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Karyotype analysis in two species of *Solanum* (Solanaceae) Sect. *Cyphomandropsis* based on chromosome banding

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To contribute to the cytogenetic characterisation of the Cyphomandra clade of *Solanum*, fluorescence banding and silver staining were performed on *Solanum fusiforme* and *Solanum stuckertii* for the first time. Both species had $2n = 24$ chromosomes. *Solanum fusiforme* had a karyotype consisting of 9m + 3sm chromosome pairs; one pair had active nucleolar organiser regions (NORs). *Solanum stuckertii* had 8m + 4sm chromosome pairs with two pairs having active NORs. The two species exhibit similar amounts (11%) of constitutive CMA + /DAPI − heterochromatin, rich in GC base pairs. This heterochromatin was distributed in a complex pattern of mainly intercalary bands. Localisation of chromosome-specific markers was successful, allowing recognition of all chromosomes of the complements. With the aim of contributing to a better understanding of the interspecific relationships within the Cyphomandra clade, we discuss our results in conjunction with previous findings for species in this clade.

Keywords: *Solanum fusiforme*; *Solanum stuckertii*; Cyphomandra clade; fluorochrome banding; silver staining

Introduction

Solanum L. (Solanaceae), with more than 1000 identified species, is one of the largest and most diverse genera of angiosperms (Hunziker 2001). Because of the large size and morphological complexity of this taxon, many infrageneric groups are not well defined. In addition, a considerable number of species are poorly known and in many cases, the boundaries and arrangement of species within the genus are controversial (Hunziker 2001; Bohs 2005). *Cyphomandra* Mart. Ex Sendtn. has been recognised as a separate genus, comprising ca. 41 species distributed from Mexico to northern Argentina (Hunziker 2001). Based on molecular data, Bohs (1995, 2001) transferred all *Cyphomandra* species to *Solanum*, including them in the sect. *Pachyphylla* (Dunal) Dunal. By contrast, Child & Lester (2001) retained the distinction between genera.

Sequence data from the chloroplast *ndhF* gene, as well as nuclear internal transcribed spacer (ITS) and waxy regions, have allowed the individualisation of at least 12 major clades within *Solanum* (Bohs & Olmstead 2001; Bohs 2005; Weese & Bohs 2007). The Cyphomandra clade (sensu Bohs 2005; Weese & Bohs 2007) is one of these well-supported major groups. It includes ca. 50 neotropical species that have been placed into three sections: *Solanum* section *Pachyphylla*, *Solanum* section *Cyphomandropsis* Bitter and *Solanum* section *Glaucophyllum* A. Child. The most consistent morphological synapomorphies of the group are the presence of

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big chromosomes (> 4 μm long) and large amounts of nuclear DNA, which have been found in all the species of the clade analysed to date (Roe 1967; Pringle & Murray 1991, 1993; Moscone 1992; Bennett & Leitch 2010). These characteristics separate these species from other members of *Solanum*, which have smaller chromosomes (< 4 μm) and a lower nuclear DNA content (Bernardello & Anderson 1990; Bernardello et al. 1994; Acosta et al. 2005; Chiarini & Bernardello 2006; Rego et al. 2009; Bennett & Leitch 2010; Melo et al. 2011).

Fluorochrome banding and silver staining were performed on the somatic chromosomes of two species of sect. *Cyphomandropsis*, *S. fusiforme* Smith & Downs, and *S. stuckertii* Bitter, with the aim of contributing to their cytogenetic characterisation. The ultimate goal of ongoing research is to contribute to species delimitation and infrageneric classification in *Solanum*.

Materials and methods

The materials examined consisted of two samples from Argentina; *S. fusiforme*, Province of Misiones, Department of Libertador General San Martín, ca. 3 de Mayo, E. A. Moscone and J. R. Daviña 218; and *S. stuckertii*, Province of Córdoba, Department of Calamuchita, El Crucero, E. A. Moscone 246. The respective voucher specimens were identified and deposited in the herbarium of the Botanical Museum of Cordoba, Argentina (CORD).

Somatic metaphases and interphase nuclei were observed in squashed root meristems obtained from seed germination. The root apices were pretreated with a saturated solution of *para*-dichlorobenzene at room temperature for 2 h, fixed in a 3:1 ethanol/acetic acid mixture for a minimum of 12 h, and stored at −20 °C until use. Root tips were macerated according to Schwarzacher et al. (1980), using an enzymatic solution of 2% cellulase (w/v) plus 2% pectinase (v/v) at 37 °C for 1 h (*S. fusiforme*) or 1 h 30 min (*S. stuckertii*). 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.

Fluorochrome chromosome banding was applied to examine the presence, type and distribution of constitutive heterochromatin. The silver impregnation procedure (AgNOR) was used to determine the active nucleolar organising regions (NORs) of somatic metaphase chromosomes and nucleoli from interphase nuclei. Triple staining with chromomycin A_3, distamycin A and 4'-6-diamidino-2-phenylindole (CMA/DA/DAPI) (i.e. CDD staining) was performed according to Schweizer (1980). Some preparations of both species were bleached by immersion in a mixture of 3:1 ethanol/glacial acetic acid for 12 h, washed in absolute alcohol for 20 min and air dried (Schweizer 1981); they were then subjected to sequential double staining with the fluorochromes DAPI and actinomycin D (DAPI/AMD) (Schweizer & Ambros 1994). Enhanced or reduced fluorescence of a chromosome segment is indicated in the text by attaching + or − to the fluorochrome or fluorochrome combination. AgNOR banding was performed according to the Ag-I Bloom & Goodpasture (1976) protocol with the modification of Kodama et al. (1980). Incubation at 60 °C was performed for 1 h (*S. fusiforme*) or 1 h 30 min (*S. stuckertii*), using nylon cloth (mesh size 0.242 mm) instead of coverslips.

Somatic chromosomes and interphase nuclei were observed and photographed with a Leica DMLB epifluorescence microscope (Leica, Heerbrugg, Switzerland) equipped with a computer-assisted Leica DC 250 digital camera system. For epifluorescence microscopy, images were captured in black and white using appropriate filter sets. Digital images were pseudocoloured using IM 1000 Leica software, then imported into Photoshop 7.0 (Adobe, San Jose, CA, USA) for final processing.

A total of 10 metaphases from five and four individuals belonging to *S. fusiforme* and *S. stuckertii*, respectively, were analysed by

fluorochrome banding. In addition, 30 and 26 metaphases from eight and six individuals belonging to *S. fusiforme* and *S. stuckertii*, respectively, were examined for AgNOR banding. For each metaphase plate, the absolute and relative lengths of short (p) and long (q) chromosome arms, chromosomes and bands (data not shown), and the absolute length of the karyotype were calculated (for relative values, haploid karyotype length = 100%). Average values are presented. For each chromosome, the centromere position was calculated by the index r (arm ratio) $= q/p$ (Levan et al. 1964). The position of intercalary bands was calculated by the index: $di = d \times 100/a$ (d = distance of band centre from the centromere, a = length of the corresponding chromosome arm) according to Greilhuber & Speta (1976). The satellite lengths were added to the length of the corresponding arms. Lengths of the secondary constrictions (NORs) were always excluded. Battaglia's (1955) terminology for satellites was used as follows: microsatellite, those with a diameter smaller than the chromosome diameter and small size; macrosatellite, those with a diameter equal to the chromosome diameter and large size. In the idiograms, chromosomes were arranged first into groups according to their increasing arm ratio (from m to st) and then, within each group, by decreasing length. Chromosome markers allowed positive identification of all chromosome pairs.

Results

The same somatic chromosome number $2n = 24$ was found in both species analysed (Table 1). The species had relatively similar karyotypes consisting of nine metacentric (m) and three submetacentric (sm) chromosome pairs in *S. fusiforme*, and eight m and four sm in *S. stuckertii*. *Solanum fusiforme* had one chromosome pair (pair 12) with NORs plus an attached satellite (micro-or macrosatellites), whereas *S. stuckertii* had two pairs with NORs (pairs 11 and 12 with micro- and

Table 1 Karyotype features of *Solanum* species studied, all with $2n = 24$.

Taxon	Haploid karyotype formula (n)	NOR-bearing pair	HKL (μm) × (SD)	Heterochromatin amount[1]				Maximum no. of bands per haploid complement[2]	Maximum no. of pairs with bands
				Total	NOR-assoc.	Interc.			
S. fusiforme	9 m + 3 sm	12 (sm)	76.09 (11.44)	10.71	1.24	6.28		24 (6)	12
S. stuckertii	8 m + 4 sm	11 + 12 (sm)	92.06 (9.67)	10.93	1.37	8.11		31 (5)	12

Note: m, metacentric; sm, submetacentric; NOR, nucleolar organizing region; HKL, haploid karyotype length; x, mean value; SD, standard deviation.
[1]Heterochromatin amount is expressed as the percentage of HKL; NOR-assoc., NOR-associated heterochromatin; Interc., intercalary heterochromatin.[2] The number of terminal bands is indicated between parentheses.

micro-or macrosatellites, respectively). In all cases, the NORs were located on the short arm.

Fluorescence banding

In both taxa analysed, the fluorescent banding patterns obtained with CMA/DA were reversed compared with DA/DAPI and DAPI/AMD staining (Figs 1C–1F). Both species exhibited 11% of constitutive CMA+/DAPI− heterochromatin (CMA positive and DAPI negative) (Table 1), suggesting the presence of GC-rich heterochromatin. The abundance of heterochromatic fluorescent segments, such as satellites and intercalary bands of different lengths in one or both arms of all chromosomes, allowed the identification of all chromosomes of the complement (Fig. 2). Centromeric bands were absent. Finally, the size of CMA+/DAPI− chromocentres present in interphase nuclei corresponded with the size of metaphase chromosome fluorescent bands (Figs 1A, 1B).

Solanum fusiforme had preferentially intercalary heterochromatic bands on the short arm of all chromosomes (Figs 1C, 1E, 2). The largest fluorescent bands were terminal and located on the short arm of pairs 10, 11 and 12. Pairs 4, 7 and 9 also had minor terminal bands. Only pairs 6, 7, 10 and 12 showed intercalary bands on their long arm. Pair 12 was unique because of its high heterochromatin content (44% of its length), which was distributed in three conspicuous bands. Pair 7 was distinguished among all chromosome pairs by having the maximum number of bands (four). The terminal bands of pairs 10 and 11 and the band of the long arm of pair 12 showed mottled fluorescence. Only pair 12 presented a heteromorphic fluorescent banding pattern between homologous chromosomes, because different satellite sizes were observed in two of five plants analysed.

Solanum stuckertii had heterochromatin in all chromosomes, usually intercalary and distributed on both arms, except pairs 1, 2 and 10, where interstitial bands were observed on only one arm (Figs 1D, 1F, 2). Terminal bands were infrequent and occurred only on the short arm of pairs 3, 4, 9, 11 and 12, in pairs 11 and 12 the bands corresponded to the NORs. Pair 3 was distinguished by having the highest number of bands (five) of all chromosome pairs, four of them located on the short arm. Among the few cases of heteromorphism in the banding pattern, a noticeable variation in the number of bands on the short arm of pair 2 was observed. For this chromosome pair, two of the individuals analysed had two bands, whereas the remaining two exhibited only the proximal one. In addition, the band on the short arm of pair 1 had different lengths in homologous chromosomes in two of the individuals examined. Finally, satellites of varying length were found in pairs 11 and 12.

AgNOR banding

AgNOR banding performed in both species showed that the number of active NORs in metaphase and the number of nucleoli in the interphase nucleus differed within each individual. In addition, the relative size of the nucleoli in the interphase nucleus and metaphase NORs was not constant, although variations in both structures showed no correlation. The NORs usually had attached satellites, which were not differentially stained with silver staining (Fig. 3).

In *S. fusiforme*, all individuals examined had a pair of chromosomes with AgNORs (pair 12) (Figs 3A, 3B). The maximum number of nucleoli in interphase nuclei impregnated with silver was two (Fig. 3A). Although most cells showed only one. The most frequent number of AgNORs found in metaphase was two (90% of metaphases analysed), and they were located on the short arm, at a subterminal position and had attached satellites of variable size (micro- or macrosatellites) (Fig. 3B).

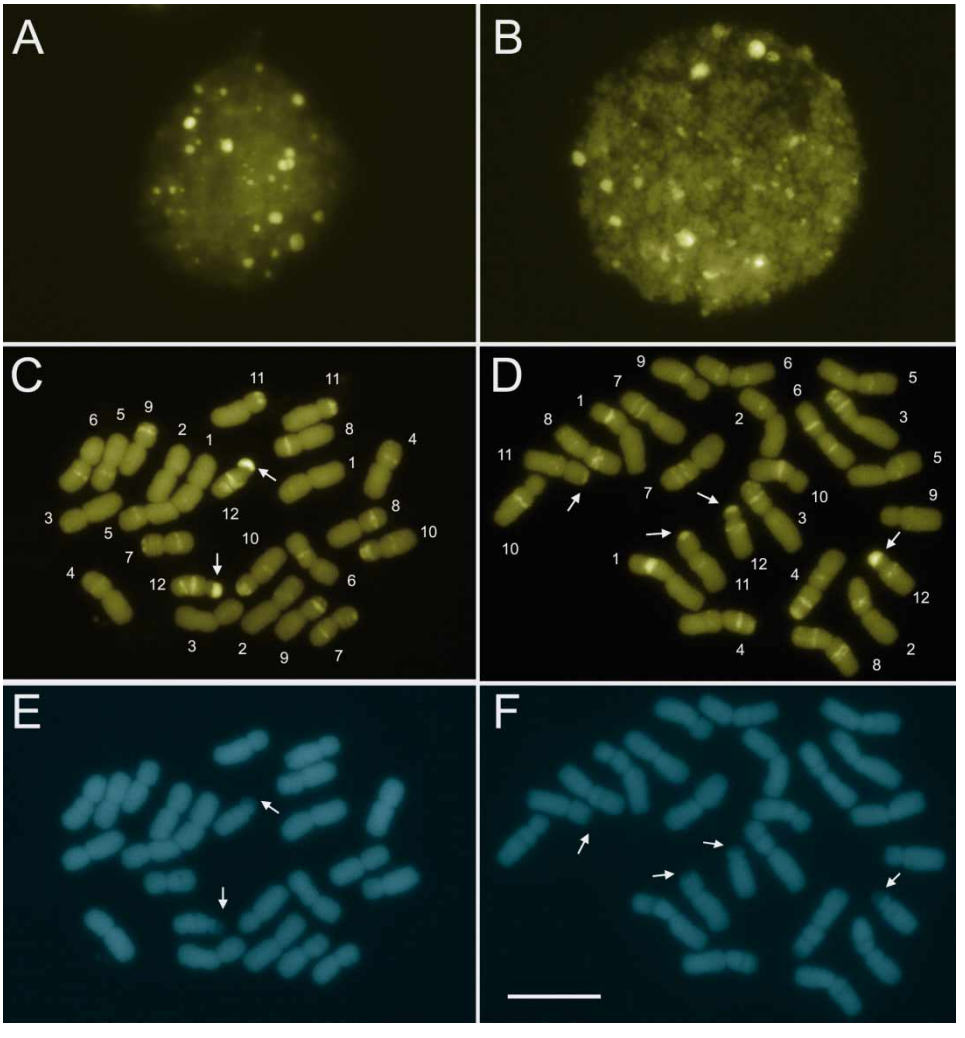

Figure 1 Interphase nuclei and somatic metaphases after double-staining with CMA/DAPI. **A,** *Solanum fusiforme* interphase nucleus stained with CMA. **B,** *Solanum stuckertii* interphase nucleus stained with CMA. **C,** *Solanum fusiforme* somatic metaphase stained with CMA. **D,** *Solanum stuckertii* somatic metaphase stained with CMA. **E,** *Solanum fusiforme* somatic metaphase stained with DAPI. **F,** *Solanum stuckertii* somatic metaphase stained with DAPI. The numbers in C and D refer to the numbering of the chromosomes in the karyotypes in Figure 2. Arrows indicate CMA + /DAPI– NOR-associated heterochromatin. Scale bar = 10 μm is the same for all figures.

For *S. stuckertii*, all individuals analysed had a maximum of two pairs of chromosomes (11 and 12) with AgNORs (Figs 3C, 3D). The maximum number of silver-stained nucleoli found was always four (Fig. 3C). Three being the most frequent number. Metaphases generally showed four AgNORs (92% of metaphases analysed), sometimes being reduced to three, because of an inactive rDNA locus at chromosome pair 11. The AgNORs were mostly

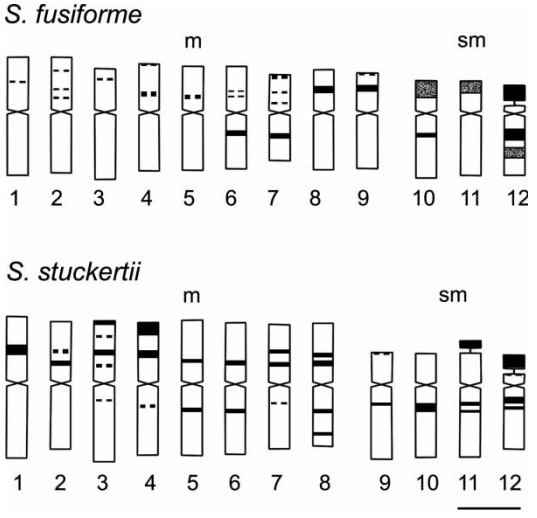

Figure 2 Idiograms of *Solanum* species studied showing heterochromatic fluorochrome banding patterns. Solid blocks indicate CMA + /DAPI– bands of homogeneous aspect; irregular coloring indicates mottled fluorescence. Scale bar = 5 μm.

subterminal and located on the short arm; in pair 11, AgNORs were followed by microsatellites and in pair 12 by macrosatellites (Fig. 3D). In some cases, NORs of both pairs appeared terminal after silver staining although satellites were always observed following fluorescence banding.

Discussion

Solanum fusiforme and *S. stuckertii* are diploid with $x = 12$ and relatively symmetrical karyotypes, presenting chromosomes that belong to category 2A, according to the system established by Stebbins (1971). This is in agreement with previous results obtained for other species of *Solanum* sect. *Cyphomandropsis*, i.e. *Solanum confusum* Morton, *Solanum glaucophyllum* Desf. and *Solanum luteoalbum* Pers. (Moscone 1989; Pringle & Murray 1993).

Fluorescence banding

Fluorescence banding allowed the localisation of specific markers in all chromosomes for *S. fusiforme* and *S. stuckertii*. The correct identification of all chromosomes of the complement permits the comparison of chromosome colinearity shared by related species and the reconstruction of ancestral karyotypes to understand the evolutionary direction of karyotypic variation.

According to the specificity of the chromomycin, the karyotypes of *S. fusiforme* and *S. stuckertii* posses GC-rich heterochromatin (Barros e Silva & Guerra 2010), as do many other *Solanum* species (cf. Sultana & Alam 2007; Brasileiro-Vidal et al. 2009; Rego et al. 2009; Melo et al. 2011; Acosta et al. unpublished).

In *S. stuckertii*, the comparison with the heterochromatic pattern previously obtained with C-banding showed no differences, except for the presence of centromeric C-bands detected by Moscone (1989), which were not observed in our study. Although both techniques reveal constitutive heterochromatin, they differed significantly in the procedures used and in the banding patterns they reveal. While C-banding requires physico-chemical pretreatment, base specific banding depends on the affinity of heterochromatin for a specific fluorochrome (Guerra 2000; Barros e Silva & Guerra 2010).

Nucleolar activity

AgNOR banding was used to reveal active rDNA sites, whose number differs between the two species. Intraspecific variation found in the number of metaphase AgNORs could be a consequence of their differential activity (Moscone et al. 1995), thus, the NORs responsible for minute nucleoli cannot be detected on the metaphase chromosomes because of the technical limitations of the AgNOR method (Sato et al. 1980). The observation of a lower number of nucleoli in some interphase nuclei could be due to their fusion (Maluszynska & Heslop-Harrison 1991; Marcon et al. 2005). Furthermore, the polymorphism observed in the size of AgNORs among individuals, cells

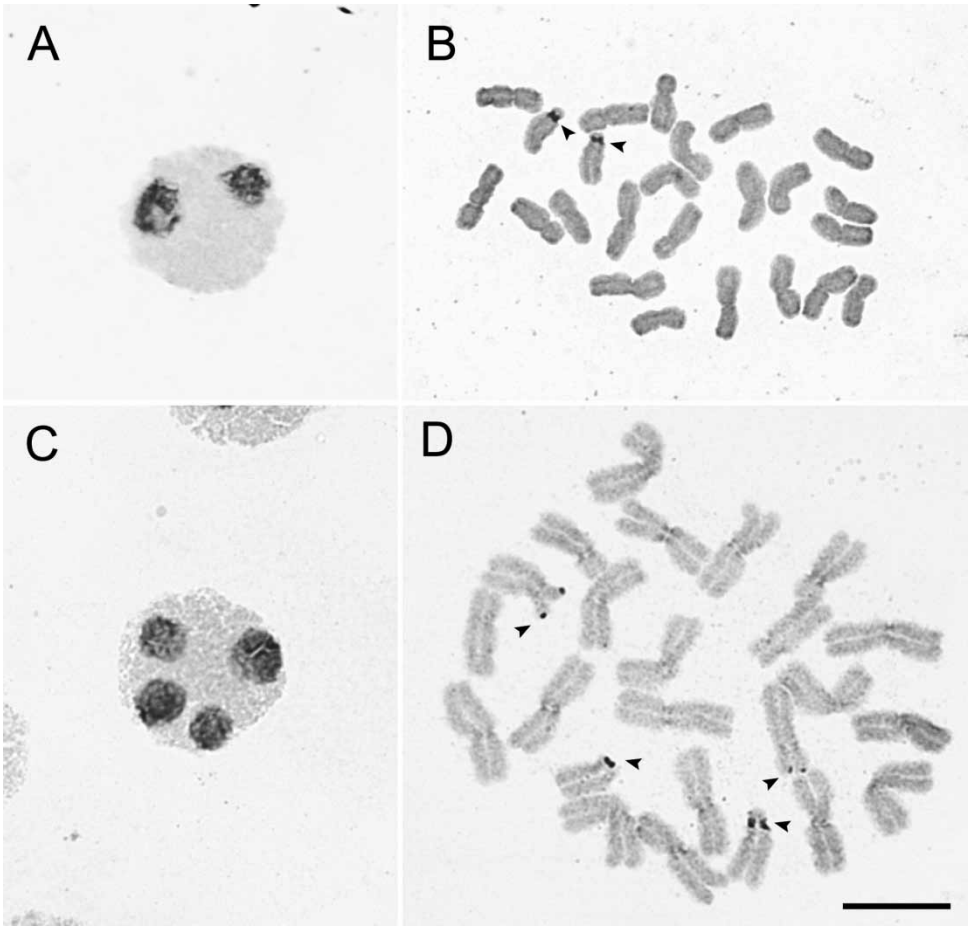

Figure 3 Interphase nuclei and somatic metaphases after AgNOR banding. **A**, *Solanum fusiforme* interphase nucleus with two Ag-positive nucleoli. **B**, *Solanum fusiforme* metaphase with two AgNORs. **C,** *Solanum stuckertii* interphase nucleus with four Ag-positive nucleoli. **D**, *Solanum stuckertii* metaphase with four AgNORs. Arrows indicate AgNORs. Scale bar = 10 μm is the same for all figures.

and even homologous chromosomes may be caused by several factors, such as the number of ribosomal genes, the transcription level and the state of chromatin condensation in the NORs (Jiménez et al. 1988; Zurita et al. 1999). The existence of secondary constrictions not detected by AgNOR due to their inactivity is possible and further fluorescence in situ hybridisation assays would allow their identification.

In *S. stuckertii*, some NORs appear to be terminal after silver staining, however, they always present satellites in fluorochrome-stained metaphases, a phenomenon that has already been observed in *Capsicum* (Moscone et al. 1995). One possible explanation for this inconsistency is that microsatellites are no longer recognisable after silver staining because of their small size.

Systematic and evolutionary analysis

Karyological studies using conventional and chromosome-banding dyes of species in the Cyphomandra clade have demonstrated a close association among *Solanum* sections

Pachypylla, *Cyphomandropsis* and *Glaucophyllum* (Moscone 1992; Bohs 2005). In fact, all species have $2n = 2x = 24$ chromosomes of large size, similar karyotypes and high nuclear DNA content (Moscone 1989, 1992; Pringle & Murray 1991, 1993; Bennett & Leitch 2010). These characteristics separate them from the remaining *Solanum* sections, which exhibit smaller chromosomes and lower DNA content (cf. Bernardello & Anderson 1990; Bernardello et al. 1994; Acosta et al. 2005; Chiarini & Bernardello 2006; Rego et al. 2009; Bennett & Leitch 2010; Melo et al. 2011).

The remarkable banding patterns obtained in both species indicate that the distribution of heterochromatin has taxonomic value for the establishment of relationships between species within the Cyphomandra clade. In *S. fusiforme* and *S. stuckertii*, intercalary bands predominate over terminals ones, as they do in *S. luteoalbum*, a taxon previously examined by C-banding (Pringle & Murray 1993), which also belongs to section *Cyphomandropsis*. In the remaining Cyphomandra species of the section *Pachyphylla* that were analysed with C-banding, terminal bands have been found to be much more frequent (Pringle & Murray 1993). *Solanum stuckertii* and *S. luteoalbum* have similar karyotypes, they are morphologically cohesive and molecular data support them in the same clade (Bohs 2007). *Solanum fusiforme* is an enigmatic taxon whose affinities are not well understood on the basis of morphology. Molecular data suggest that *S. fusiforme* is sister to other species of the Cyphomandra clade, but its position has not been resolved to date (Bohs 2007). The karyotypic characterisation obtained here supports the relationship of *S. fusiforme* with species of section *Cyphomandropsis*.

Owing to the rich and informative heterochromatic banding patterns obtained here, this study should be extended to other species of the Cyphomandra clade to elucidate chromosome relationships among them.

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