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ARTICLE

FISH and AgNor mapping of the 45S and 5S rRNA genes in wild and cultivated species of *Capsicum* (Solananceae)

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Abstract: Chromosome number and position of rDNA were studied in 12 wild and cultivated species of the genus *Capsicum* with chromosome numbers $x = 12$ and $x = 13$ (22 samples). For the first time in these species, the 5S and 45S rRNA loci were localized and physically mapped using two-color fluorescence in situ hybridization and AgNOR banding. We focused on the comparison of the results obtained with both methods with the aim of accurately revealing the real functional rRNA genes. The analyzes were 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.

Key words: Capsicum chromosomes, fluorescence in situ hybridization, AgNOR banding, 5S rRNA genes, 45S rRNA genes, physical gene mapping.

Résumé : Le nombre de chromosomes et l'emplacement de l'ADNr ont été étudiés chez 12 espèces sauvages et cultivées du genre *Capsicum* avec $x = 12$ et $x = 13$ (22 échantillons). Pour la première fois chez ces espèces, les locus codant l'ARNr 5S et 45S ont été localisés sur la carte physique tant par hybridation in situ en fluorescence bicolore que par coloration AgNOR. Les auteurs se sont concentrés sur les résultats obtenus avec ces deux méthodes afin d'identifier de manière précise les gènes fonctionnels codant pour les ARNr. Les analyses se sont appuyées sur des travaux antérieurs qui avaient démontré que les locus 18S–5,8S–25S coïncidaient principalement avec les régions hétérochromatiques riches en GC et auraient donné naissance à des ADN satellite ne contenant pas des gènes actifs. Ces données illustrent la variabilité de l'ADNr au sein des caryotypes rencontrés au sein du genre *Capsicum*, ce qui fournit une assise pour la cartographie génétique (comparée). De plus, certaines des informations obtenues seront possiblement utiles dans le cadre d'études sur l'évolution de l'ADN répété. [Traduit par la Rédaction]

Mots-clés : Capsicum chromosomes, hybridation in situ en fluorescence, coloration AgNOR, gènes de l'ARNr 5S, gènes de l'ARNr 45S, cartographie physique.

Introduction

Capsicum L. (Solanaceae) comprises up to 40 species, some of which are quite variable (Carrizo García et al. 2013); it is a small genus of the Americas that occurs in tropical and temperate areas distributed from southern Mexico to central Argentina. *Capsicum* has great economic significance, as it includes the sweet and hot chili peppers, vegetables, and spices consumed worldwide. It comprises exclusively of 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; therefore, they are assumed to be derived (Pickersgill 1971, 1991; Moscone 1990, 1993, 1999; Moscone et al. 1993, 1995, 1996*a*, 2007; Tong and Bosland 2003; Scaldaferro et al. 2013), although Pozzobon et al. (2006) hypothesized 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

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their wild relatives, which were provisionally subdivided into a "white flower group" (some species exhibit single-colored flowers, i.e., white, cream) and a "purple flower group" (pink, lilac, violet; Pickersgill 1991), although different color combinations in lobules, throat, and tube, and with spots of varying colors, complicates species delimitation (Hunziker 2001; Barboza and Bianchetti 2005). Currently, we also recognize the "yellow flower group", which comprises species from Central America and north-western areas of South America (Moscone et al. 2007; Scaldaferro 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 x = 13 (Sehr et al. 2013). The origin of x = 13 occurred in two independent events, resulting in two subgroups of the $2n$ = 26 species, with the Andean x = 13 group (*C. rhomboideum* among them) appearing in the 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 that 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; Scaldaferro 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 kinds of plants (e.g., Triticeae, Nicotiana, Brassicaceae, etc.). As a result, 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 Dvorák 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 species of *Capsicum* 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, 1996*b*, 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 has allowed the identification of all 20 species of *Capsicum* 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 species of *Capsicum*. These GC-rich regions are typically equivalent to nucleolar organizer region (NOR)-associated heterochromatin in plants (Moscone et al. 1996*a*, 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 species of *Capsicum* (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 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 4 °C for a minimum of 12 h and stored at –20 °C until use. Chromosome spreads for AgNOR and FISH were performed after digestion of the material with enzymes (2% cellulase (*w*/*v*) (Serva, Heidelberg, Baden-Wurtemberg State, Germany), 1% pectinase (*v*/*v*) at 37 °C for 40 min (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 –20 °C until use.

Table 1. List of species of the genus *Capsicum* studied, provenance, voucher number, karyotype features, and ribosomal RNA genes mapped by FISH.

Species and voucher No.[a]	Provenance[b]	2*n*	Karyotype formula[c]	Ordering no. of AgNOR-bearing pairs[c]	No. of AgNOR-bearing chromosomes[d]	Max. no. of nucleoli	No. and position of rDNA sites[e]	
							45S	5S
Capsicum annuum L.var. *annuum* NMCA 10272 cytotype 2 (10, 35)	Mexico, unknown place (c)	24	10m+1sm+1st	11 (sm), 12 (st)	4 (15.15%), 3 (42.42%), 2 (36.37%), 1 (6.06%)	4	3 [2 major, 1 small] (4p; 11–12p)	1 (6p)
NMCA 10544 cytotype 2 (2, 4)	Mexico, unknown place (c)	24	10m+1sm+1st	11 (sm), 12 (st)	4 (25%), 3 (75%)	4	3 [2 major, 1 small] (4p; 11–12p)	1 (6p)
Capsicum annuum var. *glabriusculum* (Dunal) Heiser & Pickersgill NMCA 10955 cytotype 1 (3, 6)	USA, Florida (w)	24	10m+1sm+1st	11 (sm)	2 (83.33%), 1 (16.67%)	2	1 [major] (11p)	1 (6p)
NMCA 10983 cytotype 2 (5, 9)	USA, Texas (w)	24	11m+1st	1 and 5# (m)	4 (33.33%), 3 (22.23%), 2 (11.11%), 1 (33.33%)	4	2 [major] (1p; 5q)	1 (6p)
LQ w.no. cytotype 3 (3, 14)	Peru, unknown place (w)	24	11m+1st	11 (m)	2 (79.60%), 1 (21.40%)	2	5 [1 major, 4 small] (1p, q; 4q; 9q; 11p)	1 (5p)
YSG w.no. cytotype 4 (3, 20)	Venezuela, Capital District, Caracas, Quinta Crespo, bought at the market place (w)	24	11m+1st	5# (m), 12 (st)	4 (35%), 3 (30%), 2 (25%), 1 (10%)	4	4 [2 major, 2 small] (5q; 7q; 10q; 12p)	1 (4p)
Netherlands 804750009 cytotype 5 (5, 7)	Netherlands, Nijmegen, Hortus Botanicus, Universitatis Nijmegen (w)	24	11m+1sm	12 (sm)	3 (14.29%), 2 (71.42%), 1 (14.29%)	4	3 [1 major, 2 small] (7p; 8q; 12p)	1 (2p)
PI 511885 cytotype 6 (4, 5)	Mexico, Tepehuán (w)	24	11m+1st	1, 5, and 6# (m), 12 (st)	8 (10%), 6 (20%), 5 (60%), 4 (10%)	4	6 [4 major, 2 small] (1p; 5p; 6q; 9p, q; 12p)	1 (4p)
PI 511886 cytotype 7 (4, 11)	Mexico, Tepehuán (w)	24	11m+1st	1, 2, 5#, and 8 (m)	8 (9.09%), 7 (9.09%), 6 (36.37%), 5 (27.27%), 4 (18.18%)	8	4 [4 major] (1p; 2p; 5q; 8p)	1 (4p)
Capsicum chinense Jacq. GEB 807 (3, 3)	Brazil, Pará State, Belém, bought at the market place (c)	24	11m+1st	7 (m), 12 (st)	4 (67%), 3 (33%)	3	5 [2 major, 3 small] (2p; 7p; 8q; 12p, q)	1 (6p)
Capsicum frutescens L. GEB, FC, MM 795 (3, 8)	Brazil, Minas Gerais Estate, Belo Horizonte, bought at the market place (c)	24	11m+1st	1 (m), 12 (st)	4 (50%), 3 (50%)	4	9 [2 major, 7 small] (1p; 3p, q; 4q; 5p; 8p, q; 9q; 12p)	1 (5p$^+$)

Table 1 (*continued*).

Species and voucher No.[a]	Provenance[b]	2*n*	Karyotype formula[c]	Ordering no. of AgNOR-bearing pairs[c]	No. of AgNOR-bearing chromosomes[d]	Max. no. of nucleoli	No. and position of rDNA sites[e]	
							45S	5S
Capsicum baccatum L. var. *baccatum* GEB 163 (10, 48)	Argentina, Salta province, Capital Department, Salta, cultivated on private land (w)	24	11m+1st	1, 3, and 10 (m), 12 (st)	8 (10%), 7 (13%), 6 (38%), 5 (29%), 4 (6%), 3 (4%)	7	15 [4 major, 11 small] (1p; 3p, q; 4p; 5p; 6p, q; 7q; 8p, q; 9q; 10p, q; 12p, q)	1 (5p$^+$)
var. *pendulum* (Willd.) Eshbaugh 'Cayenne' EAM & RN 211 (8, 10)	Argentina, Salta province, La Viña department, Osma (c)	24	11m+1st	1, 3, and 10 (m), 12 (st)	8 (40%), 7 (20%), 6 (10%), 5 (10%), 4 (20%)	8	14 [4 major, 10 small] (1p; 3p, q; 4q; 5p, q; 6q; 8p, q; 9q; 10p, q; 12p, q)	1 (5p$^+$)
Capsicum eximium Hunz. EAM 255 cytotype 2 (5, 12)	Argentina, Salta province, Capital department, Salta, cultivated on private land (w)	24	11m+1sm	7# (m), 12 (sm)	4 (9.09%), 3 (18.18%), 2 (63.64%), 1 (9.09%)	4	6 [2 major, 4 small] (2–3p; 4q; 7–8q; 12p)	1 (9p)
Capsicum cardenasii Heiser & Smith Netherlands 904750136 cytotype 1 (5, 9)	Bolivia (w)	24	11m+1sm	7# (m), 12 (sm)	4 (22.22%), 3 (33.33%), 2 (44.45%)	4	8–11 [2 major, 6–9 small] (1p, or p, p^, q; 2p; 4q; 6q; 7q; 9q; 11q, or p, q; 12p)	1 (9p$^+$)
AAC w.no. cytotype 2 (2, 6)	Bolivia, Murillo province, La Paz department, La Paz, bought at the market place (w)	24	11m+1sm	7# (m), 12 (sm)	4 (67%), 3 (33%)	4	18[f] [6 major, 12 small] (1p, q; 2–3p; 4q; 5p, q; 6–7q; 8–10p, q; 11q; 12p, q)	1 (9p$^+$)
Capsicum flexuosum Sendtn. GEB, FC, EMa 1034 (5, 13)	Argentina, Misiones province, Guaraní department, Guaraní land (w)	24	11m+1st	2 and 5 (m)	4 (38.46%), 3 (30.77%), 2 (30.77%)	4	14–15 [2 major, 12–13 small] (1q^; 2p; 3q^; 4q, q^; 5p, q^; 6q, q^; 7q; 8q^; 9p, q^, or q^; 12q, q^)	1 (9p$^+$)
Capsicum praetermissum Heiser & Smith EFM 05-17 cytotype 2 (4, 6)	Brazil, San Pablo State, Mogi das Cruzes (w)	24	11m+1sm	6 (m), 12 (sm)	4 (40%), 2 (60%)	4	11–13 [2 major, 9–11 small] (1p; 2p, q; 3q^; 5q^; 6p; 7p, or p, q; 9p; 10p^, q; 12p, or p, q)	1 (7p$^+$)

Table 1 (*concluded*).

Species and voucher No.[a]	Provenance[b]	2*n*	Karyotype formula[c]	Ordering no. of AgNOR-bearing pairs[c]	No. of AgNOR-bearing chromosomes[d]	Max. no. of nucleoli	No. and position of rDNA sites[e]	
							45S	5S
Capsicum rhomboideum (Dunal) Kuntze YSG 20 (2, 11)	Venezuela, Táchira state, San Cristóbal department, La Laja (w)	26	10m+1sm+2st	9 (m)	2 (36.36%), 1 (63.64%)	2	1 [major] (9p)	1 (3p)
Capsicum recurvatum Witas. GEB, MM, RSc, RM 915 (3, 12)	Brazil, Paraná state, Morretes municipality, La Graciosa (w)	26	10m+2sm+1st	12 (sm), 13 (st)	2 (25%), 1 (75%)	2	4 [2 major, 2 small] (5p^; 12p, q^; 13p)	1 (3q)
Capsicum tovarii Eshbaugh, Smith & Nickrent NMCA 90008 cytotype 2 (5, 13)	USA, New Mexico, Las Cruces, cultivated at New Mexico University (w)	24	11m+1sm	6 and 7#(m), 12 (sm)	6 (23.08%), 5 (15.38%), 4 (15.38%), 3 (15.38%), 2 (7.70%), 1 (23.08%)	6	8 [3 major, 5 small] (1q; 3p, q; 4 q; 6 p; 7q; 11 q; 12 p)	1 (9q)
Capsicum villosum Sendtn. GEB, EFi, AG, GB 1653 (5, 9)	Brazil, Rio de Janeiro state, Resende municipality, National Park Itatiaia (w)	26	9m+3sm+1t	10 and 12 (sm)	3 (22.22%), 2 (77.78%)	4	30 [2 major, 28 small] (1p, q, q^; 2p, p^, q; 3p, p^; 4p, q; 5p, q, q^; 6p, p^; 7q; 8p p^, q; 10p, q; 11p, p^, q; 12p, q; 13p, q, q^, q^)	1 (1p)

Note: Collectors: GEB, G.E. Barboza; GB, G. Bertone; AAC, A.A. Cocucci; FC, F. Chiarini; EFi, E. Filippa; AG, A. Gutiérrez; EFM, E. Forni-Martins, EMa, E. Marini; MM, M. Matesevach; RM, R. Minhot; EAM, E.A. Moscone; RN, R. Neumann; LQ, Llatas Quiróz; YSG, Y. Sánchez García; RSc, R. Scrivanti; Netherlands, Hortus Botanicus, Universitatis Nijmegen;PI accession number of the United States Department of Agriculture (USDA), Griffin, Ga., USA; NMCA accession number of the College of Agriculture and Home Economics, New México State University, Las Cruces, N. Mex., USA.

[a]w.no., without number. The number of seedlings and somatic metaphases analyzed per sample, respectively, are indicated in parentheses.

[b]c, cultivated; w, wild.

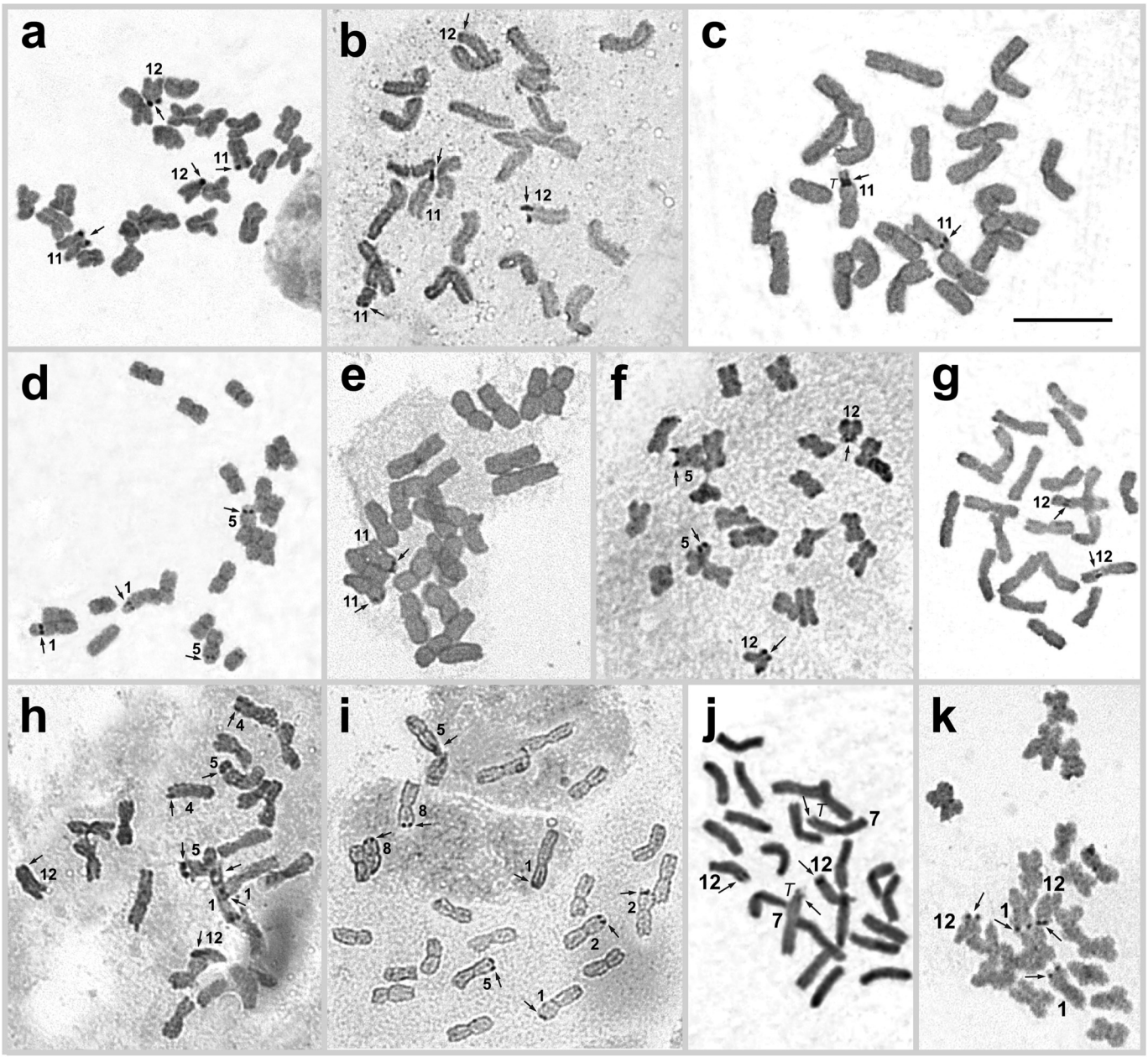
[c]m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric; #, AgNOR on the long arm. Secondary constrictions are on the short arm, except in pair No. 7 of *C. cardenasii* and *C. eximium*. References for karyotype formula and ordering number of chromosome pairs: Scaldaferro et al. (2013).

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

[e]The corresponding chromosome arms involved are indicated in parentheses. Most 45S loci are terminal (intercalary 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.

[f]Data from Scaldaferro et al. 2006.

Fig. 1. Silver-stained somatic metaphases of species of the genus *Capsicum*. (*a–b*) *Capsicum annuum* var. *annuum*: (*a*) NMCA 10272 cytotype 2, (*b*) NMCA 10544 cytotype 2; (*c–i*) *Capsicum 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*) *Capsicum chinense* GEB 807; (*k*) *Capsicum frutescens* GEB, FC, MM 795. *M*, macrosatellite; *T*, tandem satellite. Arrows indicate AgNORs. Scale bar = 10 μm.



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 the nomenclature of Battaglia (1955) 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

Fig. 2. Silver-stained somatic metaphases of species of the genus *Capsicum*. (*a*) *Capsicum baccatum* var. *baccatum* GEB 163; (b) *Capsicum baccatum* var. *pendulum* EAM and RN 211; (c) *Capsicum eximium* EAM 255 cytotype 2; (*d*–*e*) *Capsicum cardenasii*: (*d*) Netherlands 904750136 cytotype 1, (*e*) AAC w.no. cytotype 2; (*f*) *Capsicum flexuosum* GEB, FC, EMa 1034; (g) *Capsicum praetermissum* EFM 05-17 cytotype 2; (*h*) *Capsicum rhomboideum* YSG 20; (*i*) *Capsicum recurvatum* GEB, MM, RSc, RM 915; (*j*) *Capsicum tovarii* NMCA 90008 cytotype 2; (*k*) *Capsicum villosum* GEB, EFi, AG, GB 1653. *M*, macrosatellite; *T*, tandem satellite. Arrows indicate AgNORs. Scale bar = 10 μm.

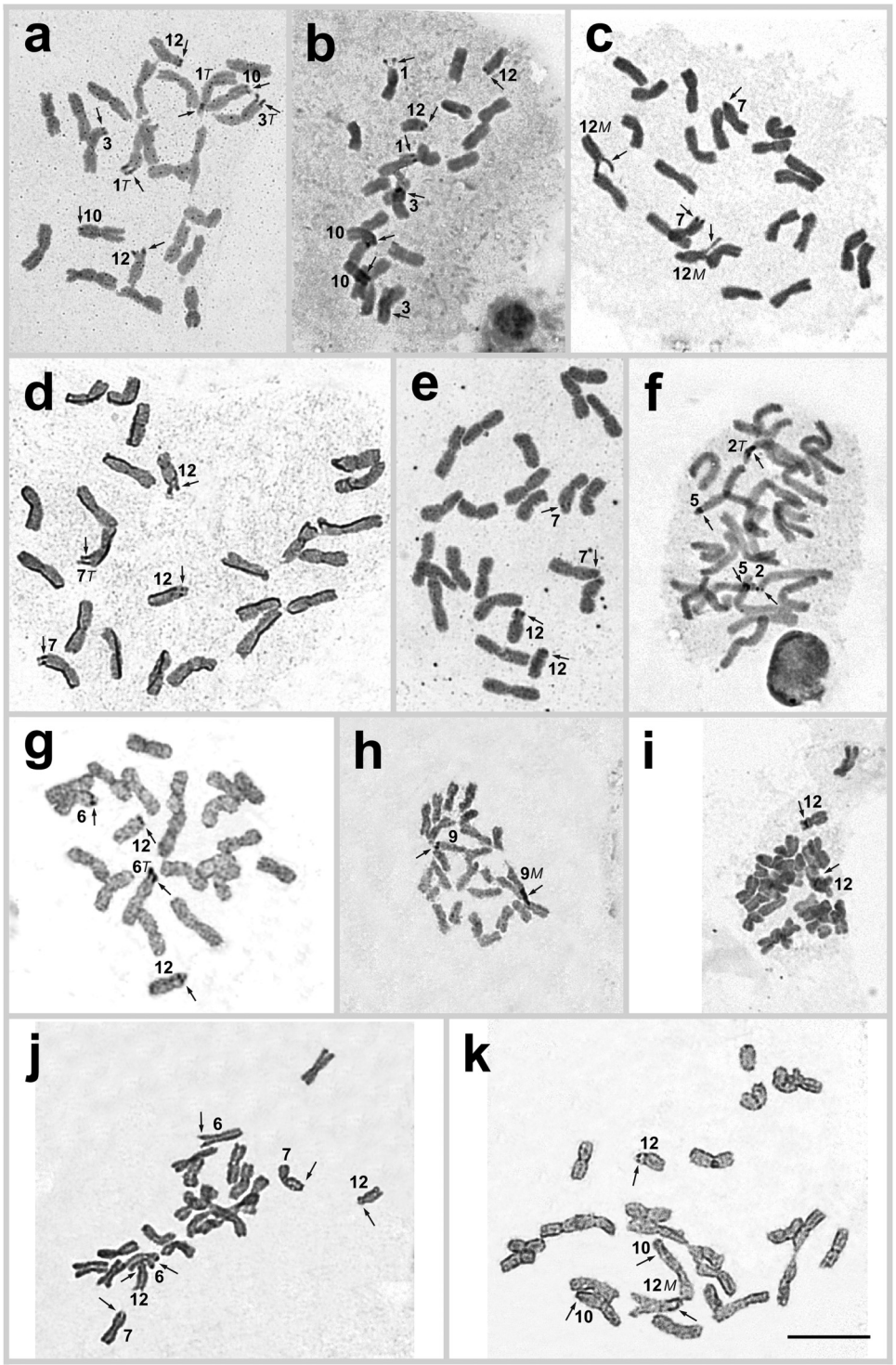
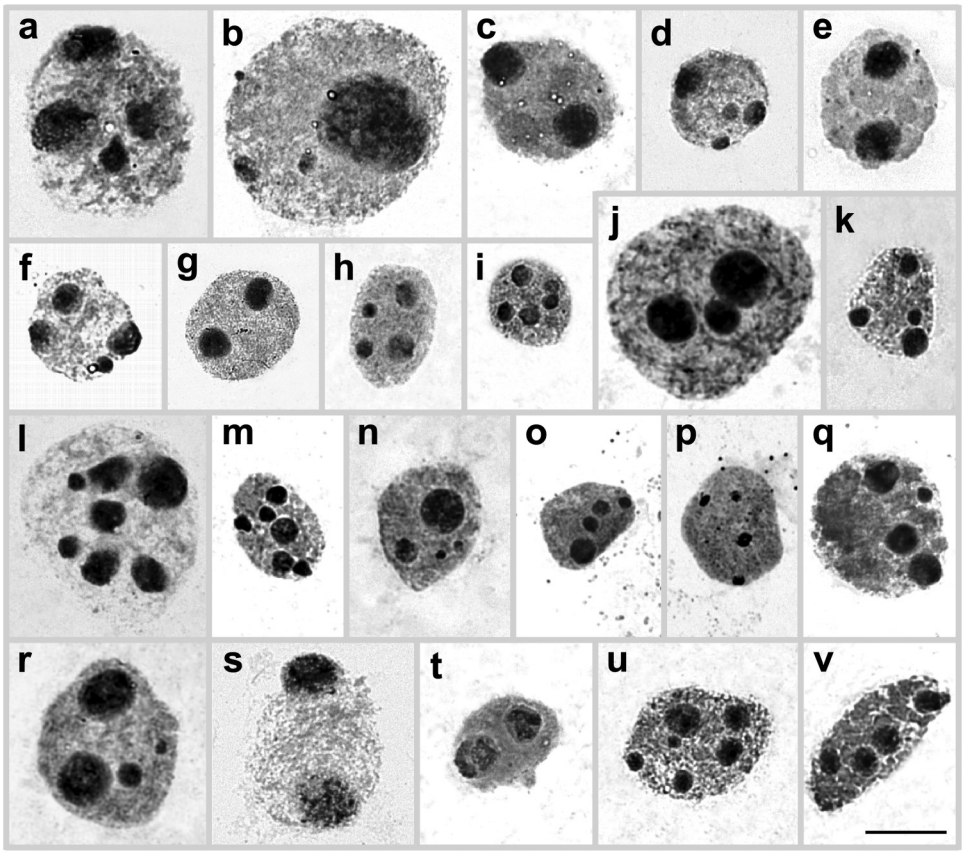


Fig. 3. Silver-stained interphase nucleus of species of the genus *Capsicum*. (*a*–*b*) *Capsicum annuum* var. *annuum*: (*a*) NMCA 10272 cytotype 2, (*b*) NMCA 10544 cytotype 2; (*c*–*i*) *Capsicum annuum* var. *glabriusculum*: (*c*) NMCA 10955 cytotype 1, (*d*) NMCA 10938 cytotype 2, (*e*) LQ w.no. cytotype 3, (*f*) YSG w.no. cytotype 4, (*g*) Netherlands 804750009 cytotype 5, (*h*) PI 511885 cytotype 6, (*i*) PI 511886 cytotype 7; (*j*) *Capsicum chinense* GEB 807; (*k*) *Capsicum frutescens* GEB, FC, MM 795; (*l*) *Capsicum baccatum* var. *baccatum* GEB 163; (*m*) *Capsicum baccatum* var. *pendulum* EAM and RN 211; (*n*) *Capsicum eximium* EAM 255 cytotype 2; (*o*–*p*) *Capsicum cardenasii*: (*o*) Netherlands 904750136 cytotype 1, (*p*) AAC w.no. cytotype 2; (*q*) *Capsicum flexuosum* GEB, FC, EMa 1034; (*r*) *Capsicum praetermissum* EFM 05-17 cytotype 2; (*s*) *Capsicum rhomboideum* YSG 20; (*t*) *Capsicum recurvatum* GEB, MM, RSc, RM 915; (*u*) *Capsicum tovarii* NMCA 90008 cytotype 2; (*v*) *Capsicum villosum* GEB, EFi, AG, GB 1653. Scale bar = 10 μm.



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. 1996*b*).

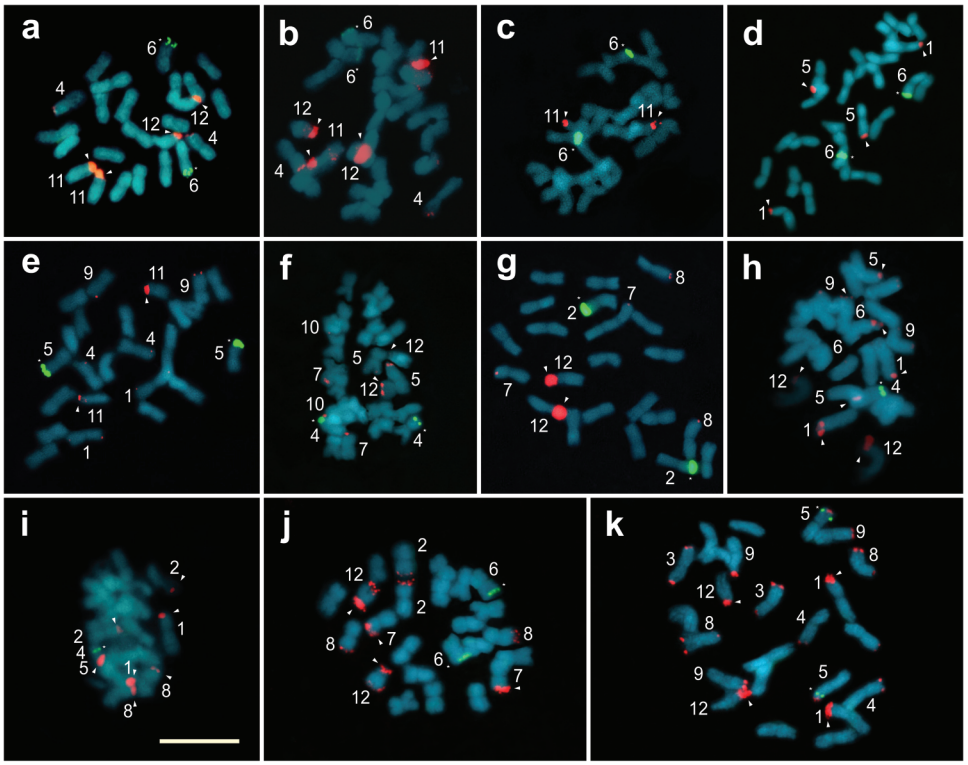
Fluorescence microscopy and image acquisition

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, Tokyio, Japan) and a JAI® CV-M4 + CL monocromatic digital camera (JAI, Barrington, N.J., 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, Calif., 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

Fig. 4. Double fluorescent in situ hybridization to metaphase chromosomes of *Capsicum* taxa ($2n = 24$) using probes for the 455 and 5S rRNA genes. (*a*–*b*) *Capsicum annuum* var. *annuum*: (*a*) NMCA 10272 cytotype 2, (*b*) NMCA 10544 cytotype 2; (*c*–*i*) *Capsicum 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*) *Capsicum chinense* GEB 807; (*k*) *Capsicum 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. Scale bar = 10 μm.



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".

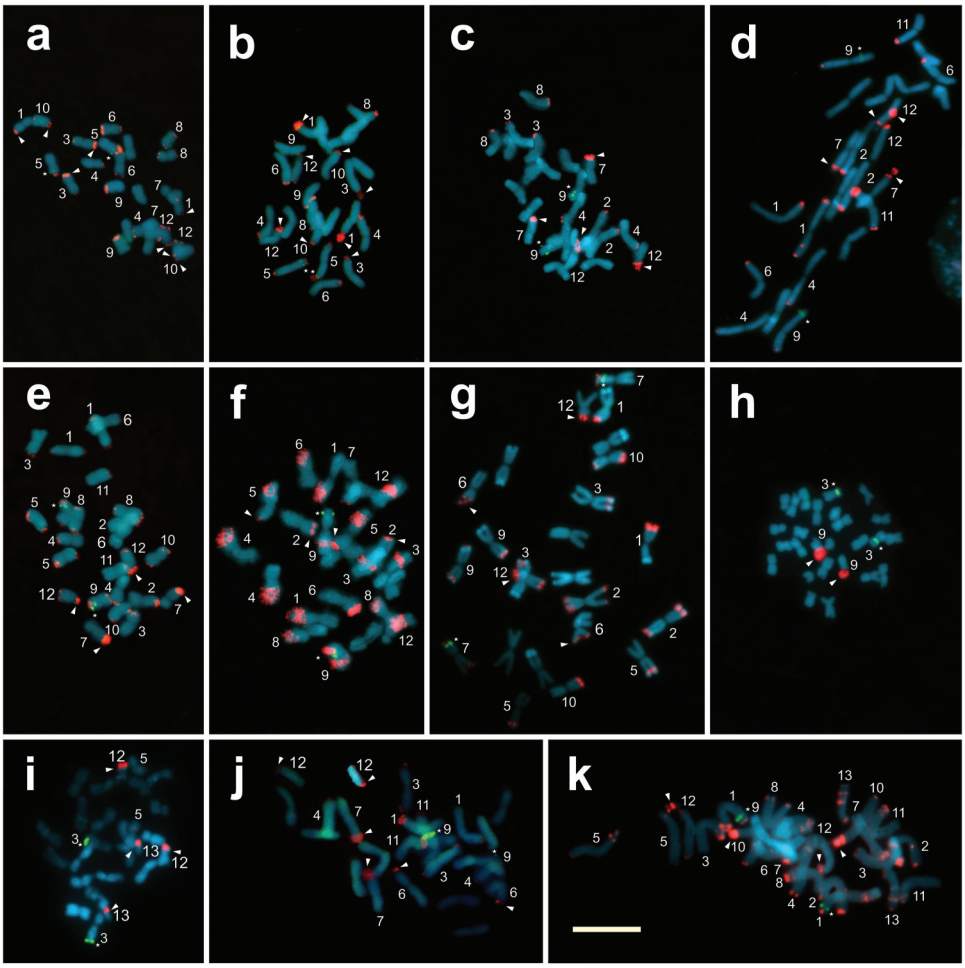
Results

Twenty two samples from 12 wild and cultivated *Capsicum* taxa were studied: *C. 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. tovarii*, 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 species of *Capsicum* 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, Figs. 1*c*, 1*e*, 1*g*) and *C. rhomboideum* (Fig. 2*h*); two AgNORs were observed in nine species: *C. annuum* var. *annuum* (cytotype 2, Figs. 1*a*, 1*b*) and *C. annuum* var. *glabriusculum* (cytotypes 2 and 4, Figs. 1*d*, 1*f*), *C. chinense* (Fig. 1*j*), *C. frutescens* (Fig. 1*k*), *C. eximium* (cytotype 2, Fig. 2*c*), *C. cardenasii* (cytotypes 1 and 2, Figs. 2*d*, 2*e*), *C. flexuosum* (Fig. 2*f*), *C. praetermissum* (Fig. 2*g*), *C. recurvatum* (Fig. 2*i*), and *C. villosum* (Fig. 2*k*); *C. tovarii* (cytotype 2) exhibited three AgNORs (Fig. 2*j*); and *C. annuum* var. *glabriusculum* (cytotypes 6 and 7, Figs. 1*h*, 1*i*) and *C. baccatum* var. *baccatum* and var. *pendulum* (Figs. 2*a*, 2*b*) showed four NORs in their haploid complements.

Fig. 5. Double fluorescent in situ hybridization to metaphase chromosomes of *Capsicum* taxa (2*n* = 24 and 2*n* = 26) using probes for the 455 and 5S rRNA genes. (*a*) *Capsicum baccatum* var. *baccatum* GEB 163; (*b*) *Capsicum baccatum* var. *pendulum* EAM and RN 211; (*c*) *Capsicum eximium* EAM 255 cytotype 2; (*d*–*e*) *Capsicum cardenasii*: (*d*) Netherlands 904750136 cytotype 1, (*e*) AAC w.no. cytotype 2; (*f*) *Capsicum flexuosum* GEB, FC, EMa 1034; (*g*) *Capsicum praetermissum* EFM 05-17 cytotype 2; (*h*) *Capsicum rhomboideum* YSG 20; (*i*) *Capsicum recurvatum* GEB, MM, RSc, RM 915; (*j*) *Capsicum tovarii* NMCA 90008 cytotype 2; (*k*) *Capsicum villosum* GEB, EFi, AG, GB 1653. 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 (Nos. 6 and 9). Scale bar = 10 μm.



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 No. 5 in cytotypes 2 and 4 of *C. annuum* var. *glabriusculum*, pair No. 6 and pair No. 4 in cytotypes 6 and 7, respectively, in the same taxa (Fig. 1); pair No. 7 in *C. eximium* (cytotype 2), *C. cardenasii* (cytotypes 1 and 2), and *C. tovarii* (cytotype 2); and pair No. 10 in *C. villosum* (Fig. 2). According to the arm position, 8 of the 12 species studied showed both terminal and subterminal satellites (Figs. 1*i*, 2*a*–2*e*, 2*g*, 2*j*). *Capsicum annuum* var. *annuum* (cytotype 2), *C. annuum* var. *glabriusculum* (cytotypes 4 and 6), and *C. rhomboideum* possessed only terminal-associated satellites (Figs. 1*a*, 1*b*, 1*f*, 1*h*, 2*h*). Finally, *C. annuum* var. *glabriusculum* (cytotypes 1, 2, 3, and 5), *C. flexuosum*, *C. recurvatum*, and *C. villosum* presented subterminal-associated satellites (Figs. 2*h*, 2*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 (Figs. 2*c*, 2*h*, 2*k*; see M NOR bearing chromosomes) and tandem satellites (Figs. 1*j*, 2*a*, 2*d*, 2*f*, 2*g*; see T NOR bearing chromosomes) in 13.64% and 22.73%, respectively. In some cases, sizes varied between homologous

Fig. 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. Scale bar = 5 μm.

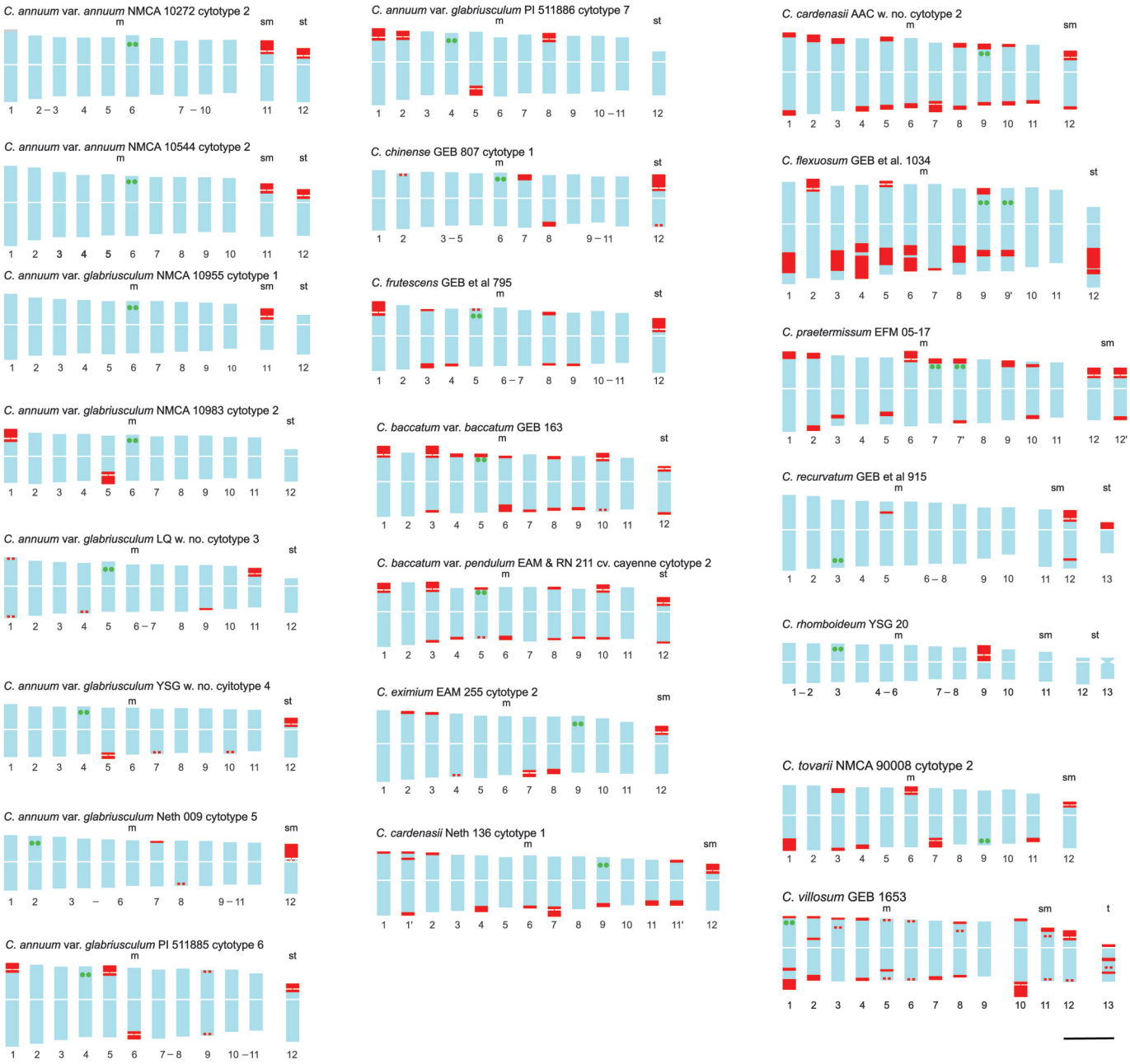


Fig. 7. Idiograms of *Capsicum* taxa showing the distribution of 45S rDNA loci (red blocks) and 5S (green blocks). Euchromatic regions appear in blue. Active 45S rDNA sites are indicated by a constriction. Chromosomes that have the same number on the idiogram are not necessarily homeologous for the different taxa. In each idiogram, chromosomes with similar measures without markers were grouped. m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric. Scale bar = 5 μm.

chromosomes, with heteromorphisms being detected (Figs. 2*d*, 2f–2*h*, 2*k*).

There was variation in the size of nucleoli in interphase nuclei and the size of AgNORs 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. 1*a*, 1*d*, 1*k*, 2*j*, 2*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. 1*h*; Table 1), in which the former was smaller than the latter (four and eight, respectively). Furthermore, unsteadiness in the number of chromosomes with AgNORs 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: 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 species of *Capsicum* analyzed, with the exception of *C. recurvatum* and *C. tovarii*, with the 5S locus placed on the long arm (Figs. 5*i*, 5*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 var. *pendulum*, and *C. frutescens*; chromosome No. 6 in *C. annuum* var. *annuum* (cytotype 2) and var. *glabriusculum* (cytotypes 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. tovarii* (cytotype 2). In addition, 5S was sintenic with a 45S locus in *C. baccatum* var. *baccatum* and var. *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; Scaldaferro 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 chromosome No. 9 of *C. rhomoboideum*; (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. futescens*, 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 of *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*; and (11) chromosome No. 11 of *C. annuum* var. *annuum* (cytotype 2) and *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 pair Nos. 1 and 11 in *C. cardenasii*, chromosome pair No. 9 in *C. flexuosum*, and chromosome pair Nos. 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. 1996*a*, 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 interspecific 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+/DAPIo regions; this technique also allowed us to

distinguish FISH signals that were not active NORs (see above).

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+/DAPIo 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. 1996*a*, 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, as 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* pair 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 pair No. 11 of *C. annuum* var. *annuum* (cytotype 2), *C. annuum* var. *glabriusculum* (cytotypes 1 and 3), and pair No. 12 of *C. annuum* var. *annuum* (cytptype 2), *C. annuum* var. *glabriusculum* (cytotypes 4, 5, and 6), *C. baccatum* var. *baccatum* and var. *pendulum*, *C. cardenasii* (cytotypes 1 and 2), *C. chinense*, *C. eximium* (cytotype 2), *C. frutescens*, *C. praettermissum*, *C. recurvatum*, *C. tovarii* (cytotype 2), and *C. villosum*.

Phylogenetic interpretations can be made based on the data mentioned, as the number of NORs present in each species. *Capsicum annuum* var. *annuum*, *C. annuum* var. *glabriusculum*, *C. chinense*, and *C. frutescens*, all belonging to white flower group, present 1–4 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 and *C. flexuosum* has two pairs, but in this case NORs are located on unconventional positions, e.g., on large m chromosomes. *Capsicum praetermissum* 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. praetermissum* 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, as they belong to the Brazilian $x = 13$ group and both have nucleolar organizers in pair Nos. 12 and 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 above).

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 Knälmann 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 a 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 Dawid 1980; Pendás 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).

These sequences have become valuable markers in the study of 5S and 18S–25S ribosomal gene localization, chromosome evolution, transgene localization, rDNA evolution associated with polyploidy, genetic and genomic relationships between species, genetic maps and linkage groups, phylogenetic, draft genome, etc., in many plants (Leitch and Heslop-Harrison 1993; Dubcovsky and Dvorák 1995; Moscone et al. 1996*b*; Fransz et al. 1998; Osuji et al. 1998; Ali et al. 2000, 2005; Hasterok et al. 2001; Matyášek et al. 2002; Shibata and Hizume 2002; Seijo et al. 2004; Datson and Murray 2006; Wang et al. 2012).

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; 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 family Brassicaceae, 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* (1–6 sites), *C. baccatum* (14–15 sites), and *C. cardenasii* (8–18 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 above).

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 se-

quence 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 a 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 Dvorák 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, as 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 and Yu (2014) confirmed that the large genome size in *Capsicum* is due to 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.

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