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Heterochromatin and rDNA patterns in *Solanum* species of the Morelloid and Dulcamaroid clades (Solanaceae)

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Abstract

The heterochromatin distribution and the position of 18-5.8-26S, and 5S rDNA loci were determined in 13 species of *Solanum* of the Morelloid and Dulcamaroid clades. The CMA/DAPI staining and FISH were employed. Two types of constitutive heterochromatin were determined: $CMA^+/DAPI^-$ associated to NOR and $CMA^+/DAPI^-$ distributed as terminal bands. In the Morelloid clade, $CMA^+/DAPI^-$ bands were found in five species while in the Dulcamaroid clade, only *S. angustifidum* presented this feature. In the Morelloid clade, two to four 18-5.8-26S rDNA loci occupied terminal positions and two rDNA 5S loci were found with variable positions (terminal, intercalary, and centromeric). In the Dulcamaroid clade, two terminal 18-5.8-26S rDNA loci were detected with the exception of *S. salicifolium* which possessed four such loci and two to four 5S rDNA loci. *Solanum crispum* is the only species possessing the 5S in synteny with 18-5.8-26S rDNA loci. Karyotype features chromosome banding pattern as well as the location of ribosomal genes which varied among the species, reflecting the chromosome differentiation and evolutionary divergence. The findings obtained contributed to the development of tools that can be used for establishing chromosomic homeologies among species and hence to clarify their taxonomic relationships.

Keywords: *Dulcamaroid, FISH, fluorescent banding, Morelloid,* Solanum

Introduction

Solanum L. comprises about 1400 species of herbs, shrubs, and woody vines with its center of diversification in South America and an estimated age of 17 mya (Bohs 2005; Särkinen et al. 2013). It is an economically important genus including major crops as *S. tuberosum* L. (potato), *S. lycopersicum* L. (tomato), and *S. melongena* L. (eggplant). On a smaller scale, there are also a number of species cultivated for their edible tubers, fruits, or leaves as well as for being a source of medicinal compounds.

This genus is systematically complex (Nee 1999; Hunziker 2001; Bohs 2005). In the last decades, several molecular studies have been published and tried to clarify their taxonomic relationships (e.g. Levin et al. 2006; Weese & Bohs 2007). Bohs (2005) using ndhF chloroplast sequences identified 13 main clades in *Solanum*. Among them, the Morelloid and Dulcamaroid clades are closely related and considered as sister groups (Bohs 2005; Weese & Bohs 2007). Their delimitation and phylogeny have been recently studied showing that both have problems in the species delimitation (Knapp 2013; Särkinen et al. 2015).

The Morelloid clade is Cosmopolitan and has ca. 75 species, most of which are endemic to the tropical Andes including the well-known *S. nigrum* L. ("black nightshade") (Edmonds 1978; Särkinen et al. 2015). The clade includes five major groups (traditionally known as sections *Solanum*, *Campanulisolanum* Bitter, *Parasolanum* A.Child, *Chamasarachidium* Bitter, and *Episarcophyllum* Bitter) that have been recircumscribed based on molecular results (Särkinen et al. 2015). Its diagnostic features are: herbaceous or sub-shrubby plants with internodal inflorescences, small flowers and fruits, and stone cells in the fruits (Edmonds 1978).

On the other hand, the Dulcamaroid clade has 45 mostly vining or scandent species including the widely distributed weed *Solanum dulcamara* L. ("bittersweet nightshade", "dulcamara"). The clade is Cosmopolitan with its center of diversity in the Andes and SE Brazil (Knapp 2013). The infrageneric traditional groups are not monophyletic and their taxonomic history is complex (Knapp 2013). Many species are morphologically variable even in a single locality: variation in habit, leaf shape and pubescence density, and type. The flowers of its

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members are generally showy, some species being ornamentals.

In situ hybridization (FISH) techniques for chromosome banding and fluorescence have demonstrated to be effective in the detection of chromosomal rearranges involved in the chromosome speciation (e.g. Srebniak et al. 2002; Melo & Guerra 2003). The double staining with the fluorochromes CMA/DAPI (Schweizer 1979) reveals constitutive heterochromatin blocks and is characterized in CG-rich regions. FISH allows the mapping of DNA sequences aiding in the identification of chromosome homologs and karyotypes in comparison to other species (Guerra 2004).

Notwithstanding, fluorescent banding and FISH studies in Solanaceae have focused in few genera such as *Cestrum* (e.g. Fregonezi et al. 2006; Fernandes et al. 2009), *Nicotiana* (Kenton et al. 1993; Lim et al. 2000), *Capsicum* (Moscone et al. 2007), *Nierembergia* (Acosta & Moscone 2010), *Lycianthes,* and *Vassobia* (Rego et al. 2009). In the genus *Solanum* and in the Morelloid, and Dulcamaroid clades in particular, the cytogenetic knowledge is relatively scanty (Rego et al. 2009; Acosta et al. 2012; Moyetta et al. 2013). Most examined species of these clades were karyologically indistinguishable based on conventionally stained mitotic chromosomes (Moyetta et al. 2013). Additional molecular karyotype analyses with banding and FISH techniques are needed in both clades to gain a better knowledge of the possible karyoevolutionary trends. Consequently, in this work, we analyzed cytogenetically 13 species of the Morelloid and Dulcamaroid clades in order to contribute to the clarification of their taxonomic and evolutionary relationships.

Materials and methods

The species studied, locations, and voucher specimens are included in Table I. Seeds were germinated and the seedlings were cultivated in the Laboratory of Cytogenetics at the Instituto Multidisciplinario de Biología Vegetal. Vouchers are kept at the Museo Botánico de Córdoba herbarium (CORD).

Chromosome banding was performed as described by Schweizer (1979) with minor modifications. Root tips were digested in an enzyme solution composed of 4% cellulose (w/v) and 40% pectinase (v/v) at 37°C and dissected in a drop of 45% acetic acid. After removal of the cover slips, the slides were aged for three days. The samples were then washed in distilled water, air dried, and stained with fluorochromes: 0.5 mg/ml chromomycin A_3 (CMA) for 1.5 h and 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 30 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1 plus 2.5 mM $MgCl_2$.

FISH was performed according to Schwarzacher and Heslop–Harrison (2000), with minor modifications. Slides were prepared as described for chromosome banding. A wheat pTa71 probe containing the 18-5.8-26S rDNA sequence (Gerlach & Bedbrook 1979) was labeled with biotin-14-dATP (Bio Nick,

Table I. Distribution of 18-5.8-26S and 5S rDNA in the studied species of Morelloid and Dulcamaroid clades of *Solanum* and collection data of the populations analyzed.

		rDNA						
		18-5.8-26SS		5S				
Taxon	KF	*n*°	Pos.	*n*°	Pos.	Sinteny	Provenance and Voucher specimens	Figures
Morelloid clade								
S. aloysiifolium Dunal	10*m* + 2*sm*	4	t	2	t	–	ARGENTINA, Salta, Barboza 2210	1a, 2c, 4a
S. americanum Mill.	10*m* + 2*sm*	2	t	2	c	–	ARGENTINA, Tucumán,Barboza 2139	1d, 2a, 4b
S. chenopodioides Lam.	10*m* + 2*sm*	2	t	2	t	–	ARGENTINA, Buenos Aires, Barboza 2292	2d, 4c
S. hastatilobum Bitter	9*m* + 3*sm*	4	t	2	t	–	ARGENTINA, Catamarca, Chiapella 2630	1e, 2h, 4d
S. palitans C.V. Morton	10*m* + 2*sm*	2	t	2	t	–	ARGENTINA, Tucumán, Barboza 2178	2g, 4e
S. pilcomayense Morong	11*m* + 1*sm*	2	t	2	t	–	ARGENTINA, Corrientes,Barboza 2279	2b, 4f
S. salicifolium Phil.	10*m* + 2*sm*	2	t	2	i	–	ARGENTINA, Córdoba, Chiarini 794	1c, 2f, 4g
S. triflorum Nutt.	10*m* + 2*sm*	2	t	2	i	–	ARGENTINA, Neuquén, Chiapella 1839	1f, 2e, 4h
Dulcamaroid clade								
S. amygdalifolium Steud.	9*m* + 2*sm* + 1*st*	2	t	2	t	–	ARGENTINA, Entre Ríos, Barboza *et al.* 1566	3a, 4i
S. angustifidum Bitter	11*m* +1*sm*	2	t	4	t,c	–	ARGENTINA, Catamarca, Barboza 3489	3b, 4j
S. crispum Ruiz & Pav.	2*m* + 9*sm* + 1*st*	2*	t	4	i,c	+	CHILE, Biobio, Chiapella 1764	3e, 4k
S. dulcamara L.	11*m* + 1*sm*	2	t	2	c	–	FRANCE, Paris, Chiarini 702	3c, 4l
S. endoadenium Bitter	8*m* + 3*sm* + 1*st*	2	t	2	t	–	ARGENTINA, Catamarca, Barboza 3476	3d, 4m

*Dispersion in A chromosomes of r DNA.
Notes: KF: karyotype formulae. Abbreviations = c: centromeric; i: intercalar; st: subterminal; and t: terminal.

Invitrogen, Carlsbad, USA) by nick translation. To localize the 5S rDNA loci, a PCR product from genomic DNA (Kitamura et al. 2001) of *Solanum stuckertii* was labeled with digoxigenin by nick translation (DIG Nick translation, Roche, Germany). Slides were incubated in 100-µg/ml RNAse, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70–100%-graded ethanol series, and air-dried. Later, 30 µl of the hybridization mixture (4–6 ng/µl of probe, 50% formamide, 10% dextran sulfate, 2XSSC, and 0.3% SDS) previously denatured at 70°C for 10 min were applied. Slides were denatured/hybridized at 90°C for 10 min, 48°C for 10 min, and 38°C for 5 min using a thermal cycler (Mastercycler, Eppendorf) and the slides were kept overnight in a humid chamber at 37°C. Post-hybridization washes were carried out in 2XSSC, 0.1XSSC, and 4XSSC/0.2% Tween 20 at 42°C. The probes were simultaneously detected with a solution composed of 5% BSA, avidin-FITC conjugate (Sigma), and anti-digoxigenin-rhodamine conjugate (Roche), and mounted with 25 µl of Vectashield (Vector Laboratories). Photomicrographs were obtained with a Zeiss Axiophot microscope (Jena, Germany) coupled with Leica DFC300FX camera (Wetzlar, Germany).

Results

Mitotic chromosomes of 13 *Solanum* species (8 of the Morelloid and 5 of the Dulcamaroid clades) were analyzed by means of CMA/DAPI banding and FISH. Table I indicates the number and position of rDNA 18-5.8-26S and 5S loci in each studied taxa.

The banding analysis performed allowed the description of two heterochromatin patterns:

(a) Bands associated with nucleolus organizing regions (NORs) ($CMA^+/DAPI^-$): in the Morelloid clade, seven species had one chromosome pair with bands except *S. aloysiifolium* (Figure 1(a)) that showed two pairs with bands, generally in submetacentric (*sm*) chromosomes; in the Dulcamaroid clade, all studied species exhibited one large pair with bands. The species with this pattern are not shown in the figures since bands are coincident with the 18-5.8-26S rDNA signals (Figures 2 and 3).

(b) Bands not associated with NORs ($CMA^+/DAPI^-$): in the Morelloid clade, four species presented terminal bands in most chromosomes (*S. aloysiifolium*, *S. americanum*, *S. hastatilobum*, and *S. salicifolium);* in the Dulcamaroid clade, only *S. angustifidum* had them (Figure 1(b)).

The FISH technique confirmed that the 18-5.8-26S sites were colocalized with the corresponding heterochromatic $CMA^+/DAPI^-$ bands. In the Morelloid clade, 18-5.8-26S rDNA loci were mostly localized in terminal position in one chromosome pair except *S. aloysiifolium* (Figures 2(c) and 4(a)) and *S. hastatilobum* (Figures 2(h), and 4(d) in two pairs.

On the other hand, the 5S rDNA loci in the Morelloid clade were detected only in one small metacentric (*m*) chromosome pair except *S. triflorum* (Figures 2(e) and 4(h)) and *S. palitans* (Figures 2(g) and 4(e)) in a *sm* pair. Most species had them in terminal positions, whereas the *S. americanum* (Figures 2(a) and 4(b)) in centromeric and *S. triflorum* (Figures 2(e) and 4(h)) and *S. salicifolium* (Figures 2(f) and 4(g)) in intercalar positions. In the *Dulcamaroid* clade, the 5S rDNA loci can vary in number (in 1–2 chromosome pairs) and position (terminal, intercalar, or centromeric) (Table I, Figures 3 and 4(i)–(m)).

All Dulcamaroid species had one 18-5.8-26S locus in one large *m* chromosome pair in a terminal position (Figures 3 and 4(i)–(m)). *Solanum crispum* presented a differential pattern: a pair of intense signals in the terminal region of the second *m* pair and a set of weaker, scarcely defined signals in most chromosomes of the complement (Figures 3(e) and 4(k)) In addition, this species is unique, showing one of the 5S sites in synteny with the intense 18-5.8-26S signals.

The size of the 18-5.8-26S and 5S sites for both clades was not always proportional to the size of the corresponding heterochromatic bands associated with NORs observed with CMA/DAPI banding. Generally, both homolog sites with similar sizes were detected being homomorphic in all taxa.

Discussion

In plants, GC-rich heterochromatin is frequently associated with NORs (Guerra 2000). Available data for the numbers and disposition of heterochromatic bands for most angiosperms studied suggest that they are variable, but a general pattern can be depicted: heterochromatin is usually located in similar chromosome regions, regardless of the distance from the centromere resulting in generalized bands with similar heterochromatin distribution in most chromosomes of a complement (Guerra 2000). GC-rich heterochromatin could be associated with NOR or be telomeric, interstitial, or proximal. In several Solanaceae genera, such as *Nicotiana* (Kenton et al. 1993; Lim et al. 2000), *Capsicum* (Moscone et al. 2007), *Cestrum* (Fregonezi et al. 2006), and *Lycium* (Stiefkens et al. 2010), bands associated with NOR or telomeric are frequent.

Terminal heterochromatic $CMA^+/DAPI^-$ bands were found in most *Solanum* species analyzed, so far in agreement with our findings. Karyotypes with constitutive heterochromatin banding patterns analyzed by C− or fluorescence banding have been obtained

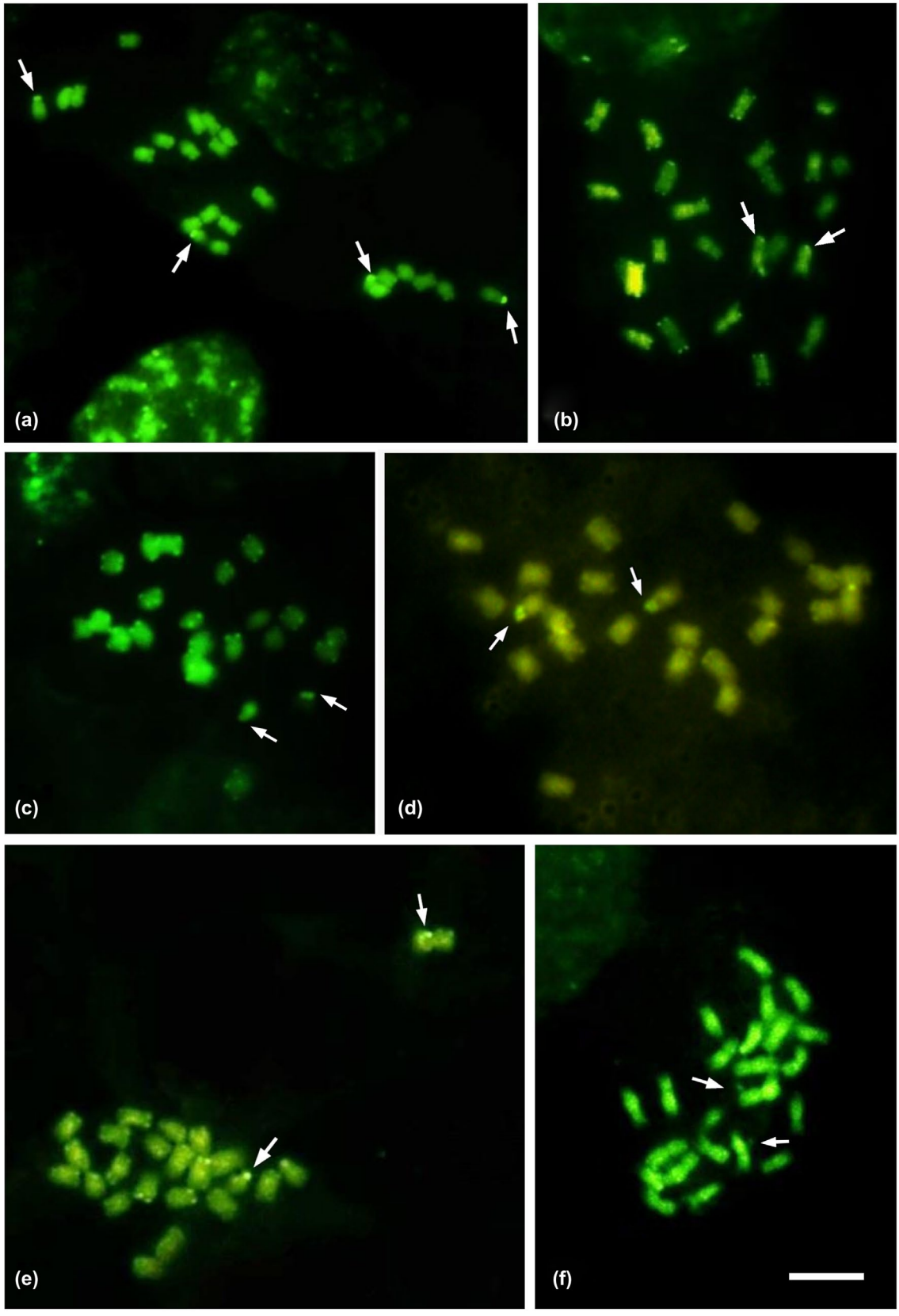

Figure 1. Chromosome banding (CMA fluorescence) in some of the studied species of *Solanum* of the Morelloid and Dulcamaroid clades. (a), *S. aloysiifolium*. (b), *S. angustifidum*. (c), *S. salicifolium*. (d), *S. americanum*. (e), *hastatilobum*. (f), and *S. triflorum*. Scale bar = 3 μm, all at the same scale. Arrows indicate $CMA^{+}/DAPI^{-}$ associated to NORs.

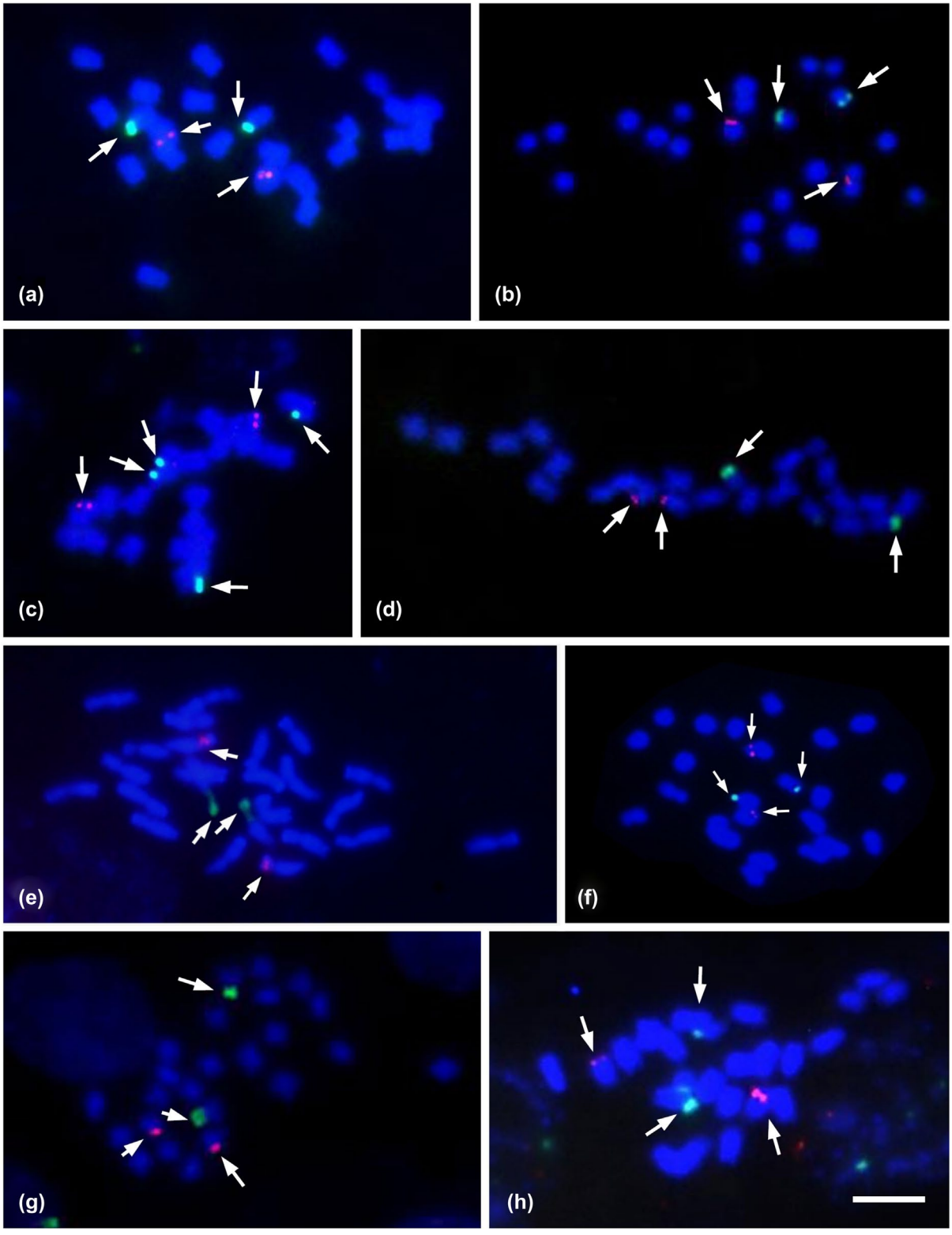

Figure 2. Fluorescence *in situ* hybridization with 5S and 18-5.8-26 rDNA probes in *Solanum* species of the Morelloid clade. (a), *S. americanum*. (b), *S. pilcomayense*. (c), *S. aloysiifolium*. (d), *S. chenopodioides*. (e), *S. triflorum*. (f), *S. salicifolium*. (g), *S. palitans*. (h), and *S. hastatilobum*. Arrows point at the rDNA signals, 18-5.8-26S in green and 5S in red. Scale bar = 3 μm, all at the same scale.

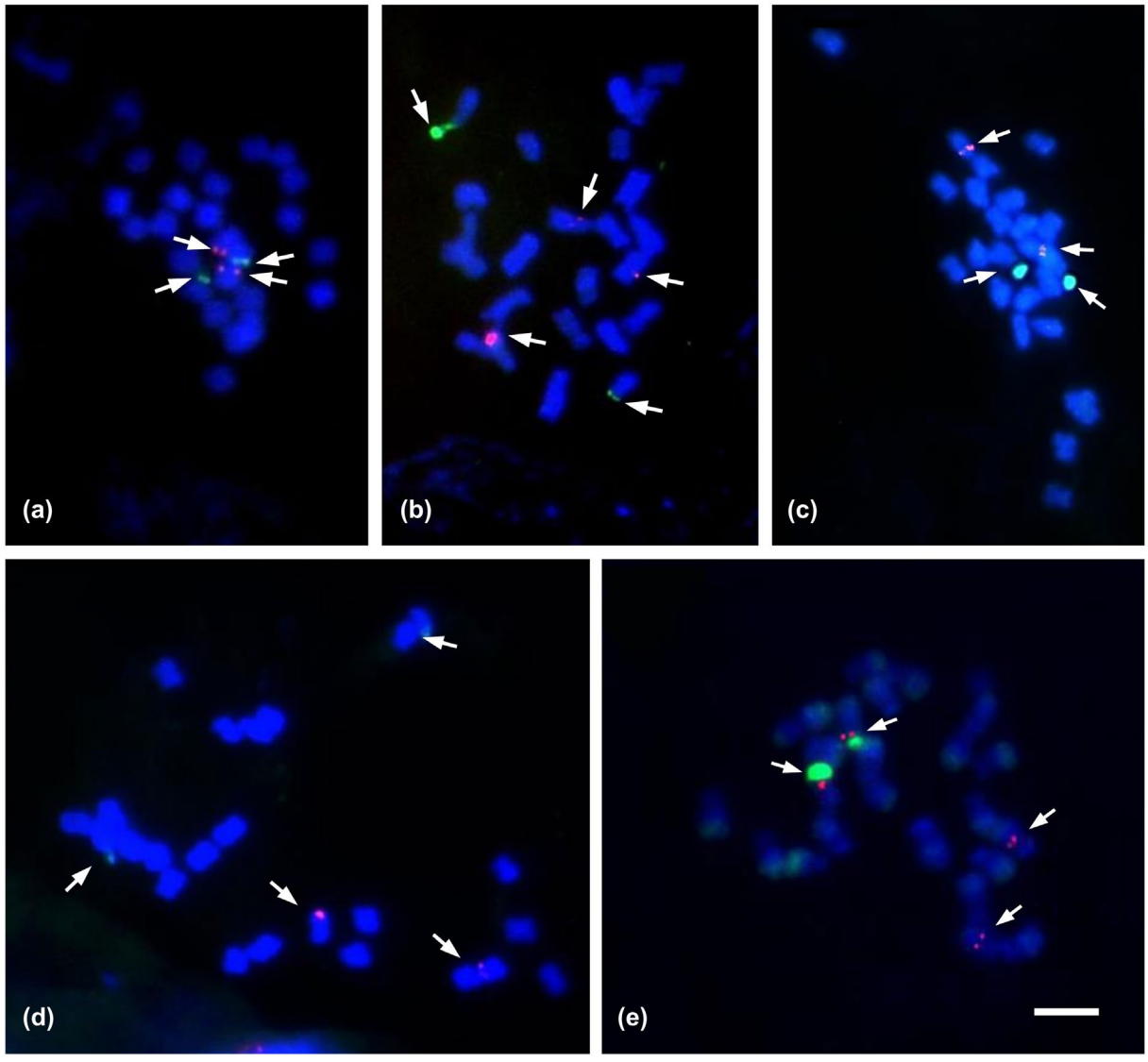

Figure 3. Fluorescence *in situ* hybridization with 5S and 18-5.8-26 rDNA probes in *Solanum* species of the Dulcamaroid clade. (a), *S. amygdalifolium*. (b), *S. angustifidum*. (c), *S. dulcamara*. (d), *S. endoadenium*. (e), and *S. crispum*. Arrows point at the rDNA signals, 18-5.8-26S in green and 5S in red. Scale bar = 3 μm, all at the same scale.

in nearly 50 species of the genus (e.g. Pijnacker & Ferwerda 1984; Pringle & Murray 1993; Sultana & Alam 2007; Brasileiro-Vidal et al. 2009; Rego et al. 2009; Melo et al. 2011; Acosta et al. 2012; Miguel et al. 2012; Chiarini et al. 2014).

The eight Morelloid species studied here agree as a whole with the banding pattern of five species previously examined (Sultana & Alam 2007; Rego et al. 2009; Melo et al. 2011; Acosta et al. 2012). *Solanum chenopodioides*, *S. palitans*, and *S. pilcomayense* showed exclusive bands associated with NORs; the same pattern was found by Melo et al. (2011) in a population of *S. nigrum*. On the other hand, we found telomeric bands not associated with NORs in *S. aloysiifolium*, *S. americanum*, *S. hastatilobum*, *S. triflorum*, and *S. salicifolium* as previously reported in different populations of *S. americanum*, *S. chenopodioides*, *S. nitidibaccatum*, *S. villosum* (as *S. luteum*), and *S. nigrum* (Sultana & Alam 2007; Melo et al. 2011). The presence or absence of heterochromatic bands not associated with NORs may vary in different populations indicating chromosomal intraspecific variability.

In the Dulcamaroid clade, we found karyotypes with bands associated to NORs in *S. crispum*, *S. dulcamara*, and *S. endoadenium* as previously detected in *S. amygdalifolium* and different populations of *S. dulcamara* and *S. endoadenium* (Rego et al. 2009; Melo et al. 2011; Acosta et al. 2012). Bands not associated with NORs had only been reported in *S. angustifidum* (our work). Acosta et al. (2012) detected one intercalary $CMA^{+}/DAPI^{-}$ band in

Figure 4. Idiograms of *Solanum* species of the Morelloid and Dulcamaroid clades. (a), *S. aloysiifolium*. (b), *S. americanum*. (c), *S. chenopodioides*. (d), *S. hastatilobum*. (e), *S. palitans*. (f), *S. pilcomayense*. (g), *S. salicifolium*. (h), *S. triflorum*. (i), *S. amygdalifoium*. (j), *S. angustifidum*. (k), *S. crispum*. (l), *S. dulcamara*. (m), and *S. endoadenium*. Scale bar = 3 µm, all at the same scale. Yellow dots indicate $CMA^{+}/DAPI^{-}$ bands, red dots indicate 5S loci, and yellow squares are 18-5.8-26S rDNA loci.

S. endoadenium, suggesting that in this population, a chromosomal rearrangement could have occurred. Heterochromatin bands would be a subject of dynamic changes and could be used to characterize species and/or populations.

Hybridization with the 18-5.8-26S rDNA probe confirmed the sites of the satellites observed by conventional staining (Moyetta et al. 2013) and fluorescent banding techniques reported in this work. This localization suggests a common distribution pattern that occurs in the terminal regions of the chromosomes and is frequent in many angiosperms (e.g. Vaio et al. 2005; Urdampilleta et al. 2006; Roa & Guerra 2012). It was found in several Solanaceae including *Capsicum* (Moscone et al. 2007), *Nicotiana* (Lim et al. 2000), *Nierembergia* (Acosta & Moscone 2010), *Cestrum* (Fregonezi et al. 2006), and *Solanum* (Rego et al. 2009; Melo et al. 2011; Chiarini et al. 2014). The presence of one chromosome pair with 18-5.8-26S loci seems to be the most frequent case in *Solanum* (Li et al. 2006; Brasileiro-Vidal et al. 2009; Rego et al. 2009; Melo et al. 2011; Chiarini et al. 2014). In the Morelloid clade, this was previously detected in four species (Rego et al. 2009; Jiang et al. 2011; Melo et al. 2011) including *S. americanum* studied here. This Cosmopolitan species was studied from three populations of different provenance yielding similar results to ours (Rego et al. 2009; Jiang et al. 2011 sub nom. *S. photeinocarpum*). In addition, we found two diploid species that had two 18-5.8-26S loci; this fact suggests that chromosomal rearrangements such as duplications and translocations may have occurred. In the Ducamaroid clade, one 18-5.8-26S site was observed in a large m pair in the five species examined so far (Rego et al. 2009; Jiang et al. 2011; Melo et al. 2011; our results).

Considering the broadest *Solanum* phylogenies (Bohs 2005; Särkinen et al. 2013, 2015), the most parsimonious hypothesis for the evolution of the 18-5.8-26S loci would be the presence

of one locus, plesiomorphic, and the existence of two loci, autapomorphic, for *S. aloysiifolium* and *S. hastatilobum*. Two 18-5.8-26S loci have also been reported for *Geminata*, *Brevantherum*, and *Potato* clades (Brasileiro-Vidal et al. 2009; Rego et al. 2009), suggesting that they would have evolved independently several times in a relatively short period of time (around 15.5 mya; Särkinen et al. 2013).

The 5S probe showed one pair of signals generally in m pairs in different positions. In six species of the Morelloid clade, they were terminally located (our results) and in two species, they were intercalar or centromerical (Rego et al. 2009; Jiang et al. 2011; our results). On the other hand, in the Dulcamaroid clade, these signals varied in their number and position in the six species studied so far: one or two pairs in centromeric, intercalary, or terminal position (Rego et al. 2009; Jiang et al. 2011; our results). The 5S locus would fastly evolve; for instance, the split between the lineages of *S. dulcamara* and *S. crispum* would have occurred around 5 mya period in which a duplication of this site had occurred. In other Solanaceae, the rDNA 5S site was found to be equally variable in number and position (e.g. Kitamura et al. 2001; Fregonezi et al. 2006; Acosta & Moscone 2010; Urdampilleta et al. 2015).

In angiosperms, the 5S and 18-5.8-26S rDNA sites are generally localized in different chromosomes (Garcia et al. 2007, 2009) including Solanaceae in which few cases of synteny have been reported, e.g. *Capsicum* (Youn-Kyu et al. 1999; Kwon & Kim 2009), *Nicotiana* (Kovarik et al. 2008), *Cestrum* (Urdampilleta et al. 2015), and *Jaborosa* (Chiarini et al. unpublished results). *Solanum* seems to follow the general pattern as we found in both the studied clades except in *S. crispum* in which one of the two pairs of 5S signals was in synteny with the 45S and in *S. habrochaites* of the *Potato* clade in which the 5S site was in synteny with one of its two 18-5.8-26S signals (Brasileiro-Vidal et al. 2009). The synteny of rDNA sites for the studied Dulcamaroid and Morelloid species seems to be a plesiomorphic character, being a chromosomal rearrangement responsible for the synteny detected in *S. crispum*.

Results obtained in this work suggest that different chromosomal rearrangements may have happened such as duplications, translocations, and inversions in a comparatively short lapse (9.6 mya when the Dulcamaroid and Morelloid clades split; Särkinen et al. 2013). The used markers can be employed to karyotypically differentiate species within each clade. For instance, *S. chenopodioides* and *S. aloysiifolium* are phylogenetically closely related (Särkinen et al. 2015) but can be distinguished by the position of the 18-5.8-26S locus.

The studied species of both clades are exomorphologically diverse. On the other hand, the Morelloid clade seems to be more homogeneous than the Dulcamaroid one with respect to heterochromatin and rDNA patterns. In this clade, loss and transposition of rDNA could have occurred independently with major chromosome modifications. Genomic differentiation would be more likely due to genetic divergence than large structural genomic divergence associated with an increase/decrease in repeated sequences with possibly transposable elements and rDNA gene families (Lönnig & Saedler, 2002; Kalendar et al. 2008). However, additional species should be examined to cast light on these patterns.

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