2C DNA content and 2*n* for 26 *Echinops* populations of 22 taxa from Turkey (Tables 1 and 2; Figures 1 and 2). The 2C DNA amounts ranged from 5.55 pg in *E. melitenensis* to 13.96 pg in *E. onopordum* (Table 2; Figure 1A and 1B). In 9 species the 2C-value was higher than the highest previously reported (10.30 pg in *E. talassicus*; Garnatje et al., 2004a; GSAD, 2014). According to the classification proposed by Leitch et al. (1998), in the section *Echinops* all species studied here, except for *E. sphaerocephalus* subsp. *albidus*, possessed an intermediate genome size (over 7 pg/2C) and in the other 2 sections a small genome size (below 7 pg/2C). The only species with an intermediate genome size in the section *Ritropsis* was *E. antalyensis* (Figure 1C).

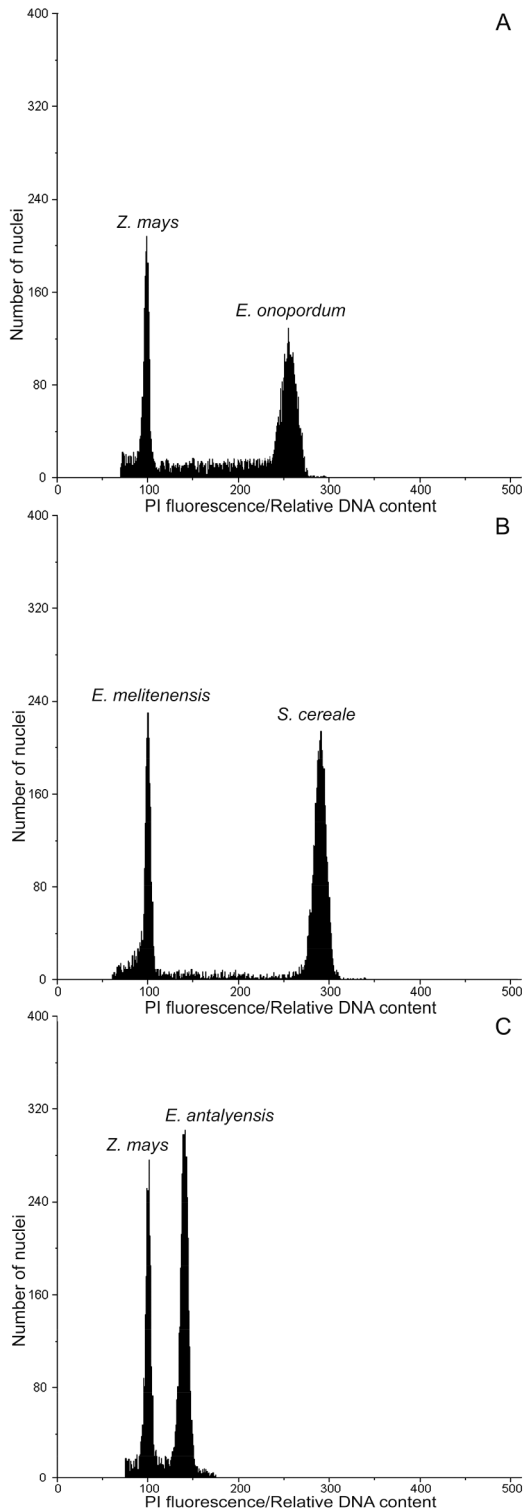

Figure 1. Selected histograms of nuclear DNA contents of *Echinops* species belonging to sections: A = *Echinops*, B = *Oligolepis*, C = *Ritropsis*.

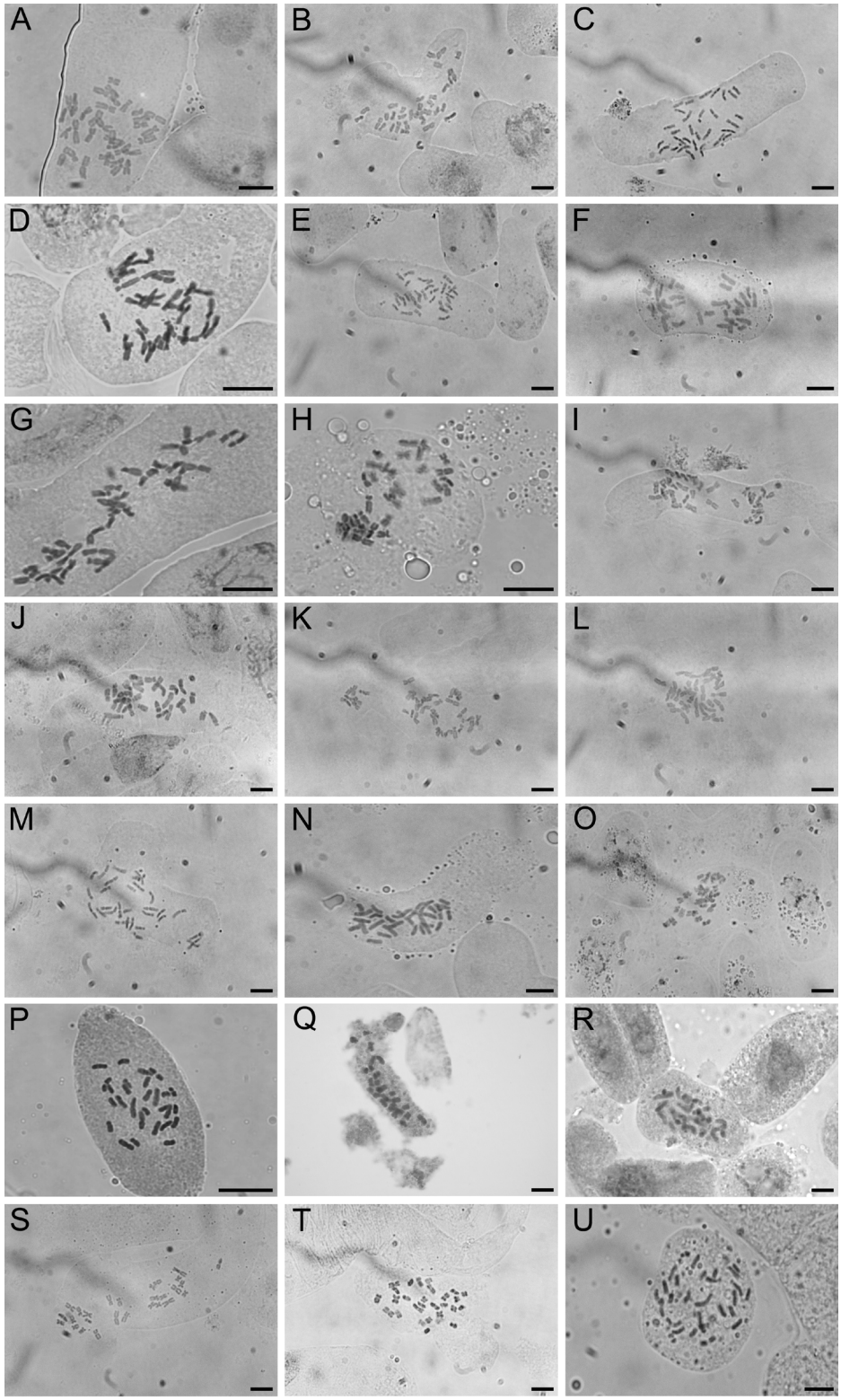

Figure 2. Chromosomes plates of *Echinops* subsp.: A = *E. emiliae*, B = *E. mersinensis*, C = *E. microcephalus*, D = *E. onopordum*, E = *E. ossicus*, F = *E. pungens* subsp. *adenoclados*, G = *E. pungens* subsp. *polyacanthus*, H = *E. pungens* subsp. *pungens*, I = *E. pungens* subsp. *transcaucasicus*, J = *E. ritro*, K = *E. sphaerocephalus* subsp. *albidus*, L = *E. sphaerocephalus* subsp. *sphaerocephalus*, M = *E. vaginatus*, N = *E. dumanii*, O = *E. melitenensis*, P = *E. phaeocephalus*, Q = *E. antalyensis*, R = *E. borae*, S = *E. orientalis*, T = *E. spinosissimus* subsp. *bithynicus*, U = *E. spinosissimus* subsp. *spinosissimus*. Scale bars = 10 μm.

Most of the species belonging to the Asteraceae are reported to have the basic chromosome number $x = 9$ (Semple and Watanabe, 2009). However, in the present research none of the *Echinops* species was found to have a chromosome number that is a multiple of 9; thus it is likely that the ancient ancestral species possessed different x values, possibly 7 or 8 (Table 2; Figure 2). Based on karyological studies on 14 *Echinops* species, Garnatje et al. (2004b) suggested $x = 8$ as the ancestral basic chromosome number. On the other hand, during polyploidization, genome downsizing often occurs (Leitch and Bennett, 2004; Leitch et al., 2008) and $2n = 34$ in *E. emiliae*, as reported here (Table 2), as well as $2n = 36$ in *E. transcaucasicus* Iljin (Sánchez-Jiménez et al., 2010), would rather suggest an ancient basic chromosome number of $x = 9$. Different chromosome numbers accompanied by similar DNA contents (e.g., *E. emiliae*, *E. heterophyllus*, and *E pungens*; Table 2) are suggestive of dysploidy (change in chromosome number through chromosomal rearrangements without considerable alteration in DNA content; Garnatje et al., 2004b; Lavia and Fernández, 2008) during *Echinops* speciation. Variable mean DNA content per chromosome (2C/2*n*), which ranged from 0.185 pg in *E. melitenensis* to 0.452 pg in *E. mersinensis*, confirms a rather complex rearrangement of chromosomes during *Echinops* evolution, including chromosome breakages, deletions, duplications, and fusions. Nevertheless, a very strong correlation between 2C DNA and 2C/2*n* still exists (Table 3).

3.1. Section *Echinops*

Despite detailed morphological and molecular studies, the section *Echinops* is not clearly defined. It contains a high number of taxa, which are scattered in phylogeny (Sánchez-Jiménez et al., 2012). Moreover, the range of DNA contents and chromosome numbers in this section is wider than in the other 2 studied here (Sánchez-Jiménez et al., 2012, present results). The number of chromosomes in Turkish species from the section *Echinops* varied ($2n = 30, 32, 34$) and species with a chromosome number higher than 30 were present only in this section; the highest $2n = 34$ was in *E. emiliae* (Table 2). Such a 2*n* value (34) is not known for any other species in the genus *Echinops* and the only known species with a higher chromosome number is *E. transcaucasicus*, from the same section ($2n = 36$; Sánchez-Jiménez et al., 2010). However, *E. emiliae* is morphologically very different from the other species within the section *Echinops* (Vural and Dadandı, unpublished). In addition, all Turkish taxa in this section are perennials, while *E. emiliae* is biennial. In addition, in the phylogenetic tree generated by Sánchez-Jiménez et al. (2010), *E. emiliae*, together with *E. elbursensis* Rech. f. and *E. strigosus* L. from the section *Psectra* Endl., were grouped separately from other species of the *Echinops*. For these reasons, we suggest that *E. emiliae* should be included more appropriately in the section *Psectra* rather than in *Echinops*.

Most of the species belonging to the section *Echinops* possess a higher genome and DNA content per chromosome than species from the other two sections (mean for the section: 2C = 11.25 pg and 2C/2*n* = 0.38 pg; Table 2). Out of the 14 taxa included in this study, genome size was previously established for only 3 of them: *E. ritro*, *E. sphaerocephalus* subsp. *albidus*, and *E. sphaerocephalus* subsp. *sphaerocephalus* (Sánchez-Jiménez et al., 2012). Their estimations were slightly different, probably because a different internal standard was used for flow cytometry and/or because of a different location from which the populations were sampled.

Two species, *E. mersinensis* and *E. onopordum*, found exclusively in a few locations in Turkey, are morphologically very different from other species of the section *Echinops* (Vural and Dadandı, unpublished), and possess higher genome sizes (present study). Moreover, molecular studies suggest that *E. onopordum* does not fit into this section (Sánchez-Jiménez et al., 2012). Therefore, we suggest that these 2 species should constitute a new section.

E. sphaerocephalus subsp. *albidus* was classified as a subspecies of *E. sphaerocephalus* by Hedge (1977). However, its DNA content and chromosome number, as estimated here, are different from those of *E. sphaerocephalus* subsp. *sphaerocephalus*, and that suggests that it should be considered a separate species. Furthermore, based on morphological traits, Bobrov (1997) proposed that this taxon be recognized as a separate one, *E. albidus*.

Table 3. Correlation between chromosome number and DNA content.

Factor 1	Factor 2	Spearman's coefficient of rank correlation	Strength of correlation	P
2*n*	2C	0.68	average	0.0001
2*n*	2C/2*n*	0.62	average	0.0008
2C	2C/2*n*	0.98	very strong	0.0000
2C	Altitude	0.60	average	0.0005

A molecular phylogenetic framework created by Sanchez-Jimenez et al. (2010) confirmed the separation of *E. sphaerocephalus* subsp. *albidus* from another *E. sphaerocephalus* subspecies.

The section *Echinops* includes species that have significantly different nuclear DNA contents while possessing the same number of chromosomes, e.g., *E. sphaerocephalus* subsp. *albidus* and *E. onopordum* (2*n* = 32, but 2.3-fold genome size difference). A similar relationship occurs for closely related species of the genus *Dianthus* (Meriç and Güler, 2008), and thus confirms that morphologically similar species with the same chromosome numbers can be identified by the estimation of nuclear DNA content.

3.2. Section *Oligolepis*

Of the 3 species studied here 2 possessed 28 chromosomes and one 30 (Table 2), which is in agreement with previous estimations for other species from this section (Sánchez-Jiménez et al., 2010). The present results confirm that 2 Turkish endemic species, *E. dumanii* and *E. phaeocephalus*, only recently described (Vural and Dadandı, unpublished; Vural et al., 2010), should be included in the section *Oligolepis*. The third species included in this section, *E. melitenensis*, possessed one of the lowest genome sizes (5.55 pg/2C) and the lowest DNA content per chromosome (0.18 pg) of all studied *Echinops* species. In addition, the mean genome size (6.32 pg/2C) and 2C/2*n* (0.22 pg) for this section was the lowest of the 3 *Echinops* sections.

3.3. Section *Ritropsis*

E. antalyensis was the only species from the section *Ritropsis* with 30 chromosomes, while all the others possessed 28 (Table 2). Genome size varied between 5.56 and 6.96 pg/2C, with the mean for the section 6.56 pg/2C. In a previous study, Garnatje et al. (2004a) reported that species from the section *Ritropsis* have 28, 30, or 32 chromosomes and a mean genome size of 6.70 pg/2C, but different species were studied. However, the same chromosome numbers and similar DNA contents were established for *E. spinosissimus* subsp. *bithynicus* and *E. spinosissimus* subsp. *spinosissimus* by Sánchez-Jiménez et al. (2012).

To verify the hypothesis that altitude can influence genome size, as reported previously for some species (Rayburn and Auger, 1990; Reeves et al., 1998), we calculated correlations between 2C DNA content and this for all studied populations, and it was average (Tables 3). Additionally, we established the DNA content of *E. orientalis* and *E. spinosissimus* subsp. *bithynicus* in populations growing at different elevations (Table 1). For these 2 species, however, there were no significant differences between populations, and thus the hypothesis was not confirmed at the intraspecific level (Table 2).

It has been suggested that in angiosperms the ancestral genome size was very small or small (Soltis et al., 2003). In relation to this, the section *Echinops* is probably the most modern section of the genus, and the section *Oligolepis* is the ancestral one (Table 2).

In conclusion, the results confirm that even closely related taxa can differ in their nuclear DNA content. Therefore, this parameter can be taxonomically significant, especially when supported by macromorphological characteristics of the species. Here, the genome sizes of 16 taxa that were not previously estimated are provided. The results confirmed that karyotypic changes, such as polyploidization, dysploidy, and chromosome breakage and elimination, took place during *Echinops* evolution. However, further cytogenetic studies of *Echinops* species would be desirable to broaden our knowledge on complex chromosome rearrangements and polyploidization during speciation of this genus.

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