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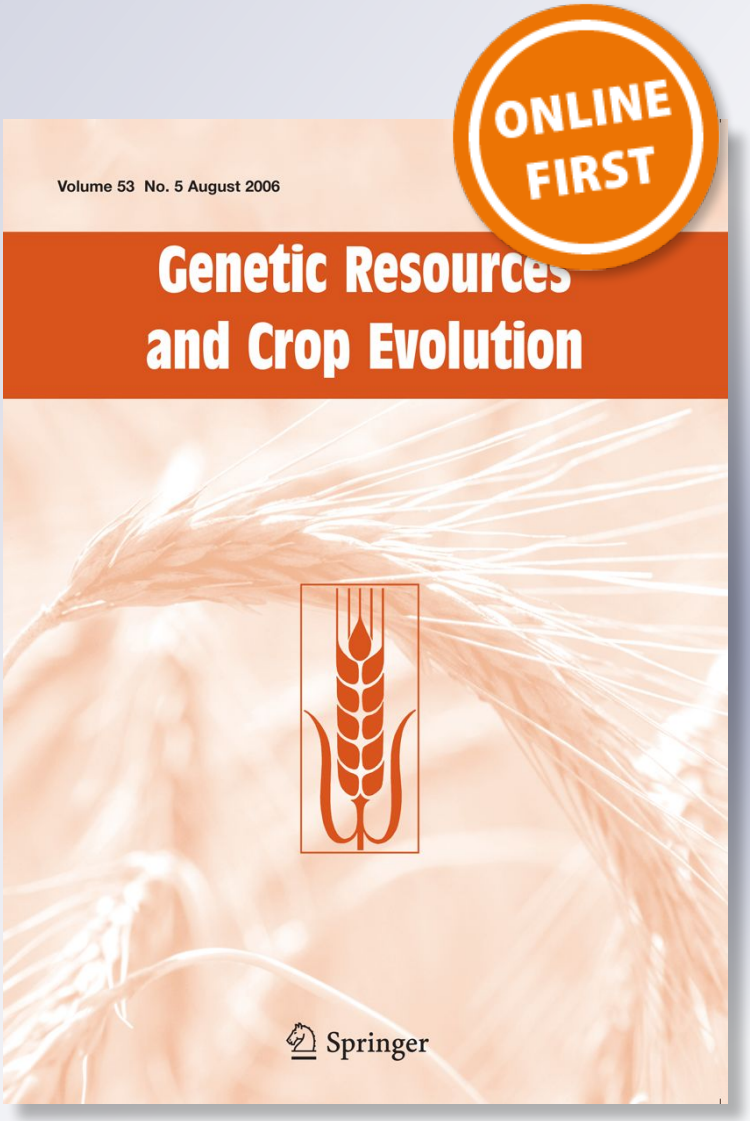
Genetic Resources and Crop Evolution
An International Journal

ISSN 0925-9864

Genet Resour Crop Evol
DOI 10.1007/s10722-012-9867-x



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RESEARCH ARTICLE

Heterochromatin type, amount and distribution in wild species of chili peppers (*Capsicum*, Solanaceae)

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Received: 29 November 2011 / Accepted: 21 May 2012

Abstract Triple staining with the fluorochromes chromomycin A3, distamycin A and 4′-6-diamidino-2-phenylindole (CMA/DA/DAPI) was applied to somatic metaphases and interphase nuclei of 11 taxa of wild chili peppers (*Capsicum*), with $2n = 2x = 24$ (*C. annuum* var. *glabriusculum*, *C. cardenasii*, *C. chacoense*, *C. flexuosum*, *C. galapagoense*, *C. eximium*, *C. praetermissum* and *C. tovarii*) and $2n = 2x = 26$ (*C. recurvatum*, *C. rhomboideum* and *C. villosum*) to analyse heterochromatin type, amount and distribution in wild members of this genus. Heterochromatic banding patterns allowed the identification of all the taxa examined and contributed to their taxonomic grouping. GC-rich heterochromatin (CMA+/DAPI−) was typical in all taxa; only *C. praetermissum* possessed also AT-rich (CMA−/DAPI+) and mixed GC- and AT-rich (CMA+/DAPI+) bands. Heterochromatin amount (expressed as % of karyotype length) ranged between 1.72 (*C. chacoense*) and 16.82 (*C. flexuosum*) and was positively correlated with karyotype length in most of the taxa examined. Heterochromatin located mainly at terminal position of chromosomes but intercalary position prevailed in *C. flexuosum*. Nucleolus organizer regions (NOR)-associated GC-rich heterochromatin was exclusively terminal and included the distal macrosatellite and a small portion on the corresponding arm. In all the taxa analysed, an equilocal heterochromatin distribution in non-homologous chromosomes of karyotype was observed, suggesting concerted evolution of heterochromatin dispersion in *Capsicum*.

Keywords *Capsicum* · Cytotaxonomy · Fluorescent chromosome banding · Karyotype evolution · Solanaceae

Capsicum is a New World genus of Solanaceae, comprising ca. 31 species, five of them domesticated and widely used as spices (pungent cultivars) or vegetables (sweet types) all over the world (Moscone et al. 2007). This genus has also medical and ornamental applications (Moscone et al. 2003, 2007).

Cytogenetic approaches to the study of *Capsicum* evidenced the universal presence of diploid karyotypes based on $x = 12$ and $x = 13$, the latter being more asymmetrical and derived than the former (Moscone 1990, 1993, 1999; Moscone et al. 1993, 1995, 1996, 2007; Pickersgill 1971, 1991; Tong and Bosland 2003; Pozzobon et al. 2006); however, Pozzobon et al. (2006) suggested that $x = 13$ would

Electronic supplementary material The online version of this article (doi:10.1007/s10722-012-9867-x) contains supplementary material, which is available to authorized users.

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Published online: 13 July 2012

be the ancestral basic number. Moscone et al. (1993) also demonstrated the presence of heterochromatic regions in chili peppers through Giemsa C-banding. Fluorochrome chromosome banding revealed that GC-rich heterochromatin located mostly at the terminal region is a common feature in the genus and that GC-rich NOR-associated heterochromatin is the rule in plants (Moscone et al. 1996, 2007). Fluorescent *in situ* hybridization to establish the number and distribution of rDNA loci in *Capsicum* showed a typical 5S rDNA intercalary locus per haploid genome (Park et al. 1999, 2000; Scaldaferro et al. 2006; Kwon and Kim 2009) and 18S-25S rDNA loci, which mostly correspond to entire GC-rich heterochromatic regions and affect genome size (Scaldaferro et al. 2006; Moscone et al. 2007; Grabiele et al. unpublished). By contrast, Ag-NOR staining revealed that the active nucleolus organizer regions (NORs) of *Capsicum* are usually fewer than 18S-25S rDNA loci in the same karyotype (Moscone et al. 1995, 2007; Scaldaferro et al. 2006; Grabiele et al. unpublished). Results of previously used cytogenetic techniques provide useful markers for chromosome identification and contribute to the taxomomic grouping of chili peppers.

The study of the genus *Capsicum* has been addressed using different techniques, such as chloroplast and nuclear DNA sequencing analyses (Walsh and Hoot 2001), and studies on restriction fragment length polymorphisms (RFLP), polymerase chain reaction (PCR), amplified fragment length polymorphisms (AFLP), randomly amplified polymorphic DNA (RAPD), RAPD markers based on touch-down polymerase chain reactions (Td-RAPD-PCR), simple sequenced repeats (SSRs) and polymorphic plastid DNA (cpDNA) markers have been published (cf. Prince et al. 1995; Paran et al. 1998; Rodriguez et al. 1999; Lefebvre et al. 2001; Buzo et al. 2002; Votava et al. 2005; Ince et al. 2010; Ibiza et al. 2011). The use of molecular data in combination with karyotype analyses should be a powerful tool for species characterization and taxonomic grouping in *Capsicum.*

Characterization of genetic diversity is essential to understand genome organization and evolution in *Capsicum*, the primary steps to successful crop improvement programmes. In this work we analysed genome variability of 11 wild members of the genus *Capsicum* by fluorescent chromosome banding, and provided a detailed characterization of their karyotype. This work contributes with basic information about the genetic diversity of the germplasm of wild species closely related to cultivated chili peppers, and presents useful data for the preservation of wild genetic resources. This approach is of value to prevent genetic deterioration.

Materials and methods

The provenance of the plant material studied is presented in Table 1. Voucher specimens were identified by Dr. Gloria E. Barboza and are deposited in the herbarium of Museo Botánico de Córdoba, Argentina (CORD).

Fluorochrome-stained chromosomes of somatic metaphases and interphase nuclei were observed in pectinase-cellulase-macerated root tip squashes obtained from seed germination. For details of dormancy breaking, mitotic arrest by *p*-dichlorobenzene, fixation, and enzyme maceration see Moscone et al. (1993).

Fluorescent chromosome banding to reveal the type and distribution of constitutive heterochromatic regions was performed using the triple staining technique (CDD) with the fluorochromes chromomycin A3, distamycin A and 4′-6-diamidino-2-phenylindole (CMA/DA/DAPI) (Schweizer and Ambros 1994). Enhanced, indifferent, or reduced fluorescence of chromosome segment is indicated in the text by attaching +, 0, or − to the fluorochrome or fluorochrome combination, respectively. Metaphase chromosomes and interphase nuclei were observed and photographed with epifluorescence in a Leica DMLB microscope equipped with the appropriate filter sets, a Leica DC250 digital camera, and the Leica IM1000 image management system.

For karyotype description, chromosomes were arranged in groups according to the position of the centromere and in order of decreasing size within each type. 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. (1996). The number of metaphases and individuals used for the karyotype analysis of each taxon is shown in Table 3 (supplementary material).

Results

Karyotype formula and length, ordering number of NOR-bearing pairs, heterochromatin amount,

Table 1 List of the *Capsicum* taxa studied, their provenance, voucher number, growth form, flower and fruit shape, and flower, fruit and seed colour

Taxon and voucher number	Growth form	Corolla shape and colour	Fruit shape and colour	Seed colour	Provenance
C. chacoense Hunz. EAM 250	Shrub (0.4–0.8 m)	Stellate; white	Ovoid or elliptic; red	Yellowish	Argentina: Salta Province, Capital Department, Salta, bought at market place
C. annuum L. var. *glabriusculum* (Dunal) Heiser et Pickersgill					
NMCA 10955	Herb or subshrub (1–2 m)	Stellate; white or cream	Elliptic; red	Yellowish	USA: Florida
NMCA 10983	Herb or subshrub (1–2 m)	Stellate; white or cream	Ovoid; red	Yellowish	USA: Texas
LQ w. no.	Herb or subshrub (1–2 m)	Stellate; white or cream	Ovoid; red	Yellowish	Peru: unknown place
YSG w. no.	Herb or subshrub (1–2 m)	Stellate; white or cream	Ovoid; red	Yellowish	Venezuela: Capital District, Caracas, Quinta Crespo, bought at market place
Netherlands 804750009	Herb or subshrub (1–2 m)	Stellate; white or cream	Ovoid; red	Yellowish	Netherlands: Nijmegen, Hortus Botanicus, Universitatis Nijmegen
PI 511885	Herb or subshrub (1–2 m)	Stellate; white or cream	Ovoid; red	Yellowish	Mexico: Tepehuan
PI 511886	Herb or subshrub (1–2 m)	Stellate; white or cream	Ovoid or spherical; red	Yellowish	Mexico: Tepehuan
C. galapagoense Hunz.					
PI 639682	Shrub (1–4 m)	Stellate; white	Spherical; red	Yellowish	The Netherlands: Nijmegen, Hortus Botanicus Universitatis Nijmegen
C. eximium Hunz.					
EAM 255	Herb, shrub or tree (0.6–4 m)	Stellate; white with violet lobules, greenish in the tube	Spherical; red	Brownish	Argentina: Salta Province, Capital Department, Salta; cultivated at a private house
C. cardenasii Heiser et Smith					
AAC w.no.	Shrub (1 m)	Campanulate; violet lobules with azure throat	Spherical; red	Brownish	Bolivia: La Paz Department, bought at market place
C. tovarii Eshbaugh, Smith et Nickrent					
NMCA 90008	Shrub (1 m)	Stellate; variable colour (purple or cream, cream with greenish spots in the lobules)	Spherical; red	Brownish	USA: New Mexico State, Las Cruces, New Mexico State University
C. praetermissum Heiser et Smith					
EFM 05/17	Herb or shrub (0.8–1.8 m)	Rotate; white with purple lobule margins and greenish spots in the throat	Spherical or elliptic; orange or red	Yellowish	Brazil: São Paulo State, Mogi das Cruzes

Table 1 continued

Taxon and voucher number	Growth form	Corolla shape and colour	Fruit shape and colour	Seed colour	Provenance
C. flexuosum Sendtn.					
GEB, FC, EMa 1034	Shrub (0.5–2 m)	Stellate; white with greenish spots in the throat	Spherical depressed; red	Blackish	Argentina: Misiones Province, Guarani Department, Predio Guaraní
C. recurvatum Witas.					
GEB, MM, RSc, RM 915	Herb or shrub (0.5–3 m)	Stellate; white with greenish spots in the throat	Spherical; yellowish green	Blackish	Brazil: Parana State, Morretes Municipality, La Graciosa
C. villosum Sendtn.					
GEB, EFi, AG, GB 1653	Subshrub or shrub (1–3 m)	Stellate; white with violet or brownish spots in the throat, greenish in the tube	Spherical; yellowish green	Blackish	Brazil: Rio de Janeiro State, Resende Municipality, Itatiaia National Park
C. rhomboideum (Dunal) Kuntze					
YSG 19	Shrub or small tree (0.8–4 m)	Rotate; yellow	Spherical; red	Brownish	Venezuela: Tachira State, San Cristobal Department, Pericos

Collectors: *GEB* G. E. Barboza, *GB* G. Bertone, *AAC* A. A. Cocucci, *FC* F. Chiarini, *EFi* E. Filippa, *EFM* E. Forni Martins, *AG* A. Gutiérrez, *EMa* E. Marini, *MM* M. Matesevach, *RM* R. Minhot, *EAM* E. A. Moscone, *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 Mexico State University, Las Cruces, NM, USA"

var. variety, *cv.* cultivar, *w.no.* without number

maximum number of bands per complement, and chromosome pairs with bands are listed for each *Capsicum* taxon in Table 2. Illustrations of interphase nuclei and somatic metaphases are presented in Figs. 1, 2, 3 and 4. Table 3 (supplementary material) provides the detailed karyotype measurements for all taxa from which the respective idiograms (Fig. 5) were derived. The correlation between heterochromatin amount, expressed as percentage of haploid karyotype length (%HKL) and HKL (μm) in *Capsicum* is shown in Fig. 6; the present data are shown together with data from Moscone et al. (2007).

All taxa with $2n = 24$ studied show rather uniform karyotypes composed of 11 m and 1 sm or st pairs, except *C. annuum* var. *glabriusculum* cytotype 1, which had 10 m, 1 sm and 1 st pairs. By contrast, taxa with $2n = 26$ present more asymmetrical karyotypes with 10 m, 2 sm and 1 st pairs, except *C. villosum*, which has 9 m, 3 sm and 1 t pairs. All karyotypes have 1 to 4 satellited (NOR-bearing) pairs.

The fluorescent chromosome banding patterns obtained by CMA/DA in the taxa analysed are in general the opposite of the DA/DAPI patterns. All the species show CMA/DA+ DA/DAPI− constitutive heterochromatin (hereafter referred to as CMA+/DAPI−, i.e., chromomycin bright and DAPI dull), occurring in macrosatellites, a variable number of other smaller and larger distal bands and intercalary bands. In addition, *C. praetermissum* has CMA/DA− DA/DAPI+ terminal and intercalary bands (hereafter referred to as CMA−/DAPI+, i.e., chromomycin dull and DAPI bright) and CMA/DA+ DA/DAPI+ mixed distal bands (hereafter referred to as CMA+/DAPI+ , i.e., chromomycin and DAPI bright). The NOR-associated heterochromatin, which is present in the satellite chromosomes, is CMA+/DAPI− and includes the distal macrosatellite and a small portion on the corresponding arm, next to the NOR. NORs are placed on short or long arms and appear as constrictions or gaps in fluorochrome-stained chromosomes; however, they sometimes appear as CMA+/DAPI 0. The centromeric heterochromatin is visible only as faint CMA+/DAPI 0 paired dots that are not always detected, as previously reported for the cultivated taxa of *Capsicum* (Moscone et al. 1996). Therefore, it was neither

Table 2 Karyotype features of the *Capsicum* taxa studied

Taxon and cytotype	2*n*	Karyotype formula (n)	Ordering no. of NOR-bearing pairs	HKL (μm) χ (sd)	Heterochromatin amount Total	NOR-assoc.	Interc.	Max. no. of bands per haploid complement	Max. no. of pairs with bands
C. chacoense									
Cytotype 1	24	11 m + 1 sm	1 (m), 12 (sm)	52.86 (4.62)	1.72	1.59	0.13	3	3
C. glabriusculum									
Cytotype 1	24	10 m + 1 sm + 1 st	11 (sm)	59.53 (3.60)	2.26	1.08	0.74	7	5
Cytotype 2	24	11 m + 1 st	1 (m) and 5 (m)	51.95 (6.61)	3.54	2.91	0.21	10	8
Cytotype 3	24	11 m + 1 st	11 (m)	55.13 (7.45)	2.33	0.74	0.31	12	8
Cytotype 4	24	11 m + 1 st	5 (m) and 12 (st)	53.56 (10.89)	6.33	0.91	0.22	18	11
Cytotype 5	24	11 m + 1 sm	12 (sm)	55.43 (8.29)	3.37	2.36	0.67	5	4
Cytotype 6	24	11 m + 1 st	1 (m), 5 (m), 6 (m) and 12 (st)	80.38 (15.53)	2.97	2.26	0.53	7	6
Cytotype 7	24	11 m + 1 st	1 (m), 2 (m), 5 (m), 8 (m)	70.05 (15.18)	3.83	3.15	0.29	7	6
C. galapagoense	24	11 m + 1 st	12 (st)	48.66 (3.22)	2.24	1.03	0.70	3	3
C. eximium									
Cytotype 2	24	11 m + 1 sm	7 (m), 12 (sm)	69.65 (6.33)	2.10	0.90	–	5	5
C. cardenasii									
Cytotype 2	24	11 m + 1 sm	7 (m), 12 (sm)	80.76 (15.80)	9.42	1.40	0.54	22	12
C. tovarii									
Cytotype 2	24	11 m + 1 sm	6 (m), 7 (m), 12 (sm)	67.02 (12.84)	4.89	0.87	–	8	8
C. praetermissum									
Cytotype 2	24	11 m + 1 sm	6 (m), 12 (sm)	76.20 (9.67)	14.92	1.81	2.15	23	12
C. flexuosum	24	11 m + 1 st	2 (m), 5 (m)	103.69 (23.42)	16.82	1.56	11.99	15	9
C. recurvatum	26	10 m + 2 sm + 1 st	11 (sm)	75.52 (15.57)	5.80	1.07	1.06	24	13
C. villosum	26	9 m + 3 sm + 1 t	12 (sm)	75.89 (7.72)	9.74	0.70	2.37	31	13
C. rhomboideum	26	10 m + 2 sm + 1 st	9 (m)	42.35 (2.34)	4.69	1.32	–	15	11

HKL haploid karyotype length; *χ* mean value; *sd* standard deviation (number of metaphases included in the measurements indicated in Table 3, supplementary material). Heterochromatin amount expressed as percentage of HKL; *NOR-assoc.* NOR-associated heterochromatin; *Interc.* Intercalary heterochromatin

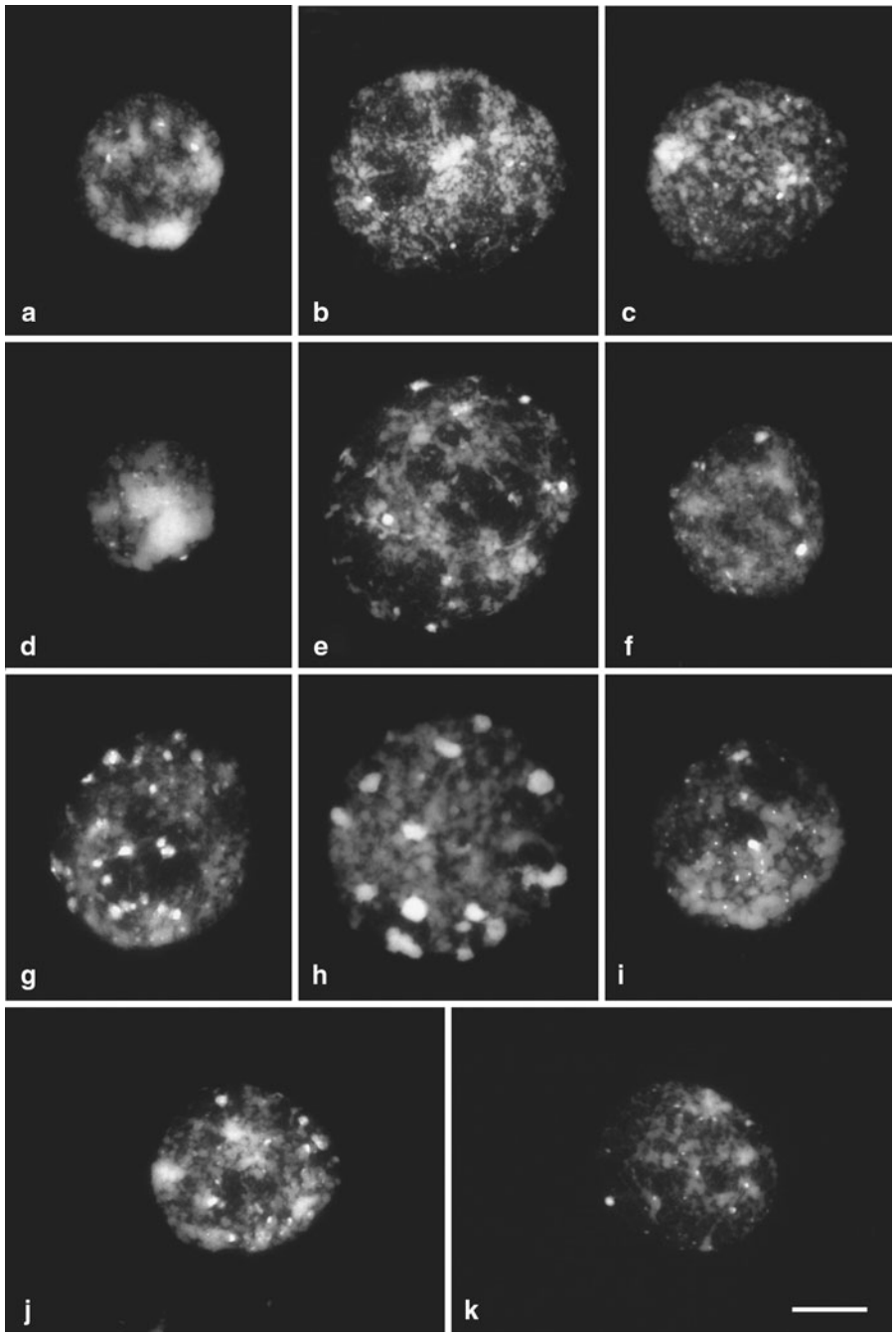

Fig. 1 CMA/DA-stained interphase nuclei of *Capsicum* taxa showing CMA+ chromocentres. **a** *C. chacoense* cytotype 1. **b** *C. annuum* var. *glabriusculum* cytotype 7. **c** *C. galapagoense*. **d** *C. eximium* cytotype 2. **e** *C. cardenasii* cytotype 2. **f** *C. tovarii* cytotype 2. **g** *C. praetermissum* cytotype 2. **h** *C. flexuosum*. **i** *C. recurvatum*. **j** *C. villosum*. **k** *C. rhomboideum*. *Bar* represents 10 μm

considered in the measurements nor presented in the idiograms (Fig. 5). In addition, heterochromatin amount varied from 1.72 % (*C. chacoense*) to 16.82 % (*C. flexuosum*) and correlated positively with the karyotype length in most of the taxa examined (Fig. 6). Furthermore, constitutive heterochromatin locates mainly at terminal position of chromosomes but intercalary position prevails in *C. flexuosum*.

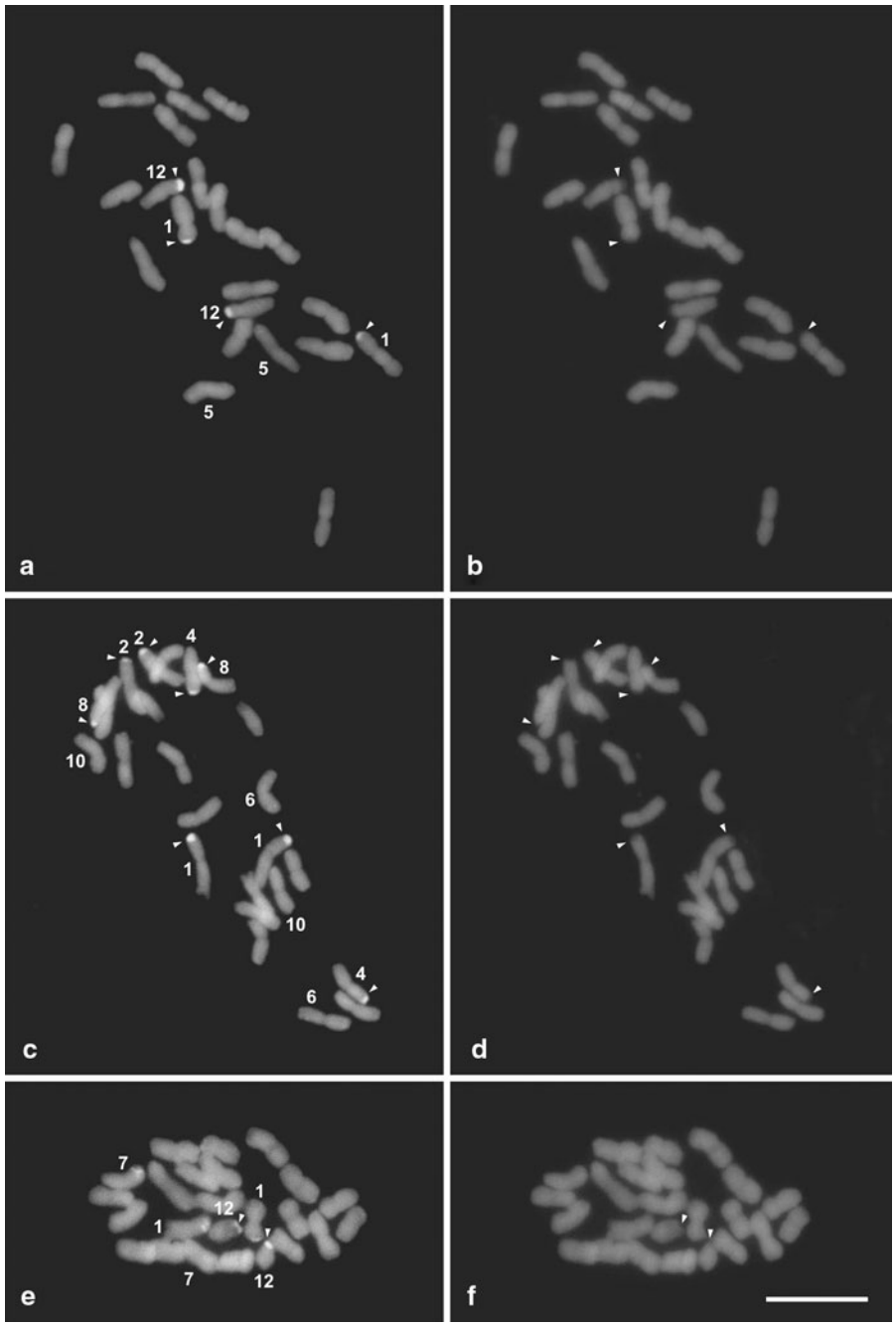

Fig. 2 Somatic metaphases of *Capsicum* species (2n = 24) triple-stained with CMA/DA/DAPI. **a**, **c**, **e** Metaphases after CMA/DA fluorescence exhibit CMA+ bands. **b**, **d**, **f** DA/DAPI fluorescence exhibit DAPI− bands. Identified homologous chromosomes are indicated with the same numbers as in the respective idiogram (Fig. 5). **a**, **b** *C. chacoense* cytotype 1. **c**, **d** *C. annuum* var. *glabriusculum* cytotype 7. **e**, **f** *C. galapagoense*. Arrowheads point out CMA+/DAPI− NOR associated heterochromatin. *Bar* represents 10 μm

Heterochromatic banding patterns allow the identification of all the taxa examined and contribute to their taxonomic grouping. In the following paragraphs, the results obtained for each *Capsicum* species are related to the data provided above; the sequence corresponds to their possible affinities.

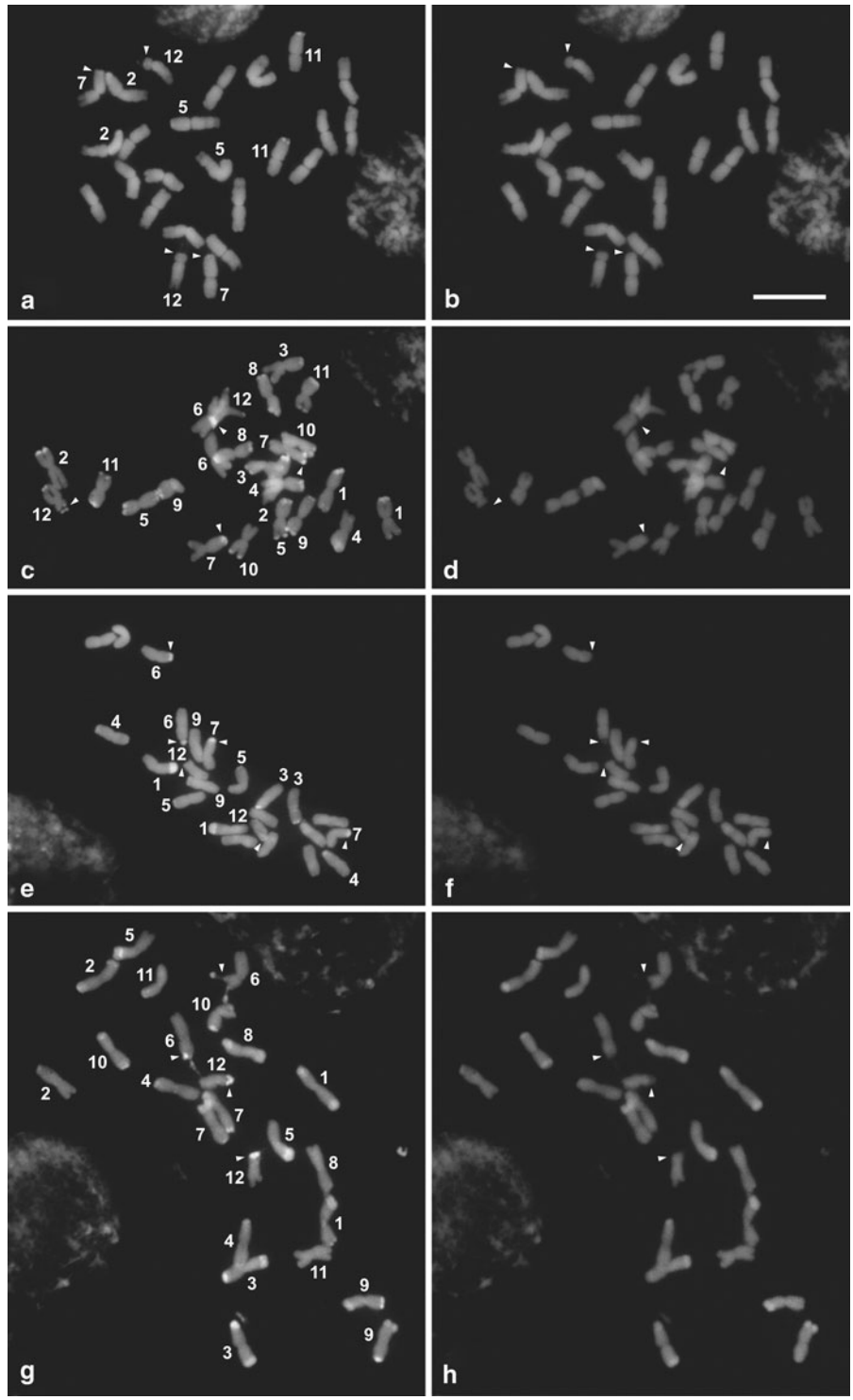

Fig. 3 Somatic metaphases of *Capsicum* species (2n = 24) triple-stained with CMA/DA/DAPI. **a**, **c**, **e**, **g** Metaphases after CMA/DA fluorescence exhibit CMA+ bands. **b**, **d**, **f**, **h** DA/DAPI fluorescence. **b**, **d**, **f** Exhibit DAPI− bands; **h** exhibit DAPI+ bands. Identified homologous chromosomes are indicated with the same numbers as in the respective idiogram (Fig. 5). **a**, **b** *C. eximium* cytotype 2. **c**, **d** *C. cardenasii* cytotype 2. **e**, **f** *C. tovarii* cytotype 2. **g**, **h** *C. praetermissum* cytotype 2. *Arrowheads* point out CMA+/DAPI− NOR associated heterochromatin. *Bar* represents 10 μm

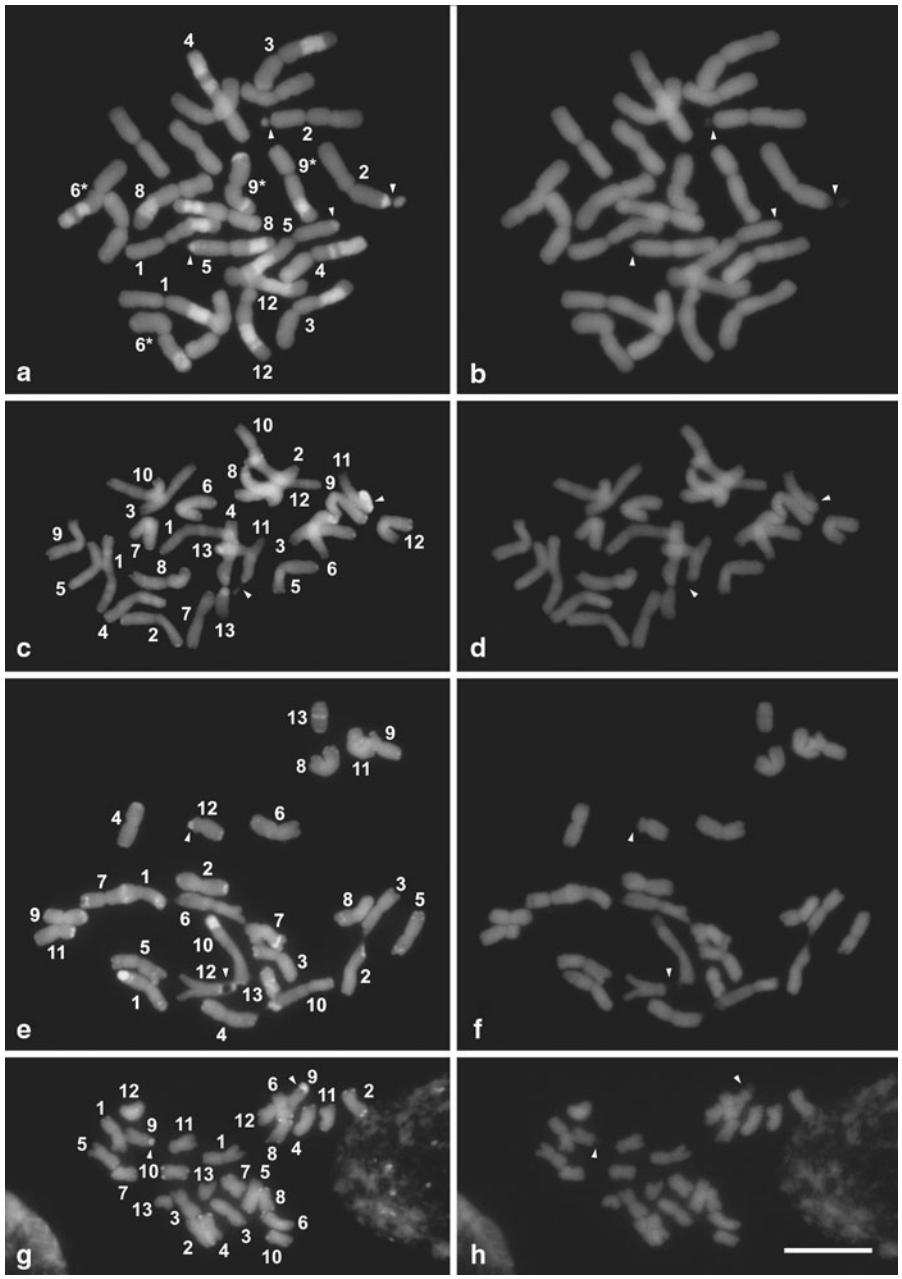

Fig. 4 Somatic metaphases of *Capsicum* species triple stained with CMA/DA/DAPI. **a**, **c**, **e**, **g** Metaphases after CMA/DA fluorescence exhibit CMA+ bands. **b**, **d**, **f**, **h** DA/DAPI fluorescence exhibit DAPI− bands. Identified homologous chromosomes are indicated with the same numbers as in the respective idiogram (Fig. 5). **a**, **b** *C. flexuosum* (2n = 24). **c**, **d** *C. recurvatum* (2n = 26). **e**, **f** *C. villosum* (2n = 26). **g**, **h** *C. rhomboideum* (2n = 26). *Asterisk* indicates heteromorphic pairs of chromosomes. *Arrowheads* point out CMA+/DAPI− NOR associated heterochromatin. *Bar* represents 10 μm

Capsicum chacoense

This species ($2n = 24$) has 11 m pairs of rather similar length (1–11) and one sm pair (12). Two pairs are satellited (1, m and 12, sm). This karyotype corresponds to cytotype 1 because cytotype 2 also exhibits two satellited pairs, but of different numbers (pairs 11, m and 12, st) (Moscone 1990; Moscone et al. 1993, 2007). *C. chacoense* together with *C. galapagoense* displays the lowest heterochromatin content and

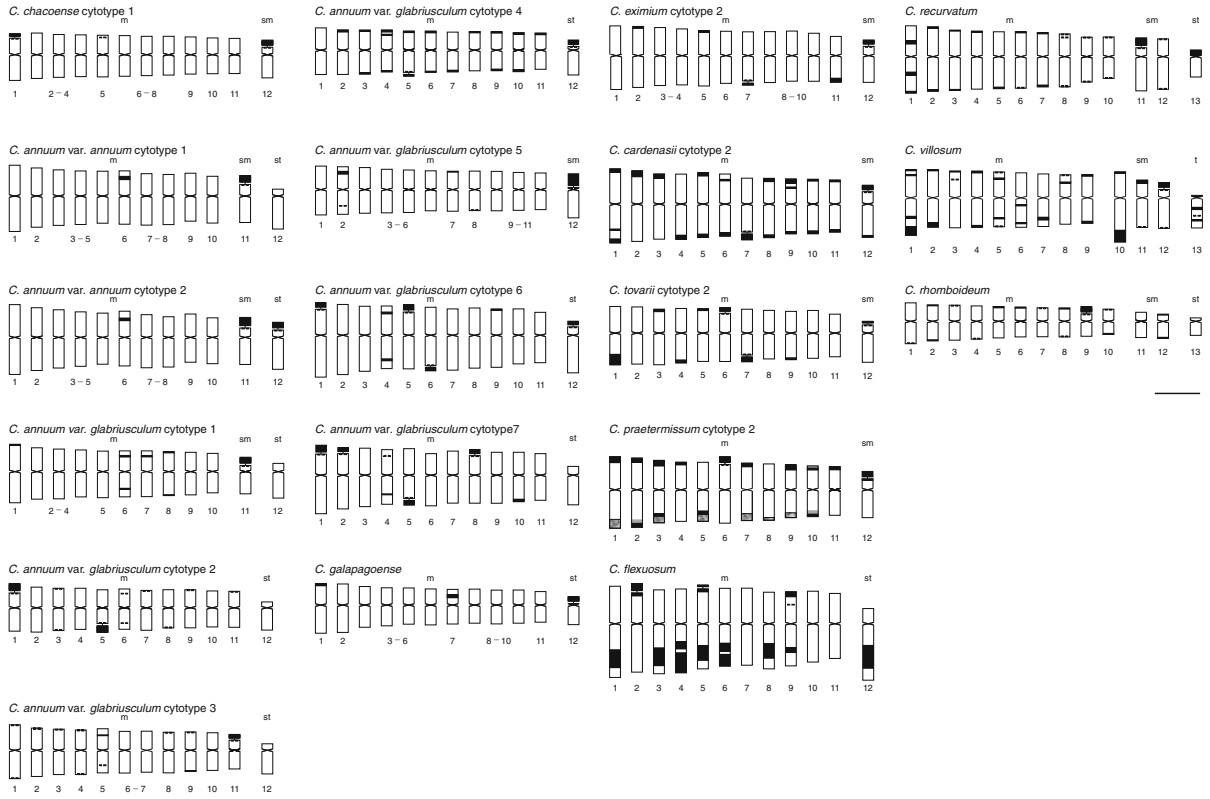

Fig. 5 Idiograms of *Capsicum* taxa showing heterochromatic fluorochrome banding patterns after triple-staining with CMA/DA/DAPI. Solid black blocks indicate CMA+/DAPI− bands of homogeneous aspect, *solid grey blocks* indicate DAPI+/CMA− bands of homogeneous aspect and *spotted blocks* indicate CMA+/DAPI+ bands of mottled appearance. Chromosomes that have the same number on the idiogram are not necessarily homologous for the different taxa. *C. annuum* var. *annuum* cytotypes 1 and 2 extracted from Moscone et al. (2007). *Bar* represents 5 μm

the simplest fluorochrome banding (Table 2; Figs. 2a, e, 5). The banding pattern of *C. chacoense* cytotype 1 is characterized by terminal NOR-associated heterochromatin on the short arm of satellite pairs 1 and 12, and an intercalary band on the short arm of pair 5. Cytotype 2 banding pattern only differs from cytotype 1 in the presence of a NOR-associated heterochromatic region in pair 11, whereas in cytotype 1 it is in pair 1 (Moscone et al. 2007). The intercalary band in pair 5 in both cytotypes probably corresponds to the 5S rDNA loci observed in pair 6 of a different plant material of *C. chacoense* cytotype 1 (AAC, EAM, FE 973; Scaldaferro et al. 2006) than that reported here. Giemsa C-banding in cytotype 1 shows a small amount of C-heterochromatin, mainly NOR-associated (EAM 104; Moscone et al. 1993), corresponding to CMA+/DAPI− NOR-associated heterochromatin of accession reported here.

Capsicum annuum var. *glabriusculum*

This taxon possesses a $2n = 24$ karyotype with 11 m pairs (1–11) of decreasing size and 1 st pair (12), except for cytotype 1, with 10 m pairs (1–10), one sm pair (11) and one st pair (12), and cytotype 5, with 11 m pairs (1–11) and one sm pair (12). Fluorescent chromosome banding performed in this variety showed only CMA+/DAPI− heterochromatin: however, a great variability was observed in HKL, karyotype asymmetry, number and position of small distal bands, intercalary bands, satellites, and NOR-associated heterochromatin. Because of the discrepancies found, below we describe the different cytotypes.

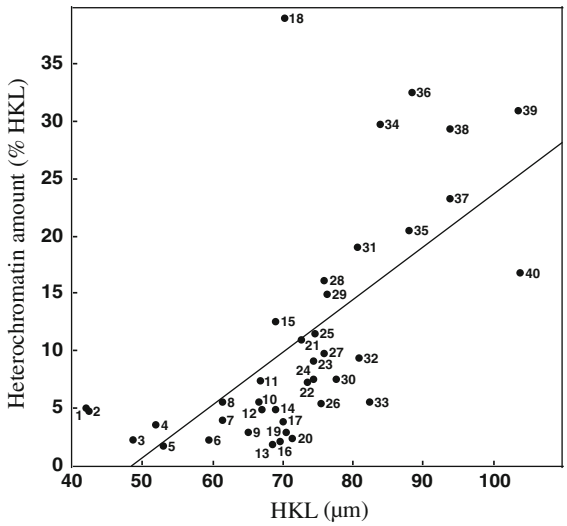

Fig. 6 Relationship between haploid karyotype length (HKL) and heterochromatin amount (expressed as percentage of HKL) in *Capsicum*. Data from the present work: 2, *C. rhomboideum*; 3, *C. galapagoense*; 5, *C. chacoense* cytotype 1; 17, *C. annuum* var. *glabriusculum* cytotype 7; 16, *C. eximium* cytotype 2; 32, *C. cardenasii* cytotype 1; 12, *C. tovarii* cytotype 2; 29, *C. praetermissum* cytotype 2; 40, *C. flexuosum*; 26, *C. recurvatum*; 27, *C. villosum*. Data from Moscone et al. (2007): 1, *C. rhomboideum*; 4, *C. annuum* var. *glabriusculum* cytotype 2; 6, *C. annuum* var. *glabriusculum* cytotype 1; 7 and 8, *C. chinense* cytotype 1 and 2, respectively; 9, *C. chacoense* cytotype 1; 10, *C. frutescens*; 11, *C. baccatum* var. *baccatum*; 13, *C. annuum* var. *annuum* cytotype 1; 14, *C. eximium* cytotype 1; 15, *C. cardenasii*; 18, *C. tovarii*; 19, *C. annuum* var. *annuum* cytotype 2; 20, *C. chacoense* cytotype 2; 21, *C. praetermissum* cytotype 1; 22 and 24, *C. baccatum* var. *pendulum* cytotype 1 and 2, respectively; 25, *C. pereirae* cytotype 1; 28, *C. pereirae* cytotype 2; 30, *C. parvifolium* cytotype 2; 31, *C. pubescens*; 33, *C. parvifolium* cytotype 1; 34, 38 and 39, *C. mirabile* cytotype 1, 2 and 3, respectively; 35 and 36, *C. campylopodium* cytotype 1 and 2, respectively; 37, *C. schottianum.* The line ($r = 0.65$; $P < 0.0001$) represents the correlation among data

Cytotype 1

This cytotype resembles cytotype 1 of *C. annuum* var. *annuum* (Moscone et al. 2007), although *C. annuum* var. *glabriusculum* displays higher heterochromatin amounts. One pair presents a terminal macrosatellite on the short arm (11). Fluorochrome banding shows distal bands on the short arms (pairs 1 and 8) and on the long arm of pair 8. Intercalary bands are present on both arms of pair 6 and on the short arm of pair 7 (Table 2; Fig. 5; Table 3 in supplementary material).

Cytotype 2

Two pairs present terminal macrosatellites on the short arm (1) or on the long arm (5). Together with other cytotypes of *C. annuum* var. *glabriusculum*, fluorochorme staining revealed more abundance of bands than in the cultivated variety. Six distal bands are present on the short arms (pairs 3, 7, 9 and 11) and on the long arms (pairs 3 and 8). Intercalary bands lie on both arms of pair 6 (Table 2; Fig. 5; Table 3 in supplementary material).

Cytotype 3

Only one pair presents terminal macrosatellite on the short arm (11). Several chromosomes exhibit fluorochrome banding; distal bands are present on the short arms (pairs 1–4, 8 and 9) and long arms (pairs 1, 4 and 9). Intercalary bands are found on both arms of pair 5 (Table 2; Fig. 5; Table 3 in supplementary material).

Cytotype 4

This cytotype presents the largest content of heterochromatin. All chromosomes possess fluorochrome bands, except pair one. Two pairs present terminal macrosatellites on the short arm (12) or on the long arm (5). The banding pattern is characterized by terminal bands on the short arms (pairs 2–6 and 8–11) or on the long arms (pairs 3, 4, 6, 7, 9 and 10). A unique intercalary band is observed on short arm of pair 4 (Table 2; Fig. 5; Table 3 in supplementary material).

Cytotype 5

This cytotype presents the lowest number of bands. Although the only terminal macrosatellite present on the short arm of pair 12, it is characterized by its large size. The fluorochrome banding pattern is simple, with a distal band on short arm of pair 7 and on long arm of pair 8. Pair 2 carries intercalary bands on both arms (Table 2; Fig. 5; Table 3 in supplementary material).

Cytotype 6

Four pairs present terminal macrosatellites on the short arm (1, 5 and 12) or on the long arm (6). Fluorochrome banding reveals that heterochromatin is mainly NOR-associated (pairs 1, 5, 6 and 12), and a single chromosome carries a distal band on its short

arm (pair 9), and intercalary bands are present on both arms of pair 4 (Table 2; Fig. 5; Table 3 in supplementary material).

Cytotype 7

Four pairs show terminal macrosatellites on the short arm (1, 2 and 8) or on the long arm (5). As in cytotyoe 6, fluorochrome banding reveals that heterochromatin is mainly NOR-associated (pairs 1, 2, 5 and 8) but a terminal band is found on the long arm of pair 10 and two intercalary bands appear in pair 4 (Table 2; Figs. 2c, 5; Table 3 in supplementary material).

Capsicum galapagoense

Capsicum galapagoense is endemic to Galapagos Islands (Ecuador); it presents $2n = 24$ with 11 m and 1 st pairs (12, satellited); two pairs (1 and 2) exhibit similar size and pairs 3–11 are also similar in length. Together with *C. chacoense*, this taxon exhibits the simplest fluorochrome banding (Figs. 2a, e, 5), with terminal NOR-associated heterochromatin in the short arm of satellited pairs 12, a terminal band in pair 1 and an intercalary band on the short arm of pair 7 (Table 3, supplementary material; Figs. 2e, 5).

Capsicum eximium

Capsicum eximium ($2n = 24$) has 11 m pairs of decreasing size (1–11) and 1 sm pair (12). Two pairs (7 and 12) possess satellites in the long and short arms, respectively. The fluorochrome chromosome banding is quite simple and heterochromatin content is the lowest within the informal purple flower group (Table 2; Figs. 3a, 5). The banding pattern is characterized by terminal NOR-associated heterochromatin in the satellited pairs (7 and 12), and three distal bands on the short arms (pairs 2 and 5) and long arm of pair 11, respectively. Another accession of *C. eximium* was analysed by fluorochrome; because it showed higher heterochromatin content and a more complex banding pattern (cytotype 1; Moscone et al. 2007) than that reported here; therefore, it was termed cytotype 2.

Capsicum cardenasii

This species endemic to Bolivia ($2n = 24$) possesses 11 m of decreasing size (1–11) and 1 sm pair (12); two pairs (7 and 12) have macrosatellites on the long and short arms, respectively. The fluorescent banding pattern is complex, with all chromosomes banded and distinguishable from each other (Figs. 3c, 5). Heterochromatin content is high and NOR-associated heterochromatin is low (pairs 7 and 12), representing a small amount of the total (Table 2); most bands are terminal but intercalary ones are observed on long arm of pair 1 and short arms of pairs 6 and 9, respectively (Figs. 3c, 5). Intercalary band in pair 9 probably corresponds to the 5S rDNA locus observed in pair 9 of a different plant material (CORD 1135; Scaldaferro et al. 2006) than that reported here. Cytotype 1 of *C. cardenasii* (CORD 1135; Moscone et al. 2007) shows lower HKL and higher heterochromatin content than the values reported here, which correspond to cytotype 2 of *C. cardenasii.*

Capsicum tovarii

Capsicum tovarii is endemic to Peru; it presents $2n = 24$ with 11 m pairs of decreasing size (1–11) and 1 sm pair (12). Short arm of pairs 6 and 12 and long arm of pair 7 are satellited. Fluorochrome banding shows exclusively terminal, NOR-associated heterochromatic bands in pairs 6, 7 and 12, a distinctive large block on the long arm of pair 1 and similar-sized bands in pairs 3, 4, 5 and 9 (Table 3, supplementary material; Figs. 3e, 5). Moscone et al. (2007) analysed the karyotype of another accession of *C. tovarii* (ATH & GB 25653) through fluorescent banding, and found strongly different results from those reported here (NMCA 90008): heterochromatin content (38.91 vs. 4.89 %, respectively) and distribution (23 terminal and 1 intercalary band per haploid genome vs. 8 terminal bands per haploid genome, respectively) and the number and position of satellites (2, pairs 10 and 12 in the former). Therefore, they correspond to two different cytotypes (1 and 2, respectively).

Capsicum praetermissum

This species ($2n = 24$) shows 11 m pairs of decreasing size (1–11) and 1 sm pair (12), with two satellited pairs (6 and 12). Heterochromatin content is high and the fluorochrome banding pattern is the most complex of the taxa analysed here, with different classes of heterochromatin consisting of terminal and intercalary bands; all chromosomes banded are distinguishable

from one another (Tables 2, Table 3 in supplementary material; Figs. 3g, h, 5). NOR-associated CMA+/DAPI− heterochromatin is found in the satellited pairs 6 and 12. Exclusive CMA+/DAPI− bands also appear at a distal position on short arms of pairs 1–4, 7, 9, 11, and at intercalary position in pairs 3, 5 (long arms) and 10 (short arm). Furthermore, CMA−/DAPI+ bands occur at intercalary location on long arm of pairs 2 and 10, and distally on the short arm of the latter. In addition, the long arm of pairs 1, 3, 5, 7, 8 and 9 exhibits mixed CMA+/DAPI+ heterochromatic bands. Moscone et al. (2007) analysed another accession (PI 441654) of *C. praetermissum*, which mainly differs from that reported here in the absence of CMA−/DAPI+ and CMA+/DAPI+ heterochromatin and also in most of the CMA+/DAPI− heterochromatin appearing as mottled; therefore, two cytotypes are recognized for *C. praetermissum* (1 and 2).

Capsicum flexuosum

Capsicum flexuosum ($2n = 24$) possesses 11 m pairs of decreasing size (1–11) and 1 st pair (12). Two pairs (2 and 5) are satellited. This species exhibits the highest values of HKL and heterochromatin content of all the taxa analysed (Table 2). Only CMA+/DAPI− heterochromatin is present; however, the fluorescent banding pattern is complex, with most of the heterochromatic regions appearing as large intercalary blocks (Table 2; Figs. 4a, 5). NOR-associated heterochromatin is found in terminal macrosatellites of pairs 2 and 5, comprising the entire satellite and a large part of the corresponding short arm; other distal bands occur on the long arm of pairs 4 and 6, and the short arm of pair 9. The latter chromosome pair also possesses a small intercalary band on its short arm, which corresponds to the 5S rDNA locus (same accession; Scaldaferro et al. unpublished). Chromosome pairs 6 and 9 show polymorphism regarding heterochromatin content and position (Table 2).

Capsicum recurvatum

This species endemic to eastern coastal Brazil possesses $2n = 26$ with 10 m pairs (1–8 of gradual and 9–10 of abrupt decreasing size), 2 sm pairs (11 and 12) of similar size, and a very small st pair (13). One pair (11) presents a macrosatellite on the short arm. Heterochromatin content is moderate and fluorochrome chromosome banding is characterized by mostly terminal bands of similar small size, except for the large heterochromatic block of pair 11 (NOR-associated) and that from the short arm of pair 13; in addition, intercalary bands are present on both arms of pair 1 and on the short arm of pair 8 (Table 2; Figs. 4c, 5).

Capsicum villosum

Capsicum villosum is endemic to eastern coastal Brazil; it exhibits the most asymmetrical karyotype of those with $2n = 26$ analysed here, with 9 m pairs (1 very large and 2–9 of decreasing size), 3 sm pairs (10 very large and 11–12 of similar size), and 1 small t pair (13). One pair (12) carries a terminal macrosatellite on the short arm. Heterochromatin content is high and the fluorescent chromosome banding is the most complex of the taxa, with $2n = 26$ analysed here, showing mostly terminal bands of similar small size, except for the large block of pairs 1, 10, 11 and the NOR-associated block of pair 12; in addition, intercalary bands appear on both arms of pairs 1 and 5, on short arm of pairs 3 and 8, and on long arm of pairs 6, 7, and 13, respectively (Table 2; Figs. 4e, 5).

Capsicum rhomboideum

This species ($2n = 26$) presents 10 m pairs (1–10) of decreasing size, 2 sm pairs (11 and 12) of similar size, and a much smaller st pair (13). One pair (9) presents a macrosatellite on the short arm. *C. rhomboideum* exhibits the lowest heterochromatin content of the taxa with $2n = 26$ analysed here; as well as the lowest chromosome length and HKL (Table 2, Table 3 in supplementary material). The fluorescent banding pattern is quite simple, with most of the chromosomes having similar-sized small terminal bands, except for pairs 11 and 13, which are not banded, and the large heterochromatic NOR-associated block on the short arm of satellite pair 9 (Table 2; Figs. 4g, 5). Another accession of *C. rhomboideum* reported previously (YSG 20; Moscone et al. 2007) had the same karyotype features than those reported here.

Discussion

Heterochromatic banding patterns in wild species of *Capsicum* evidence the natural intra- and interspecific

variability of the genus and allow the identification of all taxa examined here and those previously reported (Moscone et al. 2007). The different cytotypes observed in some of the species analysed in the present work agree with the intraspecific variability reported for the genus (Moscone et al. 2007; this work). Fluorochrome banding techniques in addition to Giemsa C-banding, AgNOR staining and FISH to rDNA and telomeres already applied in the genus (Moscone et al. 1993, 1995, 1996, 2007; Park et al. 1999, 2000; Scaldaferro et al. 2006, unpublished) are important tools for cytotaxonomy and delineation of karyotype evolution in *Capsicum*.

Heterochromatin type

According to the base-specific fluorochromes (Schweizer 1979) used for chromosome staining, in the present work four types of constitutive heterochromatin were observed in *Capsicum*, which is in agreement with Moscone et al. (1996, 2007): highly GC-rich (CMA+/DAPI−), AT-rich (CMA−/DAPI+), moderately GC-rich (CMA+/DAPI 0, i.e., chromomycin mottled bright and DAPI indifferent) and mixed GC- and AT-rich heterochromatin (CMA+/DAPI+), probably reflecting different classes of tandemly-repeated DNA sequences. Highly GC-rich heterochromatin is the rule in *Capsicum*, appearing in all taxa with $x = 12$ and $x = 13$ analysed, occurring at the macrosatellites, a variable number of other smaller and larger distal bands and intercalary bands. NOR-associated heterochromatin of *Capsicum*, which contains rDNA genes, is GC-rich, as is the universal rule in plant chromosomes (Guerra 2000). Moreover, the other three types of constitutive heterochromatin occur exclusively in *Capsicum* taxa of high heterochromatin content (Moscone et al. 1996, 2007; this work). Regarding mixed GC- and AT-rich constitutive heterochromatin, more than one unrelated satellite DNAs probably occupy the same chromosomal site, as reported for barley (Brandes et al. 1995). With reference to the moderately GC-rich heterochromatin which stains positively but mottled with CMA and reacts neutrally to DAPI, as in the previous case, probably more than one unrelated satellite DNAs occupy the same chromosomal site, although the amount of GC-rich heterochromatin may exceed that of AT-rich one.

Heterochromatin amount

Considering the fluorochrome chromosome banding already applied in the genus (Moscone et al. 1996, 2007) and the present original contribution, a wide variation in constitutive heterochromatin content can be found both within and among taxa of *Capsicum*, ranging from 1.72 to 38.91 % HKL, with a mean value of 10.90 %. In most of the taxa analysed, constitutive heterochromatin amount correlated positively with karyotype length and genome size, which is consistent with findings of Moscone et al. (1996, 2003, 2007); therefore, heterochromatin amount may be regarded as an additional component of the genome of chili peppers. These facts probably reflect that tandemly repeated sequences, which might be part of the constitutive heterochromatin rather than dispersed repeated DNA (i.e., transposable elements), play a major role in karyotype evolution of *Capsicum*.

Heterochromatin distribution

As a rule, constitutive heterochromatin locates mainly at terminal position of chromosomes in *Capsicum*, although intercalary position also occurs (Moscone et al. 1993, 1996, 2007; this work). This is particularly true for the different types of constitutive heterochromatin: high GC-rich, moderately GC-rich and mixed GC- and AT-rich. In addition, together with *C. flexuosum*, taxa of *Capsicum* with $x = 13$ from eastern coastal Brazil tend to have more intercalary GC-rich constitutive heterochromatin than taxa with $x = 12$ in terms of number and length of bands. In particular, many taxa exhibit a GC-rich small intercalary band on the short arm of an m pair, which actually corresponds to the 5S rDNA locus (Scaldaferro et al. 2006, unpublished). Furthermore, AT-rich heterochromatin shows mostly interlayer distribution in *Capsicum*, a common feature in plants with medium and large chromosome size (Guerra 2000).

Cytological evidence in the genome of many organisms reveals that different classes of hetrochromatin co-evolve, appearing in similar positions of non-homologous chromosomes of the karyotype (heterologous, sensu Bennett 1982), probably caused by unequal crossing-over or amplification and transposition events between them mediated by Rabl orientation during mitotic interphase or bouquet arrangement during meiotic prophase (Greilhuber and Loidl 1983;

Dover and Flavell 1984; Schwarzacher et al. 1984; Schweizer and Loidl 1987; Kenton 1991; Guerra 2000). In *Capscium* the different types of heterochromatin probably made up of similar tandem repeats each, display almost equilocal distribution between non-homologous chromosomes of the karyotype, suggesting concerted evolution for the heterochromatin dispersion in the genus.

Cytotaxonomy

Chromosome numbers in *Capsicum* reported here and in previous works (Moscone 1990, 1993, 1999; Moscone et al. 1996, 2007; Pickersgill 1971, 1991; Tong and Bosland 2003; Pozzobon et al. 2006) confirm the universal presence of two groups in *Capsicum*, $2n = 2x = 24$ and $2n = 2x = 26$, the latter occurring only in wild species. Moscone et al. (2007) described a probable scenario of karyotype evolution in *Capsicum*, which essentially involves changes in karyotype asymmetry, complexity of heterochromatin type, amount and distribution, genome size, and number and position of active NORs, allowing the taxonomic grouping within chili peppers and relating wild members of this genus to domesticated ones. Our novel data strongly support the diagram proposed by those authors: a diploid *Capsicum* ancestor with $x = 12$, small genome size, 11 m + 1 st chromosomes, two active NORs in the basic complement, little GC-rich heterochromatin, and simple banding pattern of a symmetrical karyotype. Then, the origin of $x = 13$ occurred in two independent events, resulting in two subgroups of the $2n = 26$ species. They share more complex chromosome complements, with karyotype formulas including more than one sm or st chromosome, and a t chromosome in *C. villosum*. In *C. rhomboideum*, heterochromatin pattern is simple, with low heterochromatin content, whereas the taxa of the other subgroup with larger chromosomes share a particular banding pattern, higher heterochromatin content, particularly *C. villosum*, which has conspicuous intercalary bands. In addition, these subgroups are supported by morphological and geographical features. *C. rhomboideum* grows in Mexico, Central America and north-western areas of South America. Instead, *C. recurvatum* and *C. villosum* grow in eastern coastal Brazil. The isolated position is also noticeable because *C. rhomboideum* has yellow flowers, with non-pungent fruits, unlike pungent fruits in the other subgroup, white flowers with greenish spots in the throat in *C. recurvatum* or white with violet or brownish spots in the throat, greenish in the tube in *C. villosum*. This hypothesis is supported by studies on phylogenetic relationships on molecular evolution using the non-transcribed spacer (NTS) of the 5S rDNA among different taxa of *Capsicum* (Grabiele 2010). Walsh and Hoot (2001) and Guzmán et al. (2009) also found that *C. rhomboideum* [*C. ciliatum* (Kunth) Kuntze] is one of the most divergent species within *Capsicum*.

Heterochromatin content and distribution in *C. tovarii* (ATH & GB 25653; Moscone et al. 2007) put this taxon close to the purple flower group of species (Pickersgill 1991). Instead, the karyotype features of the accession reported here are far from those of ATH & GB 25653 and closer to that of species of the *C. baccatum* complex; therefore, *C. tovarii* has a divergent position. Tong and Bosland (1999) recognized *C. tovarii* as a member of the *C. baccatum* complex through interspecific hybrid performance and meiotic chromosome behaviour analysis in the genus; the accession reported here (NMCA 90008) is the same as that used by those authors. Moreover, in his phylogenetic analysis of *Capsicum* using the NTS of the 5S rDNA (Grabiele 2010) found that *C. tovarii* (ATH & GB 25653) is also closer to the *C. baccatum* complex. Both cultivars are morphologically similar; hence, the differences found in heterochromatin content and distribution might be explained by the natural variability within *C. tovarii*; in addition, our novel data support its inclusion within the *C. baccatum* complex. Using RAPD markers, Ince et al. (2010) also grouped *C. tovarii* with the *C. baccatum* clade. In contrast to this definition, using AFLPs and SSRs Ibiza et al. (2011) showed that *C. tovarii* is not a clear member of the different *Capsicum* complexes, although it appeared slightly closer to the *C. annuum* complex, but with low bootstrap values.

In our cytogenetic analyses, the species of "purple-flowered group", *C. eximium* and *C. cardenasii*, in which *C. pubescens* is the core member (Pickersgill 1991), were well distinguished from each another, showing that interspecific characterization could be detected by cytogenetic tools. However, using molecular data, Ibiza et al. (2011) supported the definition of *C. eximium* and *C. cardenasii* as a single, morphologically variable species that belongs to the *C. pubescens*

complex and found these two wild species indistinguishable from each other, as they were clustered together.

Furthermore, the great variability found in the wild *C. annuum* var. *glabriusculum* compared to that of *C. annuum* var. *annuum* has evolutionary implications. Different accessions of the cultivated *C. annuum* var. *annuum* analysed with fluorochromes also show only CMA+/DAPI− heterochromatin but less complex and constant banding patterns than the variety *glabriusculum* (Moscone et al. 1996, 2007; Romero et al. unpublished; this work, Fig. 5), probably reflecting genetic bottleneck events during domestication. At least two hypotheses have postulated the origin of the cultivated variety of *C. annuum*. Pickersgill (1971, 1991) hypothesized that *C. annuum* var. *annuum* could have originated in Mexico. In our results, cytotype 1 of the wild *C. annuum* var. *glabriusculum* from Florida (USA) presents the typical karyotype of the cultivated variety. Furthermore, through archaeological findings, Perry et al. (2007) suggested South America, particularly Peru, as the most likely place where the domestication of this species would have begun. These findings are consistent with our results and refer cytotype 3 of *C. annuum* var. *glabriusculum* to cytotype 1 of *C. annuum* var. *annuum*. The data presented here together with information published in previous works (Moscone et al. 2007; Romero et al. unpublished) should be considered in efforts to elucidate the most likely site of domestication of this species.

Acknowledgments This work was made possible by doctoral scholarship granted by Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba (SECyT-UNC), and is part of the doctoral thesis of Marisel Scaldaferro.

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