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Physical mapping of the 18S-26S rDNA by fluorescent in situ hybridization (FISH) in *Camellia reticulata* polyploid complex (Theaceae)

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Abstract

Physical maps of the 18S-26S rDNA ribosomal RNA genes (rDNA) were generated by fluorescent in situ hybridization (FISH) for *Camellia reticulata* polyploid complex, including three types of ploidy of *C. reticulata* and its related species, *C. japonica*, *C. yunnanensis*, *C. pitardii* and *C. saluenensis*. The advanced method was used for preparing chromosome spreads. Eight, 12 and 18 rDNA sites were observed on the genomes of diploid, tetraploid and hexaploid *C. reticulata*. Eight, four, five, and four rDNA loci located on the chromosomes of *C. pitardii*, *C. japonica*, *C. saluenensis* and *C. yunnanensis* respectively. The number and position of rDNA sites in these species were compared for analysis. The results support some of the earlier phylogenetic speculation about this complex and suggest that some chromosomal structural rearrangements have occurred. The multiplicity, variation in size and site numbers between polyploid *C. reticulata* and its putative diploid ancestors indicate that the behavior of rDNA loci in the complex is considerably more complex than previously envisioned.

Keywords: 18S-26S rDNA; *Camellia*; Fluorescent in situ hybridization (FISH); Physical mapping

1. Introduction

Being an important flowering ornamental species, *C. reticulata* Lindl. has been cultivated in China for more than 1300 years. It is famous in the world for its large flower size, bright and charming colors, many cultivars and a long flowering season. Clarifying its origin will offer valuable theoretic evidence for the further hybrid studies.

In 1970s, Ackerman [1] and Kondo [2] found *C. reticulata* as hexaploid plant ($2n = 6x = 90$) with the basic number of chromosomes in the genus *Camellia* being $x = 15$. Recently extensive studies were carried out on the species of the genus *Camellia*, in which the chromosome basic number was confirmed then ($2n = 6x = 90$) [3–6].

Parks and Griffiths studied the *C. saluenensis–pitardii-reticulata* complex biosystematically and found them to be interrelated [7]. After that, McClung stated that the forms of *C. reticulata*, *C. saluenensis* and *C. pitardii* found in Western and Chinese gardens may be complex hybrids involving two or more of the specie as well as fractionally combined with *C. japonica* [8]. 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* [9]. The previous cytological studies with meiotic cells showed that most of the hexaploid *C. reticulata* taxa produced only bivalent pairs of all chromosomes (45_{II}) at metaphase I, while few taxa gave various chromosomes pairing configurations with some multivalents [3,6]. The above results indicate that *C. reticulata* is an allohexaploid.

Previous efforts at interspecific comparisons with the complex have employed morphological [10], karyotypic [3–6], meiotic criteria [3,6]. In situ hybridization is a powerful technique that can be applied to the study of

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chromosomes and their evolution. Fluorescent in situ hybridization (FISH) of repetitive DNA sequences, such as ribosomal RNA genes can detect differences in positions of these genes between related species [11]. In this study, we used FISH to examine the distribution of 18S-26S rDNA sites in *C. reticulata* and its related species. The major goal was to increase our understanding of the evolutionary history of rDNA loci in the *C. reticulata* complex and offer further evidence to clarify the origin of polyploid *C. reticulata*.

2. Materials and methods

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

Chromosomes were prepared using enzyme-softened root tips. The slides for chromosome spreads were air-dried and stored in the freezer at −20 °C for further treatment.

The probe used for in situ hybridization was the clone JHD2-15A containing 8.2 kb BglII+XhoI fragment of 18S-26S rDNA isolated from Arabidopsis, which was kindly provided by Professor Li Maoxue, Peking University. The probe was labeled by nick translation system (Boehringer Mannheim) using digoxigenin-11-dUTP.

Slides were incubated in 100 μg/ml DNase-free RNase A at 37 °C for 1 h followed by three washes in 2 × SSC for 5 min each. Then the slides were postfixed in CarnoyI (acetic acid:ethanol, 1:3, v/v) for 10 min, dehydrated in ethanol (70, 90 and 100%), and air-dried. Freshly denatured probe mixture was added to the denatured chromosomes and hybridized at 37 °C overnight.

For the detection of hybridization signals, 70 μl 5% BSA was added to each slide for blocking. The immunological reaction was carried out by incubating the slides in 30 μl anti-digoxigenin antibody conjugated with rhodamine (Boehringer Mannheim). Chromosomes were counterstained with 2 μg/ml DAPI (4′,6-diamidino-2-phenylindole) for 2 min followed by being rinsed in PBS buffer for 5 min. The antifading solution with glycerol was mounted on each slide.

Images were captured 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 stored on the computer disk.

For each species analyzed, 20 or more metaphase were scored. Images were trimmed to make the plates (Figs. 1 and 2).

3. Results

Fig. 1 A–C′ shows the results of FISH on the diploid, tetraploid and hexaploid of *C. reticulata*. Fig. 2 A–D′ shows those on *C. japonica*, *C. yunnanensis C. pitardii* and *C. saluenenisis*. The discriminated signals showed red colors on the blue DAPI-counterstained nuclei and chromosomes. Ordering chromosomes in this study referred to the previous results [3–6].

Diploid *C. reticulata* has eight 18S-26S rDNA sites on the nucleus and chromosomes (Fig. 1 A,A′). The fluorescent signals of all the detected loci are strong. Six of the sites are located at the terminal positions of short arms of chromosome 1, 5, 9, 14, 20 and 21 respectively. The other two are observed at the end of long arms of chromosome 18 and 19 (Table 2).

In tetraploid *C. reticulata*, there are 12 chromosomes with 18S-26S rDNA sites (Fig. 1B′). The same number of rDNA sites is found on the nucleus (Fig. 1B). For the hybridization chromosomes, the rDNA sites are in a proximal position in each case. Two of the hybridization signals are minor, each at the end of short arms of chromosome 29 and 56. The other ten are major, located at the end of long arms of chromosome 32 and 40 and short arms of chromosome 7, 37, 46, 48 51, 52, 57 and 58 respectively (Table 2).

Eighteen 18S-26S rDNA signals are observed in the nucleus and chromosomes of hexaploid *C. reticulata*

Table 1
Resources and chromosome number of *C. reticulata* polyploid complex

Number	Taxon	Chromosome number	Locality	Voucher
1	*C. reticulata* Lindl.	$2n = 6x = 90$	Huaping, Yunnan, People's Republic of China	Xiao 9906
2	*C. reticulata* Lindl.	$2n = 4x = 60$	Panzhihua, Sichuan, People's Republic of China	Xiao 0001
3	*C. reticulata* Lindl.	$2n = 2x = 30$	Panzhihua, Sichuan, People's Republic of China	Xiao 0010
4	*C. japonica* L.	$2n = 2x = 30$	Kunming, Yunnan, People's Republic of China	Xiao 0011
5	*C. yunnanensis* (Pitard es Diels) Cohen Stuart	$2n = 2x = 30$	Yanbian, Sichan, People's Republic of China	Xiao 9905
6	*C. saluenensis* Stapf ex Bean	$2n = 2x = 30$	Wuding, Yunnan, People's Republic of China	Xiao 9901
7	*C. pitardii* Cohen Stuart	$2n = 2x = 30$	Yiliang, Yunnan, People's Republic of China	L 00902

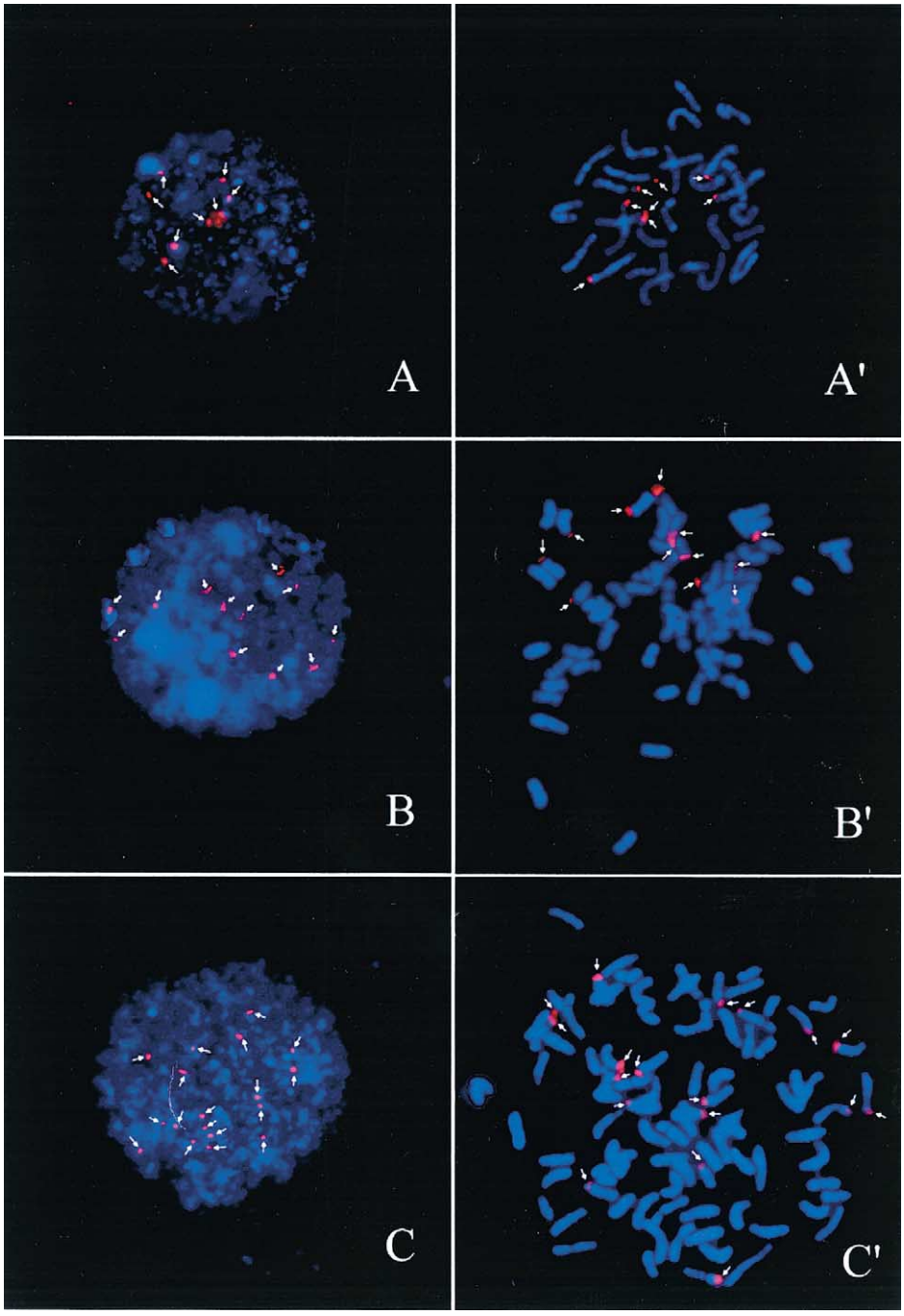

Fig. 1. Fluorescent in situ hybridization of *Camellia* interphase nuclei and metaphase chromosomes with the 18S-26S rDNA probe (red color) and blue fluorescence shows DNA counterstained with DAPI. The white arrows point the site of signals. (A, A′) The hybridization signals of diploid *C. reticulata*. (B, B′) The hybridization signals of tetraploid *C. reticulata*. (C, C′) The hybridization signals of hexaploid *C. reticulata*.

(Fig. 1 C, C′). In each case, the sites are at the chromosome ends. Four sites are located at the terminal positions of long arms of chromosome 49, 57, 74 and 75. The other 14 ones are located on the short arms of chromosome 8, 9, 12, 24, 29, 30, 37, 43, 44, 65, 69,70, 80 and 85. On chromosome 65 and 85, the two sites are relatively weaker.

Four 18S-26S rDNA FISH signals (Fig. 2 A, A′) were observed on the nucleus and chromosomes of *C. japonica*. Three major sites and one minor site are got. Two of the large signals are at the ends of short arms of two submedian-centromeric chromosomes, chromosome 3 and 4. The other major site is located at the terminal end of chromosome 22. The light signal was found at the end of short arm of one subterminal-centromeric chromosome 19 (Table 2).

C. yunnanensis have rDNA sites on four chromosomes and four signals are got in the nucleus (Fig. 2 B,

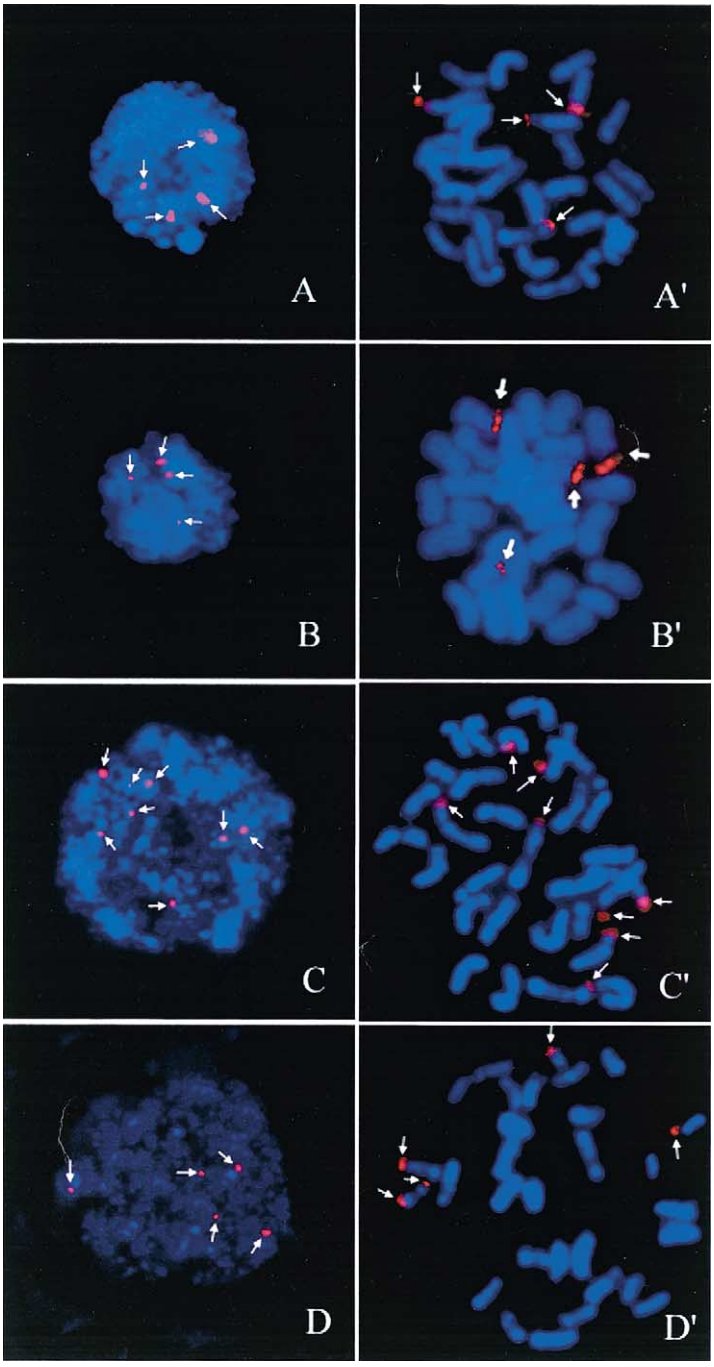

Fig. 2. (A, A′) The hybridization signals of *C. japonica*. (B, B′) The hybridization signals of *C.yunnanensis*. (C, C′) The hybridization signals of *C. pitardii*. (D, D′) The hybridization signals of *C. saluenensis*.

B′). Similarly three of them show major and strong hybridization signals, which indicate a high number of rDNA repeats at these loci, and the other signal is minor. Two of the major sites are located at the ends of the secondary constriction in chromosome 14 and 20. The third major site is on the end of long arm of submedian-centromeric chromosome 25. As far as the minor site is concerned, it is located on the long arm of one submedian-centromeric chromosome 30 (Table 2).

The 18S-26S rDNA probe hybridized to eight chromosomes on metaphase spreads of *C. pitardii* (Fig. 2 C′). Also eight signals are shown on the nucleus (Fig. 2 C). Four sites are located at the end of long arms of chromosome 2, 3, 5 and 7, and the other four ones are at

Table 2
Chromosomal locations of 18S-26S rDNA sites on chromosomes of *C. reticualta* complex

Number	Taxon	Distribution of 18S-26S rDNA		Number of sites
		Chromosomes with sites at the end of long arms	Chromosomes with sites at the end of short arms	
1	Hexaploid *C. reticulata* Lindl.	49, 57, 74, 75	8, 9, 12, 24, 29, 30, 37, 43, 44, 65, 69, 70, 80, 85	18
2	Tetraploid *C. reiticulata* Lindl.	29, 32, 40, 56	7, 37, 46, 48, 51, 52, 57, 58	12
3	Diploid *C. reticulata* Lindl.	18, 19	1, 5, 9, 14, 20, 21	8
4	*C. japonica* L.	22	3, 4, 19	4
5	*C. yunnanensis* (Pitard es Diels) Cohen Stuart	25, 30	14, 20	4
6	*C. saluenensis* Stapf ex Bean	18, 25	25, 28, 30	5
4	*C. pitardii* Cohen Stuart	2, 3, 5, 7	1, 4, 14, 25	8

the end of short arms of chromosome 1, 4, 14 and 25 (Table 2).

In *C. saluenensis*, five sites are found in the nucleus and chromosomes (Fig. 2 D, D′). They are located on chromosome 18, 25, 28 and 30, that is, one chromosome has two sites, which was ordered chromosome 25 (Table 2). In each case, the sites are at chromosome ends. On the chromosome with two sites, one is consistently less prominent than the other.

4. Discussion

According to the results, there are eight, 12 and 18 signals observed on the chromosomes of diploid, tetraploid and hexaploid *C. reticulata*. Meantime, four, four, eight and five rDNA sites are distributed in the chromosomes of diploid *C. japonica*, *C. yunnanensis*, *C. pitardii* and *C. saluenensis* respectively (Table 2). It is indicative that the number of 18S-26S rDNA sites is not consistent with the ploidy in the complex, which confirms the results of previous cytological studies with meiotic cells that they are allotetraploid and allohexaploid [3,6]. A number of hypotheses may explain the results. First, the number of copies of the rDNA repeats present at some diploids may be too few to be detected. If copy number falls below the threshold for in situ hybridization, e.g. >10 kb [12], we may not be able to reliably detect the site. Childs, Maxson, and Kedes suggested that it is possible for single rDNA sequences at different chromosomal sites to amplify quickly and form functional nucleolar organizing regions (NORs) under certain conditions [13]. The second possible explanation is, translocations with breakpoints located in the middle of 18S-26S may have bisected and thus duplicated sites following polyploidization of *C. reticulata*. Third, some new rDNA loci may have been formed in the polyploid by transposition of sequences containing rDNA repeats. Fourth, deletions may have eliminated loci in modern diploids. Or the diploids examined here may not represent the ancestral donors of the polyploid genomes. The similar hypothesis was given for the cotton [14].

From the results, we found fluorescent signals of the detected rDNA sites are all located at the terminal positions of corresponding chromosomes. In this way, we put forward the name, 'terminal NOR', instead of satellite as we consider that this name may be more appropriate than the name, satellite, for this kind of rDNA sites.

The number of 18S-26S rDNA sites in *C. pitardii* is equal to the sum of those found in diploid *C. reticulata* and both have the loci at the end of short arms of subterminal-centromeric chromosome 1 and 14. However, the positions of the other six sites are different (Table 2, Fig. 1 A′, Fig. 2 C′). There are four signals observed at the end of short arms and other two at the end of long arms in *C. pitardii*, whilst, on the chromosomes of diploid *C. reticulata* the six sites are all located on the short arms of chromosomes. The results reflect that rDNA segment varies between them, indicating the genetic differentiation between these two species. Morphologically the diploid types of *C. reticulata* were similar to its polyploid species, leaves blades are broad elliptic or elliptic, acute to acuminate. The skin of its capsule is thick, while that of *C. pitardii* is much thinner, so easy to distinguish from the morphological characters.

Comparing the karyotype formula between *C. yunnanensis* ($2n=19$ m (3SAT)+11 sm (2SAT)) and *C. japonica* ($2n=21$ m+4 sm+5 st) [4,10], we found that the difference is obvious. While according to the results here, the two species have the same number of rDNA

signals. Meantime three of them are major and the other one is minor (Fig. 2 A′, B′).

Based on the karotype formula, that of *C. pitardii* ($2n = 30 = 16$ m+10 sm+4 st) is similar to *C. saluenensis* ($2n = 30 = 18$ m+8 sm+4 st) [3,4]. It is, therefore, difficult to find the differentiation between them based on the cytological characters. FISH results show, the number and position of 18S-26S rDNA both differ, which unveils their genetic differentiation on the molecular level. On the chromosomes of *C. saluenensis*, there are five rDNA sites, two of them at the end of long arms and three at the end of short arms (Table 2, Fig. 2D′). On the two terminal ends of chromosome 25, the rDNA sites exist simultaneously. The possible explanation for this is translocation of rDNA repeat happened from one chromosome to another bearing one rDNA locus.

On the chromosomes of tetraploid and hexaploid of *C. reticulata*, there are 12 and 18 18S-26S rDNA sites respectively. There are four sites located at the ends of long arms of two median-centromeric and two submedian-centromeric chromosomes (Table 2, Fig. 1B′, C′), indicating their close relationship. Secondary constrictions are considered as NORs that are active in the preceding interphase [11]. The number of 18S-26S rDNA sites recorded here far exceeds the number of secondary constrictions shown in the studies of the karyotypes of tetraploid and hexaploid *C. reticulata* [3,6]. It means that using FISH some new NOR sites can be found.

The comparative analysis shows the number and position of 18S-26S rDNA sites are not corresponded with ploidy in *C. reticulata* complex. Physical map of 18S-26S rDNA in the genomes shows the differentiation happened in the complex. There has been a loss of NORs in the evolution of other polyploids. For instance, in the hexaploid oat, *Avena sativa*, there are no rDNA sites on the C-genome chromosomes [15]. Similarly, in tetraploid and hexaploid *Scillla autumnalis*, the rDNA sites have been deleted from the A genome [16]. One chromosome in *C. saluenensis* has two 18S-26S rDNA sites on both ends, while in polyploid *C. reticulata*, no case is like this. The genomes may differ as a result of structural rearrangements.

Grant pointed that polyploidy is a common and widespread genetic system in higher plants. Generally in a polyploid complex the tetraploid firstly forms through hybridization and polyploidization with proper internal and external factors, and then the same factors operate to promote the origin of other new tetraploids from diploids. These factors can then lead the tetraploids to produce hexaploids and octoploids [17]. Thus, based on all the results presented here and reported in the previous studies, the conclusion was drawn that hexaploid *C. reticulata* was possibly resulted from interspecific hybridization and continued polyploidization among its ancient diploid species.

The multiplicity of sites, facility of detection and relative variability of rDNA loci indicate that rDNA loci will have considerable utility in future molecular cytogenetic studies of *C. reticulata* complex and the diploid allies. As of yet, we have been unable to distinguish the chromosomes of the possible donors from those of polyploid *C. reticulata* using GISH (genomic in situ hybridization) (unpublished results). It is necessary to obtain the species specific DNA as the probe for GISH. Meanwhile, sequencing of the ribosomal spacer segments of multiple clones derived from the different species is ongoing, which would be helpful to clarify the taxonomic relationships in the complex.

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