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ORIGINAL ARTICLE

Nuclear DNA content in the polyploid complex *Turnera ulmifolia* (*Turnera* L., Passifloraceae)

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Abstract *Turnera ulmifolia* constitutes a well-studied polyploid complex with allo- and autopolyploid species ranging from 2 to 8x. Flow cytometry was used to determine nuclear DNA content, and to estimate 2C- and 1Cx-values with the aim of analysing the genome size in *Turnera* in terms of polyploid speciation. The 2C-value and 1Cx-value were evaluated in 12 species of the *T. ulmifolia* complex. Nuclear DNA content was estimated by flow cytometry of nuclei stained with propidium iodide. The 2C DNA content ranged from 1.38 to 1.83 pg in diploids, from 2.67 to 3.96 pg in tetraploids, from 2.73 to 4.31 pg in hexaploids, and from 3.53 to 5.90 pg in octoploids. The 1Cx-value ranged from 0.44 to 0.99 pg. The *Turnera ulmifolia* complex showed an increase in total DNA content in the ploidy level, but not in the expected proportion. The general tendency indicated a decrease in the 1Cx-value with increasing chromosome number, with *T. grandidentata* 4x being an outstanding exception. The 1Cx-values in the allooctoploids *T. aurelii* and *T. cuneiformis* differed by 1.6-fold from each other, probably as a result of different evolutionary histories following divergence from the last common ancestor.

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Introduction

Turnera L. is a valuable model to explore polyploid evolution (Arbo and Fernández 1983, 1987; Arbo and Espert 2009; Fernández 1987, 1997; Fernández and Arbo 1989, 1990, 1993a, b, 1996, 2000a, b; Fernández and Solís Neffa 2004; Fernández et al. 2010; López et al. 2010a, b; Solís Neffa 1996; Solís Neffa and Fernández 1993, 2000) and breeding system shifts (Barrett 1978; Barrett and Shore 1985, 1987; Labonne and Shore 2011; Labonne et al. 2010; Safavian and Shore 2010; Shore 1986; Shore and Barrett 1985a, b; Shore and Triassi 1998; Shore et al. 1994, 2006; Truyens et al. 2005) in flowering plants.

This genus is represented by about approximately 140 species native to the Americas, ranging from the southern USA to central Argentina, and two species native to Africa (Arbo 1997, 2000, 2005; Urban 1883). *Turnera* is divided into nine series (Urban 1883), the *Anomalae* Urb., *Annulares* Urb., *Capitatae* Urb., *Leiocarpae* Urb., *Microphyllae* Urb., *Papilliferae* Urb., *Salicifoliae* Urb., *Stenodictyae* Urb., and *Turnera* (=*Canaligerae*) (Urb.) Arbo. Species of *Turnera* may be herbaceous annuals or perennials, shrubs, or trees. Pollination occurs by a wide diversity of bees and butterflies (Barrett 1978). Cladistic analyses were made based on morphological characters and chromosome basic numbers in order to test the monophyly of the nine series of the genus *Turnera*. Series *Turnera* was identified as monophyletic (Arbo and Espert 2009). This series is further divided into two groups based on seed morphology—subseries *Turnera* and subseries *Umbilicatae*. The subseries *Turnera*

constitutes the polyploid complex *Turnera ulmifolia*, which is represented by 19 species that can be differentiated on the basis of morphological characters including homo- and heterostyly, flower colour, presence or absence of dark petal spots, indumentums, and seed characters. Series *Turnera* has $x = 5$ (Barrett 1978; Fernández 1987; Solís Neffa and Fernández 2000) and a fundamental karyotype of 8m + 2sm (Solís Neffa 1996; Solís Neffa and Fernández 1993). *Turnera* chromosomes are small according to the classification of Lima de Faría (1980) (Solís Neffa and Fernández 1993). The analysis of karyotype parameters of *Turnera* suggests that species can be differentiated by the number, mean length, and symmetry of their chromosomes, and the position of the nucleolus organizer regions (López et al. 2010a, b; Solís Neffa 1996; Solís Neffa and Fernández 1993, 2000).

It is known that variations in chromosome number naturally cause some variation in DNA content (Eilam et al. 2010; Greilhuber 1998; Ozkan et al. 2010; Soltis et al. 2009). Furthermore, it has been well documented in many taxa that some karyological traits such as variation in the copy number of repetitive DNA sequences are correlated with variations in nuclear DNA amount (Albach and Greilhuber 2004; Anssour et al. 2009; Springer et al. 2009). At present, The Plant DNA C-values Database (http://data.kew.org/cvalues/) contains 7,058 plant species (Bennett and Leitch 2010). Following these authors, C-value is defined as the DNA amount in the unreplicated gametic nucleus of an organism, irrespective of the ploidy level of the taxon. We will also use the term Cx-value as the DNA content of a monoploid genome with chromosome base number x (Greilhuber 2005), which we calculate by dividing the 2C-value by the ploidy level.

Knight et al. (2005) explored the distribution of the genome size among angiosperms using the Plant DNA C-values Database and concluded that the most common sizes are below 5 pg. This implies that genome size increases are rare and have occurred in only a few groups. It is also well known that there is a loss of DNA following polyploid formation. Leitch and Bennett (2004) referred to this as "genome downsizing" and proposed three possible processes for its occurrence: (1) activation of transposons, (2) homologous recombination, and (3) elimination of specific DNA sequences. Genome size data are essential in plant systematic and phylogenetic studies (Bennett and Leitch 2005a, b; Soltis et al. 2009). Any attempt to reconstruct ancestral genome sizes of angiosperms has been complicated by the fact that polyploidy is widespread throughout their evolutionary history (Soltis et al. 2005, 2009).

Flow cytometry has been widely used in various aspects of plant research, mainly to determine DNA nuclear content (Bennett and Leitch 1995, 1997, 2001, 2005a, b; Bennett et al. 1997; Hanson et al. 2001a, b; Leitch and Hanson 2002; Leitch et al. 1998, 2001, 2005; Obermayer et al. 2002; Zonneveld et al. 2005) and to explore ploidy level (De Laat et al. 1987; Sartor et al. 2009; Siena et al. 2008; Roberts et al. 2009). There are no reported measurements of DNA content in *Turnera* species, but genome size variation has been explored in species of *Passiflora* L. (Souza et al. 2004; Zonneveld et al. 2005), a closely related genus (APG III 2009).

The aims of this work are to use flow cytometry to determine nuclear DNA content in species of the subseries *Turnera* and to discuss the relationship between the species of the *T. ulmifolia* complex from the general view of the polyploid evolution that may have occurred in this group.

Materials and methods

Plant material

Analyses were performed on plants corresponding to 12 species of the *Turnera ulmifolia* complex (Table 1). *Turnera* specimens from across its distribution have been cultivated at the greenhouse in the Botanical Institute of the Northeast (IBONE) since 1982; voucher specimens were deposited in the herbarium CTES.

Methods: flow cytometric analysis of nuclear DNA content in *Turnera*

Petals, rather than leaves, were used for all measurements of *Turnera*, because of the considerable amount of secondary compounds present in *Turnera* leaves. A comparison of measurements made with leaves and petals of the same plant confirmed that results from these two tissues are comparable. To set up the internal standard, leaves of *Paspalum dilatatum* ssp *flavescens* (tetraploid, 2C = 2.43 pg) (Vaio et al. 2007) were used to measure the DNA content of leaves of *T. subulata* 2x. After that, petals of *T. subulata* 2x (2C = 1.38 pg; Table 1) were used as the internal standard for all other *Turnera* specimens. The selection of *P. dilatatum* ssp *flavescens* was made since it is the common standard used in the laboratory where the flow cytometer is situated (Laboratory of Genetics, IBONE, Corrientes, Argentina).

For flow cytometric analysis, suspensions of intact nuclei were prepared according to Otto (1990). Briefly, plant tissue was chopped with a razor blade in a petri dish containing 0.5 ml of buffer Otto I (0.1 M citric acid and 0.5% Tween 20). The chopped material was filtered through a 30 µm nylon mesh and incubated with 2 ml of buffer Otto II (0.4 M Na2HPO4.12H2O, with 1 µg/µl of propidium iodide), and then analysed by flow cytometry.

The flow cytometric estimation of nuclear DNA content was performed using a Partec PA II flow cytometer (Partec

Table 1 Taxon, collector, locality, ploidy level, 2C-value in pg, and 1Cx-value of all the *Turnera* studied

Taxon	Collector, locality	Ploidy	2C in pg.	1Cx
T. krapovickasii Arbo	Ahumada 4549, Bolivia, Tarija, O′Connor	2x	1.83 (±0.03)	0.91
	Schinini 19514, Argentina, Salta, Anta, El Quebrachal	4x	3.18 (±0.06)	0.53
	Krapovickas and Cristobal 40303, Argentina, Salta, Anta	4x	3.36 (±0.06)	0.56
	Solís Neffa 1495, Bolívia, Santa Cruz, Chiquitos	4x	3.31 (±0.08)	0.55
T. subulata Sm.	Arbo 2410, Brasil, Maranhao, Sao Luis	2x	1.38 (±0.03)	0.69
	Krapovickas & Cristóbal 37115, Brasil, Pará, Santarem	4x	2.67 (±0.04)	0.67
T. grandiflora (Urb.) Arbo	Fernández 366, Argentina, Corrientes, Paso de la Patria	2x	1.62 (±0.12)	0.81
T. grandidentata (Urb.) Arbo	Arbo 6109, Paraguay, Paraguarí	4x	3.96 (±0.06)	0.99
T. orientalis (Urb.) Arbo	Cabral 358, Argentina, Misiones, Teyú Cuaré	6x	3.57 (±0.08)	0.60
T. occidentalis Arbo & Shore	Sagástegui A 14896, Perú, Cajamarca, Contumaza, Jaguey	6x	3.50 (±0.09)	0.58
T. campaniflora Arbo, Shore & Barrett	Barret 1337, Jamaica, Quaco Rock	6x	2.76 (±0.06)	0.46
T. velutina Presl	Koch & Fryxell 78341, México, Oxaca, Santo Domingo	6x	4.34 (±0.06)	0.72
T. ulmifolia L. var. *ulmifolia*	Arbo 2698, USA, Florida	6x	2.75 (±0.08)	0.46
T. cuneiformis Juss. Ex Poir	Arbo 5449, Brasil, Bahia, Jacobina	8x	3.56 (±0.03)	0.44
T. aurelii Arbo	López and Vanii 111, Argentina, Formosa	8x	5.58 (±0.13)	0.70
T. fernandezii	Dematteis et al. 2908, Paraguay, Amambay	8x	5.94 (±0.27)	0.74

GmbH, Münster, Germany). At least 5,000 nuclei were analysed in each sample. All parameters considered (peaks, means, and coefficients of variation) were calculated using FloMax software (Partec). Nuclear genome size of each sample was then calculated using the formula:

Sample 2C DNA content = [(sample G1 peak mean)/(standard G1 peak mean)] × standard 2C DNA content (pg DNA).

Statistical analyses to estimate the genome size of each species were performed using InfoStat software (2008).

Results and discussion

The 2C-value of the species of the *Turnera ulmifolia* complex range from 1.38 pg in the diploid *T. subulata* Sm to 5.94 pg in the octoploid *T. fernandezii* Arbo. In the analysis of the relationship between 2C-value and ploidy level (Fig. 1a), three groups can be differentiated as follows: (1) diploid accessions of *T. subulata*, *T. grandiflora* (Urb.) Arbo, and *T. krapovickasii* Arbo; (2) tetraploid accessions of *T. subulata* and *T. krapovickasii*, tetraploid *T. grandidentata* (Urb.) Arbo, hexaploids *T. velutina* Presl, *T. orientalis* (Urb.) Arbo, *T. occidentalis* Arbo and Shore, and octoploid *T. cuneiformis* Juss. Ex Poir; and (3) octoploids *T. fernandezii* and *T. aurelii* Arbo. There is a trend for increasing 2C-value with increasing chromosome number, but not in the expected proportion (Fig. 1a, shaded line). In polyploid angiosperms, genome downsizing appears to be the general trend (Kellogg and Bennetzen 2004; Leitch and Bennett 2004), and *Turnera* seems not to be an exception. The 1Cx-value also reflects the genome downsizing process, ranging from 0.44 pg in *T. cuneiformis* (8x) to 0.98 pg in *T. grandidentata* (4x) (Table 1), and decreasing in inverse proportional to the ploidy level (Fig. 1b).

Some peculiarities to point out are the behaviour of certain sister species: *T. orientalis*/*T. occidentalis*; *T. ulmifolia*/*T. campaniflora*; and *T. cuneiformis*/*T. aurelii*. Allohexaploids *T. orientalis* (2C = 3.57 pg) and *T. occidentalis* (2C = 3.50 pg) show similar values of DNA content in accordance with their related morphological and cytological traits (Arbo 2005; López et al. 2010b). Also, the allohexaploids *T. ulmifolia* (2C = 2.75 pg) and *T. campaniflora* (2C = 2.76 pg) have similar DNA content, in agreement with the hypothesis of Baker and Shore (1995) concerning a common origin for both entities and the subsequent differentiation of *T. campaniflora*, considering their particular floral morphology and adaptation to different pollinators. Molecular phylogeny based on nuclear and chloroplast sequences also reinforces the close relationship between *T. orientalis* and *T. occidentalis*, and between *T. ulmifolia* and *T. campaniflora* (López 2009; Truyens et al. 2005).

Allooctoploids *T. cuneiformis* and *T. aurelii* possess a remarkable difference in their genome size (3.56 and 5.58 pg respectively). Based on cytogenetic analyses, phylogenetic studies, and geographical distribution, the hexaploid *T. orientalis* has been proposed to be one of the putative progenitors of the allooctoploids *T. aurelii* and *T. cuneiformis*, while the other progenitor remains unknown (Fernández and Arbo 1993b, 2000a; López 2009; Truyens et al. 2005). Several lines of evidence indicate that genome size evolution is dynamic, with both increases and decreases having taken place (Leitch et al. 2005; Lysak

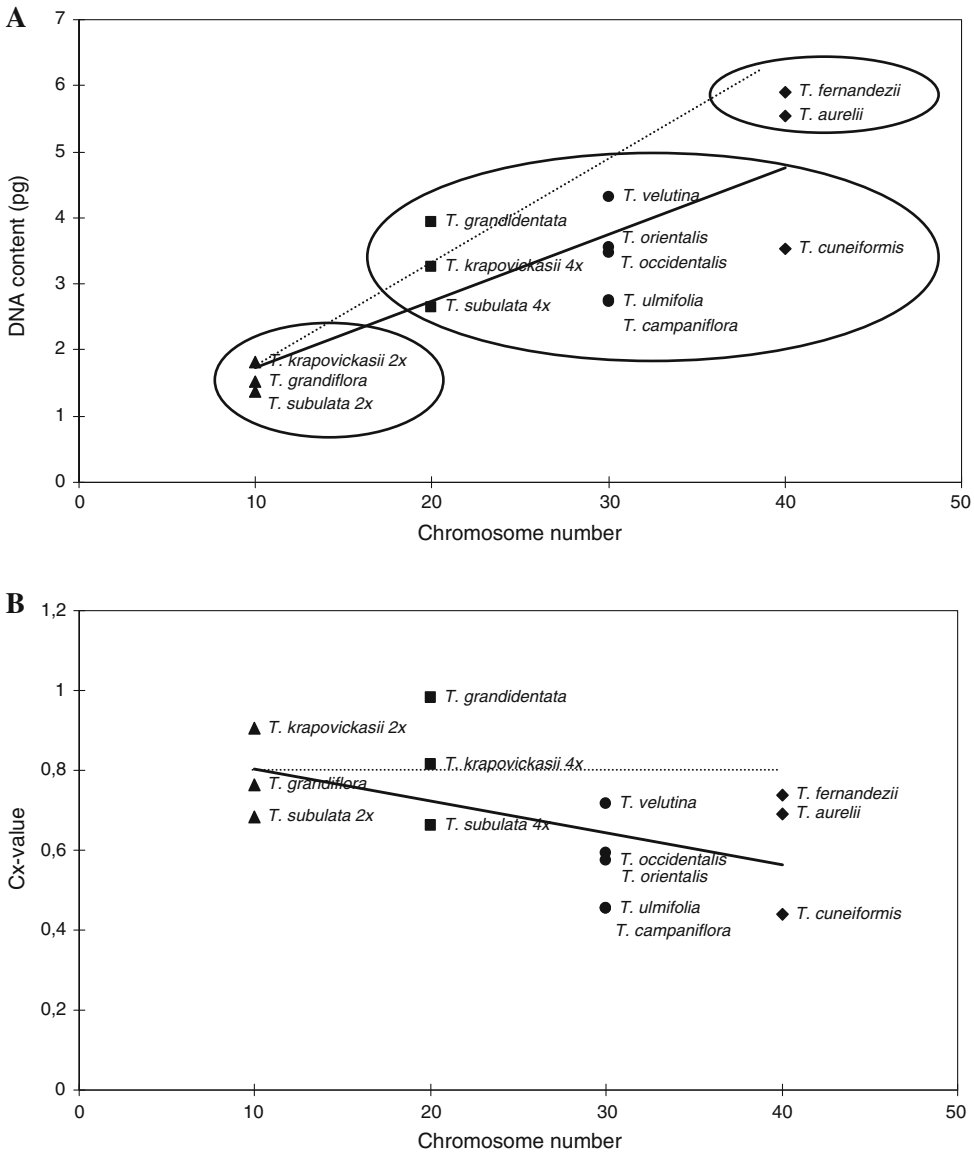

Fig. 1 **a** Scatter plot between DNA content and chromosome number. *Circles* denote three groups differentiated by DNA content (see text). **b** Scatter plot between Cx-value and chromosome. Symbols represent: *triangle*, species 2n = 2x = 10; *square*, species 2n = 4x = 20; *circle*, species 2n = 6x = 30; *diamond* 2n = 8x = 40. Tendency line observed (*continuous line*); tendency line expected (*shaded line*)

et al. 2009; Rabinowics 2000; Soltis et al. 2005; Wendel et al. 2002; Zonneveld 2010). In this sense, the ~1.6-fold difference in genome size between both octoploids could be explained by two different assumptions dealing with the constitution of the unknown progenitor, as follows: first, both species originated from a common diploid progenitor, and during speciation they underwent different divergence processes involving DNA content increase (*T. aurelii*) and decrease (*T. cuneiformis*); second, the unknown progenitor was different for each species, so that different genomic interactions may have differentially affected the processes involved in the variation of DNA content.

It is interesting to point out that the 1Cx-value of the allotetraploid *T. grandidentata* (Cx = 0.98 pg.) is the highest of all the species of *Turnera* analysed and 2.3-fold higher than the smallest one. The cytogenetic analysis of *T. grandidentata* revealed a segmental allopolyploid constitution originated by hybridisation of two different species and subsequent chromosome duplication (Fernández 1987; Fernández and Arbo 1990). A possible explanation for this high 1Cx-value is that DNA content was higher in the original progenitor species than in the diploid species studied so far. The activation of retrotransposons due to genomic stress caused by the hybrid formation may also account for an increased DNA content (Bennetzen et al. 2005; Grover and Wendel 2010). A similar situation has been described for the formation of the hybrids between *Nicotiana tabacum* and *N. plumbaginifolia* (Gerstel and Burns 1975).

Some autopolyploids were found to have lower 1Cx-values that their diploid counterparts. This was seen in *T. krapovickasii* autotetraploid accessions compared with the diploid accessions and in the autooctoploid *T. fernandezii* compared with its diploid ancestor, *T. grandiflora*. Gains or

losses of DNA in specific chromosome regions as a result of chromosome rearrangements result in karyotype asymmetry. In *T. krapovickasii*, Solis Neffa and Fernández (1993) found chromosomes longer and more asymmetric in the diploid accessions than in the tetraploids, with the number and position of the satellites being conserved. The same phenomenon was also observed in the *T. grandiflora* diploid and octoploid (re-named as *T. fernandezii*, Arbo 2005) (Solís Neffa 1996). It had been proposed that some rearrangements after polyploidization may account for changes in karyotype asymmetry (Solís Neffa and Fernández 1993). In line with this, the reduction in DNA observed in the autopoliploids *T. krapovickasii* and
T. grandiflora, with 1Cx-values slightly lower than those of their respective diploid accessions, could be explained by chromosome rearrangements or mechanisms of loss of repetitive DNA.

In brief, the results of this study suggest that both increases and decreases in DNA content have occurred during the evolution of genome size in the *Turnera ulmifolia* complex.

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