Genome size variation in some representatives of the genus *Tripleurospermum*

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Abstract

Genome size has been estimated by flow cytometry in 14 populations belonging to eight taxa (seven species, one of them with two varieties) of the genus *Tripleurospermum*. 2C nuclear DNA amounts range from 4.87 to 9.22 pg, and nuclear DNA amounts per basic chromosome set from 1.99 to 2.75 pg. Statistically significant differences depending on ploidy level, life cycle or environmental factors such as altitude have been found. Also, genome size is positively correlated with total karyotype length. The presence of rhizome is related to nuclear DNA content in these species.

Additional key words: *Asteraceae*, C-value, DNA amount, flow cytometry, *Matricariinae*, nuclear DNA content.

Introduction

The C-value of an organism, *i.e*. the amount of DNA in the unreplicated nuclear genome (Swift 1950), which is considered constant within a species, influences various cellular parameters, such as cell and nuclear volume and chromosome size, and developmental parameters like minimum generation time or duration of meiosis, among others (Price *et al.* 1981a,b, Bennett 1987). Many other important relationships have also been detected, *e.g*. with reproductive biology, ecology and plant distribution (Bennett 1998). Because of the number of biological correlations, C-value data can be considered a good predictor of phenotypic traits at multiple levels (Sparrow and Micksche 1961, Underbrink and Pond 1976). Thus, taxonomy, genome evolution, ecology, genomics, plant breeding, cell and molecular biology, conservation, physiology and development can all be better understood when C-value analysis is considered.

Genome size has been shown to vary over 1000-fold in angiosperms, ranging from *ca.* 0.10 pg in *Aesculus hippocastanum* L. to 127.4 pg in *Fritillaria assyriaca* Baker (Bennett and Smith 1976, 1991). Nonetheless, the true range of genome size variation is still unknown, and although knowledge of C-values is constantly increasing, the available data only represent approximately 1.5 % of the global angiosperm flora. Since 1976, a research group has been collecting any C-value estimate made in this period, and has assembled a database that encompasses all known information on plant C-values; it is available through an internet database (www.rbgkew.or.uk/cval/homepage.html, Bennett and Leitch 2003).

Tripleurospermum Sch. Bip. (*Asteraceae, Anthemideae, Matricariinae*) is a small genus of 38 species, and comprises plants often included in the genus *Matricaria* L. (Applequist 2002), there being some disagreements over the limits of the two genera (Bremer and Humphries 1993): some authors do not separate

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Tripleurospermum from *Matricaria* although the former has one adaxial and two lateral seed ribs and the latter four or five adaxial seed ribs (Bremer 1994). It belongs to subtribe *Matricariinae* which is the biggest in the *Anthemideae* in terms of the number of genera. It is mainly distributed in Europe (South East) and in temperate Asia (South West), with a few species also in North America and North Africa, and one widespread species (*Tripleurospermum perforatum*, often considered a synonym of *T. inodorum*, Applequist 2002). Some *Tripleurospermum* are used as ornamentals, others for medical purposes, like *T. maritimum*, which is claimed to repel fleas, beetles and other insects (Herrera 1995) or *T. decipiens*, in which saponines with a possible pharmacological application have been found (Mojab *et al.* 2003), whilst still others are invasive weeds (*T. inodorum / T. perforatum*, Buckley *et al.* 2001).

The principal aims of the study are: to increase the knowledge on C-values of this genus, to test the possibility of the existence of any relationship between genome size data and cytological, morphological or environmental factors and to analyze the scope of intraspecific and interspecific genome size variation within the genus, if any.

Materials and methods

Plants: The studied material includes eight taxa (seven species, one of them with two varieties) of the genus *Tripleurospermum* (Table 1). Vouchers for most materials are deposited in the Huseyin Inceer herbarium (Trabzon, Turkey). The chosen populations represent distinct life forms, ploidy levels and chromosome numbers. The two populations of *Tripleurospermum maritimum* were obtained from botanical gardens through Index Seminum, with known original location, whereas all other taxa were collected directly from natural populations. Seeds of *Petunia hybrida* Vilm. cv. PxPc6, used as internal standard for flow cytometric measurements, were obtained from the Institut des Sciences du Végétal (CNRS, Gif-sur-Yvette, France).

Karyology: Ripe achenes were germinated on wet filter paper in Petri dishes left in the dark at room temperature. Root tip meristems were pretreated with 0.05 % aqueous colchicine at room temperature for 2 h 15 min (diploid taxa) or 2 h 45 min (polyploid taxa), fixed in absolute ethanol and glacial acetic acid (3:1) for 2 - 4 h at room temperature and stored in the fixative at 4 ºC. Root tips were hydrolysed in 1 M HCl for 5 min at 60 ºC, stained in 1 % aqueous aceto-orcein for 2 - 12 h at room temperature, and squashed and mounted in a drop of 45 % acetic acid-glycerol (9:1). The preparations were observed with an optical microscope at a magnification of 1000×. The best metaphase plates were photographed. The counts and the calculation of total karyotype length were carried out using around five plates per population.

Flow cytometric measurements: DNA 2C-values of the tested species were estimated using flow cytometry. *Petunia hybrida* Vilm. cv. PxPc6 (2C=2.85 pg, Marie and Brown 1993) was used as an internal standard. Young, healthy leaf tissue from the target species and calibration standard (both cultivated in pots) were placed together in a plastic Petri dish and chopped in Galbraith's isolation buffer (Galbraith *et al.* 1983) with a razor blade. The amount of target species leaf (about 25 mm^2) was approximately twice that of the internal standard. The suspension of nuclei in the isolation buffer was filtered through a nylon mesh with a pore size of 30 µm, supplemented with 100 µg cm^{-3} ribonuclease A (RNase A, *Boehringer*, Meylan, France) and stained for 20 min with propidium iodide (*Sigma-Aldrich*, Alcobendas, Madrid, 60 µg cm^{-3}), the chosen fluorochrome (Johnston *et al.* 1999); tubes were kept on ice during staining and then left at room temperature until the measurement. For each population, five individuals were analyzed; two samples of each individual were extracted and measured independently. Measurements were made at the 'Serveis Cientificotècnics generals' of the Universitat de Barcelona using an *Epics XL* flow cytometer (*Coulter Corporation*, Hialeah, USA). The instrument was set up with the standard configuration: excitation of the sample was performed using a standard 488-nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC), and red (620 nm) fluorescence for propidium iodide were acquired. Optical alignment was based on optimized signal from 10-nm fluorescent beads (*Immunocheck, Epics Division, Coulter Corporation*). Time was used as a control for the stability of the instrument. Red fluorescence was projected onto a 1 024 monoparametric histogram. Gating single cells by their area versus peak fluorescence signal excluded aggregates. Acquisition was automatically stopped at 8 000 nuclei. The total nuclear DNA content was calculated by multiplying the known DNA content in *Petunia* by the quotient between the 2C peak positions of *Tripleurospermum* and the internal standard in the histogram of fluorescence intensities for the 10 runs, based on the assumption that there is a linear correlation between the fluorescence signals from stained nuclei of the unknown specimen and the known internal standard and the DNA amount. Mean values and standard deviations were calculated based on the results for the five individuals.

Statistics: Statistical analyses (analysis of variance and Pearson's product moment correlation) were carried out to evaluate the relationships between the studied variables

(DNA content and DNA per basic chromosome set *vs*. altitude and life cycle, among others). All the analyses were performed with the *Statgraphics Plus 5.0* program (*Statistical Graphics Corp*., Rockville, Maryland). In addition to the data obtained in the present study, those from the C-value database (www.rbgkew.org.uk/cval/homepage.html, Bennett and Leitch 2003) for the annual diploids *Matricaria chamomilla* (2n=18, 2C = 7.75 pg, Nagl and Ehrendorfer 1974), *M. discoidea* (2n=18, 2C = 4.90 pg, Nagl and Ehrendorfer 1974) and *M. matricarioides* (2n=18, 2C = 4.65 pg, Bennett 1972) were also used for the statistical analyses of the present work, bearing in mind the close affinity between the two genera, and the fact that many *Tripleurospermum* species had been previously classified as *Matricaria* members and *vice versa* (this closeness can be deduced from the complex synonymy of the species presented in Table 1).

Table 1. Provenance of the populations of *Tripleurospermum* studied.

Taxa	Origin of materials	Herbarium voucher
T. callosum (Boiss. & Heldr.) E. Hossain (*Chamaemelum callosum* Boiss. & Heldr.)	Turkey, Gumushane, Tekke koyu. 1100 m.	Hb. Inceer 120
T. callosum (Boiss. & Heldr.) E. Hossain (*Chamaemelum callosum* Boiss. & Heldr.)	Turkey, Rize, Ikizdere, Anzer koyu. 2200 m.	Hb. Inceer 136
T. callosum (Boiss. & Heldr.) E. Hossain (*Chamaemelum callosum* Boiss. & Heldr.)	Turkey, Bayburt, Kop Dagi. 2300 m.	Hb. Inceer 69
T. elongatum (Fischer & C. Meyer ex DC.) Bornm. (*Matricaria elongata* (Fischer & C. Meyer ex D.C.) Hand.-Mazz.)	Turkey, Gumushane, Torul. 1300 m.	Hb. Inceer 144
T. maritimum (L.) K. Koch (*Matricaria maritima* L.)	Iceland, Akureyri. Sandy banks near sea level.	Index Seminum Akureyri
T. maritimum (L.) K. Koch (*Matricaria maritima* L.)	Germany, Weimar, Hermstedt. 150 m.	Index Seminum Jena
T. melanolepis (Boiss.) Rech. f.	Turkey, Gumushane, Kose Dagi, 1700 m.	Hb. Inceer 113
T. oreades (Boiss.) Rech. f. var. *oreades* (*Matricaria oreades* Boiss.)	Turkey, Gumushane, Kose Dagi. 1800 m.	Hb. Inceer 106
T. oreades (Boiss.) Rech. f. var. *oreades* (*Matricaria oreades* Boiss.)	Turkey, Rize, Cat Koyu. 1150 m.	Hb. Inceer 109
T. oreades (Boiss.) Rech. f. var. *tchihatchewii* E. Hossain (*Chamaemelum tchihatchewii* Boiss.)	Turkey, Rize, Ikizdere, Sivrikaya, Koyu. 1750 m.	Hb. Inceer 104
T. repens (Freyn & Sint.) Bornm.	Turkey, Rize, Ikizdere, Cimil-Baskoy 1900 m.	Hb. Inceer 132
T. repens (Freyn & Sint.) Bornm.	Turkey, Rize, Ikizdere, Between Cimil and Baskoy. 1800 m.	Hb. Inceer 133
T. sevanense (Manden.) Pobed. (*Matricaria sevanensis* (Manden.) Rauschert, *Chamaemelum sevanense* Manden.)	Turkey, Gumushane, Kose Dagi. 1800 m.	Hb. Inceer 121
T. sevanense (Manden.) Pobed. (*Matricaria sevanensis* (Manden.) Rauschert, *Chamaemelum sevanense* Manden.)	Turkey, Gumushane, Kose Dagi. 1600 m.	Hb. Inceer 105a

Results and discussion

According to the existing data in the C-value database, this is the first study of seven of the eight taxa analysed. Previously, Nagl and Ehrendorfer (1974) used the Feulgen method (microdensitometry after Feulgen staining) to estimate the nuclear DNA amount of a diploid *T. maritimum* (2C = 5.50 pg). Indeed, this is the first flow cytometric investigation for DNA content assessment on the subtribe *Matricariinae* (Table 2).

Relationship with karyological characters: A statistically significant difference has been found between 2C values and ploidy level (mean 2C of diploids = 5.08 pg; mean 2C of tetraploids = 8.52 pg), as might be expected in a narrow group of species. This finding is quite clear and widespread. Fridlender *et al.* (2002) also detected a statistically significant difference between genome size and ploidy level in species from *Colchicum*. Similar relationships have been found in many other genera (*Achillea*, Dąbrowska 1992, *Artemisia*, Torrell and Vallès 2001, Garcia *et al.* 2004). In fact, genome size measurements are currently used as a reliable and fast method to establish ploidy level in groups of species in which the nuclear DNA amount of at least one diploid species is known (Vilhar *et al.* 2002). Moreover, data on

nuclear DNA amount in *Tripleurospermum* also show a positive correlation with karyotype length (r = 0.75, P < 0.01), as reported in *Echinops* by Garnatje *et al.* (2004).

We have found that genome size per basic chromosome set decreases with polyploidy: there is a significant difference (P < 0.0005) between diploids and tetraploids. For these two ploidy levels, diploids always present a higher nuclear DNA amount per basic chromosome set (mean genome sizes per basic chromosome set: 2.54 pg in diploids and 2.13 pg in tetraploids). A loss of nuclear DNA amount in the process of polyploidization is suggested to explain this observation (Leitch and Bennett 2004). Sharma and Sen (2002) considered that with polyploidization, chromosomes tend to diminish their size slightly, each one equally, and hypothesized that this 'strengthening' mechanism was a 'defence strategy' against the increased possibility of mutations with polyploidy (an increase in nuclear DNA amount increases the probability of mutations). Nuclear DNA loss per basic chromosome set in polyploids has been frequently reported, so genome downsizing following polyploid formation has been considered a widespread biological phenomenon (Leitch and Bennett 2004 and references therein). Some molecular processes for this genome downsizing have been suggested, such as the homoeologous pairing, which can lead to chromosome restructuring and deletions, and hence to a loss of DNA as a consequence of the breakdown in the postreplicative mismatch repair system (Comai 2000) or the selective gene loss (Ku *et al.* 2000, Simillion *et al.* 2002), among others. However, many of the underlying molecular mechanisms responsible for this phenomenon are still unknown.

Table 2. Nuclear DNA content and other karyological characters of the populations studied. Life cicle: A - annual, B - biennial; P - perennial; RP - rhizomatous perennial. 2C - nuclear DNA content (means ± SD of 10 samples). 1 pg = 978 Mbp (Doležel *et al.* 2003). TKL - total karyotype length. 2n - somatic chromosome number. 2C/p.l. - DNA per basic chromosome set (quotient between 2C nuclear DNA content and ploidy level). *Inceer and Beyazoglu (2005); **Nagl and Ehrendorfer (1974); *** Bennett (1972).

Taxa	Life cycle	2C [pg]	2C [Mbp]	TKL [µm]	2n	Ploidy level	2C/p.l.
T. callosum (Rize)	P	8.17±0.08	7 990.26	32.09±1.52	36	4x	2.04
T. callosum (Bayburt)	P	7.98±0.02	7 804.44	31.43±0.43*	36*	4x*	1.99
T. callosum (Gumushane)	P	8.18±0.10	8 000.04	32.09±1.33	36	4x	2.05
T. elongatum	B/P	4.87±0.14	4 765.86	14.00±0.54*	18*	2x*	2.44
T. maritimum (Germany)	A/B	5.50±0.05	5 379.00	-	18	2x	2.75
T. maritimum (Iceland)	A/B	9.22±0.18	9 017.16	-	36	4x	2.31
T. melanolepis	RP	4.88±0.04	4 772.64	13.15±0.75*	18*	2x*	2.44
T. oreades var. *oreades* (Gumushane)	RP	8.76±0.22	8 567.28	29.35±1.02	36	4x	2.19
T. oreades var. *oreades* (Rize)	RP	9.05±0.23	8 850.90	28.78±1.48	36	4x	2.26
T. oreades var. *tchihatchewii*	RP	8.91±0.57	8 713.98	24.78±0.64*	36*	4x*	2.23
T. repens (Rize, 1800 m)	P	8.54±0.44	8 352.12	39.30±1.23*	36*	4x*	2.14
T. repens (Rize, 1900 m)	P	8.32±0.18	8 136.96	40.50±1.52	36	4x	2.08
T. sevanense (Gumushane 1600 m)	P	8.26±0.30	8 078.28	26.15±0.75	36	4x	2.07
T. sevanense (Gumushane 1800 m)	P	8.34±0.21	8 156.52	25.12±1.13	36	4x	2.09
*Matricaria chamomilla***	A	7.75	7 579.50	-	18	2x	3.87
*Matricaria discoidea***	A	4.90	4 795.20	-	18	2x	2.45
*Matricaria matricarioides****	A	4.65	4 547.70	-	18	2x	2.32

Systematic implications: interspecific and intra-specific variability: The paucity of available data on C-value in subtribe *Matricariinae* makes it difficult to discuss about interspecific variation in these plants. Nevertheless, we have noted that the genus *Tripleurospermum* is quite homogeneous in terms of genome size: the ratio between maximum and minimum nuclear DNA amount and nuclear DNA amount per basic chromosome set is quite low in this group. This fact, and the absence of relevant morphological or ecological differences between the analysed plants, could also suggest homogeneity in the *Tripleurospermum* species. A high value of this ratio has been reported in other *Anthemideae* groups, such as in the genus *Artemisia* (particularly in subgenus *Artemisia*, Garcia *et al.* 2004).

Because C-value is considered constant within a species, the existence of variation in nuclear DNA amount under the specific level is controversial. Whilst some authors uphold the idea that the amount of nuclear genome can vary as a response to environmental changes (the genome plasticity or flexibility theory - Durrant and Jones 1971, Joarder *et al.* 1975, Ohri 1998), others

attribute this supposed variability to mistakes or methodological problems (Greilhuber 1997, 1998). Nonetheless, it is obvious that a certain degree of genuine intraspecific variation is always possible (Greilhuber 1998, Schmuths *et al.* 2004). Within some of the taxa of *Tripleurospermum* used in this study, different populations of the same species have been analyzed (Table 3). All but one of these species show a low percentage of intraspecific variation. Moreover, they belong to the same restricted geographical area (Turkey). Between the two varieties of *T. oreades* there also exists a low percentage of intraspecific variation, although a little bit higher than for the remaining species. In this taxon, we found a higher variation in nuclear DNA content between two populations of one variety than between both varieties (Table 3). Although the two populations analysed of *T. maritimum* belong to different ploidy levels (2x and 4x), and although the nuclear DNA loss per chromosome set in polyploids must be taken into account, the 19.56 % of intraspecific variation, calculated with the nuclear DNA per basic chromosome set, is still remarkable. The geographical distance between both populations and the remarkably different latitude of them, one coming from Germany (51 º) and the other from Iceland (65 º), could contribute to explaining the variability of the C-value in this species, as other authors have stated (Grime and Mowforth 1982, Ohri 1998).

Table 3. Nuclear DNA amount (2C) and intraspecific variability (calculated as a percentage of the quotient between the highest and the lowest nuclear DNA amount) of some populations of *Tripleurospermum.* [1]Nuclear DNA amount per basic chromosome set. [2]Percentage of the quotient between the highest and the lowest nuclear DNA amount per basic chromosome set.

Taxa	2C [pg]	Intraspecific variability [%]
T. callosum	7.98 (Bayburt) 8.17 (Rize) 8.18 (Gumushane)	2.51
T. maritimum	5.50 / 2.75[1] (Germany) 9.22 / 2.30[1] (Iceland)	19.56[2]
T. oreades var. *oreades*	8.76 (Gumushane) 9.05 (Rize)	3.31
T. oreades var. *tchihatchewii*	8.91	
T. repens	8.32 (Rize, 1900 m) 8.54 (Rize, 1800 m)	2.64
T. sevanense	8.26 (Gumushane, 1600 m) 8.34 (Gumushane, 1800 m)	0.97

Life cycle and environmental factors: Many studies have indicated a relationship between life cycle and nuclear DNA amount. It is believed that, in the framework of a genus, an increase in nuclear DNA amount implies a longer cell cycle. According to this, annual plants should have less nuclear DNA amount than perennials (Nagl and Ehrendorfer 1974, Rees and Narayan 1981, Bennett and Leitch 2003). In the present study, the *ANOVA* shows that annual and biennial taxa present a significantly higher genome size ($P < 0.05$) than perennials. It must be said, however, that most perennials of the subtribe *Matricariinae* are also tetraploid, while the majority of annuals and biennials are diploid. Thus, the increase in nuclear DNA amount of these perennial species comes from their tetraploid character rather than from their life cycle. In fact, the majority of polyploids are perennial plants (Jackson 1976). Another explanation could be that annual character is secondary and relatively recent in this group, so that the surplus DNA has not yet been eliminated from the genome. In summary, although many authors have found that perennials have significantly higher nuclear DNA amounts than annuals (Garnatje *et al.* 2004 and references therein), others have reported the opposite relationship (Martel *et al.* 1997, Jakob *et al.* 2004) or even the absence of a relationship between life cycle and genome size (Grime and Mowforth 1982, Garcia *et al.* 2004). The numerous exceptions clearly suggest that the correlation between these two parameters is not as clear as initially thought.

Nuclear DNA per basic chromosome set and altitude are negatively correlated ($r = -0.67$, $P < 0.01$) in the genus *Tripleurospermum*: there is a decrease in genome size with the altitude. This is in agreement with the negative correlation found between these two parameters in wild populations of *Arachis duranensis* (Temsch and Greilhuber 2001). On the other hand, these data do not support the observations of Bennett (1976) and Rayburn and Auger (1990), who suggested that increased nuclear DNA amount was an adaptation to altitude. An increase in nuclear DNA amount with increasing altitude was also detected in many different genera (Laurie and Bennett 1985, Godelle *et al.* 1993, Cerbah *et al.* 1999). Suda *et al.* (2003) suggested that the nuclear DNA amount of endemics to Tenerife was negatively correlated with altitude in genera distributed over a large altitudinal range, and genera with a limited range in altitude showed a positive correlation between DNA content and altitude. Again, the link between altitude and genome size is uncertain, and clearly varies diversely in different plant groups; a mechanism to explain this phenomenon is still lacking.

The diploid and tetraploid populations of *T. maritimum* show the highest nuclear DNA amounts within the diploid and tetraploid *Tripleurospermum* studied here. This species occupies sandy places near the sea, very dry areas which exhibit elevated salinity, implying relatively adverse conditions for the growth of vegetation. Various studies have shown that plants inhabiting arid or extreme environments (such as deserts and highly nitrogenated soils) tend to have an increased nuclear DNA amount in comparison with their relatives

living under more favourable environmental conditions (Garcia *et al.* 2004 and references therein). Consequently, it is conceivable that an adaptation to these extreme habitats increases genome size in *Tripleurospermum*. Nevertheless, more data will need to be collected before such a relationship can be clearly established.

Within the Turkish tetraploid species of *Tripleurospermum* studied, the rhizomatous ones were found to have a significantly higher nuclear DNA amount (P = 0.0005) than those without rhizome (mean 2C of rhizomatous ones = 8.90 pg; mean 2C of non rhizomatous ones = 8.26 pg). Rhizomes give plants the ability to colonize habitats, and it is plausible that this adaptive advantage could be related to the higher nuclear DNA amount in these species, as stated previously in relation to salinity. Possibly as a result of the presence of rhizomes, these plants show a lower incidence of sexual reproduction. Consequently, chromosomes and nuclear DNA amount are less important, and variations, such as mutations, deletions or an increase in genome size, can be easily tolerated.

Concluding remarks: This study represents the first relatively extensive survey of nuclear DNA amount in the genus *Tripleurospermum*, accounting for 20 % of the taxa. Although it does not permit absolute and definitive conclusions, it contributes as a first step towards a genome size analysis of this group of plants. A good phylogenetic context within which to analyse these data would be useful in order to study the systematics of the genus, and would help in achieving a better understanding of the results. Thus, further research in this area will be necessary.

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