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Physical mapping of rDNA and karyotype analysis in *Tulipa sinkiangensis* and *T. schrenkii*

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ABSTRACT

The karyotypes of *Tulipa sinkiangensis* and *T. schrenkii* were analyzed based on chromosome size and the physical mapping of 5S and 45S ribosomal DNAs (rDNAs) using fluorescence in situ hybridization. The genomes from both species were consistent with the uniform karyotype formula, i.e., having four pairs of submedian and eight pairs of subterminal chromosomes ($2n = 2x = 24 = 8sm + 16st$). Remarkably, the number and location of 5S rDNA loci were quite different between the two species. A single pair of 5S rDNA loci was detected on both chromosomes 5 in *T. sinkiangensis*, but the 5S rDNA loci were detected on almost all chromosomes in *T. schrenkii*, except for chromosomes 4 and 6. For both species, the 45S rDNA loci were localized to telomeric regions of the chromosome—on either the short or long arm. Interestingly, the 5S rDNA and 45S rDNA loci positions and numbers varied among different genotypes of *T. schrenkii*. We conclude that the two species are close relatives and that a series of genome modifications occurred during the diversification of these two species.

1. Introduction

Tulip (*Tulipa* spp.), belonging to the tribe Tulipeae in the family Liliaceae, is currently the most cherished ornamental bulb crop in the Netherlands and has been widely cultivated worldwide (Van Tuyl and van Creij, 2006). The genus *Tulipa* L. originates from central Asia and comprises approximately 55–139 species (Booy and Van Raamsdonk, 1998; Tang et al., 2015; Zonneveld, 2009). These species have many important and useful traits for breeding, including a variety of flower colors, strong disease resistance, hardiness, and drought tolerance, among others (Xing et al., 2017). Karyotyping is a cytological characterization of each species and is critical for studies of plant cell biology and genetics. Karyotype analysis can be useful not only for the taxonomy but also for establishing the evolutionary and genetic relationships among species of the genus *Tulipa* (Abedi et al., 2015; Marasek et al., 2006). A karyotype has been reported for more than 10 wild tulip species based on the conventional staining method. The basic chromosome number of 12 (x = 12) and the existence of polyploidy in the genus *Tulipa* had been reported (Abedi et al., 2015; Kiran et al., 2016; Marasek et al., 2006; Marasek and Okazaki, 2008; Marasek-Ciolakowska and Podwyszynska, 2008; Masoud et al., 2002; Mizuochi et al., 2007). However, there are 3–5 pairs of chromosomes with similar size and morphology, and thus it is difficult to accurately identify these chromosomes during karyotype analyses that only rely on the conventional staining method (Mizuochi et al., 2007).

Accurate chromosome identification is the basis for cytogenetic studies (Braz et al., 2018; Han et al., 2015). However, conventional staining methods can only provide information on chromosome size, centromere position, and the presence or absence of secondary constrictions. This prohibits the identification of individual chromosomes of similar size and morphology. However, the development of fluorescence in situ hybridization (FISH) provided a common platform for chromosome identification (Jiang and Gill, 2006). The application of FISH to plant chromosome characterization using 5S and 45S ribosomal DNAs (rDNAs) began in the late 1980 s and has since played an essential role in molecular cytology research (Jiang et al., 1995). Physical mapping of the 5S and 45S rDNAs provides universally applicable markers for chromosome and genome characterization in many plants (Jang et al., 2016; Li et al., 2016; Liu and Davis, 2011; Melo and Guerra, 2003; Shibata and Hizume, 2002). Simultaneous FISH of 5S and 45S rDNAs has been used to evaluate the cytological diversity of several tulip cultivars (Marasek and Okazaki, 2008; Marasek-Ciolakowska and Podwyszynska, 2008; Mizuochi et al., 2007). The most notable feature of the tulip cultivar genomes is the large number of 5S rDNA loci that

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Table 1
Chromosome characteristics in *T. sinkiangensis*.

Chr.No	Total length (Mean ± SD)(μm)	Relative length[a] (Mean ± SD) (%)	Arm ration[b]	Type
1	13.68 ± 1.80	10.85 ± 1.43	5.60	st[c]
2	13.28 ± 1.61	10.53 ± 1.28	3.49	st
3	13.26 ± 1.62	10.52 ± 1.28	4.47	st
4	11.92 ± 1.17	9.45 ± 0.93	2.55	sm[d]
5	11.20 ± 1.55	8.88 ± 1.23	3.10	st
6	10.35 ± 1.42	8.21 ± 1.12	4.50	st
7	9.82 ± 1.26	7.78 ± 1.00	3.61	st
8	9.26 ± 1.03	7.34 ± 0.81	3.22	st
9	8.77 ± 0.86	6.95 ± 0.68	2.93	sm
10	8.71 ± 1.16	6.91 ± 0.92	2.42	sm
11	8.16 ± 1.04	6.47 ± 0.82	3.31	st
12	7.71 ± 0.96	6.11 ± 0.76	2.47	sm

[a](Average length of each chromosome/average sum of the length of all chromosomes) × 100.
[b]Length of the long arm/length of the short arm.
[c]Subterminal chromosomes.
[d]Submedian chromosomes.

Table 2
Chromosome characteristics in *T. schrenkii*.

Chr.No	Total length (Mean ± SD)(μm)	Relative length (Mean ± SD) (%)	Arm ration	Type
1	14.57 ± 1.06	10.76 ± 0.77	3.99	st
2	14.31 ± 1.57	10.57 ± 1.16	4.01	st
3	13.52 ± 1.24	9.99 ± 0.91	4.54	st
4	12.83 ± 1.52	9.48 ± 1.11	2.08	sm
5	12.63 ± 1.21	9.33 ± 0.88	3.09	st
6	10.32 ± 0.77	7.62 ± 0.56	3.35	st
7	10.20 ± 0.98	7.54 ± 0.72	3.58	st
8	10.14 ± 0.88	7.49 ± 0.65	2.29	sm
9	9.73 ± 0.81	7.19 ± 0.60	2.66	sm
10	9.46 ± 0.90	6.99 ± 0.66	3.19	st
11	8.97 ± 0.79	6.62 ± 0.58	1.93	sm
12	8.72 ± 0.52	6.44 ± 0.43	3.50	st

are located on each chromosome (Marasek and Okazaki, 2008; Marasek-Ciolakowska and Podwyszynska, 2008; Mizuochi et al., 2007).

To our knowledge, the physical mapping of the 5S and 45S rDNAs is scarce in wild tulip species. *Tulipa sinkiangensis* and *Tulipa schrenkii* are both endemic Chinese species, distributed in Xinjiang, China. In addition, *T. sinkiangensis* is not only a forage crop but also an important breeding material for multiflora because of its unique multiflower characteristic (Xing et al., 2017). In this study, we used FISH for karyotype analyses based on chromosome size and the physical mapping of 5S and 45S rDNAs in *T. sinkiangensis* and *T. schrenkii*. The two species had four pairs of submedian (sm) and eight pairs of subterminal (st) chromosomes, consistent with the uniform karyotype formula ($2n = 2x = 24 = 8sm + 16st$). However, the number and location of 5S rDNA loci were quite different. A single 5S rDNA signal was detected on chromosome 5 in *T. sinkiangensis*, but the 5S rDNA signals were detected on almost all chromosomes in *T. schrenkii*, with the exception of chromosomes 4 and 6. Interestingly, the rDNAs loci varied among different genotypes of *T. schrenkii*.

2. Materials and methods

2.1. Plant materials

Two wild tulip species, namely *T. sinkiangensis* Z. M. Mao and *T. schrenkii* Regel, were used in this study. In April 2014, 20 healthy bulbs of *T. sinkiangensis* were collected in Urumchi, Xinjiang, China (latitude 43°54′28″N, longitude 87°52′28″E), and that of *T. schrenkii* were obtained from Chabuchaer, Xinjiang, China (latitude 43°34′24″N, longitude 81°09′8″E). All the bulbs were planted in a plastic tunnel in the National Tulip Germplasm Base of China, which is located in Shenyang, Liaoning, China (latitude 41°49′12″N, longitude 123°34′4″ E).

2.2. Preparation of mitotic chromosomes

Root tips (0.5–1.5 cm) cut from bulbs were pretreated with 0.7 mM cycloheximide for 8 h at room temperature, washed with distilled water, and transferred to Carnoy's fixative (3:1 ethanol:acetic acid, v/v). To prepare the chromosomes, fixed root tips were rinsed thoroughly with water and digested with an enzyme mixture containing 4% cellulase (Yakult Pharmaceutical, Japan), 2% pectinase (Sigma, USA), and 1% pectolyase (Kyowa Chemical, Japan) dissolved in 0.01 M citrate buffer (pH 4.5) at 37 °C for 1 h. Softened material was carefully placed in water and then transferred to glass slides where meristem cells were released with a pair of tweezers and macerated in 20 μL of 60% acetic acid. Then the slides were placed on a slide heater (HI 1220, Leica) at 55 °C, smeared with a dissecting needle, washed immediately with Carnoy's fixative, and air dried. Slides were screened under a phase-contrast microscope, and well-spread mitotic chromosome preparations were selected for FISH. Suitable slides were fixed in 4% paraformaldehyde dissolved in 1 × PBS for 15 min, followed by washing three times in 2 × SSC for 5 min, dehydrated in an ethanol series (70%, 90%, and 100% ethanol; 5 min each), and left to air dry.

2.3. rDNA probes and oligo probes

Plasmids encoding 5S and 45S rDNAs cloned from rice (*Oryza sativa* L.) were labeled with digoxigenin-dUTP (digoxigenin -11-dUTP, Roche 11,093,088,910) and biotin-dUTP (biotin-16-dUTP, Roche 11,093,070,910), respectively, using nick translation. Two oligos containing 59 nucleotides (designated as 5S-1 and 5S-2) were derived from the coding region of 5S rRNA from *Tulipa fosteriana* 'Red Emperor' (GenBank DQ840050). The 5S-1 probe was the deoxyribonucleotide oligomer (5′-GGGTGCGATCATACCAGACTAAGCACCGGATCCCATCAG AACTCTGCTGTTAAGCGTGC-3′) comprised of positions 1–59 of DQ840050. The 5S-2 oligo probe (5′-TTGGGCGAGAGTAGTACTAGGA TGGGTGGCCTCCTGGGAAGTCCTAGTGTTGCACTCCC-3′) consisted of positions 60–118 of DQ840050. These oligo probes were synthesized and modified at the 5′- end with 6-carboxyfluorescein by the Tsingke Company (Beijing, China).

2.4. Fish

FISH was performed according to a published protocol (Jiang et al., 1995) with minor modifications. A reaction volume of 20 μL per slide

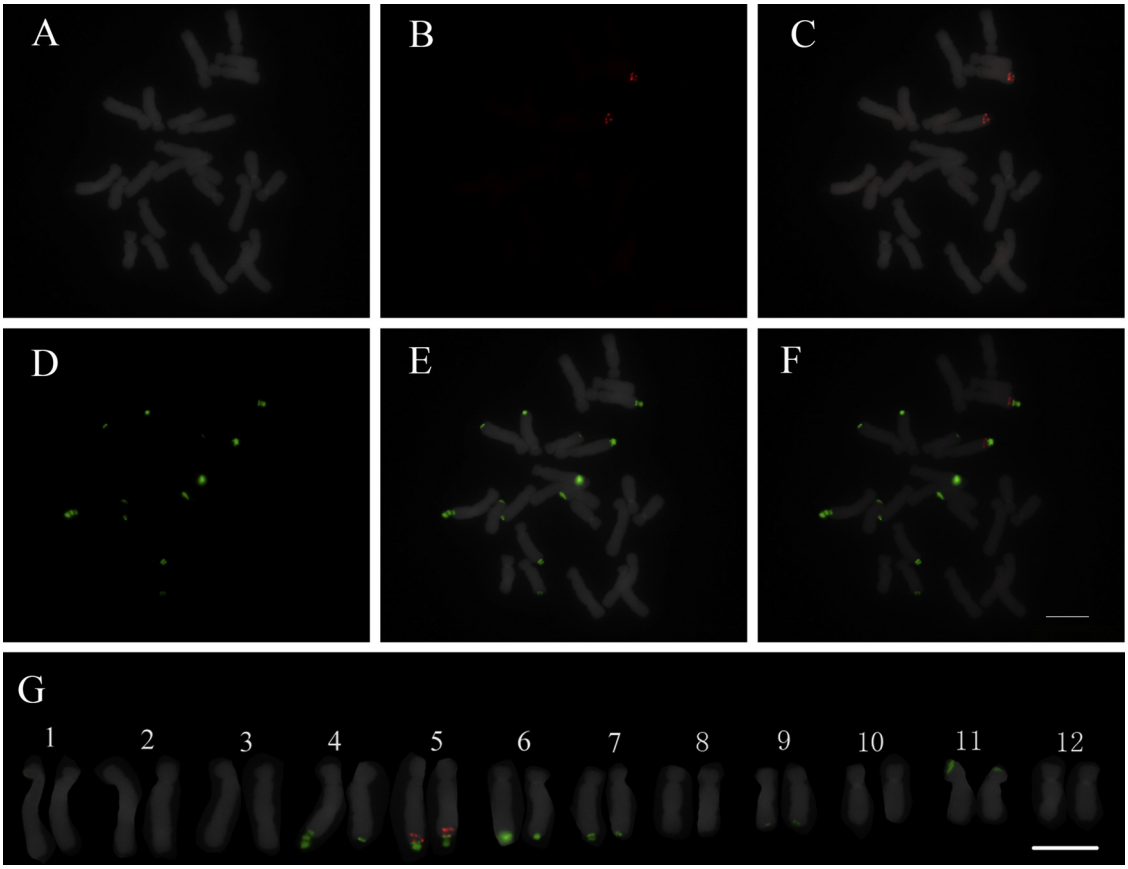

Fig. 1. Chromosome identification based on FISH using rDNA probes in *T.sinkiangensis.* (A) Grayscale image of chromosomes. (B) Signals of 5S rDNA (red fluorescence). (C) Merged picture from (A) and (B). (D) Signals of 45S rDNA (green fluorescence). (E) Merged picture from (A) and (D). (F) Merged picture from (A), (B) and (D). (G) Chromosomes extracted and arranged from (F). Scale bars = 10 μm.

contained 40 ng of each oligo probe. The hybridization mixture was denatured at 98 °C for 10 min, immersed immediately in ice for 5 min, and centrifuged briefly in a tabletop centrifuge before use. The chromosomes were denatured in 70% formamide for 6 min at 85 °C, dehydrated in a pre-chilled (−20 °C) ethanol dilution series (70%, 95%, 100%; 5 min each), and air dried. After hybridization, digoxigenin and biotin labeled probes were detected with anti-digoxigenin rhodamine (Anti-Digoxigenin-Rhodamine Fab fragments, Roche 11,207,750,910) and Alexa Fluor 488 streptavidin (Alexa Fluor 488 streptavidin, Invitrogen S11223), respectively. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, H-1200). Hybridization signals were observed using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) and images acquired using an attached CCD camera. Grayscale images were captured for each color channel and then merged. The final image contrast was processed using ADOBE PHOTOSHOP 5.0 (Adobe Systems, http:// www.adobe.com).

2.5. Karyotype analysis

Chromosome measurements were carried out on five metaphase cells using Image-Pro Plus software (Media Cybernetics, Inc.). The average relative length and arm ratio of each chromosome was determined from the recorded data, and standard deviations were calculated. Chromosomes were classified according to the nomenclature of Levan et al. (1964) and identified based on arm ratios and hybridization signal. Karyotyping was performed in accordance with Stebbins (1971). Twelve pairs of chromosomes were numbered according to their length in descending order.

3. Results

3.1. General karyotype features

For each of the two tulip species we analyzed, five well-spread mitotic metaphase plates were analyzed karyologically. The absolute length, relative length, and arm ratio of each chromosome were measured and calculated. Chromosomes were physically arranged in decreasing order with the longest chromosome pair listed as 1 and the shortest pair as 12 (Tables 1 and 2). *T. sinkiangensis* and *T. schrenkii* were diploid, having $2n = 24$ chromosomes with four pairs of submedian and eight pairs of subterminal chromosomes, consistent with the uniform karyotype formula($2n = 2x = 24 = 8sm + 16st$). The four submedian chromosomes from *T. sinkiangensis* were 4, 9, 10 and 12, whereas the four submedian chromosomes from *T. schrenkii* were 4, 8, 9 and 11. According to the classification system of Stebbins (1971), the karyotype of *T. sinkiangensis* was type 4 A, whereas that of *T. schrenkii* was type 3 A. Mean chromosome length for *T. sinkiangensis* ranged from 7.71 μm to 13.68 μm and that for *T. schrenkii* ranged from 8.72 μm to 14.57 μm. The ratio values for the length of the longest chromosome to that of the shortest chromosome for the two species were 1.77 and 1.67, respectively, which were both less than 2. The arm ratios of *T. sinkiangensis* varied from 2.42 (chromosome 10) to 5.60 (chromosome 1), whereas that of *T. schrenkii* ranged from 1.93 (chromosome 11) to 4.54 (chromosome 3). In addition, the percentage of chromosome pairs with an arm ratio of > 2.0 was 92% for *T. schrenkii* and 100% for *T. sinkiangensis*; a value > 99% constitutes the difference in karyotype classification between 3 A and 4 A. *T. schrenkii* was found to have only one chromosome pair with an arm ratio of < 2.0, i.e., a value of 1.93 for chromosome 11. Thus, although the two species were classified as

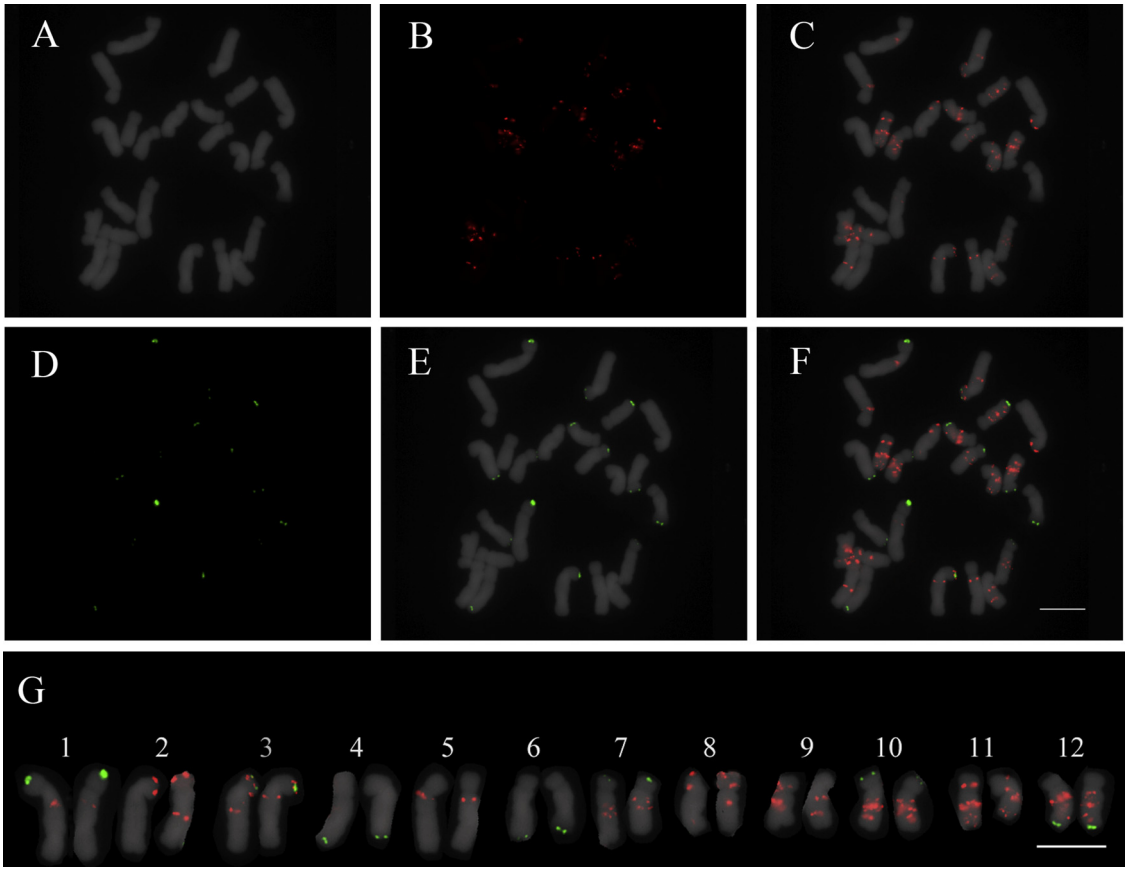

Fig. 2. Chromosome identification based on FISH using rDNA probes in *T. schrenkii.* (A) Grayscale image of chromosomes. (B) Signals of 5S rDNA (red fluorescence). (C) Merged picture from (A) and (B). (D) Signals of 45S rDNA (green fluorescence). (E) Merged picture from (A) and (D). (F) Merged picture from (A), (B) and (D). (G) Chromosomes extracted and arranged from (F). Scale bars = 10 μm.

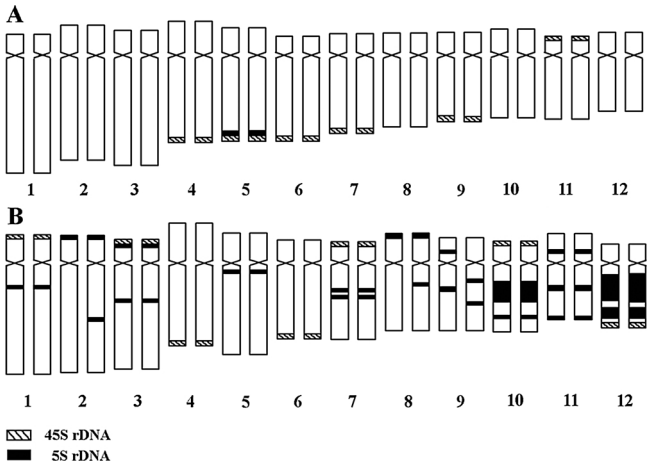

Fig. 3. Representative ideograms for metaphase chromosomes of *T.sinkiangensis* (A) and *T.schrenkii* (B) depicting the position of 5S rDNA and 45SrDNA loci.

having two distinct karyotypes, the characteristics of their chromosomes were actually quite similar.

3.2. Chromosomal distribution of 5S and 45S rDNAs

FISH was used to analyze the number and localization of 5S and 45S rDNA loci in *T. sinkiangensis* and *T. schrenkii*. At least 10 cells with good chromosome spreads and hybridization signals were assessed. For both species, the 45S rDNA signals were detected on telomeric regions of chromosomes on either the short or long arm (Figs. 1 and 2). A single pair of 5S rDNA signals was detected in *T. sinkiangensis* (Fig. 1). Surprisingly, the 5S rDNA signals of *T. schrenkii* appeared to be substantially more abundant (Fig. 2). Representative idiograms for these metaphase cells is presented in Fig. 3.

T. sinkiangensis was found to have six 45S rDNA loci and one 5S rDNA locus, which was adjacent to the 45S rDNA locus on chromosome 5 (Fig. 1). Among these 45S rDNA loci, one was localized on the short arm of chromosome 11, and the other five were localized on the long arms of chromosomes 4, 5, 6, 7 and 9. The signals on chromosome 9 were very weak (Figs. 1G, Figure 3A).

For *T. schrenkii*, seven 45S rDNA loci were located, four of which were on the short arms of chromosomes 1, 3, 7 and 10. The other three loci were on the long arms of chromosomes 4, 6 and 12. The 5S rDNA signals were detected on almost all chromosomes, except for chromosomes 4 and 6. The sizes of these 5S rDNA loci varied substantially based on the intensities of the FISH signals. Chromosome 1 contained one 5S rDNA locus on each of the long arms, and chromosome 2 had one 5S rDNA locus at the distal position of each of the short arms. However, only one chromatid of chromosome 2 exhibited one additional locus on the long chromosome arm, which was similar to chromosome 8. Chromosome 3 carried two 5S rDNA loci, one of which was located on the short arm adjacent to the 45S rDNA locus and the other located on the long arm. Chromosome 5 contained one 5S rDNA locus on the long arm. Two 5S rDNA loci were located on the long arm of chromosome 7. One chromosome 9 had 5S rDNA signals on both the short and long arms whereas its homolog had signals for two 5S rDNA loci only on the long arm. The visualized 5S rDNA signals covered approximately half the length of the long arm of chromosome 10 and three-quarters of the long arm length of chromosome 12. Chromosome 11 had one 5S rDNA locus on the short arm and two 5S rDNA loci on the

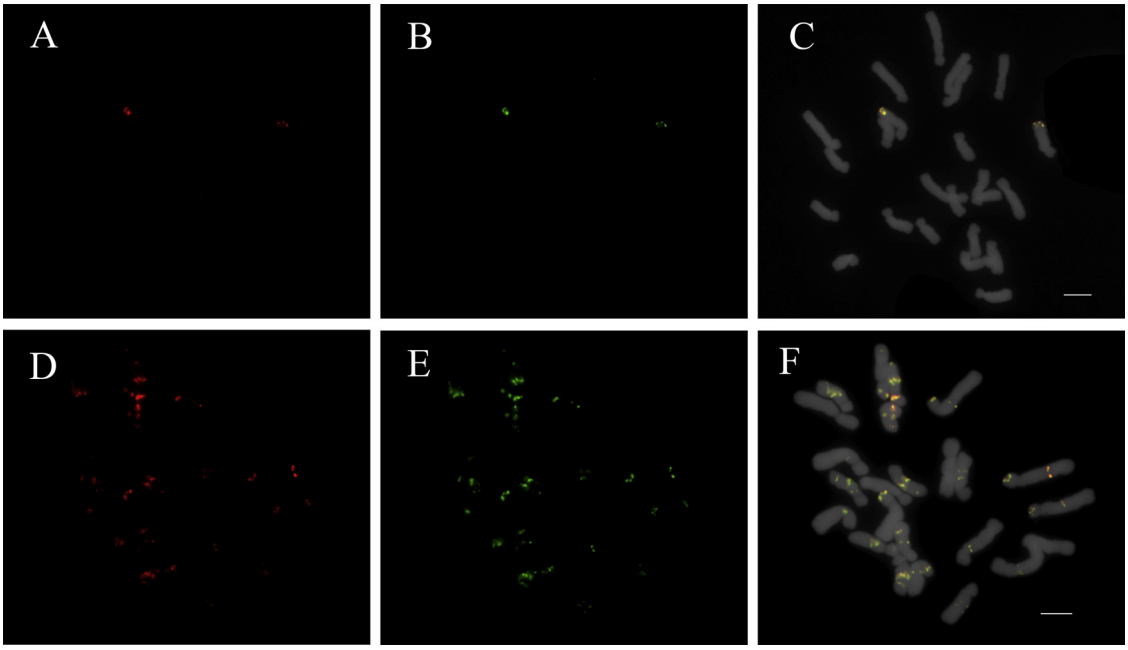

Fig. 4. Simultaneous FISH of 5S rDNA and 5S-1 probes on metaphase chromosomes of *T. sinkiangensis* and *T. schrenkii*. (A) Signals of 5S rDNA (red fluorescence) in *T. sinkiangensis*. (B) Signals of 5S-1 (green fluorescence) in *T. sinkiangensis*. (C) Merged image from (A) and (B). (D) Signals of 5S rDNA (red fluorescence) in *T. schrenkii*. (E) Signals of 5S-1 (green fluorescence) in *T. schrenkii*. (F) Merged image from (D) and (E). Scale bars = 10 μm.

long arm (Figs. 2G, Figure 3B).

To confirm that the 5S rDNA signals of *T. schrenkii* represented the true 5S rDNA sequence rather than other highly similar repetitive sequences, we synthesized two oligos containing 59 nucleotides derived from the coding region of 5S rRNA from *T. fosteriana* 'Red Emperor'. Simultaneous FISH was performed using a 5S rDNA plasmid clone and oligo probes. Fig. 4 shows that the signals derived from the plasmid clone 5S rDNA and 5S-1 oligo probe overlapped exactly in the two species, indicating that all the FISH signals represented de facto 5S rDNA loci. Two 59-nucleotide oligos, namely 5S-1 and 5S-2 (data for 5S-2 not shown), produced strong FISH signals similar to that for the 5S rDNA plasmid clone.

5. Discussion

The karyotype has been documented for a number of tulip species. The conventional staining method has revealed that most tulip chromosomes are submedian or subterminal (Kiran et al., 2016; Marasek et al., 2006; Masoud et al., 2002). In the present study, we used FISH to perform karyotypic analyses based on chromosome size and the physical mapping of 5S and 45S rDNAs in *T. sinkiangensis* and *T. schrenkii*. The results revealed that the genomes of the two species are consistent with the uniform karyotype formula with four pairs of submedian and eight pairs of subterminal chromosomes, i.e., $2n = 2x = 24 = 8sm + 16st$. Our results suggest that the two species are close relatives because they share a similar karyotype (Braz et al., 2018). The karyotype data for *T. schrenkii* were also reported by Abedi et al. (2015), but there are differences between their results and ours. They reported a karyotype consisting of three pairs of submedian and nine pairs of subterminal chromosomes ($2n = 2x = 24 = 6sm + 18st$). Moreover, they collected *T. schrenkii* bulbs from Salehabad, Khorasan, Iran, whereas we collected bulbs from Urumchi, Xinjiang, China. Therefore, the difference in karyotype data may be attributable to differences in geography and/or environment from which the samples were collected.

Physical mapping of 5S and 45S rDNA loci by FISH was previously performed for several tulip cultivars but has never been examined in any wild tulip species. For all tested cultivars, the 45S rDNA loci localized to telomeric positions on the long arm of the chromosomes (Marasek and Okazaki, 2008; Marasek-Ciolakowska and Podwyszynska, 2008; Mizuochi et al., 2007). In our study, however, the 45S rDNA sites were detected at telomeric positions either on the short or long arm of the chromosomes in both *T. sinkiangensis* and *T. schrenkii* (Fig. 1 and 2). In addition, a large number of 5S rDNA signals have been detected on each chromosome in all reported tulip cultivars (Marasek and Okazaki, 2008; Marasek-Ciolakowska and Podwyszynska, 2008; Mizuochi et al., 2007). Thus, among flowering plants, tulip species have the largest number of 5S rDNA loci (Mizuochi et al., 2007). Surprisingly, *T. sinkiangensis* has only one 5S rDNA locus located on the long arm of chromosome 5 (Fig. 1). For *T. schrenkii*, we detected 5S rDNA loci on almost all chromosomes except chromosomes 4 and 6 (Fig. 2). The number and location of 5S rDNA loci have been found to vary between standard 'Prominencs' and somaclones as well as among somaclones after long-term propagation (Marasek-Ciolakowska and Podwyszynska, 2008). We also observed that 5S and 45S rDNA loci varied among different varieties of *T. schrenkii* even though the different varieties were collected from the same location (Fig. 5). These results reveal that the rDNAs of tulip genomes are evolving rapidly, both in terms of copy number and chromosomal location.

The 5S and 45S rDNAs are clustered in high numbers as tandemly arranged repeats and thus constitute powerful cytogenetic markers for chromosome identification in all higher eukaryotes (Liu and Davis, 2011). Most significantly, the distribution and copy number of repetitive rDNA**s** directly affect the genomic organization and chromosome structure, which contributes to reproductive isolation and speciation (Zhang et al., 2015). Interestingly, our FISH data reveal that the number of 5S rDNA loci in *T. schrenkii* is much greater than that in *T. sinkiangensis* even though the two species belong to the same taxonomic section, i.e., *leiostemones*. Therefore, it is necessary to verify that the 5S rDNA signals for *T. schrenkii* indeed reflected real 5S rDNA sequences rather than other highly homologous repetitive sequences because plant genomes contain large numbers of repetitive sequences. Our results from simultaneous FISH for the 5S rDNA plasmid clone and oligo probes indicated that all the FISH signals represented authentic 5S rDNA loci (Fig. 4). The difference in number and distribution of rDNA loci between *T. sinkiangensis* and *T. schrenkii* genomes suggests that a series of genome modifications occurred during the diversification of

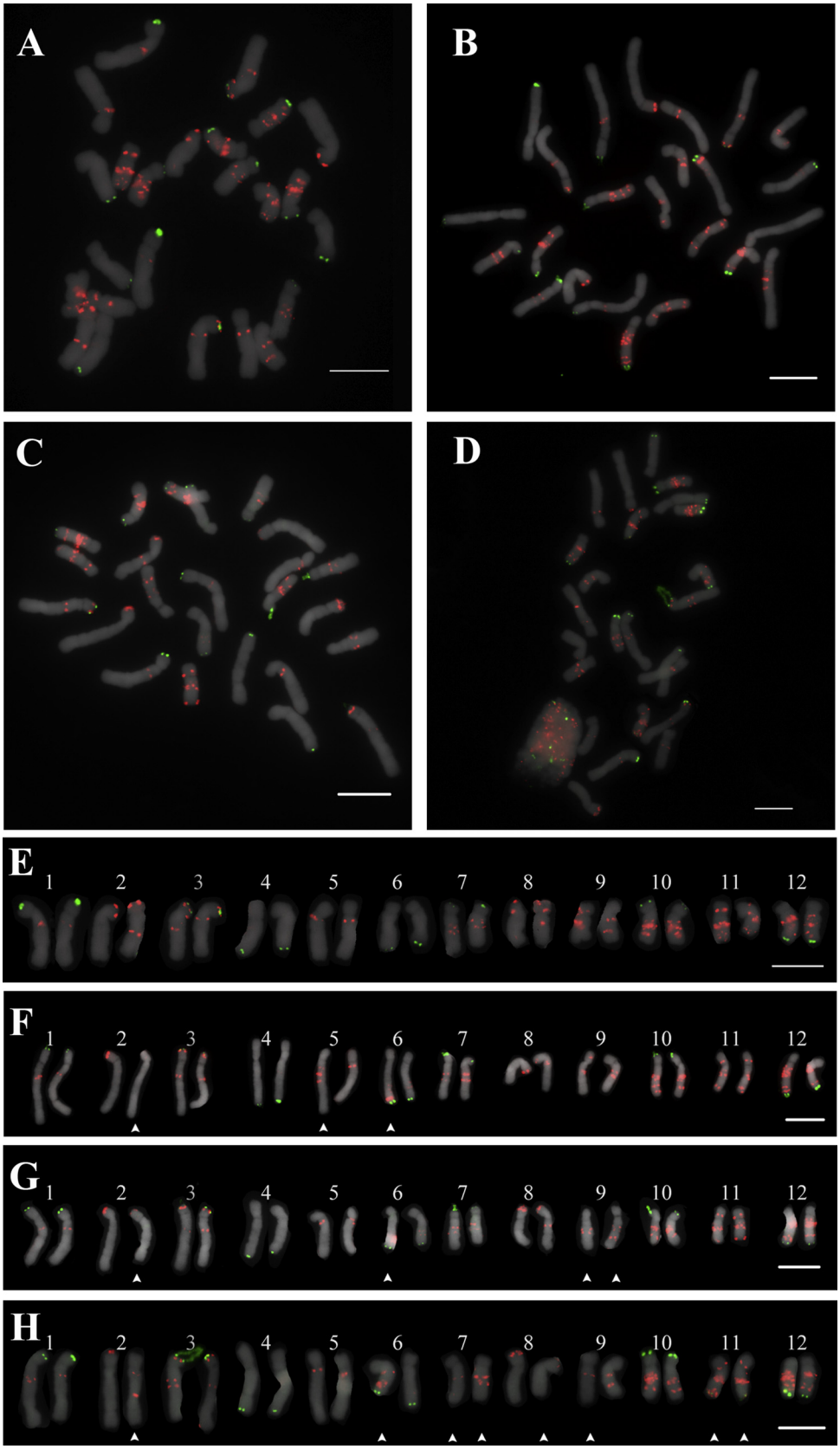

Fig. 5. Localization of 5S (red) and 45S (green) rDNA sites on four *T. schrenkii* genotypes (A–D). (E–H) Chromosomes bearing rDNA loci extracted and arranged from (A–D), respectively. White arrowheads indicate the different localization of FISH signals on these chromosomes as compared with the chromosomes in (E).Scale bars = 10 μm.

these two species.

Most *Tulipa* chromosomes have similar morphology and lack accessible landmarks for identification of individual chromosomes (Marasek and Okazaki, 2008; Mizuochi et al., 2007). The number and location of 5S and 45S rDNA signals were proved very useful as chromosome markers, which were previously used for karyotype analysis of tulips (Marasek and Okazaki, 2008; Mizuochi et al., 2007). Furthermore, in order to contribute to the understanding the rDNAs repetitive sequences and chromosomal structural evolution in tulip genomes, more species especially wild tulip species need to be analyzed.

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