

# FISH Mapping of Four *Senna* Species Revealing Genome Dynamics during Species Diversification of the Genus

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## Abstract

Ploidy changes and chromosomal rearrangements significantly influence the chromosomal structure, which is a vital driver of speciation. The number and structure of the chromosomal composition of *Senna* species has been reported to vary in several cytogenetic studies. Here, we report chromosome number and structure variations among four *Senna* species through the fluorescence *in situ* hybridization (FISH) technique, utilizing ribosomal DNA (5S, 45S rDNAs) and telomeric repeat probes. Four distinct chromosome numbers,  $2n = 26, 28, 48,$  and  $56,$  were observed in *S. obtusifolia*, *S. pallida*, *S. aphylla*, and *S. artemisioides*, respectively, with basic chromosome numbers of  $x = 12, 13,$  and  $14,$  likely corresponding to two diploid species (*S. obtusifolia*, *S. pallida*) and two tetraploid species (*S. aphylla*, *S. artemisioides*). The 5S rDNA signals varied in terms of both the number and position, and the 45S rDNA signals showed greater variation in the numbers, which ranged from two to eight. *S. obtusifolia* was distinct from the other three species in that its telomeric repeat positions varied considerably. While *S. artemisioides*, *S. pallida*, and *S. aphylla* exhibited telomeric signals primarily in the terminal regions, *S. obtusifolia* displayed these signals in the terminal, interstitial, and pericentromeric regions. In addition, interstitial telomeric repeat signals were observed in *S. pallida*. Our study demonstrates the first use of triple-color FISH to unravel molecular cytogenetic information in these *Senna* species, providing evidence of the extensive chromosomal rearrangement that occurred during the diversification of the genus *Senna*.

**Additional key words:** chromosome evolution, diploidy, dysploidy, interstitial telomeric repeat (ITR), repeat marker, tetraploid

## Introduction

Chromosomal modifications involving alterations in the number and structure of chromosomes are fundamental biological processes that often accompany speciation events; signal the occurrence of genetic diversification; and contribute to the cytological basis of ecological differentiation, adaptation, and reproductive isolation (Madlung, 2013; De Storme and Mason, 2014). The genus *Senna*, belonging to the subfamily Caesalpinaceae, provides a pertinent setting for investigating these processes. Encompassing approximately 350 herb, shrub, and tree species, some of which

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exhibit pharmacological activities and some having found commercial use (Eldemerdash et al., 2022), the *Senna* genus displays not only broad morphological diversity, including variations in the leaf size and spatial distribution (Oladeji et al., 2021), but also distinct floral morphologies and extrafloral nectaries (EFNs) (Marazzi et al., 2006). The diverse characteristics of *Senna* plants have motivated cytologists to explore their potential.

The genus *Senna* displays remarkable variability in its chromosomal number. Previous studies have reported variations in the chromosome number in *Senna* species, including  $2n = 24, 26, 28, 52,$  and  $56$  (Irwin and Turner, 1960; Rice et al., 2015; Nguyen et al., 2021). Polyploidy has been observed in *S. rugosa* with  $2n = 42, 56,$  and  $112$  (Resende et al., 2014), whereas descending dysploidy has been exemplified by different basic chromosome numbers ranging from  $x = 11$  to  $x = 14$  (Souza and Benko-Iseppon, 2004). In addition, the series *Aphyllae* exhibited three cytotypes,  $2n = 24, 48,$  and  $60$ , in *S. aphylla*, whereas *S. pachyrrhiza* displayed two cytotypes,  $2n = 24$  and  $48$  (Robbiati et al., 2022). The most common diploid chromosome number in *Senna* species is  $2n = 28$ , with a basic chromosome number of  $x = 14$  (Pellerin et al., 2019).

Alterations in repetitive DNA can lead to chromosomal rearrangements and subsequent changes in repetitive DNA through concerted evolutionary mechanisms (Louzada et al., 2020). Chromosomal markers, specifically the mobility of rDNA clusters, provide insight into microevolutionary genomic changes (Raskina et al., 2004). The 5S and 45S rDNA repeat sequences encode ribosomal RNAs essential for ribosomal function and protein synthesis, whereas telomeric repeats can reveal the evolutionary aspects of specific plant species (Waminal et al., 2018). Fluorescence *in situ* hybridization (FISH) is a valuable technique for deciphering genome structures, compositions, and chromosomal evolution, particularly in closely related species (Waminal et al., 2021).

FISH mapping studies have visualized the chromosomal structures of twenty-three *Senna* species using 5S and 45S rDNAs and telomeric repeat probes (Youn and Kim, 2018; Pellerin et al., 2019; Nguyen et al., 2021). Nine *S. tora* repeat-specific pre-labelled oligo probes (PLOPs) were designed and used to observe the chromosomal distribution among the nine *Senna* species (Ta et al., 2021; Waminal et al., 2021). However, all of these studies focused on diploid *Senna* species. Although Robbiati et al. (2022) undertook the FISH mapping of the tetraploid *S. crassiramea* species, their findings did not clearly elucidate the variation in both the chromosome number and structure.

FISH was performed using ribosomal DNA (5S and 45S rDNAs) and telomeric repeat probes to compare the chromosomal compositions and structures of four *Senna* species: *S. artemisioides*, *S. pallida*, *S. obtusifolia*, and *S. aphylla*. The results demonstrate the first utilization of triple-color FISH to reveal variations in both the chromosome number and structure in these four species, offering an important reference for understanding chromosomal evolution in the genus *Senna*.

## Materials and Methods

### Plant Samples

Accession numbers of seeds of *S. artemisioides* (DLEG 910206), *S. pallida* (DLEG 920148), *S. obtusifolia* (DLEG 910152) and *S. aphylla* (DLEG 910486) were obtained from the National Plant Germplasm System of the United States Department of Agriculture (USDA). Based on accession numbers, these seeds were collected from different regions,

specifically Australia, the United States, Peru, and Argentina (Suppl. Table 1). Following a method in Baskin et al. (1998), the seeds were subjected to a 60 min treatment with sulfuric acid (Sigma-Aldrich, MO, USA) to overcome dormancy, followed by germination in a Petri dish at 25°C. Root tips roughly 2 cm long were initially gathered, treated with 2 mM 8-hydroxyquinoline for 4 h at 18°C, and fixed with a 1:3 solution of glacial acetic acid and absolute ethanol for 24 h. The tips were soaked in 70% ethanol at 4°C for preservation.

### Chromosome Spread Preparation

Chromosomal spreads were prepared as described by Waminal et al. (2012). Meristematic root tips (~2 mm) were collected and then digested by treating them with a 1% concentration of Pectolyase Y-23 (Duchefa Biochemie, Haarlem, Netherlands) and Cellulase R-10 at a concentration of 2% (Duchefa Biochemie) at 37°C for 60 min. After the enzymatic treatment, a 1:3 solution of glacial acetic acid and absolute ethanol was used to re-suspend the pellets, and this was centrifuged at  $10,770 \times g$  and at room temperature 20–25°C (RT) for 3 min. The resulting pellet was re-suspended in a 9:1 solution of glacial acetic acid and absolute ethanol. A pipette was used to spread the pellets on pre-warmed glass slides in a humid chamber at 80°C, followed by air drying.

### FISH

The universal pre-labeled oligomer probes (PLOPs) for the 5S rDNA, 45S rDNA, and *Arabidopsis*-type telomeric repeat (TTTAGGG)<sub>n</sub> developed by Waminal et al. (2018) were used for FISH. PLOPs, typically 20–30 nucleotides long, have a specific design that ensures complementarity with a targeted sequence. This design enhances specificity, thereby augmenting the cost-effectiveness and time-efficiency of a FISH analysis (Waminal et al., 2018).

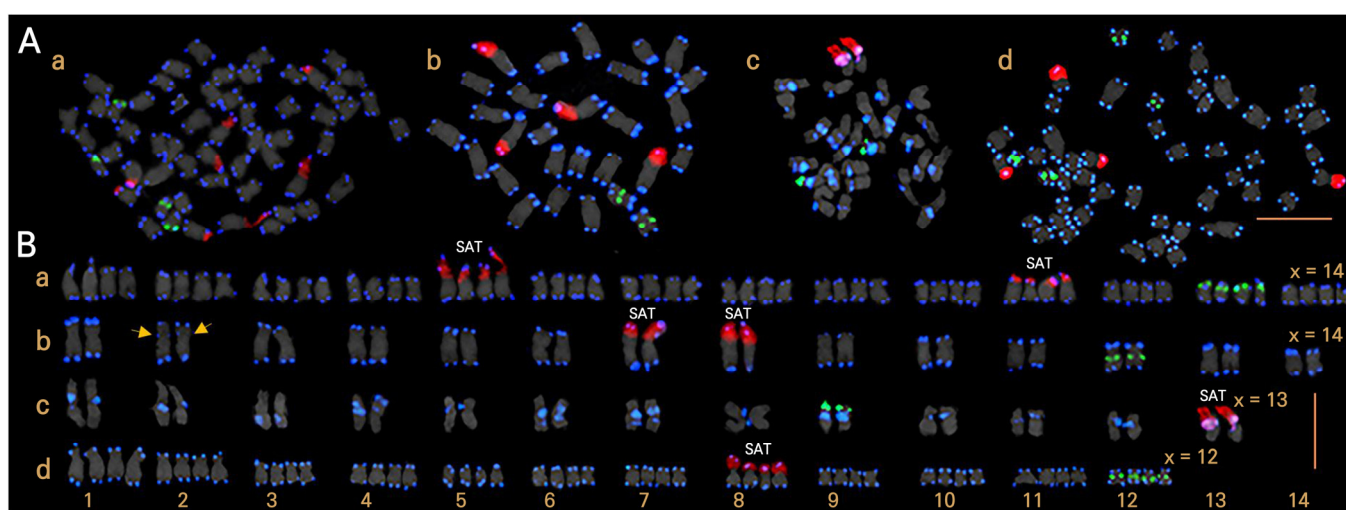
A hybridization mixture (40 µL) comprising a 50% formamide solution, a 10% solution of dextran sulfate, 2× saline sodium citrate (SSC), at Sigma water at 50 nanograms per microliter for each probe was applied to each slide. The denaturation process was carried out by exposing the slides to a temperature of 80°C for 5 min and incubating them overnight in a moist chamber at RT. Following hybridization, a series of wash steps was conducted on the slides with 2× SSC at RT for 10 min, 0.1× SSC buffer for 25 min at 42°C, and 2× SSC buffer at RT for 5 min, after which they were dehydrated by immersion in a series of ethanol solutions with increasing concentrations of 70%, 90%, and 100% for 3 min each under shaking. Next, 40 µL of a Vectashield (Vector Laboratories, CA, USA)-based solution that contained 4',6-diamidino-2-phenylindole (DAPI) was used on each slide. A BX53 fluorescence microscope (Olympus, Tokyo, Japan) coupled with a DFC365 FS CCD camera (Leica Microsystems, Wetzlar, Germany) and Cytovision ver. 7.2 software (Leica Microsystems) was used to observe and capture the chromosomal spreads.

The best mitotic metaphase spread for each species was selected and analyzed for the chromosome length and type. Chromosome lengths were measured using ImageJ software ver. 1.53 k (Wayne Rasband and contributors, National Institutes of Health, MD, USA), and the images were edited using Photoshop CS6 (Adobe Inc., San Jose, CA, USA). Chromosome pairing was performed based on the ribosomal DNA and telomeric signals, the chromosome length, and the centromere position according to Nguyen et al. (2021).

## Results

### Chromosome Numbers, Length, and Type

The four *Senna* species *S. obtusifolia*, *S. pallida*, *S. aphylla*, and *S. artemisioides* exhibited distinct chromosomal numbers ( $2n = 26, 28, 48,$  and  $56,$  respectively; Fig. 1A and Table 1). The basic chromosome number  $x = 14$  corresponded to the tetraploid *S. artemisioides* ( $2n = 4x = 56$ ) and diploid *S. pallida* ( $2n = 2x = 28$ ), whereas  $x = 13$  and  $x = 12$  corresponded to the diploid *S. obtusifolia* ( $2n = 2x = 26$ ) and tetraploid *S. aphylla* ( $2n = 4x = 48$ ), respectively. Distinct satellite (SAT) chromosomes were also identified in specific chromosomes (Fig. 1B). Eight SAT chromosomes were observed on chromosomes 5 and 11 in *S. artemisioides*, whereas *S. pallida* displayed four on chromosomes 7 and 8, and *S. aphylla* exhibited four on chromosome 8. *S. obtusifolia* possesses two SAT chromosomes on chromosome 13.



**Fig. 1.** FISH analysis demonstrating the metaphase chromosome spreads (A) and karyograms (B) of the four *Senna* species *S. artemisioides* (a), *S. pallida* (b), *S. obtusifolia* (c), and *S. aphylla* (d). Satellite (SAT) chromosomes and ITR signals (yellow arrows) were observed. The red, green, and blue signals indicate 45S rDNA, 5S rDNA, and telomeric repeats, respectively. The scale bar is 5  $\mu\text{m}$ .

**Table 1.** FISH Karyotype Analyses of the Mitotic Metaphase Chromosomes of Four *Senna* species

Species	Chr. no.		Ploid	Chr. length ( $\mu\text{m}$ )	Karyotypic formula <sup>f</sup>	rDNA signals		Telomeric signals
	2n	x				5S	45S	
<i>S. artemisioides</i>	56	14	tetraploid	1.10–2.83	11 m <sup>z</sup> + 3 sm <sup>y</sup>	4 <sup>x</sup> (13) <sup>w</sup>	8 (5, 11)	Tel <sup>v</sup>
<i>S. pallida</i>	28	14	diploid	1.72–2.85	9 m + 5 sm	2 (12)	4 (7, 8)	Tel, ITR <sup>u</sup>
<i>S. obtusifolia</i>	26	13	diploid	1.33–3.20	13 m	2 (9)	2 (13)	Tel, IRs <sup>t</sup> , pCen <sup>s</sup>
<i>S. aphylla</i>	48	12	tetraploid	0.91–2.68	10 m + 2 sm	4 (12)	4 (8)	Tel

<sup>z</sup> Metacentric.

<sup>y</sup> Submetacentric.

<sup>x</sup> Number of signals.

<sup>w</sup> Chromosome (s) bearing the signal (s).

<sup>v</sup> Tel: telomeric region.

<sup>u</sup> ITR: interstitial telomeric repeat.

<sup>t</sup> IR: interstitial region.

<sup>s</sup> pCen: pericentromere.

<sup>f</sup> Karyotype assigned according to a method previously reported by Levan et al. (1964).

The ranges of chromosome lengths among the four *Senna* species are shown in Table 1. The diploid *S. obtusifolia* exhibited the longest chromosome length, measuring 3.20  $\mu\text{m}$ , whereas the tetraploid *S. aphylla* displayed the shortest length at 0.91  $\mu\text{m}$ . In *S. aphylla*, the chromosome lengths ranged from 0.91  $\mu\text{m}$  to 2.68  $\mu\text{m}$ , whereas *S. obtusifolia* exhibited lengths varying from 1.33  $\mu\text{m}$  to 3.20  $\mu\text{m}$ . The chromosome length range for *S. artemisioides* was 1.10  $\mu\text{m}$  to 2.83  $\mu\text{m}$ , whereas *S. pallida* displayed a range of 1.72  $\mu\text{m}$  to 2.85  $\mu\text{m}$  (Suppl. Table 2 and Suppl. Table 3). Based on arm ratios, *S. obtusifolia* exclusively presented with metacentric chromosomes (Suppl. Table 4). In addition, *S. artemisioides* exhibited 11 metacentric and three submetacentric chromosomes, *S. pallida* displayed nine metacentric and five submetacentric chromosomes, and *S. aphylla* possessed ten metacentric and two submetacentric chromosomes.

### Chromosomal Location of Ribosomal DNA and Telomeric Repeat Sequences

The FISH analysis revealed notable variations in the number and position of rDNA and the telomeric signals (Fig. 1). The number of 5S rDNA signals exhibited relatively consistent patterns across the diploid species (*S. pallida* and *S. obtusifolia*), with two signals on each pair of chromosomes, whereas the tetraploid species (*S. artemisioides* and *S. aphylla*) consistently exhibited four signals. However, substantial discrepancies were observed at specific locations of the signals. In *S. pallida* and *S. aphylla*, the 5S rDNA signals were localized in the paracentromeric or centromeric regions of the short arm of chromosome 12, whereas *S. artemisioides* (chromosome 13) and *S. obtusifolia* (chromosome 9) showed these signals in the subtelomeric regions of the short arm.

Significant variations were observed in the number of 45S rDNA signals across the four *Senna* species, ranging from two to eight. These signals were consistently detected in the nucleolus organizer region (NOR) of the short arm. A minimal number of signals, specifically two, were localized in the NOR of the short arm on chromosome 13 of *S. obtusifolia*. In contrast, the tetraploid *S. artemisioides* demonstrated the maximum occurrence of eight signals which were distributed within the NOR of the short arms on chromosomes 5 and 11. *S. pallida* displayed four signals that were split between chromosomes 7 and 8, which were positioned in the NOR of the short arm. Similarly, *S. aphylla* showed four signals on chromosome 8, located in the NOR of the short arm.

Notably, significant disparities in telomeric location were particularly prominent in *S. obtusifolia*, setting it apart from the other three species. In *S. artemisioides*, *S. pallida*, and *S. aphylla*, telomeric signals were primarily observed in the terminal regions of all chromosomes, whereas *S. obtusifolia* displayed telomeric signals not only in the terminal regions, but also in the interstitial and pericentromeric regions. Specifically, chromosome 4 exhibited a solitary terminal telomeric signal, whereas the remaining chromosomes predominantly showcased telomeric signals in the interstitial and pericentromeric regions. Furthermore, faint interstitial telomeric repeat (ITR) signals were discernible on chromosome 2 of *S. pallida*.

### Discussion

The chromosomal variation, encompassing dysploidy and polyploidy with chromosome numbers of  $2n = 26, 28, 48,$  and  $56$ , observed in the four *Senna* species investigated here was consistent with previous reports (Souza and Benko-Iseppon, 2004; Rice et al., 2015; Robbiati et al., 2022). However, our study revealed an intriguing correlation

between the chromosomal variation in this case and rearrangements of ribosomal DNA and telomeric repeat sites in *Senna*.

Dysploidy refers to the gradual change in the haploid chromosome count observed in closely related species, leading to the formation of a dysploid series (Guerra, 2008). Among the cytogenetic studies of *Senna*, the most frequently observed basic chromosome number was  $x = 14$ . The primary basic number,  $x = 7$ , which was presumed to be the ancestral basic number, established a secondary basic chromosome number,  $x = 14$  (Irwin and Turner, 1960). Earlier work by Souza and Benko-Iseppon (2004) indicated that the basic chromosome number series, i.e.,  $x = 11, 12, 13$ , and  $14$ , demonstrated a dysploid tendency.

Descending dysploidy mechanisms can result in ITR, which can serve as cytological markers of chromosomal rearrangements, including chromosomal insertions (NCIs) and end-to-end translocations (EETs) (Waminal et al., 2021). ITR signals were frequently present in some diploid *Senna* species, with  $x = 14$ . We observed the presence of ITR signals in *S. pallida*, and a similar observation was made by Nguyen et al. (2021) in *S. alexandrina*, *S. corymbosa*, and *S. hirsuta*, which supports the notion that  $x = 14$  is a derived number and that other basic numbers, specifically  $x = 11, 12$ , and  $13$ , suggest the occurrence of dysploidy in the *Senna* genus (Waminal et al., 2021).

Polyploidy, the predominant chromosomal variation in plants, involves duplication or multiplication of a complete set of chromosomes. This is the most common chromosomal mechanism driving evolutionary processes in plants (Guerra, 2008). During sexual polyploidization, meiotic restitution or non-reduction results in unreduced or  $2n$  gametes, respectively, and tetraploid individuals are born by the fusion between two diploid gametes (Brownfield and Köhler, 2011). Tetraploid cytotypes exhibit twice the chromosomal complement size of diploid cytotypes (Robbiati et al., 2022). In the genus *Senna*, based on 5S rDNA signals, this may reflect the ploidy state. Two 5S rDNA signals were detected in all diploids, whereas tetraploids had four signals. However, an increase in 5S rDNA signals was observed in two diploid *Senna* species with  $x = 12$ : *S. pachyrrhiza* and *S. uniflora*, each exhibiting three to four chromosomes bearing 5S rDNA signals, (Nguyen et al., 2021; Robbiati et al., 2022).

Furthermore, the variation trend in the 5S rDNA positions among these four species was consistent with a previous study that used three chloroplast regions (rpS16, rpL16, and matK) to establish phylogenetic relationships among ninety-eight *Senna* species and to gain novel insights into the evolution of EFNs and floral symmetry (Marazzi et al., 2006). Among the seven major clades depicted in the phylogenetic tree, *S. artemisioides* and *S. obtusifolia* were grouped into clade IV, whereas *S. aphylla* and *S. pallida* were grouped into clade VI. Clade IV consisted of herbs, shrubs, and treelets that possessed EFNs on the leaves and asymmetric flowers, including the corolla, androecium, and gynoecium. Some species exhibit an oblique floral position, enantiostyly, such as *S. obtusifolia*, and hairs on the anthers of middle and abaxial stamens, which may represent a synapomorphy for clade IV, distinguishing it from other clades. *Senna* species of the series *Interglandulosae*, such as *S. pallida*, are mainly found in moist areas, whereas *Aphyllae* members, such as *S. aphylla*, inhabit dry regions. Some species of the series *Interglandulosae* have up to 14 leaflet pairs, reduced upper petals, and long-beaked anthers. *Aphyllae* species have photosynthetic stems, few leaves, and unscaled upper petals (Irwin and Turner, 1960; Marazzi et al., 2006).

The two diploid *Senna* species (*S. obtusifolia*, *S. tora*) with  $x = 13$  display a distinct feature that sets them apart from other species: the telomeric signal is located in the terminal, interstitial, and pericentromeric regions instead of only in the terminal regions. Altered telomeric repeat positions were observed in *S. obtusifolia* for the first time in this study, and

Pellerin et al. (2019) reported similar observations in *S. tora*. EETs may be responsible for the appearance of telomeric signals within interstitial and pericentromeric regions in *S. tora* were proposed by Waminal et al. (2021). Recent reports have suggested that chromosomal rearrangements (inversions, deletions, duplications, and translocations) are associated with repetitive DNA sequences in heterochromatic regions and heterochromatin-euchromatin borders (Badaeva et al., 2007). Both *S. obtusifolia* and *S. tora* are members of the section *Prosoesperma*, series *Torae* (Irwin and Turner, 1960). Based on biogeographic evidence, Randell (1995) proposed that *S. tora* was derived from *S. obtusifolia*. The FISH analysis revealed that *S. obtusifolia* and *S. tora* exhibit remarkably similar chromosomal numbers, karyotypes, rDNA, and telomeric repeat locations.

The chromosomal structure is strongly affected by ploidy changes and chromosomal rearrangements, which may lead to speciation (Madlung, 2013; De Storme and Mason, 2014). In the genus *Carex*, dysploidy plays a significant role in speciation (Lipnerová et al., 2013). Chromosomal rearrangements directly affect speciation via reproductive isolation. Two models, the ‘suppressed recombination’ and ‘hybrid sterility’ models, propose the role of chromosome rearrangement in speciation (Faria and Navarro, 2010). In the ‘suppressed recombination’ model, the suppression of recombination over inversion regions facilitates the accumulation of locally adapted alleles, aiding genetic differentiation among overlapping populations (Kirkpatrick and Barton, 2006). The ‘hybrid sterility’ model suggests that reduced fertility in individuals heterozygous for chromosomal rearrangements leads to reproductive isolation of overlapping populations (Ayala and Coluzzi, 2005). In this study, four *Senna* species were collected from different countries, revealing variations in the chromosome number and structure. These variations suggest that changes in ploidy and chromosome rearrangements have a significant effect on reproductive isolation, driving adaptation and speciation within the genus *Senna*.

The variation in the chromosome number and structure among the four *Senna* species (*S. artemisioides*, *S. pallida*, *S. obtusifolia*, and *S. aphylla*) was visualized by FISH, focusing on rDNA and telomeric repeat probes in an effort to provide potential referral sources for understanding the role of chromosomal changes in species diversification. Additionally, the cytogenetic data presented in this study could be valuable for breeding studies of *Senna* species. Future studies, including genome size measurements and chromosome painting, will provide further insights into the ploidy state and specific mechanisms underlying genetic rearrangements and will ultimately contribute to a more comprehensive understanding of speciation and evolutionary processes in *Senna* species.

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