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RESEARCH REPORT

Comparative triple-color FISH mapping in eleven *Senna* species using rDNA and telomeric repeat probes

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Abstract
Senna is a diverse and paraphyletic genus in the subfamily Caesalpinioideae (Fabaceae Lindl.) comprising various species of industrial and medicinal value. To date, the genome-based taxonomic relationship among several *Senna* species remains enigmatic. Cytogenetic information is invaluable in deciphering phylogenetic relationships and evolutionary history. However, insufficient chromosomal research for many *Senna* species impedes comparative cytotaxonomic analyses aimed at understanding their genomic evolution. To provide additional *Senna*-related molecular cytogenetic information, we karyotyped 11 *Senna* species by employing triple-color fluorescence in situ hybridization using 5S rDNA, 45S rDNA, and *Arabidopsis thaliana*-type telomeric pre-labeled oligonucleotide probes. Chromosome numbers were predominantly $2n = 28$, but $2n = 22$ (*S. marilandica*) and $2n = 24$ (*S. uniflora*) were also observed. While most species revealed only one interstitial 5S rDNA locus, except for *S. uniflora* which has two loci, a range of one to three 45S rDNA loci were detected at distal chromosomal regions. Additionally, we observed a hemizygous 45S rDNA locus in *S. auriculata*. In addition to chromosome termini, weak signals for telomeric repeats were found in interstitial regions in *S. hirsuta, S. corymbosa*, and *S. alexandrina*. These cytogenetic data can be integrated with molecular phylogenetic data for more comprehensive *Senna* cytotaxonomic analyses.

Keywords Cytogenetic markers · FISH · Genome · Karyotype · *Senna*

1 Introduction

Senna Mill., a representative genus from the family Fabaceae Lindl. (Resende et al. 2014), comprises approximately 350 morphologically diverse species of herbs, shrubs, and trees (Cordeiro and Felix 2018). *Senna* species are distributed throughout circumtropical regions with an extremely wide range of habitats (Marazzi et al. 2006; Pellerin et al. 2019). They are morphologically distinguished based on their androceu, corolla, floral architecture, bracteole, and fruit characteristics (Marazzi et al. 2006). Many *Senna* species have been recognized for their medicinal and industrial uses such as for treating diverse diseases (e.g., digestive ailments, skin disorders, respiratory illnesses, visual problems, and even heart disease) and producing compounds used in commercial goods, flavoring, perfume, pet food, and coffee (Rahman et al. 2013; Pellerin et al. 2019). Despite their economic and health benefits, a paucity of molecular cytogenetic data has impeded comparative analyses for the evaluation of *Senna* genome evolution. Although a few comparative cytogenetic studies have been reported, only a few species have data based on fluorescence in situ hybridization (FISH) of 5S and 45S rDNA and telomeric repeats (Rosato et al. 2018; Youn and Kim 2018; Pellerin et al. 2019).

Karyotype data can be used to identify species, reveal past genome rearrangements, and infer taxonomic relationships among related species (Guerra 2008; Jo et al. 2019; Chen et al. 2020). A karyotype, which is a genetically stable characteristic unique to a given species, provides the number, shape, size, and morphology of an organism's chromosome complement (Pellerin et al. 2019; Zhou et al. 2019a). Chromosomal rearrangements can alter karyotype features

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resulting in changes in chromosome number (dysploidy) or organization, which often reflect evolutionary events such as speciation (Wölk et al. 2015). Descending dysploidy occurs when chromosomal fusion leads to species with fewer chromosome numbers, whereas ascending dysploidy results from retention of centromere function after a chromosome fission (Winterfeld et al. 2020; Ta et al. 2021; Waminal et al. 2021).

FISH is an invaluable technique in karyotyping (Waminal et al. 2018; Youn and Kim 2018). The 5S rDNA and 45S rDNA sequences are commonly used as FISH probes because they are highly repetitive and widely conserved across taxonomic groups (Pellerin et al. 2018a, b; Waminal et al. 2018; Zhou et al. 2019b). The *Arabidopsis thaliana*-type telomeric repeat $(TTTAGGG)_n$, the canonical plant telomeric repeat most commonly found at chromosome termini, is also widely conserved across taxonomic groups (Watson and Riha 2010; Peska and Garcia 2020). Interspecies divergence in the chromosomal distribution of rDNA and the telomeric repeat sequences provide phylogenetically useful information for analyzing genome dynamics.

While the predominant diploid chromosome number in *Senna* is $2n = 28$ (Rice et al. 2015), descending dysploid karyotypes of $2n = 22–26$ are also commonly observed (Cordeiro and Felix 2018; Pellerin et al. 2019). Published data on chromosome number is lacking in a number of *Senna* species. To broaden the karyotype information in *Senna*, we performed triple-color FISH using rDNA and telomeric repeat sequence probes in 11 *Senna* species. To our knowledge, there are currently no reports of FISH karyotyping using rDNA and telomeric probes in these *Senna* species. This analysis revealed interspecific karyotype variations that provide insight into karyotype dynamics in *Senna*. These preliminary data will also facilitate cytogenetic mapping of major species-specific repeats, improve our understanding of taxonomic relationships and evolutionary history, and provide useful information for future *Senna* genomic research and breeding projects.

2 Materials and methods

2.1 Plant materials and chromosome preparation

Seeds of the 11 *Senna* species were purchased from the National Plant Germplasm System (NPGS, USA) and Rare Palm Seeds (RPS, Germany) (Table 1). Concentrated sulfuric acid (Sigma-Aldrich Co., St. Louis, MO, USA) was used to treat the seeds before germination to break seed dormancy and expedite germination (Baskin et al. 1998). Root tips were collected and pre-treated in 2 mM 8-hydroxyquinoline for 5 h at 18 °C then stored in 70% ethanol at 4 °C until use.

Chromosome preparation was performed according to our published protocol (Waminal and Kim 2012; Peniton et al. 2019) with minor alterations. Briefly, fixed root tips were washed in distilled water and digested in an enzyme solution containing 1% pectolyase Y-23 (Duchefa, Haarlem, The Netherlands) and 2% cellulase R-10 (Phytotechnology Laboratories, USA) for 60–90 min at 37 °C. Chromosomes were then fixed in chilled Carnoy's solution and centrifuged. Supernatants were aspirated, and the precipitates were resuspended in aceto-ethanol (9:1 v/v) and mounted onto slides in a humid chamber. After air drying, slides were soaked in 2% (v/v) formaldehyde fixative (Merck Schuchardt OHG, Hohenbrunn, Germany) for 5 min to preserve the chromosomes, quickly dipped into distilled water, and finally dehydrated in a series of ethanol concentrations (70, 90, and 100%) (Vrána et al. 2012).

Table 1 List of *Senna* species used in this study with their published chromosome information

No.	Species	Seed source	Native range	2*n*	References
1	*Senna alata* (L.) Roxb	NPGS[z]	Argentina, Australia, Belize, Bolivia, Brazil, Caribbean, Ecuador, Mexico	28	Souza and Iseppon (2004)
2	*S. alexandrina* Mill	NPGS	Brazil, Caribbean, Ecuador, India, Mexico	28	Al-Turki et al. (2000)
3	*S. auriculata* L	RPS[y]	India	28	Ohri et al. (1986)
4	*S. corymbosa* (Lam) H.S Irwin & Barneby	NPGS	Argentina, Brazil, United States, Uruguay	28	Irwin and Turner (1960)
5	*S. hirsuta* var. *leptocarpa* (Benth.)	NPGS	Brazil, El Salvador	28	Irwin and Turner (1960)
6	*S. lindheimeriana* (Scheele)	NPGS	Mexico, United States	28	This study
7	*S. marilandica* (L.) Link	NPGS	United States	22	This study
8	*S. notabilis* (F.Muell) Randell	RPS	Australia	28	Randell (1970)
9	*S. polyphylla* (Jacq) H.S Irwin & Barneby	RPS	Brazil, Caribbean, Guyana, Mexico, United States	28	This study
10	*S. siamea* (Lam.) H.S Irwin & Barneby	RPS	Brazil, Cambodia, Caribbean, Ecuador	28	Souza and Iseppon (2004)
11	*S. uniflora* (Mill.) H.S Irwin & Barneby	RPS	Brazil, Cambodia, Caribbean, Ecuador, Mexico	24	This study

[z]National Plant Germplasm System (NPGS, USA), [y]RPS = Rare Palm Seed Company (RPS, Germany)

2.2 Fluorescence in situ hybridization (FISH)

FISH was performed according to Waminal and Kim (2012) with some modifications. Pre-labeled oligoprobes (PLOPs) for 5S rDNA, 45S rDNA, and *Arabidopsis*-type telomeric sequences are described in Waminal et al. (2018). Hybridization solutions consisted of 50% formamide, 10% dextran sulfate, 2 × saline sodium citrate buffer (SSC), 50 ng/μL of each PLOP, and nuclease-free water to a total volume of 40 μL. Slides were denatured at 80 °C for 5 min, then placed in a humid chamber at 37 °C for at least 45 min. Slides were then washed carefully in 2 × SSC and dehydrated in a series of ethanol concentrations (70, 90, and 100%) for 3 min each at room temperature. Finally, chromosomes were counterstained with DAPI premixed in Vectashield antifade solution. Chromosome images were captured using a BX53 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a DFC365 FS CCD camera (Leica Microsystems, Wetzlar, Germany), and analyzed with Cytovision ver. 7.2 software (Leica Microsystems). Images were finalized using Photoshop CS6 (Adobe Inc., San Jose, California, USA).

2.3 Karyotyping

We used at least three metaphase spreads with the best morphology for total chromosome length (TCL, 2*n*) measurements using Image J software ver. 1.51 k (Schneider et al. 2012). Homologous chromosomes were paired and arranged based on rDNA and telomeric repeat FISH signals, chromosome length, and centromere position. Chromosome type was classified according to the criteria of Levan et al. (2009). Karyograms and idiograms were generated using Adobe Photoshop CS6.

3 Results

3.1 Chromosome counts

The 11 species could be grouped into three groups according to their diploid chromosome number, $2n = 22$, 24, and 28 (Table 2). Because $2n = 28$ is considered the predominant chromosome number in *Senna* (Cordeiro and Felix 2018), and was most frequently represented in our data, all species with different 2*n* numbers were regarded as descending dysploidy karyotypes (Winterfeld et al. 2020; Ta et al. 2021; Waminal et al. 2021).

Chromosome morphology, differential intensities, and chromosomal distribution of rDNA and telomeric repeats enabled the identification of homologous chromosomes in the 11 *Senna* species. The total chromosome length (TCL) among these 11 species ranged from 54.16 to 133.2 μm (Table 2). *S. uniflora* had the longest chromosomes, whereas *S. notabilis* had the shortest chromosomes. The average chromosome length (TCL/2*n*) also differed among the species, indicating that cyclic changes in genome size may have occurred during genus diversification (Seijo and Fernández 2003).

Homologous chromosome complements of the 11 species included metacentric, submetacentric, and sub-telocentric

Table 2 Triple-target FISH karyotype analysis of 11 *Senna* species

No.	Species	2*n*	TCL (μm)	TCL/2*n* (μm)	Arm ratio (L/S)	rDNA signals		Telomeric signals	Karyotypic formula
						5S	45S		
1	*S. alata*	28	54.16	1.93	1.20	1[a] (13)[b]	3 (2, 7,11)	+	14 m
2	*S. alexandrina*	28	100.8	3.60	1.50	1 (1)	3 (6, 11, 12)	+ (2)[d]	10 m[x] + 4 sm[y]
3	*S. auriculata*	28	69.62	2.49	1.31	1 (3)	3 (5, 6[c], 7)	+*[e]	13 m + 1sm
4	*S. corymbosa*	28	75.54	2.70	1.92	1 (13)	1 (11)	+ (3)	7 m + 6 sm + 1st[z]
5	*S. lindheimeriana*	28	75.24	2.69	1.68	1 (13)	1 (11)	+	8 m + 5 sm + 1st
6	*S. hirsuta*	28	70.97	2.53	1.41	1 (13)	1 (2)	+ (3)	12 m + 2 sm
7	*S. marilandica*	22	54.70	2.49	1.81	1 (10)	1 (9)	+	6 m + 5 sm
8	*S. notabilis*	28	48.04	1.72	1.60	1 (13)	3 (2, 4, 5)	+	8 m + 6 sm
9	*S. polyphylla*	28	59.10	2.11	1.93	1 (13)	3 (2, 5, 7)	+	4 m + 10 sm
10	*S. siamea*	28	74.15	2.65	1.84	1 (4)	3 (2, 5, 6)	+	7 m + 6 sm + 1st
11	*S.uniflora*	24	133.2	5.55	1.98	2 (7, 9)	2 (11, 12)	+	4 m + 8 sm

[a]Number of signals in a haploid chromosome set

[b]Chromosomes bearing rDNA signals

[c]Hemizygous locus

[d]Numbers in parentheses denote number of chromosomes with interstitial telomeric repeats (ITRs)

[e]Extremely weak signals

[x]m = metacentric, [y]sm = submetacentric, [z]st = subtelocentric

chromosomes. Only *S. alata* had all metacentric chromosome pairs. Other species included metacentric and submetacentric chromosomes. In addition, *S. siamea, S. corymbosa,* and *S. lindheimeriana* had one pair each of sub-telocentric chromosomes (Table 2, Fig. 3).

3.2 Chromosomal distribution of rDNA and telomeric probes

The rDNA probes displayed varied chromosomal distributions and signal intensities across the 11 *Senna* species (Fig. 1). All species presented a single 5S rDNA locus, except for *S. uniflora*, which possessed two loci (Fig. 2). The 5S rDNA signals were frequently detected in the penultimate chromosome number and were generally localized to proximal chromosome regions (Figs. 2 and 3). The number and intensity of 45S rDNA signals varied considerably among the species. Three pairs were detected in *S. alata, S. alexandrina, S. auriculata, S. notabilis, S. polyphylla,* and *S. siamea*. Two pairs were found in *S. uniflora*, and one pair each was found in *S. corymbosa, S. hirsuta* var. *leptocarpa, S. lindheimeriana,* and *S. marilandica*. Most of these signals were found in the terminal regions of the short arms of the respective chromosomes. A hemizygous 45S rDNA locus was observed in chromosome 6 of *S. auriculata* (Fig. 2), and we did not observe any juxtaposition between 5S rDNA and 45S rDNA signals in any species.

The *Arabidopsis*-type telomeric repeat hybridized to the terminal regions of all chromosomes in all species (Table 2, Figs. 1, 2, and 3). In addition, some chromosomes also displayed weak interstitial telomeric repeat (ITR) signals in peri-centromeric regions in *S. alexandrina, S. corymbosa,* and *S. hirsuta* (Fig. 2). A pair of ITR signals was detected on chromosome 1 in all three species. The remaining pairs were localized to chromosome 12 in *S. alexandrina*, 2 and 6 in *S. corymbosa*, and 9 and 12 in *S. hirsuta* (Fig. 2).

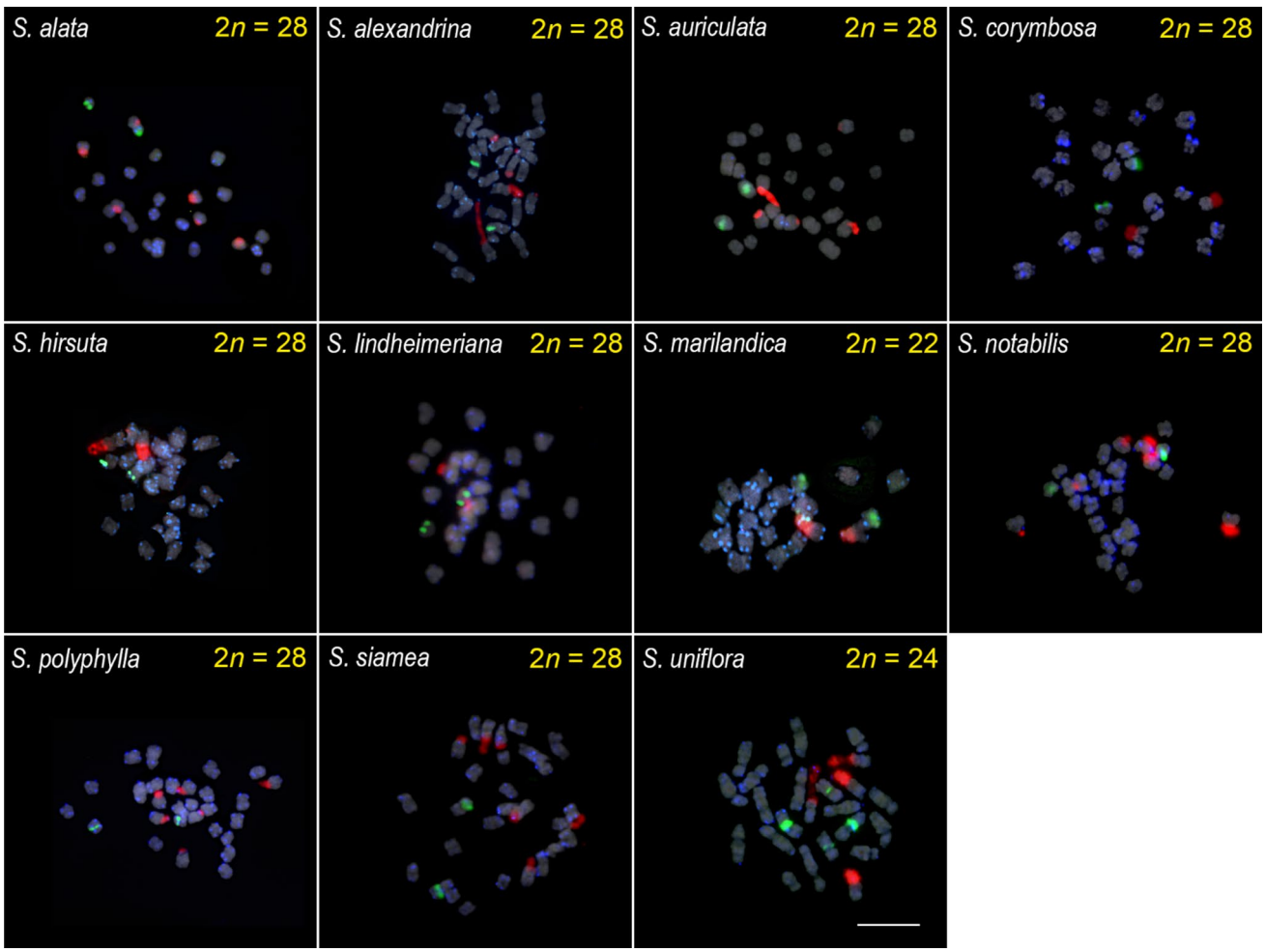

Fig. 1 Triple-color FISH images of 11 *Senna* species. The predominant chromosome number was $2n = 28$. The exceptions were *S. marilandica* and *S. uniflora* which have $2n = 22$ and $2n = 24$, respectively. One pair of 5S rDNA (green) and one to three pairs of 45S rDNA (red) were detected. Scale bar = 10 μm

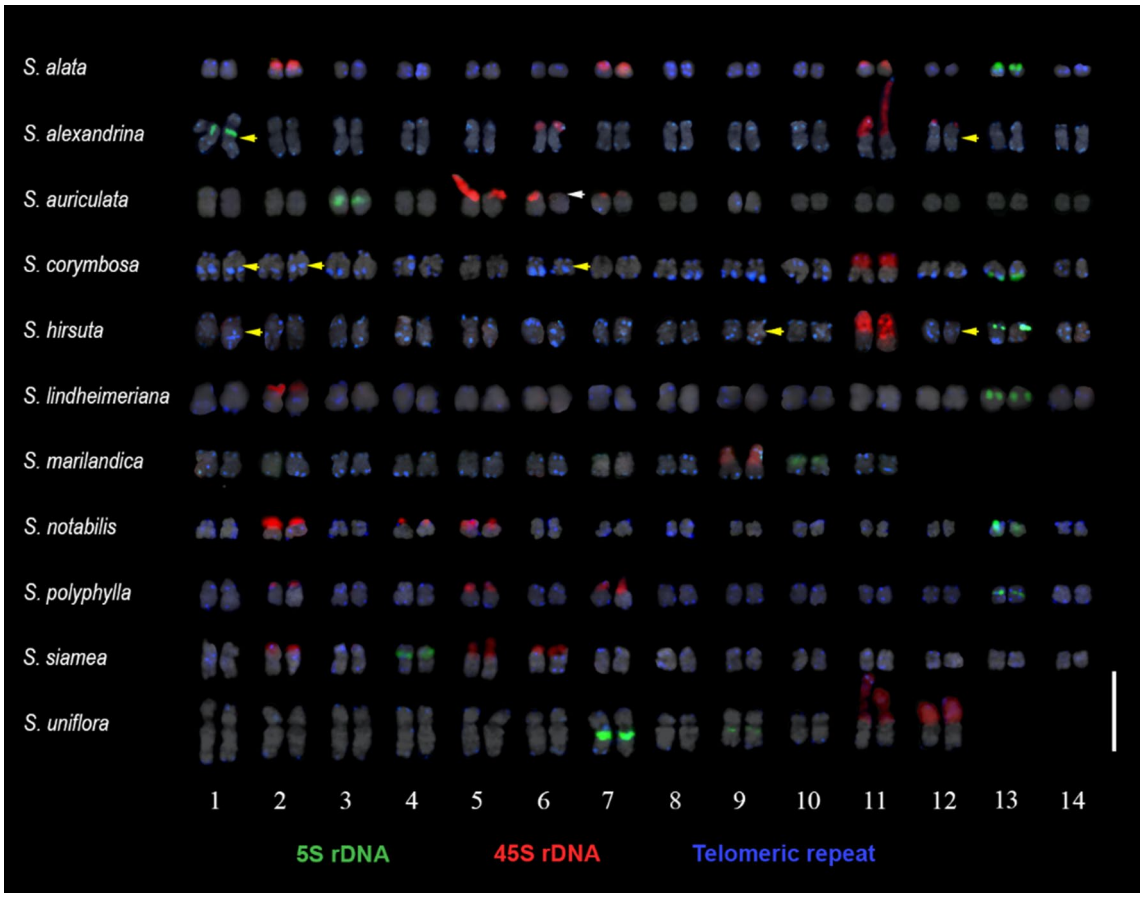

Fig. 2 FISH karyograms of the 11 *Senna* species. Green, red, and blue signals indicate 5S rDNA, 45S rDNA, and telomeric repeats, respectively. Yellow arrowheads point to ITR signals. The white arrowhead points to the hemizygous 45S rDNA locus in *S. auriculata*. Scale bar = 10 μm

4 Discussion

Data on chromosome number and FISH-based karyotype in *Senna* are relatively scarce (Cordeiro and Felix 2018). In our previous work, we presented FISH karyotypes using 5S and 45S rDNA and telomeric repeats in 12 *Senna* species, including *S. tora* whose genome has been recently sequenced (Youn and Kim 2018; Pellerin et al. 2019; Kang et al. 2020; Ta et al. 2021; Waminal et al. 2021). To complement previous data and improve our understanding of karyotype diversity in *Senna*, we further analyzed the karyotypes of 11 additional *Senna* species. To our knowledge, this is the first report on the chromosome numbers of *S. lindheimeriana, S. marilandica, S. polyphylla*, and *S. uniflora.*

Most *Senna* species investigated in this study were diploid, with a predominant chromosome number of $2n = 28$, corresponding with previous reports (Souza and Iseppon 2004; Cordeiro and Felix 2018). However, *S. marilandica* ($2n = 22$) and *S. uniflora* ($2n = 24$) showed descending dysploid karyotypes, which may have resulted from post-polyploidy dysploidization after a polyploidization of ancient karyotypes with $2n = 14$ (Biondo et al. 2012; Shchapova 2013; Winterfeld et al. 2020). Similar processes have also occurred in several other plants such as *Brassica*, *Cucumis*, *Nothoscordum*, *Brachyscome*, and *Senna tora* (Maluszynska and Heslop-Harrison 1993; Watanabe et al. 1995; Koo et al. 2010; Pellerin et al. 2019; Waminal et al. 2021). Indeed, changes in chromosome count may have played a role in the occurrence of reproductive isolation and speciation in *Senna* (Freyman and Höhna 2018).

Using FISH, we observed interspecific differences in the signal patterns of our markers, indicating species specificity and the usefulness of our probes in distinguishing each species. A hemizygous 45S rDNA pattern similar to that observed in the short arm of chromosome 6 in *S. corymbosa* has been observed in other *Senna* and non-*Senna* species (Lan and Albert 2011; Mancia et al. 2015; Waminal et al. 2016; Pellerin et al. 2019). This hemizygous locus may be explained by homology-mediated unequal crossing over between non-allelic homologous repeat units, which significantly shortened one site, making it undetectable by FISH (Pellerin et al. 2019).

Most species displayed a single locus proximal distribution of 5S rDNA, except for *S. uniflora*, which showed two

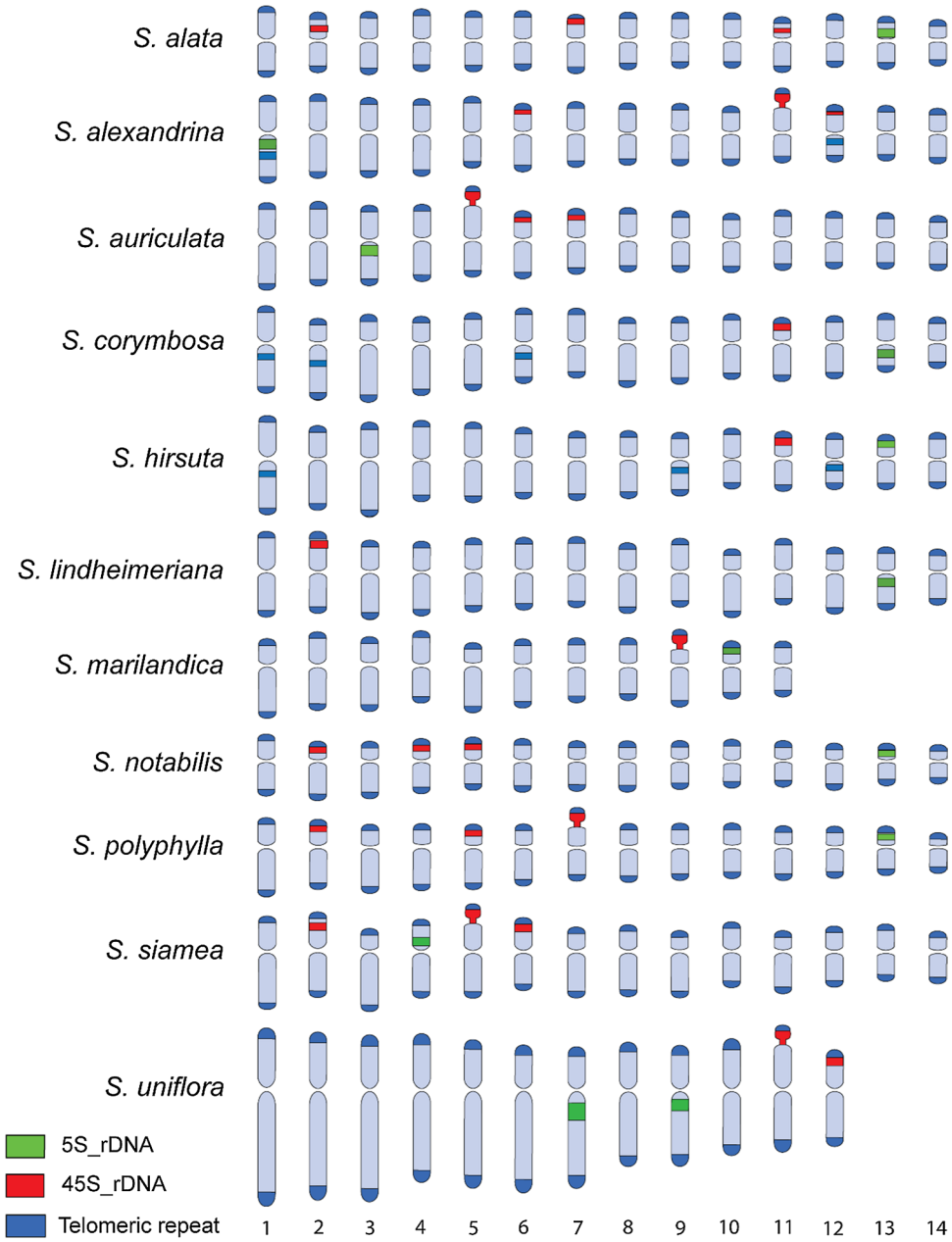

Fig. 3 Idiogram of the 11 *Senna* species. Red, green, and blue bars represent 45S rDNA, 5S rDNA, and telomere repeat, respectively

loci. These results corroborate the reduced copy number and interstitial distribution of 5S rDNA often observed in flowering plants (Roa and Guerra 2012, 2015). Our results also revealed that 5S and 45S rDNA are not linked in the same chromosomal region; thus, genomic rearrangements by conversion and crossing-over should occur with greater frequency (Waminal and Kim 2012). Independent localization suggests that 5S and 45S rDNA experienced distinct evolutionary processes (Mantovani et al. 2005). Variations in the distribution pattern of rDNA repeats in groups of related species have been explained via structural rearrangement events such as translocations, inversions, duplications, and deletions. All of these events commonly result in structural changes in the karyotype (Silvestri et al. 2020).

Although telomeric sequences are normally located at chromosomal termini (Fuchs et al. 1995), some ITRs were detected in either three or two chromosome pairs in *S. hirsuta, S. corymbosa,* and *S. alexandrina* (Fig. 2). ITRs have been observed in a few chromosomes in several *Senna* species, especially in *S. tora*, where they are extensively amplified in all chromosomes (Pellerin et al. 2019). ITR signals have also been discovered in animals and some other plant species (Uchida et al. 2002; He et al. 2013; Souza et al. 2016). ITR size, number, and distribution could vary inter- or intra-specifically. Although the origin and evolution of

ITRs remain largely unexplored in plants, some proposed mechanisms to explain ITR formation include unequal gene conversion, chromosomal fusion, crossing-over, DNA replication, transposition of telomeric repeats by mobile elements, or the translocation of an ITR during genetic recombination (He et al. 2013; Aksenova and Mirkin 2019). The ITRs observed in *Senna* species suggest that telomere-mediated inter-chromosomal rearrangements are a major pathway in the evolutionary dynamics of most *Senna* species (Sousa et al. 2014). This observation is supported by the high frequency of descending dysploids in *Senna*, as dysploidy often arises from inter-chromosomal rearrangements including end-to-end translocations and nested chromosome insertions (Winterfeld et al. 2020; Ta et al. 2021; Waminal et al. 2021). Recent studies have shown that ITRs are dynamic elements that play essential roles in telomere maintenance and the regulation of gene expression through interactions with telomeres (Ruiz-Herrera et al. 2008; Aksenova and Mirkin 2019).

If these ITRs are formed by chromosomal fusion with reciprocal translocation, the product of this translocation would be a submetacentric chromosome with a weakly detectible ITR, plus a single chromosome and a small fragment (Schubert and Lysak 2011). However, we did not find such small chromosomes in *Senna*. Another mechanism, called a fusion–fission cycle or a Robertsonian rearrangement, has been used to explain ITRs in other plants (Schubert et al. 1995). With these mechanisms, both centromeric and telomeric sequences are retained, although one of the centromeres and the interstitial telomeric sequences must be inactivated for proper mitosis (Sousa et al. 2014).

We observed that *S. corymbosa* and *S. hirsuta* had similar numbers and distributions of 5S rDNA, 45S rDNA, telomeric repeats, and even ITR signals (Figs. 2 and 3). This similarity was also observed in *S. occidentalis* (Pellerin et al. 2019; Ta et al. 2021), suggesting a closer relationship between *S. hirsuta*, *S. corymbosa*, and *S. occidentalis*. Based on FISH signal similarity, we speculate that *S. alata*, *S. alexandrina*, *S. auriculata*, *S. notabilis*, *S. polyphylla*, and *S. siamea* are closely related, whereas *S. corymbosa* is closely related to *S. hirsuta* and *S. lindheimeriana* in another clade. Molecular phylogenomic data will further clarify these relationships.

5 Conclusion

FISH karyotypes of 11 *Senna* species were established using three-color probes targeting 5S rDNA, 45S rDNA, and telomeric repeat sequences. The interspecific karyotypic variation in the species studied constitutes useful data for identifying each species and elucidating interspecific relationships in *Senna*. FISH karyotype analysis using major species-specific repeats as probes, and phylogenomic analyses using chloroplast genomes may provide a clearer picture of the genome dynamics in *Senna*. The determination of highly abundant repeats using next-generation sequencing data and application of such markers in more *Senna* species are essential for further studies.

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Declarations

Conflict of interests The authors declare that they have no conflicts of interest.

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