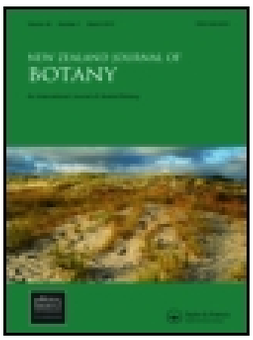

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RESEARCH ARTICLE

# Differentiation of *Nolana* and *Sclerophylax* (Solanaceae) by means of heterochromatin and rDNA patterns

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**ABSTRACT**

Within Solanaceae, *Nolana* L. f. and *Sclerophylax* Miers are two genera that have deserved special attention because of their rare anatomical characteristics. However, they are poorly known from the cytogenetic perspective. In order to discuss their chromosome features in an evolutionary context, classical staining, chromomycin $A_3$/4′-6-diamidino-2-phenylindole (CMA/DAPI) fluorescent banding and in situ hybridisation (FISH) with probes for the 18-5.8-26S and the 5S rDNA loci were applied to root tips of germinating seeds. All the species presented the chromosome number 2n = 24. Karyotypes were highly symmetric, with most chromosomes being metacentric and with a maximum of three submetacentric pairs in *N. divaricata*. The CMA/DAPI banding technique, assayed for the first time in *Sclerophylax*, showed $CMA^+/DAPI^-$ bands associated with nucleolar organiser regions (NORs) in the first metacentric chromosome pair in each species. The FISH technique (applied to four species of *Sclerophylax* and, for the first time, in one species of *Nolana*) showed that the 18-5.8-26S loci coincide with $CMA^+/DAPI^-$ bands. Three *Sclerophylax* species presented two pairs of 5S signals, whereas *S. adnatifolia* showed three. The rDNA loci resulted as asyntenic in *Sclerophylax*, but were localised in the same chromosome in *N. divaricata*. Despite the morphological peculiarities of *Nolana* and *Sclerophylax*, the chromosome number and karyotype features are consistent with the position of the two genera within the 'x = 12 clade', while the number and position of rDNA loci established the chromosome rearrangements, suggesting different evolutionary pathways with respect to their closest relatives, *Lycium* and *Jaborosa*.

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Heterochromatin; karyotype; *Nolana*; rDNA; *Sclerophylax*; South America

## Introduction

The Solanaceae is a cosmopolitan family that includes c. 2700 species (Olmstead & Bohs 2007; Särkinen et al. 2013), some of them having great economic, ethnobotanic, experimental and ornamental value. Potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) are species of this family originally from the South American Andes, together with another 16 genera (Hunziker 2001).

The phylogeny of the Solanaceae is relatively resolved and the limits of the family have been expanded to also embrace the Sclerophylacaceae, Nolanaceae, Goetzeaceae and the

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Duckeodendraceae (Olmstead et al. 2008; Särkinen et al. 2013). This scheme is supported by strong evidence and it is interesting to find supplementary chromosomal synapomorphies that complement it. In spite of the relevance of cytology for the diversification and systematics of this family (e.g. Tu et al. 2005; Moscone et al. 2007; Chiarini et al. 2016), chromosome studies are still lacking in many of the Solanaceae. In particular, several genera and many species (e.g. two thirds of the c. 1400 species of *Solanum*) in the so called 'x = 12 clade' are cytogenetically unknown. To fill this gap we have carried out a study on two unexplored genera, *Nolana* L. f. and *Sclerophylax* Miers, in order to understand their systematic relationships and chromosome evolution.

*Nolana* is one of the four largest genera within Solanaceae. It embraces 89 species mainly distributed in arid regions of Chile and Peru, in coastal environments dependent for water on fog called lomas formations (Dillon et al. 2007, 2009). *Nolana* species are annual or perennial herbs or shrubs, with showy flowers (*N. paradoxa* Lindl. is cultivated as ornamental) with a gynoecium formed of five strongly lobed carpels (Tago-Nakawaza & Dillon 1999; Freyre et al. 2005). *Nolana* is easily recognised by the unusual sclerified fruits divided into many mericarps, each one with several seeds (Bruno 1994), a feature which is rare in the family (Knapp 2002). This character is an autapomorphy (a uniquely derived character) of the genus, and it was the principal reason why some authors have maintained the group at the family level (Nolanaceae; Hunziker 2001). However, according to molecular studies, *Nolana* is a natural group within the Solanaceae (Tu et al. 2008), *Sclerophylax* being its sister clade, and it is also closely related to *Lycium* L. and *Jaborosa* Juss. (Dillon 2005; Särkinen et al. 2013). Concerning cytogenetics, chromosome counts in five species and the karyotype of *N. crassulifolia* Poepp. with the formula $11m + 1sm$, is the only information available for the entire genus (Bolkhovskikh et al. 1969; Chiarini et al. 2010).

*Sclerophylax* is a small genus that embraces 12 species endemic to dry regions in western and central Argentina (only two species occur outside Argentina: *S. spinescens* Miers, in Paraguay, and *S. lorentzianus* Hoffm., in Uruguay). *Sclerophylax* species are herbs, almost glabrate or pubescent, succulent and mostly annual, with breakable, winding stems of circular-triangular section. They have crystals of calcium oxalate in roots, stems and leaves. Flowers are perfect, axillary, solitary and sessile, with an asymmetrical calyx and a slightly zygomorphic corolla, generally white or blue (Di Fulvio 1961; Barraza 1994); the ovary is superior, bicarpellate, bilocular and contains two to three ovules. *Sclerophylax* was excluded from the Solanaceae based on its distinctive gynoecium and fruit morphology: the fruit is dry, indehiscent, with a membranous pericarp enclosing two to three small straight seeds, and it is usually embedded into the stem and crowned by the spiny calyx. Thus, the genus was alternatively included in Boraginaceae, Hydrophyllaceae or in its own family, Sclerophylacaceae, but finally studies of molecular phylogeny have demonstrated that *Sclerophylax* belongs in the Solanaceae (Olmstead et al. 2008). With respect to the cytogenetics, to date it is only known that the basic chromosome number is x = 12 and the karyotypes are constituted mostly by metacentric (*m*) and submetacentric (*sm*) chromosomes (Di Fulvio 1961).

*Lycium* and *Jaborosa*, two of the genera most closely related to *Sclerophylax* and *Nolana*, have been the focus of detailed chromosome studies that curiously revealed different evolutionary paths, apparently related to key geological events (Stiefkens et al. 2010; Blanco et al. 2012; Chiarini et al. 2016). However, no species of *Nolana* or *Sclerophylax*

have been studied up to now using, for instance, fluorochrome banding or fluorescence in situ hybridisation (FISH) procedures, which are useful in detecting the chromosome rearrangements involved in speciation (e.g. Hasterok et al. 2006; Chacón et al. 2012). Chromomycin $A_3$/4′-6-diamidino-2-phenylindole (CMA/DAPI) staining reveals heterochromatin blocks, which are one of the most remarkable chromosome components because of their apparent lack of genes, their unknown function and their differential stainability, thus constituting a source of variability for comparative purposes (Guerra 2000). FISH permits homologous chromosomes in a complement to be identified and allows comparison between related species, thus answering chromosomal evolutionary questions (e.g. Chacón et al. 2012; Chiarini et al. 2014). The most common FISH markers are ribosomal genes (5S and 18–5.8–26S rDNA), which are abundant and highly conserved in higher plants (Heslop-Harrison & Schwarzacher 2011).

Considering this background, the aim of this work was to study the mitotic chromosomes of *Sclerophylax* and *Nolana* by means of classic staining, CMA/DAPI banding and FISH with probes for the ribosomal genes 5S and 18–5.8–26S rDNA, in order to establish chromosome numbers, karyotypes, heterochromatin patterns, and the number and position of rDNA sites, as well as to discuss the resulting information in an evolutionary context.

## Material and methods

Voucher specimens of the studied material are deposited at Museo Botánico de Córdoba (CORD). The provenance of the plant material is as follows:

*Sclerophylax adnatifolia* Di Fulvio: ARGENTINA, prov. Jujuy, dpt. Tumbaya, 23°46′18″ S, 65°56′51″ W, Barboza et al. 4382.

*Sclerophylax arnottii* Miers: ARGENTINA, prov. La Rioja, dpt. Gobernador Gordillo, 30° 04′23″ S, 66°47′47″ W, Barboza et al. 4202.

*Sclerophylax kurtzii* Di Fulvio: ARGENTINA, prov. La Rioja, dpt. Famatina, 28°51′16.7″ S, 67°36′30″ W, Barboza et al. 4235.

*Sclerophylax spinescens* Miers: ARGENTINA, prov. Córdoba, dpt. San Justo, Miramar, Laguna Mar Chiquita, 30°55′02.45″ S, 62°40′58.88″ W, Chiarini 1267.

*Nolana divaricata* (Lindl.) I.M. Johnst.: CHILE, Region II Antofagasta, 25°33′16.2″ S, 70° 21′34.3″ W, Barboza et al. 2971.

*Nolana villosa* (Phil.) I.M. Johnst.: CHILE, Region II Antofagasta, Paposo, 25°00′21.8″ S, 70°27′55.8″ W, Barboza et al. 2979.

*Nolana stenophylla* I.M. Johnst.: CHILE, Region II Antofagasta, Paposo, 25°00′21.8″ S, 70° 27′55.8″ W, Barboza et al. 2978.

*Nolana patula* (Phil.) M.O. Dillon: CHILE, Region III Atacama, 26°09′14.9″ S, 70°27′43.4″ W, Barboza et al. 2970.

It was necessary to rupture the sclerified fruits of the species in both genera in order to release the seeds, which were very reluctant to germinate, especially the *Nolana* species. Mitotic chromosomes were examined in root tips obtained from germinating seeds. Roots were pretreated in saturated *p*-dichlorobenzene in water for 2 h at room temperature, fixed in 3:1 ethanol:acetic acid, washed in distilled water, digested for 45 min at 37 °C

with Pectinex SP ULTRA (Novozymes) and squashed in a drop of 45% acetic acid. After coverslip removal in liquid nitrogen, the slides were stored at –20 °C.

For mitotic counts and karyotypes, slides were stained with Giemsa (Guerra 1983). At least 10 metaphases of each species were photographed with phase contrast in a Zeiss Axiophot microscope. Photographs were used for taking the following measurements of each chromosome pair: s (short arm); l (long arm); and c (total chromosome length). The arm ratio (r = l/s) was then calculated and used to classify the chromosomes as recognised by Levan et al. (1964). In addition, the total haploid chromosome length of the karyotype (TL) based on the mean chromosome length was calculated. Karyotype asymmetry was estimated using Romero Zarco's (1986) indices ($A_1$ = intrachromosomal asymmetry index, and $A_2$ = interchromosomal asymmetry index). Idiograms were based on the mean values for each species.

For fluorescent banding, slides were stained with a drop of 0.5 mg/mL chromomycin $A_3$ (CMA) in McIlvaine buffer, pH 7.0 and distilled water (1:1) containing 2.5 mM $MgCl_2$ for 90 min, and subsequently stained with 2 µg/mL 4′-6-diamidino-2-phenylindole (DAPI) for 30 min, and finally mounted in McIlvaine's buffer-glycerol v/v 1:1 (Schweizer 1976; Schweizer & Ambros 1994). The amount of heterochromatin was expressed as a percentage of the total length of the haploid karyotype.

The location and number of rDNA sites were determined by FISH using two probes: the p*Ta*71 containing the 18-5.8-26S gene of wheat (Gerlach & Bedbrook 1979) labelled with biotin-14-dATP (BioNick, Invitrogen Carlsbad) and a 5S rDNA fragment obtained by polymerase chain reaction (PCR) from *Solanum stuckertii* Bitter (Chiarini et al. 2014), labelled with digoxigenin-11-dUTP (DigNick, Roche). The FISH protocol was according to Schwarzacher & Heslop-Harrison (2000), with minor modifications. The preparations were incubated in 100 µg/mL RNAase, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70%–100% graded ethanol series, and air-dried. On each slide, 15 µL of hybridisation mixture was added (4–6 ng/µL of probe, 50% formamide, 10% dextran sulfate, 2× saline sodium citrate and 0.3% sodium dodecyl sulfate), previously denatured at 70 °C for 10 min. Chromosome denaturation/hybridisation was undertaken at 90 °C for 10 min, 48 °C for 10 min and 38 °C for 5 min using a thermal cycler (Mastercycler, Eppendorf), and slides were placed overnight in a humid chamber at 37 °C. The 18-5.8-26S probe was detected with avidin-FITC conjugate (Sigma-Aldrich), the 5S probe was detected with antidigoxigenin-rhodamine (Roche), and then counterstained and mounted with 25 µL antifade Vectashield (Vector Lab.) containing 1.5 µg/mL of DAPI.

At least 10 metaphases of each species from at least three different individuals were photographed with a Zeiss Axiophot microscope equipped with epifluorescence and a digital image capture system. The free software ImageJ (http://rsbweb.nih.gov/ij/) was used for merging the images.

## Results

The mitotic chromosome number in all the species studied is 2n = 24. Idiograms based on average chromosome measurements are shown in Figure 1, and chromosome variables are summarised in Table 1. In the general context of angiosperms (Guerra 2000), chromosomes here studied are relatively small to medium sized (Figure 2, Table 1), with a chromosome size varying from 3.20 to 6.77 µm. The shortest chromosome was measured

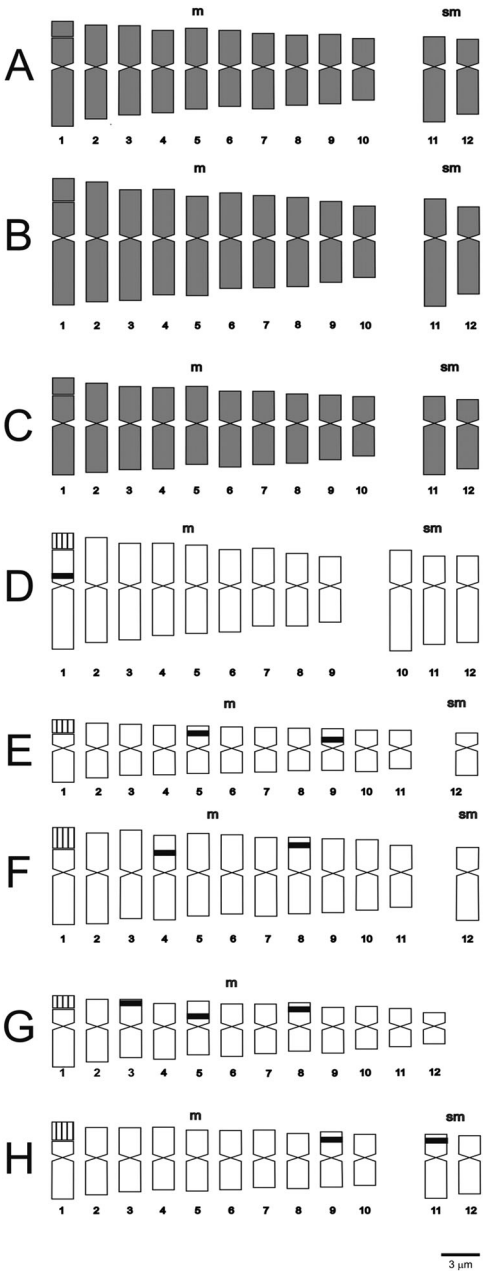


**Figure 1.** Idiograms of *Nolana* and *Sclerophylax*. **A,** *N. stenophylla*; **B,** *N. patula*; **C,** *N. villosa*; **D,** *N. divaricata*; **E,** *S. kurtzii*; **F,** *S. arnottii*; **G,** *S. adnatifolia*; **H,** *S. spinescens*. Chromosomes are ordered from the longest to the shortest within each category, from *m* to *sm*. Species only studied with classical staining are shown in grey. Black blocks are 5S signals and hatched blocks indicate the 18-5.8-26S signals plus a $CMA^{+}/DAPI^{-}$ NOR associated band in the same position.

in pair 12 of *S. adnatifolia* (2.3 µm) and the longest was in pair 1 of *N. patula* (8.67 µm). This species also presents the highest value for total haploid genome length (TL = 81.32 µm), while *S. kurtzii* presents the lowest (38.47 µm).

**Table 1.** Chromosome variables of species of *Sclerophylax* and *Nolana*. Karyotype formulae, total haploid genome length in μm (TL), average chromosome length in μm (c) ± standard deviation, average arm ratio (R) ± standard deviation, intrachromosomal asymmetry index ($A_1$) and interchromosomal asymmetry index ($A_2$).

| | | | | | | | | No. of pairs of FISH signals | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Species | Karyotype formula | c | R | TL | $A_1$ | $A_2$ | Percentage of heterochromatin | 18-5.8-26S loci | 5S loci | Co-localisation of rDNA genes |
| *S. adnatifolia* | 12 m | 3.37 ± 0.42 | 1.28 ± 0.06 | 41.70 ± 5.09 | 0.19 | 0.21 | 2.89 | 1 | 3 | no |
| *S. arnottii* | 11 m + 1 sm | 5.46 ± 0.60 | 1.25 ± 0.03 | 65.52 ± 7.29 | 0.17 | 0.12 | 2.15 | 1 | 2 | no |
| *S. kurtzii* | 11 m + 1 sm | 3.20 ± 0.33 | 1.22 ± 0.03 | 38.47 ± 3.96 | 0.15 | 0.14 | 2.56 | 1 | 2 | no |
| *S. spinescens* | 10 m + 2 sm | 4.17 ± 0.63 | 1.26 ± 0.07 | 50.12 ± 7.62 | 0.17 | 0.11 | 2.51 | 1 | 2 | no |
| *N. divaricata* | 9 m + 3 sm | 6.13 ± 1.44 | 1.39 ± 0.06 | 73.60 ± 17.38 | 0.23 | 0.15 | | 1 | 1 | yes |
| *N. stenophylla* | 10 m + 2 sm | 5.25 ± 1.02 | 1.31 ± 0.06 | 63.07 ± 12.33 | 0.21 | 0.14 | | | | |
| *N. villosa* | 10 m + 2 sm | 5.25 ± 0.43 | 1.35 ± 0.08 | 63.02 ± 5.25 | 0.23 | 0.14 | | | | |
| *N. patula* | 10 m + 2 sm | 6.77 ± 1.26 | 1.30 ± 0.06 | 81.32 ± 15.20 | 0.20 | 0.16 | | | | |

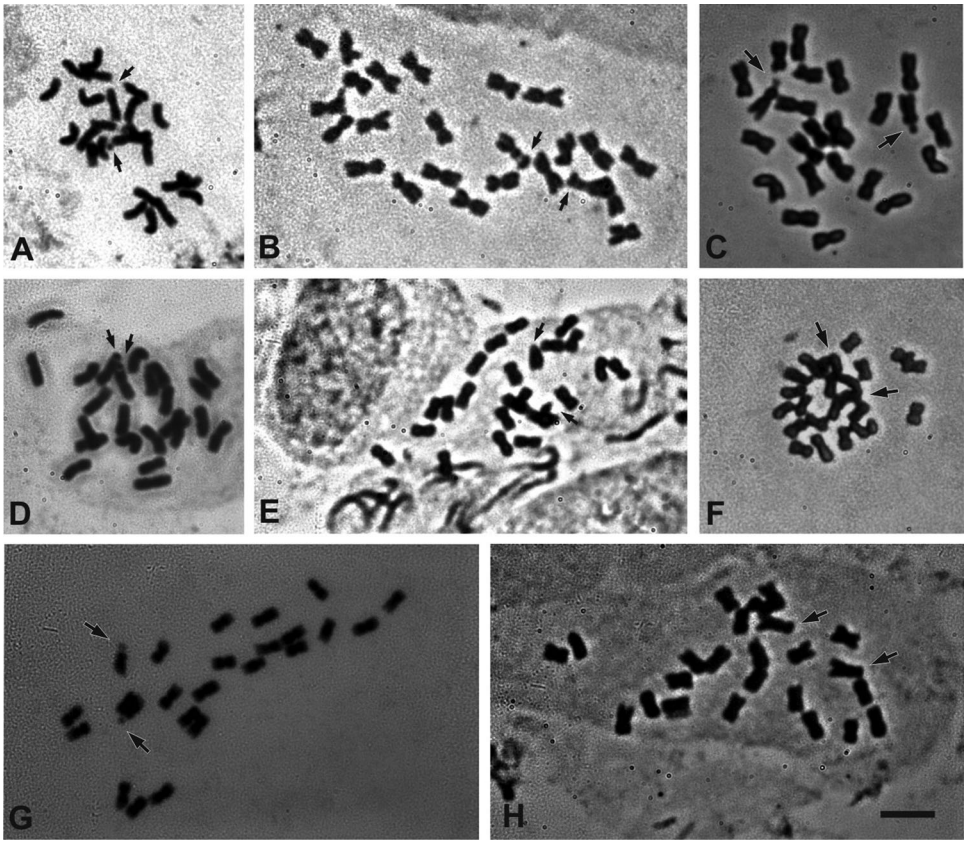


**Figure 2.** Photomicrographs of mitotic metaphases of *Sclerophylax* and *Nolana* stained with Giemsa. **A,** *N. divaricata*; **B,** *N. patula*; **C,** *S. arnottii*; **D,** *N. villosa*; **E,** *N. stenophylla*; **F,** *S. adnatifolia*; **G,** *S. spinescens*; **H,** *S. kurtzii*. Arrows point to satellites. All at the same scale, bar = 5 µm.

All species show secondary constrictions (Figure 2), which belong to the microsatellite type of Battaglia (1955). Karyotype formulae are composed of nine to 12 *m* chromosome pairs, plus zero, one, two or three *sm* pairs (Table 1). According to Romero Zarco (1986), karyotypes are symmetrical (Table 1), that of *S. adnatifolia* being the most symmetrical and that of *N. divaricata* the least.

In all the species analysed with the CMA/DAPI technique (*S. adnatifolia, S. arnottii, S. kurtzii* and *S. spinescens*), $CMA^{+}/DAPI^{-}$ bands associated with nucleolar organiser regions (NORs) were detected, always located in the largest *m* pair (Figure 3). The heterochromatin percentage varied from 2.15% in *S. arnottii* to 2.89% in *S. adnatifolia* (Table 1).

Ribosomal DNA genes were studied with FISH in five species: *S. adnatifolia*, *S. arnottii*, *S. kurtzii*, *S. spinescens* and *N. divaricata*. In all cases the probe for the 18-5.8-26S gene hybridised in the secondary constriction on the short arm of the largest *m* pair, coinciding with a $CMA^{+}/DAPI^{-}$ NOR associated band. On the other hand, signals for the 5S gene varied in number and position according to the species (Figure 4, Table 1).

In *N. divaricata*, one 18-5.8-26S signal is located in the same chromosome that also bears the 5S gene, although the two signals are not co-localised (i.e. they are syntenic, contiguous in the same chromosome, but they are not embedded to each other).

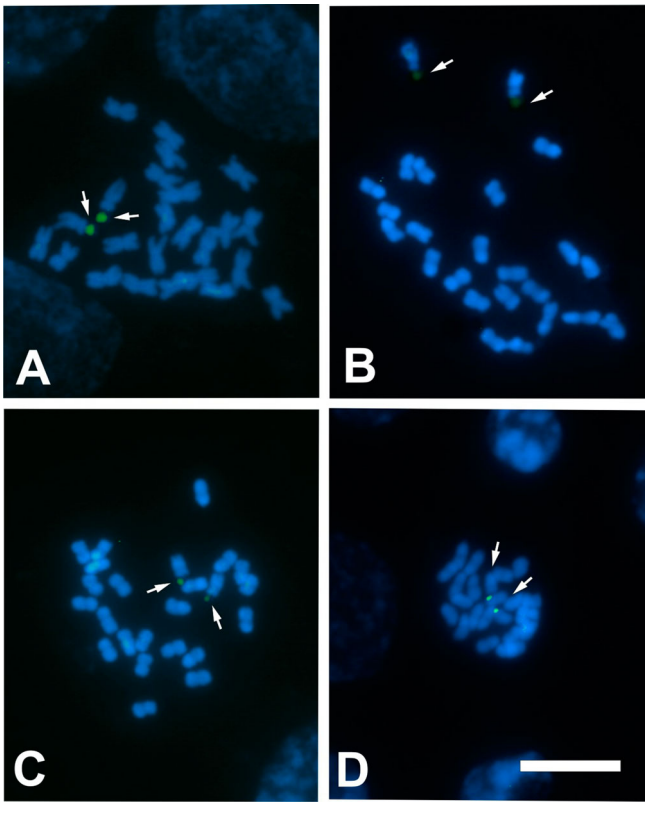


**Figure 3.** Photomicrographs of mitotic metaphases of *Sclerophylax* stained with CMA/DAPI. **A,** *S. kurtzii*; **B,** *S. arnottii*; **C,** *S. spinescens*; **D,** *S. adnatifolia.* Arrows point to $CMA^{+}/DAPI^{-}$ bands (green signals). All at the same scale, bar = 10 µm.

## Discussion

### *Chromosome number*

In both *Nolana* and *Sclerophylax* all species presented 2n = 24, which confirm previous reports (Di Fulvio 1961; Chiarini et al. 2010) and is consistent with the data available for the Solanoideae or 'x = 12 clade' (Hunziker 1979; Olmstead et al. 2008). All the analysed species resulted as diploids, suggesting that neither polyploidy or aneuploidy/dysploidy have played any significant role in the diversification of these two genera. However, more species need to be counted (especially for *Nolana*) to confirm this assertion.

According to Särkinen et al. (2013), *Sclerophylax* + *Nolana* + *Lycium* form a clade, whose sister clade is the Hyoscyameae (*Hyoscyamus* L., *Atropa* L., *Anisodus* Link. ex Spreg., *Scopolia* Jacq., *Physochlaina* G. Don and *Przewalskia* Maxim.). Whereas *Sclerophylax* and *Nolana* are South American, *Lycium* inhabits both South and North America, as well as the Old World (Miller et al. 2011). However, the Hyoscyameae are exclusive to the Old World, with some genera endemic to China (Tu et al. 2010; Sanchez-Puerta & Abbona 2014). This is important for comparative purposes, since the continental separation could have led to different evolutionary pathways, including at the chromosomal level. In fact,

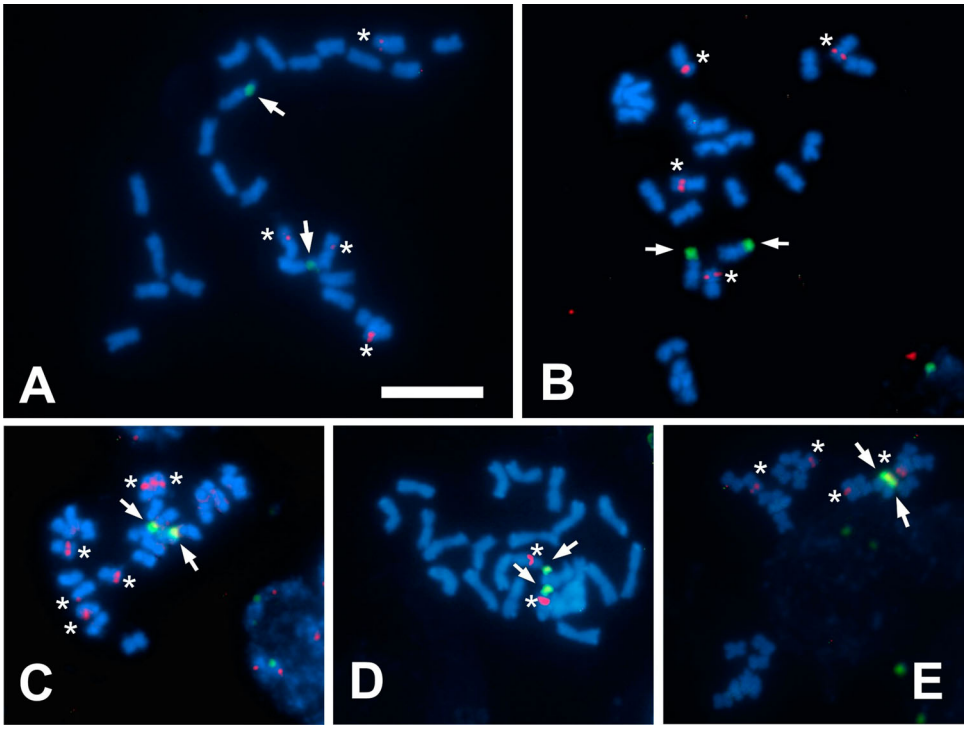


**Figure 4.** Fluorescent in situ hybridisation (FISH) in metaphasic chromosomes of *Sclerophylax* and *Nolana*. **A,** *S. arnottii*; **B,** *S. spinescens*; **C,** *S. adnatifolia*; **D,** *N. divaricata*; **E,** *S. kurtzii*. Arrows point to 18-5.8-26S signals (green) and asterisks indicate 5S signals (red). All at the same scale, bar = 10 µm. Notice that in *N. divaricata* the 18-5.8-26S and 5S signals are in the same chromosome but they are not embedded to each other.

most Hyoscyameae are polyploids or have derived basic numbers (2n = 28, 34, 42, 44, 48, 68, 72, 84, 96) probably originated by dysploidy from a polyploid ancestor (Tu et al. 2005), whereas *Lycium*, *Jaborosa*, *Sclerophylax* and *Nolana* are mostly diploids with 2n = 24 (Stiefkens & Bernardello 1996; Bernardello et al. 2008; Chiarini & Barboza 2008; Chiarini et al. 2010; Stiefkens et al. 2010).

### *Karyotypes*

In the species studied, the chromosomes are small compared with other angiosperms in general (Guerra 2000), but medium sized with respect to the Solanaceae (Badr et al. 1997). Chromosomes of *Nolana* are larger than those of *Sclerophylax*, and also larger than those of closely related genera, such as *Lycium* (Stiefkens & Bernardello 1996, 2000, 2002; Stiefkens 2001; Bernardello et al. 2008; Stiefkens et al. 2009, 2010; Blanco et al. 2012) and *Jaborosa* (Chiarini & Barboza 2008). The four species of *Nolana* studied here all presented symmetrical karyotypes, similar to that previously reported for *N. crassulifolia* (Chiarini et al. 2010), and *Sclerophylax* also has low asymmetry indices. Symmetrical karyotypes are the rule in *Lycium* where most species have complements with 10–12 *m* chromosomes. On the contrary, *Jaborosa* displays a wider

karyotype diversity, with some species having up to six *sm* pairs and two to three *st* pairs (Chiarini & Barboza 2008; Chiarini et al. 2016). Another similarity between *Nolana*, *Sclerophylax* and *Lycium* is the possession of satellites positioned in the short arm of the largest *m* chromosome pair, which differs from *Jaborosa* species that present more than one pair and in variable positions (e.g. Stiefkens & Bernardello 1996; Bernardello et al. 2008; Chiarini & Barboza 2008; Chiarini et al. 2010; Stiefkens et al. 2010; Blanco et al. 2012). All these chromosome features bring *Nolana* and *Sclerophylax* closer to *Lycium* and separate them from *Jaborosa*, which would have followed a different evolutionary path at the chromosomal level, perhaps caused by a different biogeographic history.

Concerning heterochromatin, in the Solanaceae the number and the size of bands are variable, but the overall pattern is relatively preserved, such as in *Lycium* (Stiefkens et al. 2010; Blanco et al. 2012). The species studied of *Sclerophylax* are all similar in having a low percentage of heterochromatin, with only one $CMA^+/DAPI^-$ band associated with NOR located at the first *m* pair, which is co-localised with the 18-5.8-26S loci. The same pattern has been observed in *Lycium* (Stiefkens et al. 2009, 2010; Blanco et al. 2012), as well as in other Solanaceae of the x = 12 clade not so closely related, such as *Solanum* L. (Chiarini et al. 2014), *Capsicum* L. (Moscone et al. 1996, 2007), *Lycianthes* (Dunal) Hassl. and *Vassobia* Rusby (Rego et al. 2009). On the contrary, the heterochromatin percentage is more variable in *Jaborosa* species, with many of them having additional $CMA^+/DAPI^-$ bands that are not associated with NORs.

It has been observed that asymmetric karyotypes seem to be associated with a greater amount of heterochromatin in species of the *Solanum* sect. *Acanthophora* (Chiarini et al. 2013). Coincidently, in the species studied here, the karyotypes are symmetrical and the amount of heterochromatin is low. Contrary cases have been found only in species of the Cactaceae (Las Peñas et al. 2008).

The rDNA 18-5.8-26S loci are part of the structure of NORs, which are generally in the secondary constriction of chromosomes (Dubcovsky & Dvorak 1995) and consist of tandem repeat units. The FISH technique, conducted in four species of *Sclerophylax* and, for the first time, in *Nolana*, revealed that the 18-5.8-26S site is located in the first *m* pair and co-localised with a $CMA^+/DAPI^-$ band in both genera. This localisation is usual for the Solanaceae and for most plant species where 18-5.8-26S always occurs in the terminal regions of the chromosomes (Lim et al. 2000; Fregonezi et al. 2006; Kwon & Kim 2009). Meanwhile, 5S rDNA units are composed of sequences of 120 highly conserved base pairs organised in tandem at specific locations of the genome (Appels & Honeycutt 1986). *Sclerophylax* species differ from each other in terms of the location and number of sites of 5S rDNA loci. The probe hybridised in two chromosome pairs in *S. kurtzii*, *S. spinescens* and *S. arnottii*, and in three in *S. adnatifolia*. This is different from what happens in *Lycium* where only a single pair of signals per complement has been seen (Blanco et al. 2012). Concerning *N. divaricata*, the synteny of the rDNA loci 18-5.8-26S and 5S is remarkable. One hypothesis to explain this situation would be an accidental insertion of 5S rDNA within or near the 18-5.8-26S by means of retrotransposons or other mobile elements (Drouin & De Sa 1995; Lönnig & Saedler 2002; Altinkut et al. 2006). This phenomenon has also been observed in other species such as some Asteraceae (Garcia et al. 2007) and Amaryllidaceae (Chang et al. 2009). The presence of both rDNA signals on the same chromosome can be interpreted as evidence of a chromosomal rearrangement (translocation) since the 18-5.8-26S and 5S signals are located on different

chromosomes in the sister clades (*Lycium* and *Sclerophylax*) (Tang et al. 2008). The synteny in *N. divaricata* can be considered as a synapomorphy, although studies on a larger number of species would be needed to establish whether this is a characteristic of the genus or if it is particular to the species.

### Morphological diversification and chromosome differentiation

In *Sclerophylax*, both morphological and karyotype features are fairly uniform, whereas in *Nolana*, although morphological differences exist, such as the habit, corolla shape, leaf type, presence or absence of glandular hairs, etc (Johnston 1936; Di Fulvio 1961), they are not accompanied by any remarkable karyotype changes (Chiarini et al. 2010; this study), although in this case it would be necessary to analyse a greater number of species cytogenetically in order to establish a general pattern.

In contrast, *Lycium* shows a vast morphological variability (e.g. the fruit can be a berry or a drupe, the corollas can be rotate or tubular, the leaves are fleshy or membranous, orbicular, lanceolate or linear; Bernardello 1986; Bernardello & Chiang Cabrera 1998), but the karyotypes are very similar (Stiefkens & Bernardello 1996, 2002; Stiefkens et al. 2010; Blanco et al. 2012) which has been called 'karyotypic orthoselection'. Likewise, *Jaborosa* presents notable morphological variability (especially floral characters), but in this case it is accompanied by a karyotype diversification (Chiarini & Barboza 2008; Chiarini et al. 2013).

One possible explanation for these karyotype variations may arise from establishing the divergence times among the genera. According to Särkinen et al. (2013), the clade that embraces *Lycium*, *Sclerophylax* and *Nolana* originated about 16 million years ago (mya), a period during which the chromosome number and karyotypes have remained more or less constant (2n = 24, karyotypes with most *m* chromosomes), unlike what happened in Hyoscyameae where polyploidy and disploidy have occurred (Tu et al. 2005, 2010), or *Jaborosa* where karyotypes are much more asymmetric (Chiarini & Barboza 2008; Chiarini et al. 2013). *Sclerophylax* split from *Nolana* and *Lycium* about 12.16 mya whereas the latter two genera separated from each other at ±10.57 mya (Särkinen et al. 2013). This would explain the fact that both *Nolana* and *Lycium* present only one 5S rDNA site (Blanco et al. 2012), whereas *Sclerophylax*, which diverged earlier, has two or three sites. Since in most of the *Lycium* species studied so far the two rDNA loci are asyntenic, it is presumed that this would be the ancestral state, and during the last 10 million years the rearrangement that led to the synteny in *N. divaricata* would have occurred.

Moreover, it is noticeable that *S. adnatifolia* has recently separated (± 4 mya) from the other species of the genus (Särkinen et al. 2013) suggesting that the change in number and position of the 5S rDNA loci is relatively fast, and the times of variation of this locus are different from those of the 18-5.8-26S locus, which would be more stable. A similar situation, where the two rDNA loci vary differentially, has been described in *Aloe* L. (Asphodelaceae) where the karyotype formula is retained, but in this genus the pattern of the 5S signal is similar between the species and the 18-5.8-26S sites are more variable (Adams et al. 2000).

The data provided by this study (chromosome number, karyotypes, heterochromatin patterns, number and position of rDNA sites) reinforce the position of *Nolana* and *Sclerophylax* within the 'x = 12 clade', but point to a different evolutionary pathway with

respect to their closest relatives, *Lycium*, *Jaborosa* and the Hyoscyameae. The features examined here allow the differentiation of *Nolana*, *Sclerophylax* and *Lycium*; however, studies on a larger number of species (especially in *Nolana*) would be required in order to propose patterns that would definitely separate them from other genera of the Solanaceae. However, all the variables analysed do enable eight species to be singled out clearly.

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