



Genomic *in situ* hybridization identifies genome donors of *Camellia reticulata* (Theaceae)

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ABSTRACT

Camellia reticulata (Theaceae genus *Camellia*) is a world-famous, ornamental flowering plant. More interestingly, it has a polyploid series varying from $2n = 2x = 30$, $2n = 4x = 60$ to $2n = 6x = 90$, with a basic chromosome number of $x = 15$. The hypothetical allopolyploid origin and parental genomes of these polyploid types remains unknown. Genomic *in situ* hybridization (GISH) was used to study the genome organization and evolution of *C. reticulata*. Total genomic DNA from closely-related diploid species (*C. pitardii* and *C. saluenensis*), with the chromosome number $2n = 2x = 30$, were labeled and hybridized in the presence of blocking DNA onto metaphase spreads of *C. reticulata*. The *C. pitardii* probe painted part of the tetraploid and hexaploid *C. reticulata* genomes, whereas the *C. saluenensis* probe delineated part of the hexaploid *C. reticulata* genome. The results provide compelling evidence for the allopolyploid origin of *C. reticulata* genomes and demonstrate that 1) the diploid *C. reticulata*, *C. pitardii* and *C. saluenensis* are the progenitors of polyploid *C. reticulata*, 2) hybridization between diploid *C. reticulata* and diploid *C. pitardii* gave birth to allotetraploid *C. reticulata*, and 3) subsequent hybridization between allotetraploid *C. reticulata* and diploid *C. saluenensis* formed the allohexaploid *C. reticulata*.

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1. Introduction

Camellia reticulata Lindl. is a world-famous, ornamental flowering species notable for its large flowers, bright and charming colors, various cultivars and long flowering season. It has a polyploid series of $2x = 30$, $4x = 60$ and $6x = 90$, with a basic chromosome number of $x = 15$. Moreover, its diploid, tetraploid and hexaploid-type populations are exclusively and widely distributed across Southwest China [1–3]. Thus, clarifying the origin of the polyploid *C. reticulata* will offer valuable insight into the role of polyploidization in plant speciation.

Previous cytological studies of meiotic cells showed that most of the hexaploid and tetraploid *C. reticulata* populations formed exclusively bivalent pairs at metaphase I, with only a few cultivars showing some multivalents [1,2]. These studies indicate that hexaploid and tetraploid *C. reticulata* are most likely to be of allopolyploid origin. Parks and Griffiths [4] studied the *C. saluenensis*–*pitardii*–*reticulata* complex biosystematically and found them to be interrelated. Later, McClung [5] postulated that the forms of *C. reticulata*, *C. saluenensis* and *C. pitardii* found in West-

ern and Chinese gardens may be complex hybrids involving two or more species as well as fractionally combined with *C. japonica*. Based on the results of extensive crossing-compatibility studies in the genus *Camellia*, Parks speculated that polyploid *C. reticulata* possibly formed a complex with *C. saluenensis*, *C. pitardii* and *C. japonica* [6]. According to morphological characteristics, the four species mentioned above (*C. reticulata*, *C. saluenensis*, *C. pitardii* and *C. japonica*) were classified into the same subgenus in the genus *Camellia* by different taxonomists [3,7,8]. Physical mapping of 18s–26s rDNA in these species provided evidence for the allopolyploid nature of *C. reticulata* genome organization, and it showed that the hexaploid *C. reticulata* possibly resulted from interspecific hybridization and continued polyploidization among its ancestral diploid species [9].

Genomic *in situ* hybridization, i.e., fluorescence *in situ* hybridization using total genomic DNA as probes, is a very effective molecular cytogenetic method that can provide clear distinction between genomes [10]. When being used jointly with procedures for blocking (competitive *in situ* hybridization with unlabelled DNA), GISH discriminates among closely related species in plants. This technique is especially effective in identifying parental genomes in interspecific hybrids such as in *Lilium* [11], *Nicotiana* [12] and *Brassica* [13]. Moreover, it has also been successfully applied to identify ancestral genomes in natural allopolyploid hybrids such as cotton [14], birch [15] and milium [16].

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Table 1

Sources and chromosome number of the materials used in this study.

Number	Taxon	Voucher	Chromosome number	Locality
1	<i>C. reticulata</i> Lindl.	Xiao 0010	$2n = 2x = 30$	Panzhihua, Sichan, China
2	<i>C. reticulata</i> Lindl.	Xiao 0001	$2n = 4x = 60$	Panzhihua, Sichan, China
3	<i>C. reticulata</i> Lindl.	Xiao 9906	$2n = 6x = 90$	Huaping, Yunnan, China
4	<i>C. pitardii</i> Cohen Stuart	L 00902	$2n = 2x = 30$	Yiliang, Yunnan, China
5	<i>C. saluenensis</i> Stapf ex Bean	Xiao 9901	$2n = 2x = 30$	Wuding, Yunnan, China

Although previous efforts have given a strong hypothesis about the allopolyploid origin of polyploid *C. reticulata* and suggested its most probable progenitors, the parental genomes of these polyploid types remain elusive. Genomic *in situ* hybridization is a powerful technique in verifying the origin of polyploid taxa and may provide preliminary insights into the extent of genomic rearrangements. In this study, GISH was applied to validate the hypothesized allopolyploid origin and to identify the genome donors of the tetraploid and hexaploid *C. reticulata*.

2. Materials and methods

2.1. Plant materials

Table 1 lists the origin and ploidy of the species used in the study. The mature seeds were collected from plants grown in the wild or grown in the Kunming Botanical Garden, Kunming Institute of Botany, Chinese Academy of Sciences. All voucher specimens are deposited in the Herbarium of the Kunming Institute of Botany (KUN).

2.2. Chromosome preparation

Root tips (0.5–1.5 cm) were pre-treated with 0.05% colchicine for 2.5 h at room temperature, fixed in 3:1 (v/v) mixture of ethanol and glacial acetic acid and stored at -20°C . The root tips were then washed in enzyme buffer (0.01 M citric acid/sodium citrate, pH 4.8) for 20 min and digested in an enzyme mixture comprised of 2% (w/v) cellulase Onozuka-RS (Yakult Co., LTD., Japan) and 1% (w/v) pectolase Y-23 (Kyowa Chemical Products Co., LTD., Japan) for 0.5 h at 37°C . Meristems were squashed in a drop of 45% acetic acid with a coverslip, and the preparations were frozen on dry ice. The coverslips were removed, and the chromosome preparations were dehydrated, air-dried and stored at 4°C until use.

2.3. Preparation of probe and block DNA

Total genomic DNA was extracted from leaves using the standard CTAB method [17]. The DNA was boiled to yield 300- to 700-bp fragments and labeled by nick translation with digoxigenin-11-dUTP according to the manufacturer's instructions (Roche). Block DNA was autoclaved for 5 min to obtain 100- to 300-bp fragments.

2.4. Genomic *in situ* hybridization and detection

Genomic *in situ* hybridization was performed as described by Hasterok et al. [18] with minor modifications. Slides were pre-treated with RNase A (100 $\mu\text{g}/\text{ml}$) in $2 \times \text{SSC}$ for 1 h at 37°C , washed in $2 \times \text{SSC}$ and then incubated in pepsin (5 $\mu\text{g}/\text{ml}$) for 20 min (Wako Pure Chemical Industries, Ltd. Japan). After washing with $2 \times \text{SSC}$, samples were post-fixed in 1% formaldehyde in PBS buffer for 10 min, washed again in $2 \times \text{SSC}$ and then dehydrated in absolute ethanol. The hybridization mixture consisted of target DNA (150 ng), 50% deionized formamide, 10% dextran sulfate, $2 \times \text{SSC}$, 1% SDS, and salmon sperm blocking DNA (80- to 100-fold more than the labeled probes). To reduce cross-hybridization of the genomic

probes, sheared (autoclaved for 5 min at 103.5 kPa) and unlabeled total nuclear DNA from diploid *C. reticulata* (70-fold more than the labeled genomic probe) was added as blocking DNA. Chromosome preparations and pre-denatured probes were denatured at 75°C for 10 min and allowed to hybridize overnight in a humid chamber at 37°C . Post-hybridization washes were carried out for 15 min in $2 \times \text{SSC}$ at room temperature, followed by washes in $0.1 \times \text{SSC}$ at 42°C for 30 min. The digoxigenin probe was immunodetected using a standard protocol with anti-digoxigenin antibody conjugated to rhodamine (Roche). The chromosomes were counterstained with 1 $\mu\text{g}/\text{ml}$ 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in Vectashield (Vector Laboratories, Inc., Burlingame, CA).

2.5. Image capturing and processing

Images were taken using the CytoVision system (Applied Imaging) and a ZEISS Axioplan2 microscope. Fluorescent signals were captured separately as 8-bit black and white images through appropriate excitation filters, normalized, and merged to a 24-bit color image. Signal-tagged nuclei and chromosomes were captured. The overlay images were saved on the computer disk. For each species, 20 or more metaphases were scored. The images were trimmed to make the plates (Figs. 1–3).

3. Results and discussion

Initially, the diploid *C. reticulata* probe was applied on the chromosome preparations of diploid, tetraploid and hexaploid *C. reticulata* without blocking DNA (Fig. 1A–F). At diploid *C. reticulata*, the hybridized signals were very bright on both interphase nuclei and metaphase chromosomes (Fig. 1A and B). GISH signals were distributed throughout the entire interphase nuclei. Almost all metaphase chromosomes gave strong hybridization signals along the entire chromosomal arms, with the exception of a few chromosomes that lacked signals at their distal ends. At tetraploid, the number and intensity of hybridization signals on interphase nuclei and metaphase chromosomes were lower than those on diploid *C. reticulata* (Fig. 1C and D). A few chromosomes were partially painted with hybridization signals, indicating that the tetraploid *C. reticulata* is an allotetraploid. Regarding hexaploid *C. reticulata*, the hybridization signals on interphase nuclei and metaphase chromosomes were less than the former two (Fig. 1E and F), and the hybridization patterns on metaphase chromosomes were more complex than those in the tetraploid. GISH showed recombinant chromosomes of both hexaploid and tetraploid *C. reticulata*, which suggests that intergenomic rearrangements had occurred between the parental genomes. More recombinant chromosomes in hexaploid *C. reticulata* revealed that more intergenomic rearrangements had occurred. The results confirm the allopolyploid origin of both tetraploid and hexaploid *C. reticulata* genomes and suggest that it is due to congress of diploid *C. reticulata* and other parent species.

In subsequent experiments, total genomic DNA from *C. pitardii* was labeled and used as a probe. Fig. 2 shows the results of the GISH analyses. Chromosomes originating from *C. pitardii* were painted with uniform red fluorescence, whereas chromosomes originating

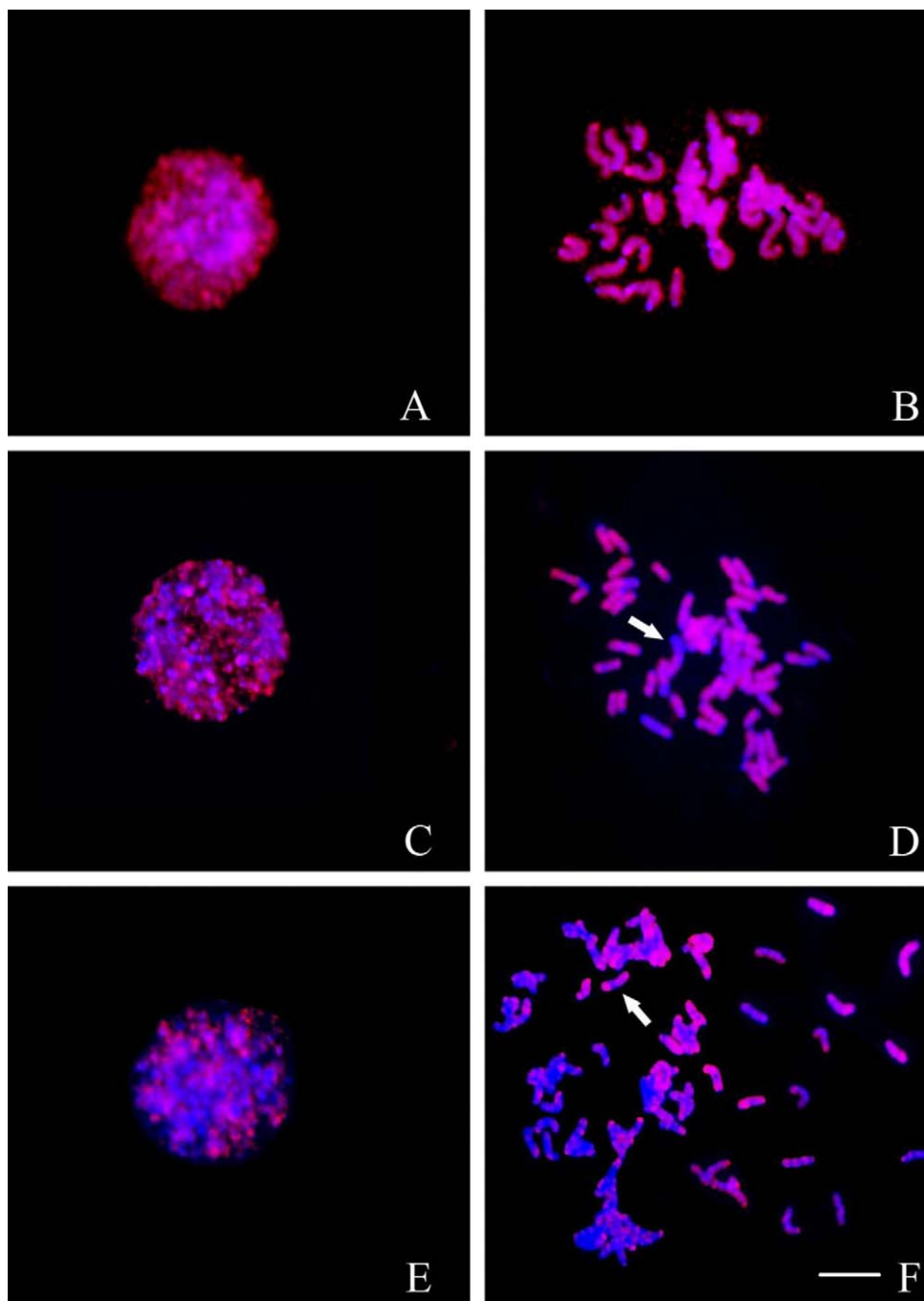


Fig. 1. Genomic *in situ* hybridization on *C. reticulata* interphase nuclei and metaphase chromosomes using diploid *C. reticulata* genomic DNA as a probe. Red fluorescence indicates hybridization signal to the probe. Chromosomes and nuclei are counterstained with DAPI (blue). (A and B) Diploid *C. reticulata*. (C and D) Tetraploid *C. reticulata*. (E and F) Hexaploid *C. reticulata*. Bar = 5 μ m. The arrows indicate the recombinant chromosomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

from diploid *C. reticulata* exhibit blue segments for less homology to the *C. pitardii* genome in these regions. First, GISH without blocking DNA was performed on diploid *C. reticulata*. The distribution patterns of the GISH signals provided initial information about the relationship between the two species. In the interphase nuclei, the GISH signals are punctate (Fig. 2A). The GISH signals are also dots during metaphase and are located at the ends of diploid *C. reticulata* chromosomes (Fig. 2B). Then, GISH to tetraploid and hexaploid *C. reticulata* were performed with unlabeled DNA from diploid *C. reticulata* as blockings. Compared with diploid *C. reticulata*, greater proportions of the nuclei of tetraploid and hexaploid *C. reticulata* were painted with GISH signals using the *C. pitardii*

genomic DNA as a probe (Fig. 2C and E). Furthermore, the GISH patterns on the metaphase chromosomes of these three species were different (Fig. 2B, D and F). In tetraploid *C. reticulata*, GISH results distinguished 35 *C. pitardii* chromosomes, 23 *C. reticulata* chromosomes and 2 recombinant chromosomes with both red (*C. pitardii*) and blue (*C. reticulata*) segments on the same chromosome (Fig. 2D). These GISH results support the hybrid origin of tetraploid *C. reticulata* and clearly show that hybridization between diploid *C. reticulata* and diploid *C. pitardii* gave birth to allotetraploid *C. reticulata*. The distribution of GISH signals on the chromosomes of hexaploid *C. reticulata* showed similar patterns when using the genomic DNA of diploid *C. reticulata* (Fig. 1F) or *C. pitardii* (Fig. 2F)

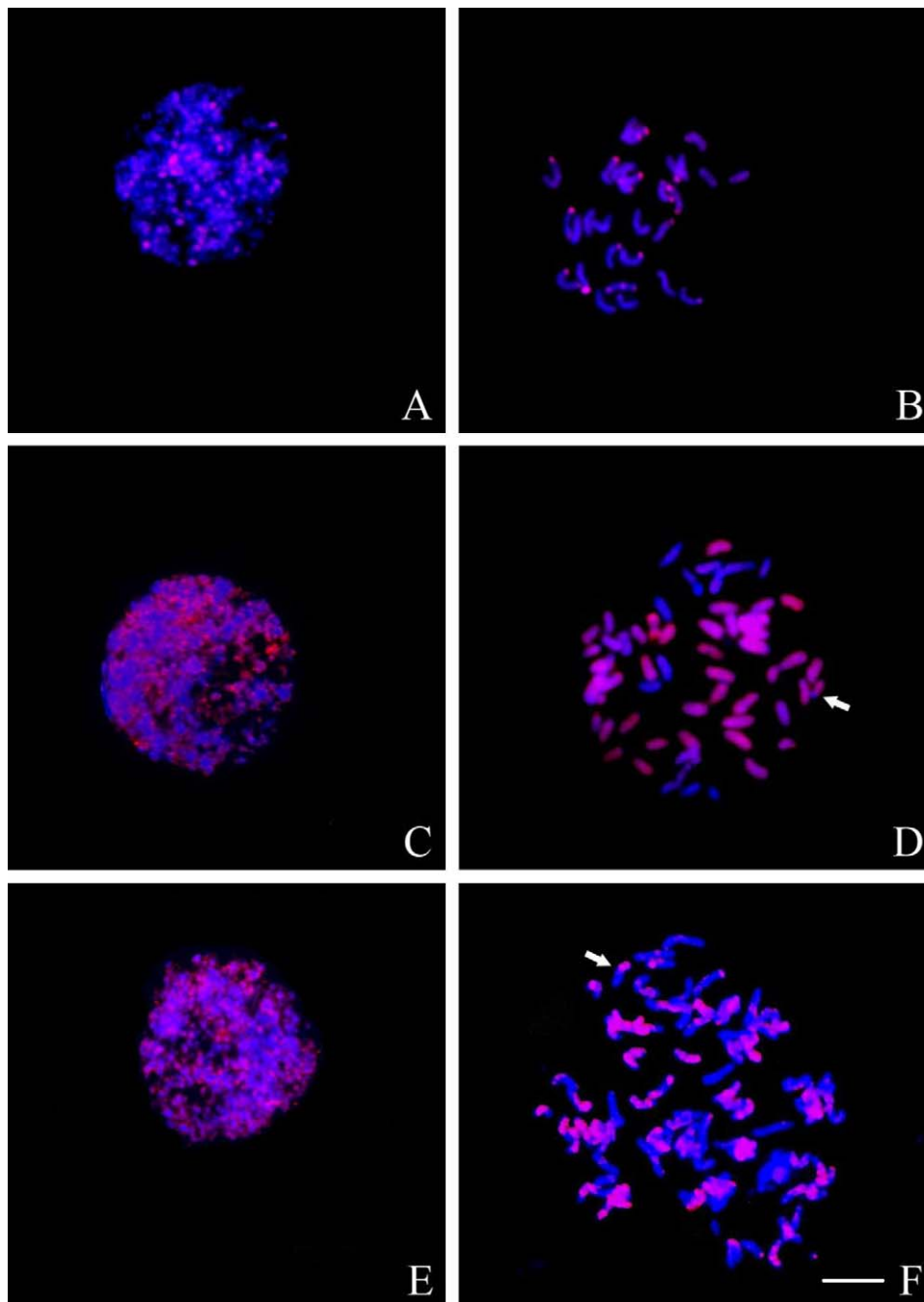


Fig. 2. Genomic *in situ* hybridization on *C. reticulata* mitotic interphase nuclei and metaphase chromosomes using *C. pitardii* genomic DNA as a probe. Red fluorescence indicates hybridization signal to the probe. Chromosomes and nuclei are counterstained with DAPI (blue). (A and B) Diploid *C. reticulata*. (C and D) Tetraploid *C. reticulata*. (E and F) Hexaploid *C. reticulata*. Bar = 5 μ m. The arrows indicate the recombinant chromosomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

as a probe. The results further confirm that *C. pitardii* is contained in polyploid *C. reticulata* chromosomes and that there is congress of the parent species.

Last, the *C. saluenensis* probe was hybridized onto the chromosome preparations of diploid, tetraploid and hexaploid *C. reticulata* (Fig. 3A–F). When the *C. saluenensis* probe was hybridized to diploid *C. reticulata*, no blocking DNA was used, whereas unlabeled DNA from diploid *C. reticulata* was applied as blocking DNA when the *C. saluenensis* probe was hybridized to tetraploid and hexaploid *C. reticulata*. When using digoxigenin-labeled genomic DNA from *C. saluenensis* as a probe, distinct GISH signals presented limitedly at

the telomeric regions of the chromosomes of diploid and tetraploid *C. reticulata* (Fig. 3B and D). In hexaploid *C. reticulata*, more than half of the chromosomes are recombinant chromosomes (Fig. 3F). The results confirm that *C. saluenensis* is one of the hexaploid *C. reticulata* chromosome components and reveal that considerable structural rearrangements have occurred between the parental species.

In a previous report [19], the genomic DNA of *C. japonica*, which is considered to be closely related to *C. reticulata*, was used as a probe of GISH to the chromosome spreads of *C. reticulata*. In this experiment, GISH signals restrictedly presented at the telomeric

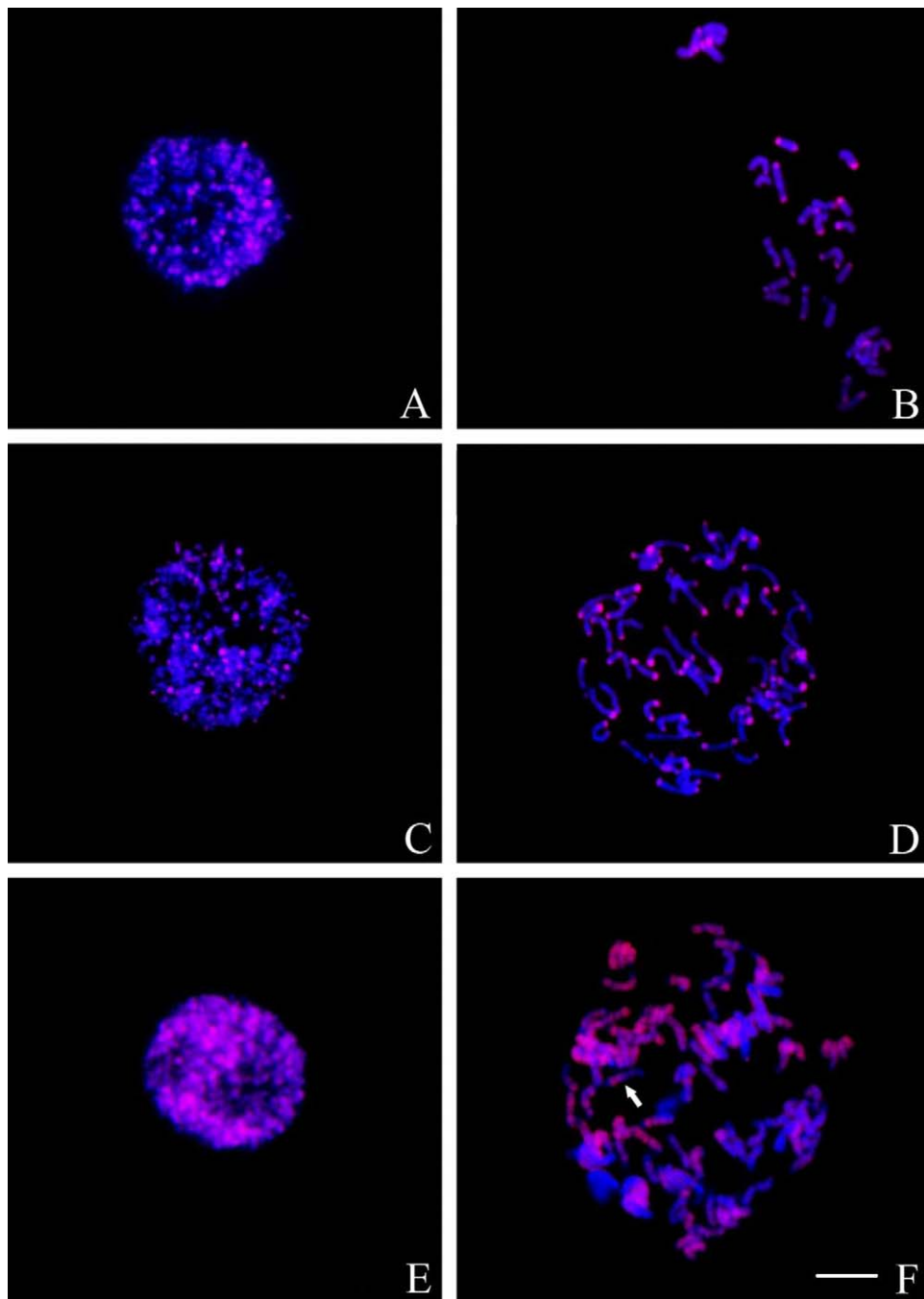


Fig. 3. Genomic *in situ* hybridization on *C. reticulata* mitotic interphase nuclei and metaphase chromosomes using *C. saluenensis* genomic DNA as a probe. Red fluorescence indicates hybridization signal to the probe. Chromosomes and nuclei are counterstained with DAPI (blue). (A and B) Diploid *C. reticulata*. (C and D) Tetraploid *C. reticulata*. (E and F) Hexaploid *C. reticulata*. Bar = 5 μ m. The arrows indicate the recombinant chromosomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

regions of chromosomes of diploid, tetraploid and hexaploid *C. reticulata*. These results confirmed that *C. japonica* is not the progenitor of *C. reticulata*.

The telomeric regions of diploid *C. reticulata* chromosomes were painted using genomic DNA of either *C. pitardii* or *C. saluenensis*, without diploid *C. reticulata* as blocking DNA. However, the telomeric regions of hexaploid *C. reticulata* chromosomes could not be painted by either *C. pitardii* (Fig. 2F) or *C. saluenensis* (Fig. 3F) with diploid *C. reticulata* as blocking DNA. The distribution of GISH signals on telomeric regions of diploid *C. reticulata* (Figs. 2B and 3B) and of tetraploid *C. reticulata* (Fig. 3D) revealed the differentiation of the genomic organization in these species; diploid *C. reticulata* and

C. pitardii, diploid *C. reticulata* and *C. saluenensis*, and tetraploid *C. reticulata* and *C. saluenensis* shared homology only at their telomeric regions.

Previous studies have suggested that *C. reticulata* is a polyploid complex originating from the hybridization of the diploid *C. reticulata* and other relative species [2,9]. This paper unambiguously confirms this hypothesis and provides insight into the evolution of this polyploid complex. Our results demonstrate that hybridization between diploid *C. reticulata* and diploid *C. pitardii* gave birth to allotetraploid *C. reticulata* and that subsequent hybridization between allotetraploid *C. reticulata* and diploid *C. saluenensis* formed the allohexaploid *C. reticulata*.

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