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<td>Date Submitted by the Author:</td>
<td>03-Nov-2015</td>
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<td>Complete List of Authors:</td>
<td>Scaldaferro, Marisel; Universidad Nacional de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales; Instituto Multidisciplinario de Biología Vegetal (IMBIV), CONICET, Romero-da Cruz, María; Instituto de Biología, Universidade Estadual de Campinas-UNICAMP, Brasil. Cecchini, Nicolás; Molecular Genetics and Cell Biology The University of Chicago 929 East 57th Street GCIS Room W519P Chicago Moscone, Eduardo Alberto; IMBIV</td>
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<td>Keyword:</td>
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FISH and AgNor-mapping of the 45S and 5S rRNA genes in wild and cultivated *Capsicum* species (Solanaceae)

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Abstract

Chromosome number and position of rDNA were studied in 12 wild and cultivated *Capsicum* species with \( x = 12 \) and \( x = 13 \) (22 samples). The 5S and 45S rRNA loci were localized and physically mapped using two-color fluorescence *in situ* hybridization and AgNOR banding for the first time in those species. We focused on the comparison of the results obtained with both methods with the aim of accurately revealing the real functional rRNA genes. We based on a previous work that reported that the 18S-5.8S-25S loci mostly coincide with GC-rich heterochromatic regions and likely have given rise to satellite DNAs, which are not active genes. These data show the variability of rDNA within karyotypes of the genus *Capsicum*, providing anchor points for (comparative) genetic maps. In addition, the obtained information might be useful for studies on evolution of repetitive DNA.

**Keywords**: *Capsicum* chromosomes, Fluorescence *in situ* hybridization, AgNOR banding, 5S rRNA genes, 45S rRNA genes, Physical gene mapping
Introduction

*Capsicum* L. (Solanaceae) comprises up to 40 species, some of which are quite variable species (Carrizo García et al. 2013); it is a small American genus that occurs in tropical and temperate areas distributed from Southern Mexico to Central Argentina. *Capsicum* has great economic significance, because it includes the sweet and hot chili peppers, vegetables and spices consumed worldwide. It comprises exclusively diploid species with two basic chromosome numbers, \( x = 12 \) and \( x = 13 \). The karyotypes of the latter are more asymmetrical than those of the former, and therefore are assumed to be derived (Pickersgill 1971, 1991; Moscone 1990, 1993, 1999; Moscone et al. 1993, 1995, 1996a, 2007; Tong and Bosland 2003; Scaldaferrro et al. 2013), although Pozzobon et al. (2006) hypothesised that \( x = 13 \) is the ancestral basic number of the genus.

In earlier studies, corola color has been utilized in a practical way to characterize the cultivated species and their wild relatives, which were provisionally subdivided into a "white" (some species exhibit single-colored flowers, i.e. white, cream) and a "purple flowered group" (pink, lilac, violet; Pickersgill 1991), although different color combinations in lobules, throat and tube, with spots of varying colors complicates species delimitation (Hunziker 2001; Barboza and Bianchetti 2005). Currently we also recognize the "yellow flowered group", which comprises species from Central America and north-western areas of South America (Moscone et al. 2007; Scaldaferrro et al. 2013).
The phylogenetic relationships presented to date propose *Capsicum* monophyletic, diploid and including four clades with two different chromosome base numbers, $x = 12$ and 13 (Sehr et al. 2013). The origin of $x = 13$ occurred in two independent events, resulting in two subgroups of the $2n = 26$ species, the Andean $x = 13$ group (*C. rhomboideum* among them) appearing in most basal position (Walsh and Hoot 2001; Olmstead et al. 2008; Guzmán et al. 2009), and clearly related the Brazilian $x = 13$ group (*C. recurvatum* and *C. villosum* among them). Then occurred a single phylogenetic return to $x = 12$ indicated by several, closely following and basal $x = 12$ clades, e.g. *C. flexuosum*. They mark phylogenetic steps towards the speciose $x = 12$ core complex of the genus which includes *C. pubescens* group (*C. eximium* and *C. tovarii* among them), *C. baccatum* group, and *C. annuum* group (*C. annuum*, *C. chinense* and *C. frutescens* among them; Sehr et al. 2013).

Physical mapping of 5S and (or) 18S–5.8S–25S (45S) rRNA genes by fluorescence *in situ* hybridization (FISH) provides valuable chromosome landmarks. These landmarks have proven to be important in understanding the evolution and diversification of the genus *Capsicum* (Park et al. 1999, 2000; Scaldaferrro et al. 2006; Kwon and Kim 2009); in the same way these sequences have been widely employed to study 5S and 18S-25S ribosomal genes localization, chromosome evolution, transgene localization, rDNA evolution, genetic maps, linkage groups, phylogenetic, etc., in many kind of plants (e.g. *Triticeae*, *Nicotiana*, *Brassicaceae*, etc.). Resulting from that, a variation in the distribution of ribosomal genes has been reported in numerous studies, e.g. in
Nemesia (Datson and Murray 2006), Triticeae (Leitch and Heslop-Harrison 1993; Dubcovsky and Dvorak 1995), Brassicaceae (Ali et al. 2005), etc.

In Capsicum, FISH revealed the presence of the unique 5S rDNA intercalary locus and one to several 18S-25S rDNA loci per haploid genome (Park et al. 1999, 2000; Scaldaferro et al. 2006; Kwon and Kim 2009). The latter loci mostly coincides with GC-rich heterochromatic regions and likely has given rise to satellite DNAs in some species of the genus (e.g. C. pubescens; Scaldaferro et al. 2006), thus affecting total genome size (Scaldaferro et al. 2006, 2013; Moscone et al. 2007). Karyotypes of numerous Capsicum species were analyzed using fluorochrome banding, Giemsa C-banding, AgNOR staining, as well as the localization of rDNAs and telomeric sequences using FISH (Moscone et al. 1993, 1995, 1996b, 2007; Park et al. 2000; Scaldaferro et al. 2006, 2013). These methods are important established tools for cytotaxonomy and delineation of karyotype evolution in Capsicum. Patterns of heterochromatin distribution allowed the identification of all the 20 Capsicum species examined to date, although some intra- and interspecific variation has been documented (Moscone et al. 2007; Scaldaferro et al. 2013).

Fluorochrome banding has revealed that GC-rich heterochromatin is located mostly at the terminal regions of some chromosomes and is a common feature of all Capsicum species. These GC-rich regions are typically equivalent to nucleolar organizer region (NOR)-associated heterochromatin in plants (Moscone et al. 1996a, 2007; Scaldaferro et al. 2013). The NORs are an evolutionarily very important but poorly studied component of the genome of chili peppers.
In the present study we report the number, size and physical mapping of active NORs by AgNOR banding and the physical mapping of the active and inactive 45S rRNA genes and 5S rRNA genes by FISH within the karyotypes of 12 Capsicum species (22 samples). In addition, we analyzed the relationships between the active AgNOR sites and the number and position of 45S sites, and compared the results of both FISH and AgNOR banding methods, with the aim of accurately revealing the functional 45S rRNA genes in the genus. A physical map with active and inactive 45S rRNA genes and 5S rRNA genes of 12 Capsicum taxa chromosomes was constructed.

Materials and methods

Plant material—The provenance of the plant material studied is presented in Table 1. In the Table and Figures, the species were arranged in general, according to their karyotypic affinities. Voucher specimens were identified by Dr. Gloria E. Barboza and are deposited in the herbarium of Museo Botánico de Córdoba, Argentina (CORD).

Chromosome preparations—Somatic chromosomes were examined; root tips (5-10 mm long) were collected and pre-treated with p-dichlorobenzene-saturated solution in the dark at room temperature for 2 h, then fixed in a freshly prepared 3:1 mixture (ethanol: glacial acetic acid) at 4ºC for a minimum of 12 h and stored at -20ºC until use. Chromosome spreads for AgNOR and fluorescence in situ hybridization (FISH) were performed after digestion of the material with enzymes
[2% cellulase (weight/volume, w/v) (Serva, Heidelberg, Baden-Wurtemberg State, Germany), 1% pectinase (volume/volume, v/v) at 37ºC for 40 minutes (Sigma, Munich, Baviera State, Germany)] (Schwarzacher et al. 1980). The meristems were squashed in a drop of 45% acetic acid and, after removal of the coverslip with CO$_2$, slides were air-dried, aged for 1-2 days at room temperature and stored at -20ºC until use.

**AgNOR banding**—Silver impregnation to detect NOR was performed after the silver-incubation procedure (Ag-I) (Bloom and Goodpasture 1976), setting the slides flooded with the Ag solution in moisture-tight plastic container, with modifications of Kodama et al. (1980), using nylon cloth (mesh size 0.243 mm) instead of coverslips (Figs. 1-3).

Satellites were termed according to Battaglia’s (1955) nomenclature with some modifications: microsatellite (diameter smaller than the chromosome diameter), macrosatellite (diameter equal to chromosome diameter and length equal to or smaller than that of the corresponding chromosome arm), linear satellite (diameter equal to chromosome diameter and length greater than that of the corresponding chromosome arm), and tandem satellite (two segments [plus two secondary constrictions] of variable diameter and length, one terminal and the other intercalary, in the same chromosome arm).

**Probe labeling and fluorescence *in situ* hybridization**—FISH was performed with 5S rDNA and 18S–25S rDNA repeated sequences (Figs. 4, 5), using the following DNA probes: pXV1, a 349-base pair (bp) fragment of the 5S rRNA gene
repeated unit from *Beta vulgaris*, including the adjacent intergenic spacer (Schmidt et al. 1994); 5S fragment obtained by PCR from genomic DNA of *C. annuum* L. var. *annuum* with the primers 5SrDNA-3 and 5SrDNA-4 (Kitamura et al. 2001); R2, a 6.5-kilobase (kb) fragment of the 18S–5.8S–25S rDNA repeat unit from *Arabidopsis thaliana*, including internal transcribed spacers ITS1 and ITS2 and a short segment of the intergenic region (IGR) (Wanzenböck et al. 1997); and pTa71, unit repetition fragment of rRNA 18S-5.8S-25S (45S) genes of *Triticum* (Gerlach and Bedbrook 1979). The 5S probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) and the 45S probes with biotin-11-dUTP (Sigma), both by nick translation.

Pre-treatment of slides and probe denaturation, conditions for *in situ* hybridization, post-hybridization washings, blocking, and indirect detection by fluorochrome conjugated antibodies [anti-biotin conjugated with TRITC (tetramethyl-rhodamine isothiocyanate, Dakopatts nº R270, Glostrup, Hovedstaden Region, Denmark) (red); and anti-digoxigenin conjugated with FITC (fluorescein isothiocyanate, Dakopatts nº F135, Glostrup, Hovedstaden Region, Denmark)] were performed as previously described (Moscone et al. 1996b).

**Fluorescence microscopy and image acquisition**—Metaphase chromosomes were observed and photographed, depending on the procedure, with transmitted light or epifluorescence using an Olympus BX61 microscope equipped with the appropriate filter sets (Olympus, Shinjuku-ku, Tokyo, Japan) and a JAI® CV-M4+CL monocromatic digital camera (JAI, Barrington, NJ, USA). Red, green, and blue images were captured in black and white using appropriate filters for TRITC,
FITC, and DAPI excitation, respectively. Digital images were imported into Photoshop 7.0 (Adobe, San Jose, California State, USA) for pseudo-colored and final processing.

**Karyotype**—For karyotype description, chromosomes were arranged in groups according to the position of the centromere and in order of decreasing size within each type. In each idiogram, chromosomes not identified for possessing similar measures without markers, were grouped. Chromosome terminology followed Levan et al. (1964) and satellites were classified according to Battaglia (1955). The idiograms were based on chromosome measurements of fluorochrome banded metaphase plate photomicrographs, according to Moscone et al. (2007) and Scaldaferro et al. (2013). Karyotype variants below the species level were considered as "cytotypes".

**Results**

Twenty two samples from 12 wild and cultivated *Capsicum* taxa were studied: *Capsicum annuum* var. *annuum* and var. *glabriusculum*, *C. chinense*, *C. frutescens*, *C. baccatum* var. *baccatum* and var. *pendulum*, *C. eximium*, *C. cardenasii*, *C. flexuosum*, *C. praetermissum*, *C. rhomboideum*, *C. recurvatum*, *C. tovari* and *C. villosum* (Table 1). Chromosome number, karyotype formula, number of AgNOR-carrying chromosomes at metaphase (together with the ordering number of the AgNOR-bearing pairs), maximum number of nucleoli of interphase nuclei for each taxon and cytotype, and number and position of rDNA sites of 12
Capsicum species studied in this work are included in Table 1. Illustrations of silver-stained somatic metaphases and interphase nucleoli are given in Figs. 1-3. In addition, 45S and 5S rDNA repeated sequences were mapped by FISH on metaphase chromosomes of the considered species (Figs. 4, 5). A comparison of each Capsicum taxa chromosome bearing NORs, stained with AgNOR and labeled by FISH, is presented in Fig. 6. The respective idiograms are shown in Fig. 7.

AgNOR mapping

The Capsicum taxa studied had a maximum number of active NORs at metaphase from one to four in the haploid complement with the following distribution: one AgNOR was found in two species: C. annuum var. glabriusculum (cytotypes 1, 3 and 5, Fig. 1c, e, g) and C. rhomboideum (Fig. 2h); two AgNORs were observed in nine species: C. annuum var. annuum (cytotype 2, Fig. 1a, b), C. annuum var. glabriusculum (cytotypes 2 and 4, Fig. 1d, f), C. chinense (Fig. 1j), C. frutescens (Fig. 1k), C. eximium (cytotype 2, Fig. 2c), C. cardenasii (cytotypes 1 and 2, Fig. 2d, e), C. flexuosum (Fig. 2f), C. praetermissum (Fig. 2g), C. recurvatum (Fig. 2i) and C. villosum (Fig. 2k); C. tovari (cytotype 2) exhibited three AgNORs (Fig. 2j); C. annuum var. glabriusculum (cytotypes 6 and 7, Fig. 1h, i) and C. baccatum var. baccatum and var. pendulum (Fig. 2a, b) showed four NORs in their haploid complements.

Most AgNORs of the analyzed species (82.98%) were located on the short arm of the corresponding chromosome, although some taxa, such as C. annuum
var. *glabriusculum* (cytotypes 2, 4, 6 and 7), *C. eximium* (cytotype 2), *C. cardenasii* (cytotypes 1 and 2), *C. tovarii* (cytotype 2) and *C. villosum*, also exhibited one nucleolar organizer on the long arm in different chromosome pairs (17.02%); pair 5 in cytotypes 2 and 4 of *C. annuum* var. *glabriusculum*, pair 6 and pair 4 in cytotypes 6 and 7, respectively, in the same taxa (Fig. 1); pair 7 in *C. eximium* (cytotype 2), *C. cardenasii* (cytotype 1 and 2) and *C. tovarii* (cytotype 2), and pair 10 in *C. villosum* (Fig. 2). According to the arm position, eight of the 12 species studied showed both terminal and subterminal satellites (Figs. 1i, 2a, b, c, d, e, g, j). *C. annuum* var. *annuum* (cytotype 2), *C. annuum* var. *glabriusculum* (cytotypes 4 and 6) and *C. rhomboideum* possessed only terminal-associated satellites (Figs. 1a, b, f, h, 2h). Finally, *C. annuum* var. *glabriusculum* (cytotypes 1, 2, 3 and 5), *C. flexuosum*, *C. recurvatum* and *C. villosum* presented subterminal-associated satellites (Fig. 2h, k). Only *C. annuum* var. *glabriusculum* presented the three alternatives in their cytotypes (only terminal, only subterminal or both).

NOR-associated satellites displayed different sizes among species, individuals and often among cells from the same plant. Different proportions of microsatellites, macrosatellites and tandem satellites were recorded among species. Microsatellites were observed in 95.45% of the taxa analyzed (Figs. 1, 2), whereas macrosatellites (Fig. 2c, h, k; see *M* NOR bearing chromosomes) and tandem satellites (Figs. 1j, 2a, d, f, g; see *T* NOR bearing chromosomes) in 13.64% and 22.73%, respectively. In some cases, sizes varied between homologous chromosomes, with heteromorphisms being detected (Fig. 2d, f, g, h, k).
There was variation in the size of nucleoli in interphase nuclei and the size of AgNOR in late metaphase, although there was no correlation in size variation of both structures.

It was also found that silver nitrate binds not only the NOR and the nucleoli, but sometimes also the chromosome centromere (Figs. 1a, d, k, 2j, k).

The number of AgNORs in metaphase matched the maximum number of nucleoli observed in the silver impregnated interphase nucleus in most of the taxa examined (Table 1), except in *C. annuum* var. *glabriusculum* (cytotype 6, Fig. 1h; Table 1), in which the former was smaller than the latter (four and eight, respectively). Furthermore, unsteadiness in the number of chromosomes with AgNOR among species was observed in all analyzed cells. For example, a high percentage of metaphases showed the maximum number of AgNORs among species; in others, most metaphases showed the minimum of AgNORs, and finally, distribution of intermediate values was mostly evidenced (Table 1).

The number and position of AgNORs in the taxa examined were in agreement with data on secondary constrictions in fluorochrome-stained chromosomes from the same accessions (Moscone et al. 2007; Scaldaferro et al. 2013). In these cases, heterochromatic satellites (CMA+/DAPI- or CMA+/DAPIo) allowed safe identification of chromosomes carrying secondary constrictions.

**Fluorescence in situ hybridization (FISH): cytological mapping of the 45S-5S rRNA genes**
The 45S-5S ribosomal repeated sequences were mapped by FISH in every examined sample. The distribution pattern of the 18S-5.8S-25S rRNA (rDNA 45S) gene family differed considerably among species. The number of 45S loci varied between 1 and 30 pairs in diploid complement of the studied taxa, whereas there was a unique 5S site in all cases (Figs. 4, 5; Table 1). The 5S FISH signal usually had short arm interstitial location on a large or median metacentric chromosome in every Capsicum species analyzed, with the exception of C. recurvatum and C. tovari, with the 5S locus placed on the long arm (Figs. 5i, j, 7). The idiograms showed the 5S sites as follows: chromosome nº 1 in C. villosum; chromosome nº 2 in C. annuum var. glabriusculum (cytotype 5); chromosome nº 3 in C. rhomboideum and C. recurvatum; chromosome nº 4 in C. annuum var. glabriusculum (cytotypes 4, 6 and 7); chromosome nº 5 in C. annuum var. glabriusculum (cytotype 3), C. baccatum var. baccatum and pendulum, C. frutescens; chromosome nº 6 in C. annuum var. annuum (cytotype 2) and var. glabriusculum (cytotype 1 and 2) and C. chinense; chromosome nº 7 in C. praetermissum; and chromosome nº 9 in C. eximium (cytotype 2), C. cardenasii (cytotypes 1 and 2), C. flexuosum and C. tovari (cytotype 2). In addition, 5S was sintenic with a 45S locus in C. baccatum var. baccatum and pendulum, C. cardenasii, C. frutescens, C. flexuosum, C. praetermissum and C. villosum (Fig. 7). In most cases, CMA/DAPI banding on the same species (Moscone et al. 2007; Scaldaferrro et al. 2013) helped us to find the correct location of this gene.

The cytological mapping of rRNA gene clusters revealed possible intra and interspecific chromosome homeologies, in which the shared chromosome
characteristics indicate some original ancestral homology, as follows (Fig. 7): 1) chromosomes nº 12 of *C. annuum* var. *annuum* (cytotype 2), *C. annuum* var. *glabriusculum* (cytotypes 4, 5 and 6), *C. chinense* (cytotype 1), *C. frutescens*, *C. baccatum* var. *baccatum* and var. *pendulum*, *C. eximium* (cytotype 2), *C. cardenasii* (cytotypes 1 and 2), *C. praetermissum*, *C. tovarii* (cytotype 2), *C. recurvatum* and *C. villosum*, and nº 9 of *C. rhomboideum*; 2) chromosomes nº 1 of *C. villosum*; nº 2 of *C. annuum* var. *glabriusculum* (cytotype 5); nº 3 of *C. recurvatum* and *C. rhomboideum*; nº 4 of *C. annuum* var. *glabriusculum* (cytotypes 4, 6 and 7); nº 5 of *C. annuum* var. *glabriusculum* (cytotype 3), *C. frutescens*, and *C. baccatum* var. *baccatum* and var. *pendulum*; nº 6 of *C. annuum* var. *annuum* (cytotype 2), *C. annuum* var. *glabriusculum* (cytotypes 1 and 2), *C. chinense* (cytotype 1), and *C. eximium* (cytotype 2); nº 7 of *C. praetermissum* and nº 9 of *C. cardenasii* (cytotypes 1 and 2), *C. flexuosum* and *C. tovarii* (cytotype 2); 3) chromosome nº 1 of *C. annuum* var. *glabriusculum* (cytotypes 2, 6 and 7), *C. frutescens*, *C. baccatum* var. *baccatum* and var. *pendulum*, and nº 2 of *C. flexuosum*; 4) chromosome nº 4 of *C. frutescens*, *C. baccatum* var. *baccatum* and var. *pendulum*, *C. eximium* (cytotype 2), *C. cardenasii* (cytotypes 1 and 2), *C. flexuosum* and *C. tovarii* (cytotype 2); 5) chromosome nº 5 of *C. annuum* var. *glabriusculum* (cytotypes 2, 4 and 7), and nº 6 of *C. annuum* var. *glabriusculum* (cytotype 6); 6) chromosome nº 5 of *C. flexuosum* and nº 6 of *C. praetermissum* and *C. tovarii* (cytotype 2); 7) chromosome nº 6 de *C. baccatum* var. *baccatum* and var. *pendulum*, *C. cardenasii* (cytotypes 1 and 2), and *C. flexuosum* and nº 8 of *C. eximium* (cytotype 2); 8) chromosome nº 7 of *C. eximium* (cytotype 2), *C. cardenasii* (cytotypes 1 and 2), and *C. tovarii* (cytotype 2); 9) chromosomes nº 3 of
C. baccatum var. baccatum and var. pendulum, C. eximium (cytotype 2), C. cardenasii (cytotypes 1 and 2), C. praetermissum and C. tovarii (cytotype 2); 10) chromosome nº 10 of C. baccatum var. baccatum and var. pendulum, C. cardenasii (cytotype 2), and C. praetermissum; 11) chromosome nº 11 of C. annuum var. annuum (cytotype 2), C. annuum var. glabriusculum (cytotypes 1 and 3).

FISH patterns found on 45S ribosomal family loci evidenced high similarity in number, position and size to those of specific fluorescent banding. However, some discrepancies were noted, such as the occurrence of a site on long arm of chromosome nº 7 that was not observed with triple staining CDD, in C. flexuosum. Moreover, a small signal appeared on the long arm of chromosome nº 5 of C. baccatum var. pendulum, which was not recognized by fluorescent banding (Moscone et al. 2007).

In C. cardenasii (cytotype 1), C. praetermissum and C. flexuosum, some heteromorphisms were found: chromosome pairs nº 1 and 11 in C. cardenasii, chromosome pair nº 9 in C. flexuosum, and chromosome pairs nº 7 and 12 in C. praetermissum (Figs. 5, 7).

Discussion

Nucleolar activity
The results obtained from AgNOR banding were considerably informative, together with other banding techniques already implemented in Capsicum (Moscone et al. 1996a, 2007; Scaldaferro et al. 2013), in terms of the identification of chromosomes and the recognition of number and position of NORs in the studied samples. This technique demonstrates its value for evidencing intra- and inter-specific chromosome variation in the genus. Silver impregnation allowed us to detect active rDNA sites that were not evidenced by fluorochrome banding, although they might have been recognized as CMA+DAPI- or CMA+DAPlO regions; this technique also allowed us to distinguish FISH signals that were not active NORs (see Distribution of 5S and 45S rDNA loci section).

In Capsicum, AgNORs are frequently accompanied by satellites that are not always differentially dyed with silver nitrate. NORs appear as a constriction in chromosomes stained with fluorescent dyes in every case (Scaldaferro et al. 2013). NORs and associated satellites are shown as CMA+DAPI- or CMA+DAPlO heterochromatic bands with fluorochrome banding technique, revealing that they are of GC-rich constitution. In Capsicum, NORs and their associated heterochromatin are rich in GC base pairs (Moscone et al. 1996a, 2007; Scaldaferro et al. 2013) as is the rule in plants (Sinclair and Brown 1971). Therefore, all NORs are considered descendants of one ancestral NOR (Berg and Greilhuber 1993).

In some of the examined taxa the number of AgNORs in metaphase disagrees with the maximum number of nucleoli in interphase nuclei. In C. annuum var. glabriusculum (cytotype 6), C. baccatum var. baccatum and C. chinense the
first number was higher than the second. This is probably due to nucleolar association, merging the nucleolus during interphase (Nicoloff et al. 1977; Sato et al. 1981; Lacadena et al. 1984), which is also influenced by the non-random position of NORs in the cell (Jordan et al. 1982). Moreover, we should consider the occurrence of interchromosomal nucleolar dominance where nucleolar organizers from different chromosome pairs compete in making up the nucleoli (Flavell and O’Dell 1979; Nicoloff et al. 1979). Furthermore, low transcriptional activity is attributed when AgNOR number is lower than nucleoli number (C. annuum var. glabriusculum cytotype 5, and C. villosum), in particular rDNA sites (small NORs) that are not detected by metaphase chromosome silver staining, but that may produce micronucleus in interphase (Sato et al. 1980).

The observed infraspecific variations with varying numbers of AgNORs in metaphase chromosomes resulted from the differences in rDNA locus activity, because detection with silver nitrate was not possible when loci were inactive (Moscone et al. 1995).

In this study the presence of nucleolar organizers on the long arm enables us to detect chromosome homeologies: in C. annuum var. glabriusculum pairs nº 5 of cytotypes 2, 4, 6 and 7 are considered homeologues. In C. tovarii (cytotype 2), C. eximium (cytotype 2) and C. cardenasii (cytotypes 1 and 2), there is homeology in chromosomes nº 7 which, as the examples cited above, carry a nucleolar organizer on the long arm. Similarly, the following chromosomes are postulated as homeologues, but due to the presence of AgNORs on the short arm: chromosome pairs nº 11 of C. annuum var. annuum (cytotype 2), C. annuum var. glabriusculum
(cytotype 1 and 3), and nº 12 of C. **annuum** var. **annuum** (cytotype 2), C. **annuum** var. **glabriusculum** (cytotypes 4, 5 and 6), C. **baccatum** var. **baccatum**, C. **baccatum** var. **pendulum**, C. **cardenasii** (cytotypes 1 and 2), C. **chinense**, C. **eximium** (cytotype 2), C. **frutescens**, C. **praettermissum**, C. **recurvatum**, C. **tovari** (cytotype 2) and C. **villosum**.

Phylogenetic interpretations can be made based on the data mentioned, as the number of NORs present in each species. C. **annuum** var. **annuum**, C. **annuum** var. **glabriusculum**, C. **chinense** and C. **frutescens**, all belonging to white flower group, present 1 to 4 NOR pairs, showing the greatest dissimilarities in the cytotypes of the wild variety of C. **annuum**. Another group is composed of purple flower species C. **cardenasii**, C. **eximium** and C. **tovarii**. They have similarities in the position and number of AgNORs, although C. **tovarii** (cytotype 2) presents an additional rDNA site. In the group of white flowers with greenish spots in the throat, C. **baccatum** var. **baccatum** and var. **pendulum** have four pairs of nucleolar organizers, C. **flexuosum** has two pairs but in this case NORs are located on unconventional positions, e.g. on large m chromosomes. C. **praettermissum** does not belong to the previous groups, although it was considered to be a variety of C. **baccatum** by Hunziker (2001). Data presented by Moscone et al. (2007) support the specific rank of C. **praettermissum** and an intermediate position between C. **baccatum** and the purple flower group. This species presents only two pairs of NORs, which is very significant because its specific position is far from C. **baccatum**. Finally, C. **recurvatum** and C. **villosum** show homeologies in their NOR position; these species are also phylogenetically distant from the above groups,
since they belong to the Brazilian $x = 13$ group and both have nucleolar organizers in pairs no 12, and in pair no 13 in *C. recurvatum* and in pair no 10 in *C. villosum*. 

*Capsicum rhomboideum* is the most distant taxa, belonging to the yellow flower group and with $x = 13$ (Andean $x = 13$ group). Its NOR position, a unique organizer region in pair no 9 (an m chromosome), is further evidence of the remoteness of this group. All these data have been corroborated with FISH using the corresponding rDNA probes (see Distribution of 5S and 45S rDNA loci section).

Records of size differences in NOR-associated satellites between cell and individuals, and even between homologous chromosomes revealed the presence of microsatellites, macrosatellites and tandem satellites in the species studied. These polymorphisms between homologous chromosomes may have various origins, like a different number of ribosomal genes, a dissimilar transcriptional activity, a distinct condensation level of chromatin in the NOR, or tandem NOR due to duplications as observed in barley (Linde-Laursen 1984) and in chili peppers (Moscone et al. 1995).

No correlation between the size of the AgNOR in metaphase chromosomes and the size of nucleoli in the interphase nuclei was noticed; this finding is consistent with findings reported in a previous study in the same genus by Moscone et al. (1995), and this is a phenomenon that is well established in plants (Burger and Knällmann 1980; Hizume et al. 1982; Linde-Laursen 1984). Regarding the specificity of silver nitrate staining, we found that silver nitrate binds to the NOR, the nucleoli and sometimes shows a tendency to bind chromosome
centromere in some taxa, although its specificity has not been confirmed, as in *Allium* (Schubert 1984).

**Distribution of 5S and 45S rDNA loci**

The number and position of secondary constrictions, satellites, AgNOR bands and 45S rDNA sites are karyotype characters often used in cytotaxonomy (Baeza and Schrader 2005; Xu et al. 2007; García et al. 2009, among hundreds). All of them are related to the highly conserved ribosomal 45S RNA genes, with the former three markers being dependent on the transcription of these genes, whereas the latter is independent of transcription and may also detect non-functional rDNA sites (Kovarik et al. 2008).

FISH is an important tool used in physical gene mapping. Ribosomal genes are highly repetitive sequences or tandem arrangements found in small number of sites (loci) in the species genome. In higher eukaryotes, ribosomal RNA genes (rDNAs) are arranged in two different families, the nucleolus forming major rDNA (45S rDNA) family transcribed by RNA polymerase I and non-nucleolus forming, and minor rDNA (5S rDNA) family transcribed by RNA polymerase III. The major family is composed of clusters of multiple copies of tandemly repeated units that consist of a transcribed zone with coding regions for 18S, 5.8S and 28S rRNA genes separated by internal transcribed spacers (ITS 1 and ITS 2) and surrounded by non-transcribed spacer (NTS) sequences (Long and David 1980; Pendas et al. 1993). The minor family is composed of multiple copies and arranged in tandem
arrays, which comprise a highly conserved 120-bp long coding sequence with a variable non-transcribed spacer (NTS; Hemleben and Werts 1988; Kellogg and Appels 1995).


In Capsicum, FISH analysis of 5S and 45S rRNA gene family shows significant differences in number, size and distribution among the species studied. Physical mapping of the 5S locus indicates a single site of this rDNA in a conserved position, mostly intercalary and in an m median chromosome in the genus. In the Solanaceae family there are genetic maps including 5S rRNA gene (Mueller et al. 2005), although their physical counterpart is unknown. In Capsicum, established linkage groups do not include the 5S rRNA gene (Livingstone et al. 1999; Lefebvre et al. 2001).

The present results disagree with those obtained in other organisms, in which the number of 5S loci broadly differs from that found in Capsicum. In other plant species number varies between two sites (e.g. Nicotiana, Allium; Matyášek et al. 2002).
2002; Shibata and Hizume 2002), three sites (e.g. *Arabidopsis thaliana*; Fransz et al. 1998), four sites (e.g. *Hordeum*; Leitch and Heslop Harrison 1993), and eight sites (e.g. *Musa*; Osuji et al. 1998). In the Brassicaceae family the maximum number of 5S loci found was six sites (Ali et al. 2005). All these values greatly exceed the only existing site in every *Capsicum* taxa studied until now. In addition, *Beta vulgaris* (Schmidt et al. 1994) and *Paspalum* in its diploid state (Vaio et al. 2005) share with peppers the unilocus condition of the site. The most parsimonious explanation for the 5S rDNA distribution is that the ancestor of the group had a single locus on a medium to large chromosome, probably chromosome nº 6, considering its ancestral species *C. chacoense* (Scaldaferro et al. 2006).

The character number and position of 45S rDNA loci are useful for morphological identification of similar chromosome sites and operate as evolutionary markers between species. The variability of these analyzed characters is remarkable, showing a wide range from one pair in *C. rhomboideum* up to 30 pairs in *C. villosum*. Within each species, 18S-5.8S-25S rDNA locus number and position remain constant, except some variations, e.g. *C. annuum* (1 to 6 sites), *C. baccatum* (14 to 15 sites) and *C. cardenasii* (8 to 18 sites). Smaller landmarks are very variable, unlike the major ones, which hold number and position constant within each species and within each cytotype in *C. annuum*. Major sites are coincident with NORs that were previously identified by AgNOR banding (Fig. 6) (see *Nucleolar activity section*).

In most plant genera it has been observed that a diploid species generally contains one pair of NORs (Raina and Khoshoo 1971). Only in very few cases might
a diploid taxon contain more than two NORs. *In situ* hybridization studies have identified several other rDNA loci but on chromosomes that are devoid of NORs. However, the signals at those sites are generally considered to be inactive sites that do not synthesize ribosomal RNA. Even in those diploid species with more than two NORs, it has been generally found by FISH that, of these, only two remain active (Raina and Mukai 1999). In *Carthamus*, the distinctive variability in the distribution, number and signal intensity of hybridization sites for 18S-26S and 5S rDNA loci was often considered to distinguish the 14 *Carthamus* taxa. Active 18S-26S rDNA sites were generally associated with NOR loci on the nucleolar chromosomes (Agrawal et al. 2013).

The repetitive sequences of the 18S-5.8S-25S gene family show a variable distribution triggered by profound changes, including locus loss and gain, and sequence dispersion, which are involved in the genomic evolution of the genus *Capsicum*. Chromosome evolution often takes place via structural rearrangements, such as inversions and translocations, homologous and non-homologous unequal crossing-over, gene conversion and transpositional events (Danna et al. 1996; Thomas et al. 1996). These mechanisms were the ones responsible for the variation in size, number and position of rDNA sites (Hall and Parker 1995; Sharma and Raina 2005). Evidence suggests that positioning and remodeling rDNA sites could be related to the rDNA gene shuffling or transposable elements playing an important role in plant genome evolution (Dubcovsky and Dvorák 1995; Raskina et al. 2004; Datson and Murray 2006).
Although *in situ* hybridization is not a truly quantitative technique, signal size does reflect differences in copy number (Maluszynska and Schweizer 1989; Schwarzacher and Heslop-Harrison 1991). Hence, the rRNA loci of most taxa analyzed probably have similar number of units repeated in tandem. However, two taxa probably have a dissimilar number: in *C. annuum* var. *glabriusculum* (cytotype 1, chromosome pair nº 11) and in *C. flexuosum* (chromosome pair nº 2). This phenomenon has been recognized in plants (Flavell and Smith 1974; Rogers and Bendich 1987; Heslop-Harrison and Schwarzacher 2011).

Many studies have reported variation of ribosomal gene locus distribution, e.g. in *Nemesia* (Datson and Murray 2006), Triticeae (Leitch and Heslop-Harrison 1993; Dubcovsky and Dvorak 1995) and Brassicaceae (Ali et al. 2005). In *Capsicum*, the FISH landmarks of the 45S gene family resemble specific fluorescent banding, although not fully in number, position or size (Moscone et al. 2007; Scaldaferro et al. 2013). A relationship between 45S rDNA probes used in this study and GC-rich heterochromatic regions should be considered. Lim et al. (2004) reported the isolation and characterization of a repetitive sequence composed of A1/A2 units (GC-rich sub-repetition) that occurs as part of the IGS of 26S-18S rDNA and independently as a high-copy satellite repeat not associated with rDNA in the genomes of *Nicotiana*, Tomentosae section (sensu Knapp et al. 2004) and tobacco. Park et al. (2012) investigated the evolution of constitutive heterochromatin in detail, because this region was identified as most of the pepper genome structure in *Capsicum*. They showed that constitutive heterochromatin in pepper was actively expanded 20.0–7.5 million years ago through a massive accumulation of single-type
Ty3/Gypsy-like elements that belong to the Del subgroup. Interestingly, derivatives of the Del elements played important roles in the expansion of constitutive heterochromatic regions. This process represents a characteristic mechanism for genome expansion in plant species through expansion of constitutive heterochromatic regions, which does not involve a genome-wide duplication event. Most recently, Qin et al. (2014) confirmed that the large genome size in Capsicum is due to the LTR expansion.

Our findings about the localization of 45S probes and their relationship with heterochromatic regions and active NORs also suggest their additional role in Capsicum genome diversity.

Acknowledgements

This research was supported by grants from the University of Córdoba (SECyT-UNC), and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

References

Agrawal, R., Tsujimoto, H., Tandon, R., Rama Rao, S., Raina, S. N. 2013. Species-
genomic relationships among the tribasic diploid and polyploid *Carthamus* taxa based on physical mapping of active and inactive 18S–5.8S–26S and 5S ribosomal RNA gene families, and the two tandemly repeated DNA sequences. Gene 521: 136-144.


Bloom, S.E., Goodpasture, C. 1976. An improved technique for selective silver


Fransz, P., Armstrong, S., Alonso-Blanco, C., Fisher, T.C., Torres-Ruiz, R., Jones,


Kodama, Y., Yoshida, M.C., Sasaki, M. 1980. An improved silver staining technique


Lim, K.Y., Skalicka, K., Koukalova, B., Volkov, R.A., Matyasek, R., Hemleben, V.,
satellite homologous to intergenic 26-18S rDNA spacer in the evolution of
*Nicotiana*. Genetics **166**: 1935-1946.

vulgare* L. Heredity **100**: 33-43.

Genome mapping in *Capsicum* and the evolution of genome structure in the
Solanaceae. Genetics **152**: 1183-1202.

Biochemistry **49**: 727-764. doi:10.1146/annurev.bi.49.070180.003455

Maluszynska, J., Schweizer, D. 1989. Ribosomal RNA genes in B chromosomes of
*Crepis capillaris* detected by non-radioactive *in situ* hybridization. Heredity **62**:
59-65.

rDNA unit arrays in the plant genus *Nicotiana* (Solanaceae). Genome **45**: 556-
562.

Moscone, E.A. 1990. Chromosome studies on *Capsicum (Solanaceae)* I.
Karyotype analysis in *C. chacoënse*. Brittonia **42**: 147-154.
Moscone, E.A. 1993. Estudios cromosómicos en Capsicum (Solanaceae) II.


Pozzobon, M.T., Schifino-Wittmann, M.T., Bianchetti, L.B. 2006. Chromosome
numbers in wild and semidomesticated Brazilian *Capsicum* L. (Solanaceae) species: do $x = 12$ and $x = 13$ represent two evolutionary lines? Botanical Journal of the Linnean Society **151**: 259-269.


Protoplasma 105: 77-85.


Wanzenböck, E.-M., Schöfer, C., Schweizer, D., Bachmair, A. 1997. Ribosomal transcription units integrated via T-DNA transformation associate with the nucleolus and do not require upstream repeat sequences for activity in

Figure 1. Silver-stained somatic metaphases of *Capsicum* species. a-b. *C. annuum* var. *annuum* a. NMCA 10272 cytotype 2 b. NMCA 10544 cytotype 2 c-i. *C. annuum* var. *glabriusculum* c. NMCA 10955 cytotype 1 d. NMCA 10983 cytotype 2 e. LQ w.nº cytotype 3 f. YSG w.nº cytotype 4 g. Netherlands 804750009 cytotype 5 h. PI 511885 cytotype 6 i. PI 511886 cytotype 7 j. *C. chinense* GEB 807 k. *C. frutescens* GEB, FC, MM 795. *M* = macrosatellite; *T* = tandem satellite. Arrows indicate AgNORs. Bar = 10 µm.


Figure 3. Silver-stained interphase nucleus of *Capsicum* species. a-b. *C. annuum* var. *annuum* a. NMCA 10272 cytotype 2 b. NMCA 10544 cytotype 2 c-i. *C. annuum* var. *glabriusculum* c. NMCA 10955 cytotype 1 d. NMCA 10938 cytotype 2 e. LQ w.nº cytotype 3 f. YSG w.nº cytotype 4 g. Netherlands 804750009 cytotype 5 h. PI 511885 cytotype 6 i. PI 511886 cytotype 7 j. *C.
Figure 4. Double fluorescent in situ hybridization to metaphase chromosomes of *Capsicum* taxa (2n = 24) using probes for the 45S and 5S rRNA genes. 

**a-b.** *C. annuum var. annuum a. NMCA 10272 cytotype 2 b. NMCA 10544 cytotype 2**

**c-i.** *C. annuum var. glabriusculum c. NMCA 10955 cytotype 2 d. NMCA 10983 cytotype 2 e. LQ w.nº cytotype 3 f. YSG w.nº cytotype 4 g.**

Netherlands 804750009 cytotype 5 h. PI 511885 cytotype 6 i. PI 511886 cytotype 7 j. *C. chinense* GEB 807 k. *C. frutescens* GEB, FC, MM 795.

Arrows indicate the 45S hybridization to the biotin-labelled 45S probe, which was detected with TRITC-conjugated antibodies. A five pointed star indicates hybridization to the digoxigenin-labelled 5S probe, which was detected with FITC-conjugated antibodies. Bar = 10 µm.

Figure 5. Double fluorescent in situ hybridization to metaphase chromosomes of *Capsicum* taxa (2n = 24 and 2n = 26) using probes for the 45S and 5S rRNA

Arrows indicate the 45S hybridization to the biotin-labelled 45S probe, which was detected with TRITC-conjugated antibodies. A five pointed star indicates hybridization to the digoxigenin-labelled 5S probe, which was detected with FITC-conjugated antibodies. In f, one chromosome is missing (no 7). In k, two chromosomes are missing (no 6 and 9). Bar = 10 µm.

**Figure 6.** Close-ups of the individual chromosome pairs of each *Capsicum* taxa simultaneously stained with AgNOR and FISH, showing the size and morphology of NORs with both techniques. Bar represents 5 µm.

**Figure 7.** Idiograms of *Capsicum* taxa showing the distribution of 45S rDNA loci (red blocks) and 5S (green blocks). Euchromatic regions appear in blue. Active 45S rDNA sites are indicated by a constriction. Chromosomes that have the same number on the idiogram are not necessarily homeologous for the different taxa. In each idiogram, chromosomes with similar measures without markers were grouped. Bar represents 5 µm.
Table 1. List of *Capsicum* species studied, provenance, voucher number, karyotype features, and ribosomal RNA genes mapped by FISH.

<table>
<thead>
<tr>
<th>Species and voucher number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Provenance&lt;sup&gt;b&lt;/sup&gt;</th>
<th>2n</th>
<th>Karyotype formula&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Ordering nº of AgNOR-bearin</th>
<th>No. of AgNOR-bearing chromosomes&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Max. no. of nucleoli</th>
<th>No. and position of rDNA sites&lt;sup&gt;e&lt;/sup&gt;</th>
<th>45S</th>
<th>5S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. annuum</em> L. var. <em>annuum</em> NMCA 10272 cytotype 2 (10, 35)</td>
<td>Mexico, unknown place (c)</td>
<td>24</td>
<td>10 m + 1 sm + 1 st</td>
<td>11 (sm), 12 (st)</td>
<td>4 (15.15 %), 3 (42.42 %), 2 (36.37 %), 1 (6.06 %)</td>
<td>4</td>
<td>3 [2 major, 1 small] (4p; 11-12p)</td>
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<tr>
<td>NMCA 10544</td>
<td>Mexico, unknown place (c)</td>
<td>24</td>
<td>10 m + 1 sm + 1 st</td>
<td>11 (sm), 12 (st)</td>
<td>4 (25 %), 3 (75 %)</td>
<td>4</td>
<td>3 [2 major, 1 small] (4p; 11-12p)</td>
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<td>NMCA 10955</td>
<td>USA, Florida (w)</td>
<td>24</td>
<td>10 m + 1 sm + 1 st</td>
<td>11 (sm)</td>
<td>2 (83.33 %), 1 (16.67 %)</td>
<td>2</td>
<td>1 [major] (11p)</td>
<td>1 (6p)</td>
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<td>cytotype 1</td>
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<tr>
<td>NMCA 10983</td>
<td>USA, Texas (w)</td>
<td>24</td>
<td>11 m + 1 st</td>
<td>1 and 5# (m)</td>
<td>4 (33.33 %), 3 (22.23 %), 2 (11.11 %), 1</td>
<td>4</td>
<td>2 [major] (1p; 5q)</td>
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<td>cytotype 2</td>
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<td>2 (79.60 %), 1 (21.40 %)</td>
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<td>5 [1 major, 4 small] (1p, q; 4q; 9q; 11p)</td>
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<td>11 m + 1 st</td>
<td>5# (m), 12 (st)</td>
<td>4 (35 %), 3 (30 %), 2 (25 %), 1 (10 %)</td>
<td>4</td>
<td>4 [2 major, 2 small] (5q; 7q; 10q; 12p)</td>
<td>1 (4p)</td>
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<td>Netherlands, Nijmegen, Hortus Botanicus, Universitatis</td>
<td>24</td>
<td>11 m + 1 sm</td>
<td>12 (sm)</td>
<td>3 (14.29 %), 2 (71.42 %), 1 (14.29 %)</td>
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<td>3 [1 major, 2 small] (7p; 8q; 12p)</td>
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<tr>
<td>PI 511885</td>
<td>Mexico, Tepehuan (w)</td>
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<td>11 m + 1 st</td>
<td>5, 6 (10%), 6 (20%), 5 (60%), 4 (10%)</td>
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<tr>
<td>PI 511886</td>
<td>Mexico, Tepehuan (w)</td>
<td>24</td>
<td>11 m + 1 st</td>
<td>5, 6 (9.09%), 7 (9.09%), 6 (36.37%), 5 (27.27%), 4 (18.18%)</td>
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<td>C. chinense Jacq.</td>
<td>Brazil, Pará State, Belém, bought at</td>
<td>24</td>
<td>11 m + 1 st</td>
<td>7 (m), 12 (st)</td>
<td>4 (67%), 3 (33%)</td>
<td>5 [2 major, 3 small]</td>
<td>1 (6p)</td>
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<td>Brazil, Minas Gerais Estate, Belo Horizonte, bought at the market place (c)</td>
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<tr>
<td>GEB, FC, MM 795</td>
<td>Brazil, Minas Gerais Estate, Belo Horizonte, bought at the market place (c)</td>
<td>24</td>
<td>11 m + 1 st</td>
<td>1 (m), 12 (st)</td>
<td>4 (50 %), 3 (50 %)</td>
<td>4</td>
<td>9 [2 major, 7 small]</td>
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<tr>
<td><strong>C. baccatum L. var. baccatum</strong></td>
<td>Argentina, Salta province, Capital Department, Salta, cultivated on</td>
<td>24</td>
<td>11 m + 1 st</td>
<td>1, 3 and 10 (m), 12 (st)</td>
<td>8 (10 %), 7 (13 %), 6 (38 %), 5 (29 %), 4 (6 %), 3 (4 %)</td>
<td>7</td>
<td>15 [4 major, 11 small]</td>
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<tr>
<td>GEB 163 (10, 48)</td>
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</table>

(c) indicates the market place where the plant was purchased.
<table>
<thead>
<tr>
<th>Variety</th>
<th>Location</th>
<th>Accession</th>
<th>Height (m)</th>
<th>Node Size</th>
<th>Nodes (%)</th>
<th>Nodes Count</th>
<th>Node Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>var. <em>pendulum</em> (Willd.) Eshbaugh</strong> cv. “Cayenne” EAM &amp; RN 211 (8, 10)</td>
<td>Argentina, Salta province, La Viña department, Osma (c)</td>
<td>24</td>
<td>11 m + 1 st</td>
<td>1, 3 and 10 (m), 12 (st)</td>
<td>8 (40%), 7 (20%), 6 (10%), 5 (10%), 4 (20%)</td>
<td>8</td>
<td>14 [4 major, 10 small] (1p; 3p, q; 4q; 5p, q; 6q; 8p, q; 9q; 10p, q; 12p, q)</td>
</tr>
<tr>
<td><strong>C. eximium</strong> Hunz. EAM 255 cytotype 2 (5, 12)</td>
<td>Argentina, Salta province, Capital department, Salta, cultivated on</td>
<td>24</td>
<td>11 m + 1 sm</td>
<td>7# (m), 12 (sm)</td>
<td>4 (9.09%), 3 (18.18%), 2 (63.64%), 1 (9.09%)</td>
<td>4</td>
<td>6 [2 major, 4 small] (2-3p; 4q; 7-8q; 12p)</td>
</tr>
<tr>
<td>Taxon</td>
<td>Collection Details</td>
<td>n</td>
<td>Cytotype Details</td>
<td></td>
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<td>-------</td>
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</tr>
<tr>
<td><strong>C. cardenasii</strong>&lt;br&gt;Heiser &amp; Smith&lt;br&gt;Netherlands</td>
<td>904750136&lt;br&gt;cytotype 1&lt;br&gt;(5, 9)</td>
<td>24</td>
<td>11 m + 1 sm</td>
<td>7# (m), 12 (sm)</td>
<td>4 (22.22 %), 3 (33.33 %), 2 (44.45 %)</td>
<td>4</td>
<td>8-11 [2 major, 6-9 small]&lt;br&gt;(1p, or p, p^, q; 2p; 4q; 6q; 7q; 9q; 11q, or p, q; 12p)</td>
</tr>
<tr>
<td><strong>AAC w.nº</strong>&lt;br&gt;cytotype 2&lt;br&gt;(2, 6)</td>
<td>Bolivia, Murillo province, La Paz department, La Paz, bought at the market place (w)</td>
<td>24</td>
<td>11 m + 1 sm</td>
<td>7# (m), 12 (sm)</td>
<td>4 (67 %), 3 (33 %)</td>
<td>4</td>
<td>** 18 [6 major, 12 small]&lt;br&gt;(1p, q; 2-3p; 4q; 5p, q; 6-7q; 8-10p, q; 11q; 12p, q)</td>
</tr>
<tr>
<td>Species</td>
<td>Collectors</td>
<td>Location</td>
<td>Slides</td>
<td>Slides</td>
<td>Classifications</td>
<td>Chromosomes</td>
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<tr>
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<td>--------</td>
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</tr>
<tr>
<td>C. flexuosum</td>
<td>Sendtn.</td>
<td>Argentina, Misiones province, Guaraní department, Guaraní land (w)</td>
<td>24</td>
<td>11 m + 1 st</td>
<td>2 and 5 (m)</td>
<td>4 (38.46 %), 3 (30.77 %), 2 (30.77 %)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>GEB, FC, EMa</td>
<td></td>
<td></td>
<td></td>
<td>14-15 [2 major, 12-13 small]</td>
<td>1</td>
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<tr>
<td></td>
<td>1034 (5, 13)</td>
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<td></td>
<td></td>
<td>(1q^; 2p; 3q^; 4q, q^; 5p, q^; 6q, q^; 7q; 8q^; 9p, q^, or q^; 12q, q^)</td>
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<td></td>
</tr>
<tr>
<td>C. praetermissum</td>
<td>Heiser &amp; Smith</td>
<td>Brazil, San Pablo state, Mogi das Cruzes (w)</td>
<td>24</td>
<td>11 m + 1 sm</td>
<td>6 (m), 12 (sm)</td>
<td>4 (40 %), 2 (60 %)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>EFM 05-17</td>
<td></td>
<td></td>
<td></td>
<td>11-13 [2 major, 9-11 small]</td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td>cytotype 2</td>
<td></td>
<td></td>
<td></td>
<td>(1p; 2p, q; 3q^; 5q^; 6p; 7p, or p, q; 9p; 10p^; q;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4, 6)</td>
<td></td>
<td></td>
<td></td>
<td>6q, q^; 7q, or q^; 12q, q^)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Collection Details</td>
<td>Chromosome Number</td>
<td>Ratio</td>
<td>Percentage</td>
<td>Additional Information</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>C. rhomboideum</em></td>
<td>YSG 20 (2, 11)</td>
<td>26</td>
<td>9</td>
<td>2 (36.36 %), 1 (63.64 %)</td>
<td>1 major (9p)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3p</td>
<td></td>
</tr>
<tr>
<td><em>C. recurvatum</em></td>
<td>GEB, MM, RSc, RM 915 (3, 12)</td>
<td>26</td>
<td>12 (sm), 13 (st)</td>
<td>2 (25 %), 1 (75 %)</td>
<td>4 [2 major, 2 small] (5p^; 12p, q^; 13p)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Witas*, Brazil, Paraná state, Morretes municipality, La Graciosa (w)
<table>
<thead>
<tr>
<th>Species</th>
<th>Location Details</th>
<th>Chromosomes Count</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. tovari</em></td>
<td>USA, New Mexico, Las Cruces, cultivated at New Mexico University (w)</td>
<td>24</td>
<td>11 m + 1 sm, 6 and 7# (m), 12 (sm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 (23.08 %), 5 (15.38 %), 4 (15.38 %), 3 (15.38 %), 2 (7.70 %), 1 (23.08 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 [3 major, 5 small] (1q; 3p, q; 4 q; 6 p; 7q; 11 q; 12 p)</td>
</tr>
</tbody>
</table>

| *C. villosum*    | Brazil, Rio de Janeiro state, Resende municipality, National Park Itatiaia (w) | 26                | 9 m + 3 sm + 1 t, 10 and 12 (sm) |
|                  |                                                       |                   | 3 (22.22 %), 2 (77.78 %) |
|                  |                                                       |                   | 4 [2 major, 28 small] (1p, q, q^; 2p, p^, q; 3p, p^; 4p, q; 5p, q, q^; 6p, p^; 7q, 8p p^, q; 10p, p^) |

| Metadata         |                                                       |                   | 1 |


\(^a\) var. variety, cv. cultivar; w.no. without number. The number of seedlings and somatic metaphases analyzed per sample, respectively, are indicated in parentheses.

\(^b\) c, cultivated; w, wild.
c m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric; # AgNOR on the long arm. Secondary constrictions are on the short arm, except in pair no. 7 of *C. cardenasii* and *C. eximium*. References for karyotype formula and ordering number of chromosome pairs: Scaldaferro et al. (2013).

d Percentages of metaphases with respective numbers of NOR-bearing chromosomes are given in parentheses.

the corresponding chromosome arms involved are indicated in parentheses. Most 45S loci are terminal (intcalary 45S loci are indicated by ^) and 5S loci are intercalary. Synteny of 5S site to 45S site is denoted by +. p, short arm; q, long arm.

* References for nº and position of rDNA sites

** Data from Scaldaferro et al. 2006