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ARTICLE

Evidence of the occurrence of structural chromosome changes at the initial diploid diversification of the autopolyploid *Turnera sidoides* L. (Passifloraceae) complex

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Abstract: *Turnera sidoides* is an autopolyploid complex of obligate outcrossing perennial herbs. It includes five subspecies and five morphotypes in which diploid to octoploid cytotypes were found. Based on phenetic analyses of the complex and karyotype data of polyploid cytotypes, it has been hypothesized that morphological and chromosome differentiation of *T. sidoides* occurred at the diploid level. To test this hypothesis, we present the first detailed chromosome analysis of diploid populations of three subspecies and four morphotypes. $CMA^+/DAPI^-$ bands were restricted to secondary constrictions (except in the *andino* morphotype) and varied in number and position among taxa. By contrast, DAPI staining was uniform in all the materials investigated. The number and position of 45S rDNA loci were coincident with the $CMA^+/DAPI^-$ bands associated with secondary constrictions. Only one pair of 5S rDNA loci was detected in all the taxa (except in subsp. *holosericea*), but its position was variable. The identified chromosome markers varied among the three subspecies analyzed, but they were more conserved among the morphotypes of subsp. *pinnatifida*. Cluster analysis of these chromosome markers supports the current taxonomic arrangement of diploids and demonstrates that structural chromosome changes would have led or accompanied the initial differentiation of *T. sidoides* at the diploid level.

Key words: diploid, chromosome differentiation, *Turnera sidoides*.

Résumé : Le *Turnera sidoides* représente un complexe d'espèces autopolyploïdes comprenant des plantes pérennes à allofécondation stricte. Cette espèce compte cinq sous-espèces et cinq types morphologiques au sein desquels des cytotypes diploïdes et octoploïdes ont été rencontrés. Sur la base d'analyses phénétiques au sein de ce complexe et de données caryotypiques chez les cytotypes polyploïdes, il a été proposé que la différenciation morphologique et caryotypique chez le *T. sidoides* serait survenue à l'état diploïde. Pour tester cette hypothèse, les auteurs présentent la première analyse chromosomique détaillée de populations diploïdes au sein de trois sous-espèces et de quatre types morphologiques. Les marquages $CMA^+/DAPI^-$ étaient limités aux constrictions secondaires (sauf au sein du type morphologique *andino*) et tant le nombre que la position des bandes étaient variables entre les taxons. Au contraire, le marquage au DAPI était uniforme chez tous les échantillons examinés. Le nombre et la position des locus d'ADNr 45S coïncidaient avec les bandes $CMA^+/DAPI^-$ associées aux constrictions secondaires. Seule une paire de locus d'ADNr 5S a été détectée chez tous les taxons (sauf chez la sous-espèce *holoserica*), mais sa position était variable. Les marqueurs permettant l'identification des chromosomes variaient entre les trois sous-espèces étudiées, mais ils étaient davantage conservés au sein des types morphologiques de la sous-espèce *pinnatifida*. Une analyse de groupement de ces marqueurs chromosomiques a montré que des changements structuraux au sein des chromosomes auraient mené ou accompagné la différenciation initiale au sein du *T. sidoides* à l'état diploïde. [Traduit par la Rédaction]

Mots-clés : diploïde, différenciation chromosomique, *Turnera sidoides*.

Introduction

Turnera sidoides L. (Passifloraceae) is a complex of obligately outcrossing perennial herbs distributed in almost all the Chaquenean Phytogeographical Domain (Arbo 1985; Solís Neffa 2000). It includes five subspecies and five morphotypes in which diploid to octoploid cytotypes were found.

Karyotype features of *T. sidoides* have been studied only in polyploid populations using conventional cytogenetic techniques, except in subsp. *pinnatifida* in which diploid

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populations of two morphotypes were analysed (Solís Neffa and Fernández 2002). The deduced basic karyotypes from the polyploid cytotypes made in that report showed that in spite of the high degree of intraspecific uniformity and the small chromosome size, the subspecies may be differentiated by the karyotype formula, as well as by the number, type, and position of satellites. Comparing the karyotypes of diploid and polyploid cytotypes of *T. sidoides* subsp. *pinnatifida*, those authors evidenced that the particular karyotype features of this subspecies remain independent from the ploidy level (Solís Neffa and Fernández 2002). On this basis, it was proposed that parallel morphological and karyotypic changes would have happened during the diversification of the *T. sidoides* complex (Solís Neffa and Fernández 2002). Furthermore, considering that morphological differentiation occurred at the diploid level (Solís Neffa 2007; Panseri 2012), it has been hypothesized that karyotype differentiation of *T. sidoides* complex would also have occurred at the diploid level. However, a comprehensive analysis including diploid populations of the subspecies and morphotypes of *T. sidoides* is still lacking. Also, the available information is still insufficient to infer the evolutionary relationships among subspecies and morphotypes and to understand the role that structural chromosome changes may have had during the diversification of *T. sidoides*.

In several plant species, which show small and morphologically similar chromosomes, such as those of *T. sidoides* (Solís Neffa and Fernández 2002), chromosome banding and fluorescence in situ hybridization (FISH) provided useful information for karyotype characterization and evolutionary studies (Zoldos et al. 1999; Schrader et al. 2000; Ran et al. 2001; Baeza Perry and Schrader 2004; Pedrosa-Harand and Guerra 2004, Robledo and Seijo 2010). The detection of constitutive heterochromatin by the simultaneous using of chromomycine A3 (CMA) and 4′,6-diaminio-2-phenylindole (DAPI) fluorochromes, which preferentially binds to GC-rich and AT-rich DNA, respectively, were particularly informative for the karyotype characterization of related species (Guerra et al. 2000; Cabral et al. 2006; Robledo et al. 2009). Further, the application of FISH to detect the chromosomal location of the ribosomal RNA genes (rDNA) provided variable patterns that aids in the identification of homologous chromosomes and in tracing evolutionary pathways of karyotype change (Ansari et al. 1999; Moscone et al. 1999; Heslop-Harrison 2000; Krishnan et al. 2001; Guerra 2004; Srisuwan et al. 2006).

In this paper, we present the first chromosome analysis of representative diploid populations of different taxa of *T. sidoides* by CMA/DAPI banding technique and by FISH of 45S and 5S rDNA to provide useful information for a better characterization of the subspecies and morphotypes and to infer the mechanisms involved in the chromosome differentiation of the complex at the diploid level.

Materials and methods

Turnera sidoides

Turnera sidoides is the species of the genus *Turnera* with the most southern distribution in America, ranging from southern Bolivia, Paraguay, and Brazil to Uruguay and Argentina, where it reaches 39°S. It includes five taxonomic subspecies (*T. sidoides* subsp. *carnea* (Cambess.) Arbo, *T. sidoides* subsp. *holosericea* (Urban) Arbo, *T. sidoides* subsp. *integrifolia* (Griseb) Arbo, *T. sidoides* subsp. *pinnatifida* (Juss. ex Poir.) Arbo, and *T. sidoides* subsp. *sidoides*). These subspecies differ in their geographical distribution and exhibit a wide morphological variability (Arbo 1985). Populations of *T. sidoides* subsp. *pinnatifida* were further grouped into five different morphotypes (*andino*, *chaqueño*, *mesopotamico*, *pampeano*, and *serrano*) based on the degree of incision of the leaf blade, the color of the flowers, and geographical distribution (Solís Neffa 2010).

Besides the morphological variation, different ploidy levels with $x = 7$ were found in *T. sidoides* (Fernández 1987; Solís Neffa and Fernández 2001; Solís Neffa et al. 2004; Roggero Luque 2010; Elías et al. 2011; Speranza et al. 2007; Kovalsky and Solís Neffa 2012). In *T. sidoides* subsp. *carnea*, subsp. *holosericea*, and subsp. *pinnatifida* diploid, tetraploid ($2n = 4x = 28$) and hexaploid ($2n = 6x = 42$) cytotypes were found. Furthermore, spontaneous triploid cytotypes ($2n = 3x = 21$) were found in subsp. *carnea* and subsp. *pinnatifida* (Elías et al. 2011; Kovalsky and Solís Neffa 2012). *Turnera sidoides* subsp. *integrifolia* has a polyploid series with ploidy levels from diploid to octoploid ($2n = 8x = 56$), while only tetraploids were found in subsp. *sidoides*. From the study of meiosis and pollen viability it was suggested that polyploids in the complex would have an autopolyploid origin (Fernández 1987; Solís Neffa 2000). These observations were supported by results obtained from the analysis of the effect of polyploidy in some morphological, cytological, isoenzymatic, and biochemical features (Solís Neffa 2000; Solís Neffa and Fernández 2002; Solís Neffa et al. 2003).

From an evolutionary point of view, *T. sidoides* is a polytypic species in process of diversification (Solís Neffa and Fernández 2001). Cytogeographic analyses of more than 100 populations (Solís Neffa and Fernández 2001; Speranza et al. 2007; Solís Neffa et al. 2004; Roggero Luque 2010; Elías et al. 2011) demonstrated that most of the subspecies distribution is covered by tetraploid cytotypes, which occasionally overlaps. Hexaploids, octoploids, and odd-polyploids are rare and usually located at marginal areas. More important to this study is that diploid populations are very rare (except in subsp. *pinnatifida*, that although rare, they were more frequently recorded) and occupy extremely restricted and disjunct areas. That is, only one diploid population was recorded for subsp. *holosericea* and subsp. *integrifolia*, two for subsp. *carnea*,

Table 1. Populations of *Turnera sidoides* studied including collector, accession number, and provenance.

Turnera sidoides	Morphotype	Collector, accession ~~number~~, and provenance
subsp. *carnea* (Cambess.) Arbo	—	SN and S 960. Argentina, Corrientes, Dpt. Mercedes, Miriñay River and route 123. 29°33′42.9″S, 57°30′40.3″W, 54 m.a.s.l.[a,b,c]
subsp. *holosericea* (Urban) Arbo	—	SN and S 2073. Uruguay, Dpt. Tacuarembó, Quebrada Grande Stream. 32°05′34″S, 56°06′13″W, 145 m.a.s.l.[a,c]
subsp. *pinnatifida* (Juss. ex Poir.) Arbo	*andino*	SN et al. 827. Argentina, Jujuy, Dpt. Capital, Yala. 24°07′31″S, 65°23′56″W, 1467 m.a.s.l.[a]
		SN et al. 839. Argentina, Salta, Dpt. Capital. 24°43′12″S, 65°24′33″W, 1246 m.a.s.l.[a]
	chaqueño	SN et al. 1025. Bolivia, Dpt. Tarija, Villa Montes. 20°34′33″S, 63°16′35″W, 850 m.a.s.l.[a]
		SN et al. 1986. Argentina, Salta, Dpt. Pluma de Pato. 23°22′21″S, 63°05′53″W, 237 m.a.s.l.[a]
		SN and S 2224. Argentina, Salta, Los Blancos. 23°30′10.9″S, 62°49′37.7″W, 225 m.a.s.l.[a]
	pampeano	H 378. Argentina, Buenos Aires, San Cayetano, 38°19′60″S, 59°37′0″W, 98 m.a.s.l.[a]
	serrano	SN and S 967. Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′50.83″S, 64°29′36.24″W, 1186 m.a.s.l.[a]
		SN and Sp 2000. Uruguay, Dept. Lavalleja, Aguas Blancas. 34°30′46.7″S, 55°20′53″W, 109 m.a.s.l.[c]

Note: H, D.H. Hojsgaard; S, J.G. Seijo; SN, V.G. Solís Neffa; Sp, P.R. Speranza.
[a]Accessions for which chromosome preparations were obtained form root tips.
[b]Accessions for which chromosome preparations were obtained from anthers of young buds.
[c]Accessions for which chromosome preparations were obtained from ovaries of matured flowers.

and none for subsp. *sidoides*. Most of them separated by hundreds of kilometers and habit in very different environments (Solís Neffa unpublished). This scenario greatly prevents the gene flow among diploid populations of *T. sidoides* and imposes a high degree of genetic isolation.

Plant material

Information about the taxa studied, the provenance of the materials, collectors, and collection numbers are listed in Table 1. The voucher specimens are deposited at the Herbarium of the Instituto de Botánica del Nordeste, Corrientes, Argentina (CTES), and vouchers from Bolivian specimens were also deposited at the National Herbarium of Bolivia, La Paz (LPB). Diploid cytotypes of all subspecies and morphotypes were analyzed, except for subsp. *sidoides* (not documented for the taxon) and subsp. *integrifolia* because the only recorded diploid population could not be found anymore in the original locality.

Most of the analyses were made using roots of germinated seeds; however, for some accessions, in which seed were not available or were very few, chromosome preparations were made from ovaries of matured flowers and (or) from anthers of young buds obtained from plants growing in greenhouse conditions. Preparations of mitotic chromosomes were made on root tips or ovaries pretreated in distilled water cooled to 1 °C for 24 h, fixed in absolute ethanol : acetic acid (3:1) (Solís Neffa and Fernández 2001), and stored at –20 °C in the same solution. For meiotic preparations, flower buds were directly fixed in absolute ethanol : acetic acid (3:1) and stored at –20 °C. The plant tissue was enzymatically digested in 2% cellulose – 10% pectinase and squashed in 45% acetic acid. The coverslips were removed with CO_2, the slides were air dried and stored at –20 °C until use (Geber and Schweizer 1987).

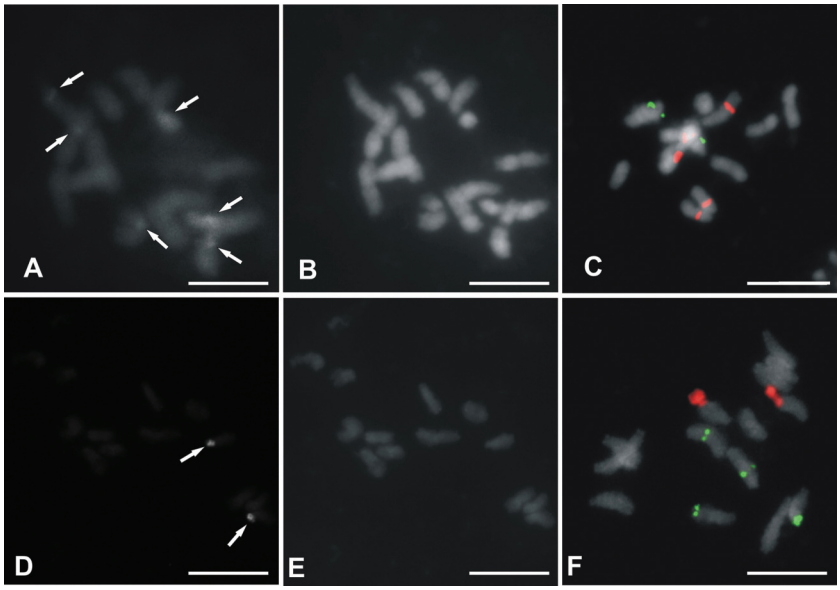
CMA/DAPI banding

CMA/DAPI banding patterns were analyzed on chromosome spreads according to Schweizer (1976) with minor modifications. Chromosome preparations were stained with CMA for 90 min, washed in running water, stained with DAPI for 30 min, and finally mounted in Vectashield (Vector Laboratories, Burlingame, Calif., USA). Slides were kept in darkness for 3–5 days at 4 °C before observation with a epifluorescense microscope. After image acquisition, some preparations were destained in absolute ethyl alcohol : glacial acetic acid (3:1), dehydrated in alcoholic series (70%, 95%, and 100%), air dried, and then kept at –20 °C until used for FISH experiments.

Fluorescent in situ hybridization

Probes for the 5S and 45S rDNA were developed from genomic DNA of *Arachis hypogaea* (Robledo and Seijo 2008). Nick translation with digoxigenin–11-dUTP or biotin-11-dUTP was used to label the probes. The preparation of the hybridization mixture, pretreatment of chromosomal preparations, conditions for in situ hybridization, post-hybridization washes, blocking, and detection by fluorochrome conjugated antibodies were performed according to Moscone et al. (1996*a*) and Chalup et al. (2012). The first set of antibodies (Sigma-Aldrich) consisted of anti-biotin produced in mouse and anti-digoxigenin conjugated

Fig. 1. CMA/DAPI banding and FISH with 45S (red signals) and 5S rDNA (green signals) probes in mitotic metaphases of *Turnera sidoides* subsp. *carnea* (A–C) and *T. sidoides* subsp. *holosericea* (D–F): (A and D) CMA staining, (B and E) DAPI staining, and (C and F) FISH with 45S and 5S rDNA probes. Arrows indicate CMA$^+$ bands. Scale bar = 5 μm. [Colour figure available online.]



to fluorescein-5-isothiocyanate (FITC) produced in sheep. The second set (Sigma) consisted of anti-mouse conjugated to tetramethyl-rodamine isothiocyanate (TRITC) produced in rabbit and anti-sheep conjugated to FITC produced in rabbit. Preparations were counterstained and mounted with Vectashield medium containing 2 mg/mL of DAPI.

Fluorescence microscopy and image acquisition

Chromosome preparations were observed and photographed with a Leica DMRX epifluorescence microscope equipped with a computer-assisted digital camera system. Red, green, and blue images were captured in black and white using the appropriate excitation filters for TRITC, FITC, and DAPI, respectively. Digital images were then pseudo-colored and combined using the Adobe Photoshop version 7.0 (Adobe, San Jose, Calif., USA).

Data analysis

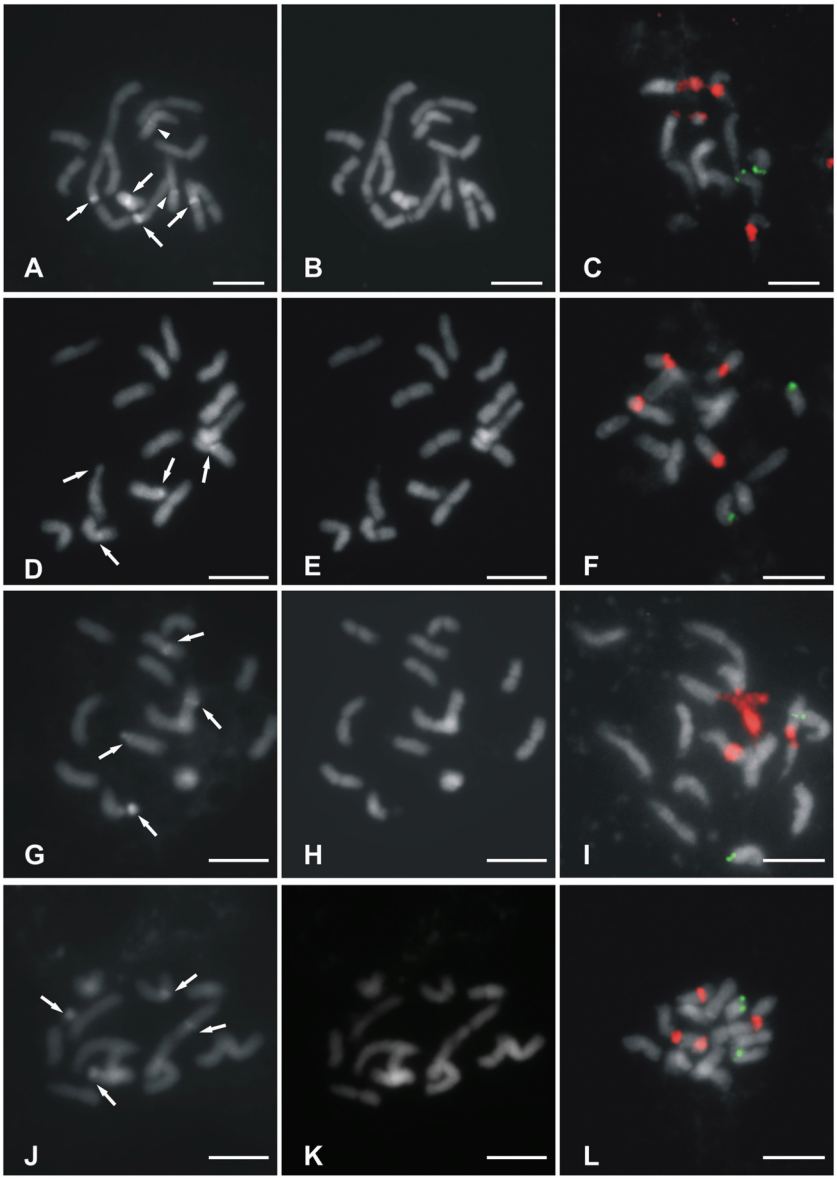
Statistical analyzes were performed using the Infostat software, version 2014 (Di Rienzo et al. 2014). To assess the similarity among subspecies and morphotypes based on the patterns of heterochromatic bands and rDNA loci, a cluster analysis was performed. A data matrix of 6 taxa × 6 characters was constructed from which a distance matrix was obtained using the Gower index. The taxa were clustered using the unweighted pair-group method (UPGMA) and arithmetic averages (Sokal and Sneath 1963). The cophenetic correlation coefficient (*r*) was computed as a measure of the distortion introduced during the clustering (Sokal and Rohlf 1962). The results of this analysis were compared to those obtained from morphological data (leaves: elliptic, obovate; the incision degree of the leaf blade: entired, pinnatifid, pinnatisected; secondary veins: incurved, excurved; indumentum: lax, lanate-sericeus, hirsute, stellated; and color of the flower: light pink, dark pink, salmon, ornage, yellow). To do this, a data matrix of 6 taxa × 5 morphological characters was constructed from which a distance matrix was obtained using the Gower index. The taxa were clustered using the unweighted pair-group method (UPGMA) and arithmetic averages (Sokal and Sneath 1963).

Results

The results obtained from the experiments of CMA/DAPI banding and FISH in diploid populations of *T. sidoides* are shown in Figs. 1–3. Since the patterns of heterochromatin and rDNA loci were conserved among populations of each taxon analyzed, karyotype data for each subspecies and morphotypes are summarized in Table 2 for the subspecies and morphotypes.

In the subspecies and morphotypes analyzed, all the CMA$^+$/DAPI$^-$ bands detected were associated with the secondary constrictions (Fig. 1), except in the *andino* morphotype of subsp. *pinnatifida* that showed one pair of CMA$^+$/DAPI$^-$ bands in a chromosome pair without secondary constrictions (Table 2). *Turnera sidoides* subsp. *carnea* showed interstitial CMA$^+$/DAPI$^-$ bands in three different chromosome pairs (Figs. 1A, 1B), while *T. sidoides* subsp. *holosericea* showed one chromosome pair with terminal bands (Figs. 1D, 1E). In *T. sidoides* subsp. *pinnatifida*, the *chaqueño*, *pampeano*, and *serrano* morphotypes presented CMA$^+$/DAPI$^-$ bands in two pairs of chromosomes, one pair at the interstitial position and the other at the terminal position (Fig. 2). The additional pair of CMA$^+$/DAPI$^-$ bands in the *andino* morphotype was interstitial (Fig. 2A). In all the subspecies and morphotypes analyzed, DAPI staining was uniform in all the chromosomes of the complements and no DAPI$^+$ bands were detected.

Fig. 2. CMA/DAPI banding and FISH with 45S (red signals) and 5S rDNA (green signals) probes in mitotic chromosomes of *Turnera sidoides* subsp. *pinnatifida*: (A–C) *andino* morphotype, (D–F) *chaqueño* morphotype, (G–I) *pampeano* morphotype, (J–L) *serrano* morphotype. (A, D, G, and J) CMA banding, (B, E, H, and K) DAPI banding, and (C, F, I, and L) FISH with 45S and 5S rDNA probes. Arrows indicate CMA$^+$ bands and arrowheads indicate additional CMA$^+$ bands. Scale bar = 5 μm. [Colour figure available online.]



FISH experiments showed that the 45S ribosomal DNA loci were localized in the secondary constrictions in all subspecies and morphotypes; however, the number and their position (interstitial or terminal) where particular for each subspecies (Figs. 1C, 1F, 2C, 2F, 2I, 2L; Table 2). Notoriously, in the terminal 45S loci, the signal not only covered the secondary constriction but also the micro- (in subsp. *pinnatifida*) and macrosatellite (in subsp. *holosericiea*). All the *T. sidoides* taxa presented one 5S rDNA loci at the terminal position, except *T. sidoides* subsp. *holosericea* that presented two pairs of loci, one at the interstitial and the other at the terminal position (Table 2; Fig. 1F). The counterstaining with DAPI after pretreatment for FISH revealed small heterochromatic bands at telomeres and centromeres in some of the taxa analyzed (Figs. 1F, 2F). Moreover, the heterochromatin CMA$^+$/DAPI$^-$ were also highlighted with DAPI counterstaining after FISH experiments.

The phenograms obtained from cluster analysis based on morphological data and the patterns of heterochromatic bands and rDNA loci are shown in Fig. 3. A high correlation between the cophenetic matrix and the distance matrix was observed in both analysis (r = 0.98 and 0.99, respectively). The phenogram obtained from morphological data (Fig. 3A) showed that cluster I includes subsp. *carnea* and subsp. *holosericea*, while cluster II includes the morphotypes of subsp. *pinnatifida*. Of them, the *chaqueño*, *andino*, and *serrano* morphotypes grouped

Fig. 3. Phenograms obtained from cluster analysis (UPGMA) based on morphological data (A) and the patterns of heterochromatin bands and ribosomal loci (B) in diploid materials of *Turnera sidoides* subsp. *carnea* and subsp. *holosericea* and, the morphotypes (*andino*, *chaqueño*, *serrano*, and *pampeano*) of *T. sidoides* subsp. *pinnatifida*.

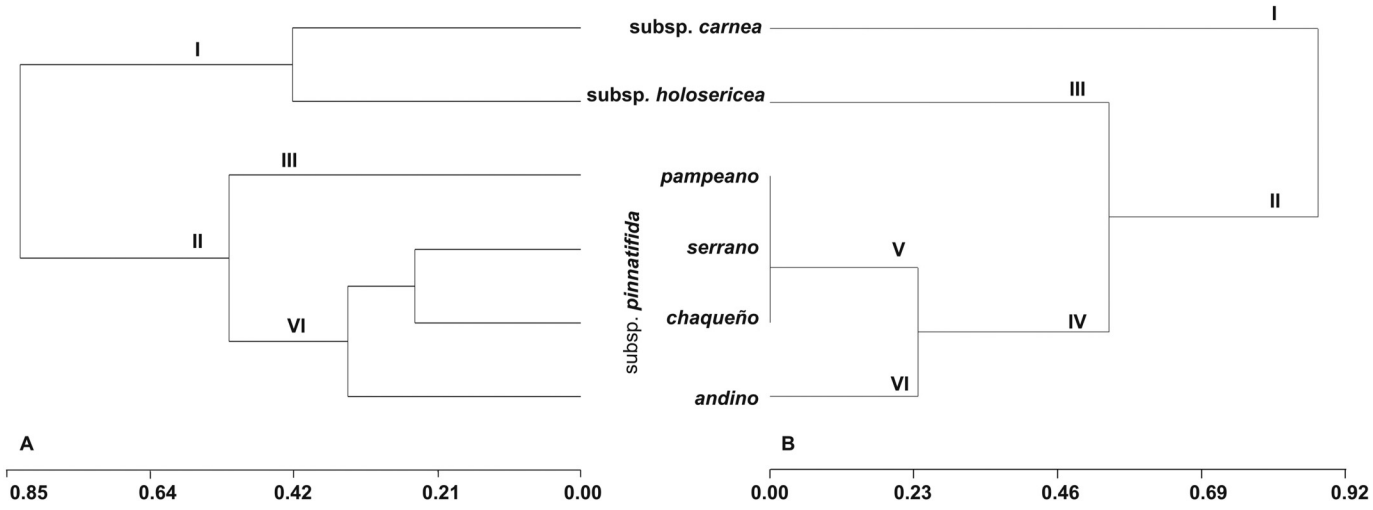


Table 2. Patterns of secondary constrictions, heterochromatic bands, and 45S and 5S rDNA loci observed in diploid populations of *Turnera sidoides*.

			No. and position of rDNA loci		No. and position of heterochromatic bands	
Turnera sidoides	Morphotype	No. of secondary constrictions	45S rDNA	5S rDNA	$CMA^+/DAPI^-$	$CMA^-/DAPI^+$
subsp. *carnea* (Cambess.) Arbo	—	3	3i	1t	3i	0
subsp. *holosericea* (Urban) Arbo	—	1	1t	2 (1i+1t)	1t	0
subsp. *pinnatifida* (Juss. ex Poir.) Arbo	*andino*	2	2 (1i+1t)	1t	3 (2i+1t)	0
	chaqueño	2	2 (1i+1t)	1t	2 (1i+1t)	0
	pampeano	2	2 (1i+1t)	1t	2 (1i+1t)	0
	serrano	2	2 (1i+1t)	1t	2 (1i+1t)	0

Note: I, interstitial; t, terminal.

separately from the *pampeano* one. The phenogram obtained from chromosomal data (Fig. 3B) showed that subsp. *carnea* is the first to be separated from the other two, followed by subsp. *holosericea*. Within subsp. *pinnatifida*, the *chaqueño*, *pampeano*, and *serrano* morphotypes grouped separately from the *andino* one.

Discussion

In this paper the patterns of heterochromatic bands and 45S and 5S rDNA loci of 10 diploid populations representative of three subspecies and four morphotypes of the *T. sidoides* autopolyploid complex are presented for the first time. The detected regions of heterochromatin and ribosomal loci unambiguously allowed the identification of three to four of the seven chromosome pairs present in the species, becoming excellent markers for the study of chromosomal evolution in the complex.

The presence of GC rich heterochromatin associated with secondary constrictions detected in the subspecies and morphotypes of *T. sidoides* is consistent with the patterns observed for most plants (Schweizer and Loidl 1987; Guerra 2000; De Carvalho and Guerra 2002; Hamon et al. 2009; Cuco et al. 2005). It has been demonstrated that the NOR-associated heterochromatin is clearly differentiated from other heterochromatin types because, although it may contain repeated sequences not related to the NORs (Cuadrado and Jouve 1994; Garnatje et al. 2004; Acosta et al. 2012), it is mostly composed of 45S rDNA genes (Appels et al. 1998). The fact that in *T. sidoides* the CMA^+ bands were restricted to the secondary constrictions (except one pair of bands in the *andino* morphotype) and that the whole size of the bands were covered by the 45S rDNA, suggest that all the CMA^+ heterochromatin is mostly composed of rDNA genes. Moreover, the hybridization of the whole or most of the microsatellites (in subsp. *pinnatifida* and subsp. *holosericea*) with the 45S rDNA probe evidenced that they are also mainly composed of this sequences. The association between CMA^+ heterochromatin with the distribution of 45S rDNA detected in all the diploid taxa analysed here demonstrate that all the 45S rDNA loci have a high level of chromatin condensation and hence, it remains transcriptionally silent. However, the coincidence between the number of

45S rDNA loci and the number of secondary constrictions also evidenced that each 45S rDNA loci detected by FISH has transcriptionally active rDNA genes that contribute to the nucleoli organization.

In the particular case of the *andino* morphotype of subsp. *pinnatifida*, the fact that the CMA$^+$/DAPI$^-$ additional bands were not found associated with 45S rDNA loci evidenced that they may be composed of GC-rich DNA different from rDNA genes. Such bands are usually much rarer than the CMA$^+$ bands associated with NORs in most plant groups, but in some taxa, GC-rich bands are very diversified and highly dominant or even exclusive, as in the genus *Capsicum* (Moscone et al. 1996*b*) and in the Aurantioideae subfamily of Rutaceae (Guerra et al. 2000). Despite the distribution and number of these bands, they constitute excellent chromosome markers for the establishment of homologies, and in *T. sidoides* they allowed the identification not only of one chromosome pair but also to distinguish the *andino* morphotype of subsp. *pinnatifida*.

Uniform DAPI staining in all the taxa of *T. sidoides* suggests that large AT-rich heterochromatic blocks are not present in their karyotypes. The presence of small highlighted blocks of chromatin in the centromeres and telomeres of *T. sidoides* subsp. *holosericea* and subsp. *pinnatifida* by counterstaining with DAPI after FISH experiments evidenced the existence of heterochromatin, but they were composed by sequences without a differential nucleotide composition. This kind of banding pattern, which is revealed after FISH experiments, was reported to be similar to the C-banding (Moscone et al. 1999); and this pattern arises as a consequence of the differential digestion of chromatin by proteinase during pretreatment of chromosomes.

The number of 45S rDNA loci detected in the diploid cytotype of *T. sidoides* subsp. *pinnatifida* is consistent with the number of SAT-chromosomes reported for this taxon with the same ploidy level using Feulgen techniques (Solís Neffa and Fernández 2002). However, the numbers of ribosomal sites here detected for other diploid taxa are different from the number of SAT chromosomes cited for the basic karyotypes deduced from autopolyploid cytotypes with classical cytogenetic techniques (Solís Neffa and Fernández 2002) For instance, in subsp. *carnea*, the number of 45S rDNA loci (three) here detected per haploid complement in the diploid cyotype was greater than the number of SAT-chromosomes (one) observed per haploid complement in two tetraploid populations. This fact suggests that most of the expected 45S rDNA sites in the polyploid cytotypes of this subspecies have been silenced as occurred in many auto- and allopolyploid species (Chen and Pikaard 1997; Seijo et al. 2004; Książczyk et al. 2011). On the contrary, the number of 45S rDNA loci (one) observed per haploid complement in the diploid cyotype of subsp. *holosericea* was lower than the number of SAT chromosomes (two) per haploid complement deduced from an hexaploid cytotype. Even though the existence of secondary constrictions not associated with NORs has been detected in several groups of organisms (Viinikka 1975; Berg and Greilhuber 1993; Guarido et al. 2012) we cannot discard the existence of intra-subspecific variability of the 45S rDNA or the possibility that the hexaploid cytotype of subsp. *holosericea* analysed by classical cytogenetic were an inter-subspecific hybrid. The latter hypothesis is supported by the fact that the area where the hexaploid material of *T. sidoides* subsp. *holosericea* was collected corresponds to a contact zone between this subspecies (one 45S rDNA) and subsp. *carnea* (three 45S rDNA), in which several individuals with mixed characteristics were found (Roggero Luque et al., submitted).

Evolutionary relationships between subspecies and morphotypes

Clustering (UPGMA) of subspecies and morphotypes of *T. sidoides* obtained in this work based on the similarity of the patterns of heterochromatin bands and 45S and 5S rDNA loci support the published taxonomical grouping (Arbo 1985).

At the inter-subspecific level, subsp. *carnea* and subsp. *holosericea* are similar in terms of leaf morphology but can be distinguished by the type of indumentum (Arbo 1985). Classical cytogenetics of polyploids evidenced that these subspecies have karyotypes with a similar degree of assymetry but differ in the number and morphology of the SAT chromosomes (Solís Neffa and Fernández 2002). Our results provide chromosome markers that clearly distinguish both subspecies, as they differ in the number and position of CMA$^+$/DAPI$^-$ heterochromatic bands and in the number and position of 45S and 5S rDNA loci. These markers evidenced a clear cytogenetic differentiation between them.

The conserved pattern of heterochromatin (except for one extra band in the *andino* morphotype) and FISH signals found in the four analyzed morphotypes of subsp. *pinnatifida* is consistent with the genetic uniformity observed using cpDNA molecular markers (Speranza et al. 2007), and these findings support the current hypothesis of a relatively recent divergence time among them (Solís Neffa 2010). As a whole, subsp. *pinnatifida* is clearly distinguished from subsp. *carnea* and subsp. *holosericea* by their pinnatifid to pinnatisect leaves and by the leaf indumentum (Arbo 1985). In agreement, subsp *pinnatifida* differs from the other two subspecies by its CMA$^+$/DAPI$^-$ banding pattern and the number and position of the rDNA loci. The existence of one interstitial and one terminal 45S loci is consistent with the classical cytogenetic data, which showed that this subspecies has one pair of macro- and one pair of microsatellites (Solís Neffa and Fernández 2002).

The above discussion evidenced that in spite that polyploidy has played a prominent role in the evolution of the *T. sidoides* complex (Solís Neffa and Fernández

2001; Elías et al. 2011), an important structural chromosome differentiation occurred at the diploid level. The presence of diploids in most of the subspecies and morphotypes support the hypothesis that the initial morphological and chromosome differentiation of the complex occurred at the diploid level (Solís Neffa 2007). On the basis of molecular marker analyses, it was proposed that the differentiation of morphotypes and subspecies of *T. sidoides* would have occurred during the cold-drought climatic phases of the Last Maximum Glacial (Speranza et al. 2007; Solís Neffa 2010; Moreno 2014). In this scenario, fragmentation of the original area would have allowed the fixation of molecular, chromosomal, and morphological changes at the diploid level, leading the complex differentiation. Further expansion of the complex populations mainly occurred through autopolyploid cytotypes bearing the chromosome changes fixed in the isolated diploid populations.

Conflict of interest

The authors declare that they have no conflict of interest.

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