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Research paper

Karyotype analysis of *Piptanthus concolor* based on FISH with a oligonucleotides for rDNA 5S

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ABSTRACT

The karyotype of *Piptanthus concolor* was analysed via fluorescence in situ hybridization (FISH) using oligonucleotide probe for ribosomal DNA (rDNA) (5S rDNA, 41 bp). *P. concolor* had 2n = 18 chromosomes. The karyotype was composed of 3 pairs of metacentric chromosomes (including one pair of satellites), 3 pairs of submetacentric chromosomes, and 3 pairs of subtelocentric chromosomes. 5S rDNA loci were detected in 8 chromosome pairs. The density and location of signals for both 5S rDNA varied among individual chromosomes. High-intensity 5S signals were detected in the short arms of chromosomes 7 and 8, and weak signals were also detected in the long arms of the same chromosomes. For the satellite chromosomes, 17 and 18, the 5S rDNA signal was located at the end of the long arm. The third pair of chromosomes, 9 and 10, also significantly differed from other chromosomes with respect to the 5S rDNA signals observed in the subterminal region. The fourth pair of chromosomes, 11 and 12, had no 5S rDNA signal. The other pairs of chromosomes also exhibited individual signals. Our results provide information that may be beneficial for future cytogenetic studies and could contribute to the physical assembly of the *Piptanthus* genome.

1. Introduction

Karyotypes provide important information regarding chromosomal aberrations, cellular function, taxonomic relationships, and prior evolutionary events of plant species because, in contrast to other characteristics, chromosomal information is consistently retained in plants. In particular, for the genus *Piptanthus*, karyotype analysis of the chromosome is fundamental for achieving a classification that accurately reflects evolution (Turner, 1980; Liu and Wang 1996). Karyotype analysis has been widely adopted to clarify species by counting the number, length, arm ratios, and centromere positions of chromosomes during somatic metaphase (Rho et al., 2012; Lu et al., 2011). For the shrub *Zanthoxylum bungeanum*, karyotype analysis has not been possible because the hardness of germinating seeds renders metaphase chromosomes unobtainable. In addition, metaphase chromosomes are smaller in this species than in other plant species (unpublished paper). However, Wang et al. (2015) elucidated the ribosomal DNA (rDNA) distribution pattern of *Rubus* via fluorescence in situ hybridization (FISH) with probes targeting 5S rDNA; this approach may be applicable for shrubs. FISH analyses in which 5S rDNA are used as molecular markers to identify chromosomes have been performed for many woody plants, such as *Hippophae rhamnoides* (Puterova et al., 2017), *Gossypium* species (Gan et al., 2013), and *Pinus* species (Cai et al., 2006).

However, FISH has rarely been used to assess *Piptanthus* species. The genus *Piptanthus* was first proposed by Sweet (1828) to accommodate a single species. *Piptanthus concolor* was described by Craib (1916). For an extended period, chromosome counts had only been reported for one *Piptanthus* species, *Piptanthus nepalensis*, for which 2n = 18 (Federov, 1969). Subsequently, researchers performed karyotype analysis for *Piptanthus nepalensis* (Liu and Wang 1996) and *Piptanthus tomentosus* (Lu et al., 2011). However, most karyotype analyses have consisted of simple idiograms organized by chromosome length and centromere position and have not involved the use of FISH. In addition, karyotype analysis suffers from several technical restrictions with respect to extending beyond gathering chromosome information using conventional staining procedures. Furthermore, it can be difficult to discriminate between chromosomes in karyotypes due to similarities in chromosome shape and high chromosome heterozygosity at the mitotic metaphase stage. Therefore, in this work, we utilized the FISH method to conduct a more accurate karyotype analysis of *P. concolor*.

2. Materials and methods

2.1. Plant materials and chromosome preparation

P. concolor Harrow ex Craib seeds (2n = 2x = 18) collected from

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Xiangcheng County, Sichuan Province, China, were germinated on sterile wet filter paper under controlled temperature and light conditions (14 h light at 25 °C; 10 h dark at 20 °C). When root tips had reached a length of 1.5-2.0 cm, they were excised, fully immersed in nitrous oxide for 4 h and then stored in 70% ethanol (Kato et al., 2004). Root-tip meristem, approximately 1 mm, was cut and treated with cellulose and pectinase and then dropped in suspension onto slides (Komuro et al., 2013). After the slides had air dried, they were examined using an Olympus CX21 microscope (Olympus, Japan) and then stored at −20 °C for further use.

2.2. Probe DNA preparation

The oligonucleotide probe was used for FISH analyses, which was for 5S rDNA and contained the 41 bp fragment 5′ TCAGAACTCCG AAGTTAAGCGTGCTTGGGCGAGAGTAGTAC3′, was developed based on repeat sequences available in the public database GenBank (accession nos. KX359334.1, CP019093.1, HQ270775.2, KU587438.1, AH010627.2). The 5S rDNA oligonucleotide was tested as a novel probe. Oligonucleotide probe was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The synthetic oligonucleotide was 5′ end-labelled with 6-carboxyfluorescein (6-FAM) or 6-carboxyfluorescein (6-TAMRA). The synthesized probe was diluted using 1 × TE solution and maintained at a concentration of 10 μM and then stored at −20 °C.

2.3. FISH and karyotype analysis

FISH with multiple probes was performed as previously described by Hao et al. (2013). The chromosome preparations were fixed with 4% (w/v) paraformaldehyde, washed with 2 × saline sodium citrate (SSC), and dehydrates using an ethanol series before air drying. Deionized formamide (FA; 60 μL) was added to the chromosome preparations, which were then denatured for 2 min at 80 °C and placed in an ethanol series at −20 °C before air drying. A hybridization mixture (10 μL) containing 0.35 μL oligonucleotide, 4.825 μL of 2 × SSC and 4.825 μL of 1 × TE was applied to each chromosome preparation. The preparations were covered with glass coverslips, and the chromosomes and probes hybridized at 37 °C in a humidity chamber for 1–2 h. The preparations were then rinsed twice for 5 min with 2 × SSC at room temperature and finally with dd H_2O. the air-dried chromosomes preparations were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, USA). Slides were examined using an Olympus BX-51 microscope coupled to a Photometric SenSys Olympus DP70 CCD camera (Olympus, Japan). Raw images were processed in Photoshop version 7.1 (Adobe Systems Incorporated, San Jose, CA, USA) using only functions that affect the entire image equally. Karyotype analysis was performed using NucType version 1.5. Karyotype idiograms were constructed using Excel 2013 based on the relative chromosome lengths.

Chromosome nomenclature was determined using length, from longest chromosome (#1) to shortest chromosome (#18). The arm ratio of each chromosome was calculated (over 3 metaphases) by dividing the length of the long arm by the length of the short arm. The forms of chromosomes were classified as indicated in Table 1 based on the classification method used by Levan et al. (1964) and Li and Chen (1985). The chromosome ratio was calculated by dividing the length of the longest chromosome by the length of the shortest chromosome. The cytotype was determined using the chromosome ratio and the percentage of chromosomes with an arm ratio greater than 2:1 (Stebbins 1971) (Table 2).

Table 1
The forms of chromosomes, as determined using the arm ratio.

Arm ratio	Location of centromere	Abbreviation
1.00	median point	M
1.01–1.70	median region	m
1.71–3.00	submedian region	sm
3.01–7.00	subterminal region	st
> 7.00	terminal region	t
∞	terminal point	T

Table 2
The cytotype, as determined based on chromosome ratio and the percentage of chromosome s with an arm ratio greater than 2:1.

Chromosome ratio	Percentage of chromosome whose arm ratio is greater than 2:1			
	0.00	0.01–0.50	0.51–0.99	1.00
< 2:1	1A	2A	3A	4A
2:1–4:1	1B	2B	3B	4B
> 4:1	1C	2C	3C	4C

3. Results

3.1. FISH with 5S rDNA probe

Images of mitotic metaphase plates of *P. concolor* visualized via FISH are presented in Fig. 1. Three to five additional replications (not shown) produced results consistent with those reported here with respect to chromosome number and the numbers and locations of 5S rDNA signals. In agreement with previous chromosome counts for *Piptanthus* species, eighteen chromosomes were observed in *P. concolor*. Two satellite chromosomes were labelled by 5S (white arrows). Two chromosomes displayed four 5S-labelled signals (yellow arrows), including two extremely intense signals (yellow arrows). Two chromosomes had weak 5S signals (cyan arrows). Two chromosomes had no 5S signals (red arrows). The remaining ten chromosomes had relatively intense 5S signals (Fig. 1).

5S rDNA loci were detected in 8 chromosome pairs (Fig. 2a, c). The densities and locations of 5S rDNA signals varied among individual chromosomes. High-intensity 5S rDNA signals were detected in the short arms of chromosomes 7 and 8, and weak signals were also detected in the long arms of the same chromosomes. For the satellite chromosomes, 17 and 18, a 5S rDNA signal was located at the end of the long arm. The third pair of chromosomes, 9 and 10, also significantly differed from other chromosomes in that 5S rDNA signals were observed in the subterminal region. The fourth pair of chromosomes, 11 and 12, had no detectable 5S rDNA signals.

3.2. Karyotype analysis

The results of karyotype analysis showed that for *P. concolor*, 2n = 2x = 18, with a basic chromosome number x = 9. The relative lengths of the FAM-labelled mitotic metaphase chromosomes in Fig. 2a and b ranged from 4.03 for chromosome 18 (the shortest chromosome) to 7.21 for chromosome 1 (the longest chromosome); the corresponding relative lengths for TAMRA-labelled chromosomes ranged from 4.19 to 6.64 (Fig. 2c and d).

The chromosome length ratios (longest/shortest) for FAM-labelled and TAMRA-labelled chromosomes were 1.79 and 1.59, respectively (Table 3). The proportion of FAM and TAMRA-labelled chromosomes with an arm ratio greater than 2:1 was 0.66. Thus, *P. concolor* belongs to the 3A cytotype. In terms of chromosome constitution based on type [2n = 2x = 6 m (2SAT) + 6sm + 6st] (Table 3), *P. concolor* exhibited 3 pairs of metacentric chromosomes (including a pair of satellites, chromosomes 17 and 18), 3 pairs of submetacentric chromosomes, and 3 pairs of subtelocentric chromosomes (Fig. 2b and d).

4. Discussion

Recent studies have indicated that there are nine *Piptanthus* species