



Research article

A refined *Panax ginseng* karyotype based on an ultra-high copy 167-bp tandem repeat and ribosomal DNAs



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ABSTRACT

Background: *Panax ginseng* Meyer (Asian ginseng) has a large nuclear genome size of > 3.5 Gbp in haploid genome equivalent of 24 chromosomes. Tandem repeats (TRs) occupy significant portions of the genome in many plants and are often found in specific genomic loci, making them a valuable molecular cytogenetic tool in discriminating chromosomes. In an effort to understand the *P. ginseng* genome structure, we characterized an ultrahigh copy 167-bp TR (Pg167TR) and explored its chromosomal distribution as well as its utility for chromosome identification.

Methods: Polymerase chain reaction amplicons of Pg167TR were labeled, along with 5S and 45S rDNA amplicons, using a direct nick-translation method. Direct fluorescence *in situ* hybridization (FISH) was used to analyze the chromosomal distribution of Pg167TR.

Results: Recently, we reported a method of karyotyping the 24 chromosome pairs of *P. ginseng* using rDNA and DAPI (4',6-diamidino-2-phenylindole) bands. Here, a unique distribution of Pg167TR in all 24 *P. ginseng* chromosomes was observed, allowing easy identification of individual homologous chromosomes. Additionally, direct labeling of 5S and 45S rDNA probes allowed the identification of two additional 5S rDNA loci not previously reported, enabling the refinement of the *P. ginseng* karyotype.

Conclusion: Identification of individual *P. ginseng* chromosomes was achieved using Pg167TR-FISH. Chromosome identification is important in understanding the *P. ginseng* genome structure, and our method will be useful for future integration of genetic linkage maps and genome scaffold anchoring. Additionally, it is a good tool for comparative studies with related species in efforts to understand the evolution of *P. ginseng*.

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1. Introduction

The presence of repetitive elements (REs) is the main factor responsible for the huge variations in nuclear genome size among angiosperms [1,2]. REs are categorized as either dispersed repeats, i.e., transposable elements (TEs) or tandem repeats (TRs). As their names denote, dispersed REs are often loosely distributed throughout the genome [3], whereas TRs are organized in a head-to-tail fashion in distinct chromosomal regions [4,5].

Satellite DNAs (satDNAs) are a special class of TRs that consist of monomers, often of 150–400 bp but occasionally reaching a few thousand base pairs, whose repeat array size can extend to several hundred megabase pairs [2,6]. The satDNAs include the ribosomal RNA gene families (5S and 45S rDNA), centromeric and subtelomeric TRs, and heterochromatin “knobs” [5–12]. The resulting distinct chromosomal distribution of satDNAs allows them to be used as efficient cytogenetic markers in identifying homologous and homeologous chromosomes, thus facilitating karyotyping and comparative cytogenetics among closely related taxa [13–16].

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Fluorescence *in situ* hybridization (FISH) is a powerful tool to physically localize genes and REs in chromosomes [17]. Probes can be prepared through random priming [18], polymerase chain reaction (PCR) [19], or nick translation [20] labeling methods, which incorporate haptens or fluorochromes in indirect and direct methods, respectively [17].

Panax ginseng Meyer (Asian ginseng) is a perennial herb highly valued for its ginsenosides, which are reputed to have a wide range of medicinal effects [21–23]. *P. ginseng* is the most widely studied species in the genus *Panax*, but most studies have focused on the plant's pharmacological effects [22,23]. Nevertheless, there has been a growing interest in *P. ginseng* molecular cytogenetic and genomic studies in recent years [3,24].

P. ginseng has a large haploid nuclear genome size of > 3.5 Gbp in 24 chromosomes [3,24–26]. The large genome size compared with other species in the genus, is attributed to two rounds of whole genome duplications that occurred 24.6–32.8 million years and 1.6–3.3 million years ago prior to its divergence with *Panax quinquefolius* [27]. Consequently, both *P. ginseng* and *P. quinquefolius* are known tetraploids having $2n = 4x = 48$ chromosomes. Despite the shared whole genome duplications, *P. quinquefolius* has

approximately 4.9 Gbp haploid genome equivalent, which is about 1.4 Gbp larger than that of *P. ginseng*, although both have the same number of chromosomes (24 chromosome pairs). The big variation in genome size between this two closely related *Panax* species, *P. ginseng* and *P. quinquefolius*, is an interesting feature to be elucidated further.

A large proportion of the *P. ginseng* genome is covered by REs [3]. In our previous analysis of three repeat-rich *P. ginseng* BAC clones, we identified a 167-bp TR [3]. Here, we present the sequence characteristics, chromosomal distribution, and cytogenetic marker potential of this *P. ginseng* TR (Pg167TR). Additionally, we demonstrated the efficiency of direct nick-translation labeling of FISH probes to detect smaller 5S rDNA loci.

2. Materials and methods

2.1. PCR amplification of Pg167TR array and 5S rDNA

Primers flanking the entire Pg167TR locus in BAC PgH005J07 (KF357942) (5'-ATT TGA GTT TGT ATT CTT CAA GTT AGG TG-3' and 5'-AAC TGG ACA CAA AGA TCC ATG TTA TTC-3') were designed

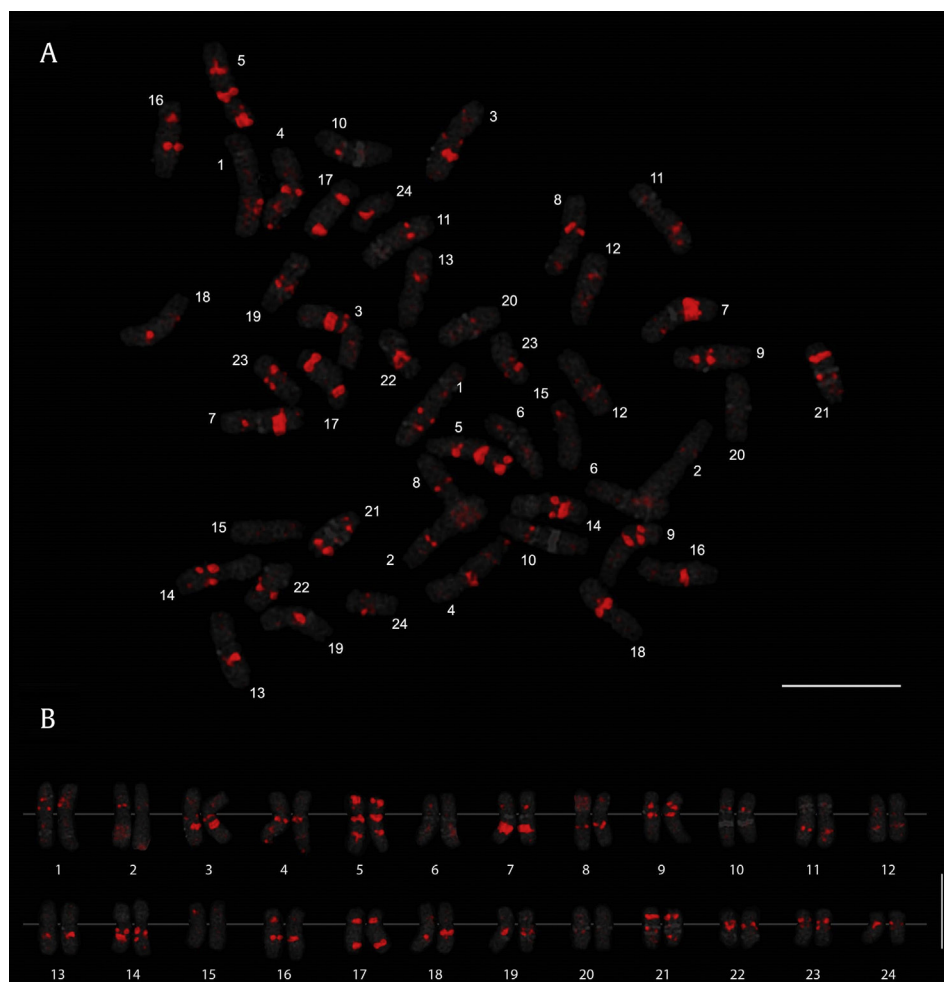


Fig. 3. Genomic distribution of Pg167TR and identification of homologous chromosomes. (A) FISH analysis—using the BAC-amplified 1,862-bp Pg167TR PCR product as a probe—shows distinct chromosome distribution and abundance, allowing for easy identification of chromosomes. (B) Karyogram of ginseng using only Pg167TR signals and the recognizable DAPI bands. This allows refinement of the previously reported ginseng karyotype. Bars, 10 μ m. DAPI, 4',6'-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; PCR, polymerase chain reaction.

Table 1
Summary of Pg167TR distribution in 24 *Panax ginseng* chromosomes

Chr. No.	Chromosome features
1	2 medium-intense Pg167TR loci on 1S, 1 being pericentromeric & the other intercalary. Another 2 weak Pg167TR loci on 1L flanking the 1L DAPI band.
2	1 pericentromeric Pg167TR locus on 2S.
3	Large & intense Pg167TR on intercalary 3L, with small & weak loci on pericentromeric 3L & intercalary 3S.
4	3 Pg167TR loci on 4L, pericentromeric, intercalary (about 50% from centromere & telomere), & telomeric, with the pericentromeric signal being the most intense.
5	This chromosome is easily distinguishable owing to its intense Pg167TR signals localized in the pericentromeric & intercalary regions of 5L & subtelomeric region of 5S. Another weak Pg167TR signal can be seen just proximal to the 5S intense signal.
6	Weak Pg167TR signal proximal to the intercalary DAPI band on 6L. Another 2 weak Pg167TR signals on 6S, 1 at the intercalary region, & 1 at the pericentromeric area.
7	This is easily distinguishable for its large & intense Pg167TR signal at the intercalary region of 7L, 1 of the most intense signals in the genome. Additional Pg167TR signal is localized at the intercalary region of 7S.
8	Intercalary 8L Pg167TR signal.
9	Pg167TR signals localized at the pericentromeric regions of 9S & 9L.
10	Only 1 Pg167TR signal at the centromeric region.
11	1 intercalary Pg167TR signal proximal to the subtelomeric DAPI band on 11L.
12	Intercalary 12L DAPI band. PgDel2-rich, concentrated at the centromeric up to the intercalary regions of 12L. Weak pericentromeric 12S & intercalary 12L Pg167TR signals.
13	1 intercalary Pg167TR signal on 13L, proximal to the DAPI band.
14	2 intense Pg167TR loci can be found in the intercalary region of 14L, which sometimes overlap & can be seen as 1 large signal in some spreads.
15	Weak intercalary Pg167TR signal on 15S.
16	1 intense intercalary Pg167TR signal proximal to the DAPI band on 16L, another weak signal on 16S.
17	Intense Pg167TR signals that correspond to the DAPI bands on both arms. This chromosome is easily distinguishable owing to the intense Pg167TR signals.
18	Intercalary 18L & weak pericentromeric 18S Pg167TR signals.
19	2 closely localized intercalary 19L Pg167TR signals that sometimes overlap to be seen as 1 signal. Another weak intercalary Pg167TR signal seems to colocalize with the 19S DAPI band.
20	1 weak 20L Pg167TR signal just distal to the 20L DAPI band.
21	Intense centromeric Pg167TR locus is observable, plus 2 other weaker loci on 21L, 1 in between the DAPI bands, & 1 at the subtelomeric area.
22	2 Pg167TR loci, 1 at the pericentromeric region of each arm, that can overlap to be seen as 1 signal.
23	2 Pg167TR loci, 1 at the pericentromeric region of each arm, that can overlap to be seen as 1 signal. This looks similar to chromosome 22 except for the DAPI band that is absent here.
24	2 closely localized Pg167TR at the pericentromeric area of 24L.

DAPI, 4',6-diamidino-2-phenylindole.

using the online tool Primer3 [28]. Another set of primers was designed from the internal region of the Pg167TR repeat unit to amplify Pg167TR from genomic DNA (gDNA) template (5'-GAG GCG GGT TTT GAC CTA TT-3' and 5'-CCA CGC AAA CAC ACA CGT A-3'). The PCR mixture (total volume of 50 μ L) consisted of 2.5 U *ExTaq* DNA polymerase, 5 μ L 10 \times *ExTaq* buffer, 2.5mM each dNTP (RR001A; TaKaRa Bio Inc., Kusatsu, Shiga, Japan), 0.3 μ M each primer, < 500 ng BAC PgH005J07 or *P. ginseng* gDNA template, and DNase-free water (W4502; Sigma–Aldrich, St. Louis, MO, USA). The thermocycling conditions were as follows: initial denaturation at 95°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 4 minutes, and a final extension at 72°C for 10 minutes. The 5S rDNA coding region from *Brassica oleracea* was amplified to be used as a probe with primers and amplification conditions reported previously [29].

2.2. Restriction digest

The Pg167TR array from BAC PgH005J07 was searched *in silico* using CLC Main Workbench ver. 7.6.4 (Qiagen, Aarhus, Denmark) for restriction sites appearing at regular intervals among repeat units. PCR products were digested with *TaqI* (ER0671; Thermo Fisher Scientific Inc., Waltham, MA, USA) following the

manufacturer's instructions. High-resolution fragment analysis was done using a Fragment Analyzer (Advanced Analytical Technologies, Inc., Ankeny, IA, USA).

2.3. FISH analysis

Root mitotic chromosome spreads were prepared from stratified seeds provided by the Korea *P. ginseng* Corporation Natural Resources Research Institute (Daejeon, Korea), according to the methods described by Waminal et al [24]. Pg167TR and *B. oleracea* 5S rDNA amplicons were labeled with Texas Red-5-dUTP (NEL417001EA; Perkin Elmer, Waltham, MA, USA), and a 9-kb fragment of 45S rDNA (18S–5.8S–25S) [30] was labeled with Alexa Fluor 488-5-dUTP (C11397; Invitrogen, Waltham, MA, USA) through direct nick-translation labeling. FISH procedures were performed as described previously [24]. Briefly, slides were immediately used for FISH after fixation with 4% paraformaldehyde, without pepsin and RNase pretreatment. Images were captured with an Olympus BX53 fluorescence microscope equipped with a Leica DFC365 FS CCD camera, and processed using Cytovision version 7.2 (Leica Microsystems, Wetzlar, Germany). Further image enhancements and creation of the idiogram were performed in Adobe Photoshop CC.

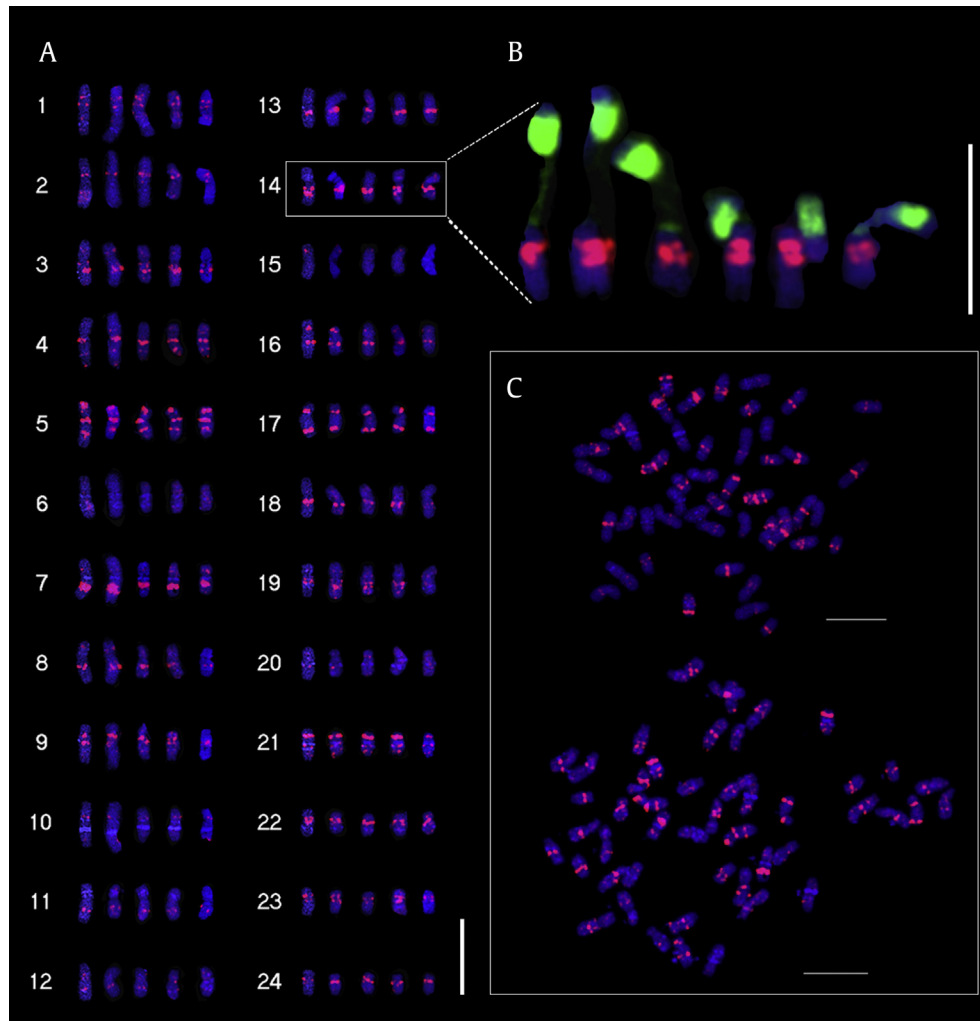


Fig. 4. Cytogenetic mapping of Pg167TR. (A) Identification of ginseng chromosomes from several root mitotic chromosome spreads using Pg167TR. (B) Consistent rDNA and Pg167TR patterns for chromosome 14 demonstrates reliability of Pg167TR for chromosome identification. (C) Two mitotic chromosome spreads with Pg167TR signals used for chromosome identification. Bars, 10 μm .

3. Results and discussion

3.1. PCR and sequence analysis of Pg167TR array

Repeat unit analysis of the Pg167TR locus in BAC PgH005J07 (nt 10,048–11,650) with Tandem Repeat Finder [31] revealed 9.6 repeat units in the array (Fig. 1A) [3]. Sequence alignment of the nine repeat units showed high GC content at the nucleotide positions 80–100 (Fig. 1B), and *in silico* restriction digestion revealed *TaqI* restriction sites at regular intervals, which would result in fragments ranging from 149 bp to 169 bp (Figs. 2A, 2C, and 2D). PCR with primers flanking Pg167TR in BAC PgH005J07 amplified the main 1,682-bp target with additional ladder-pattern amplicons, and a smear pattern of high molecular weight amplicons. The same primer set with gDNA template amplified a similar pattern as found with the BAC template (data not shown), indicating that such amplification of this Pg167TR locus is independent of the PCR template. In addition, PCR with primers designed from the internal region of the Pg167TR unit and using gDNA template to amplify other units in the genome also amplified a smear pattern from about 150 bp to much longer fragments. This smear pattern in both

BAC and gDNA indicates that there are multiple annealing sites, as is generally the case for REs (Fig. 2B). Further analysis may help us understand the nature, origin, and impact of Pg167TR in the *P. ginseng* genome, i.e., whether it is associated with other TEs as in the case of other satDNAs [5,32].

Restriction digestion of both BAC and gDNA amplicons with *TaqI* enzyme supported the *in silico* prediction of restriction size fragments, indicating that the amplicons are Pg167TR-associated (Figs. 2A–2D). A major satDNA in *P. ginseng* with features that resemble those of Pg167TR was previously reported [33]. Although these authors observed a 170-bp repeat unit (compared with up to 168 bp for Pg167TR), validation could not be carried out because precise sequence information was not available (personal communication). Nevertheless, they reported that this repeat is one of the most abundant satDNA repeats in *P. ginseng* and showed a gel ladder pattern after digestion with *HinI* that was similar to our *TaqI* digestion results. To explore this similarity further, we carried out *in silico* restriction analysis of Pg167TR, which revealed *HinI* restriction sites near the *TaqI* sites (Fig. 2A), suggesting that the satDNA identified in the previous report is most likely Pg167TR.

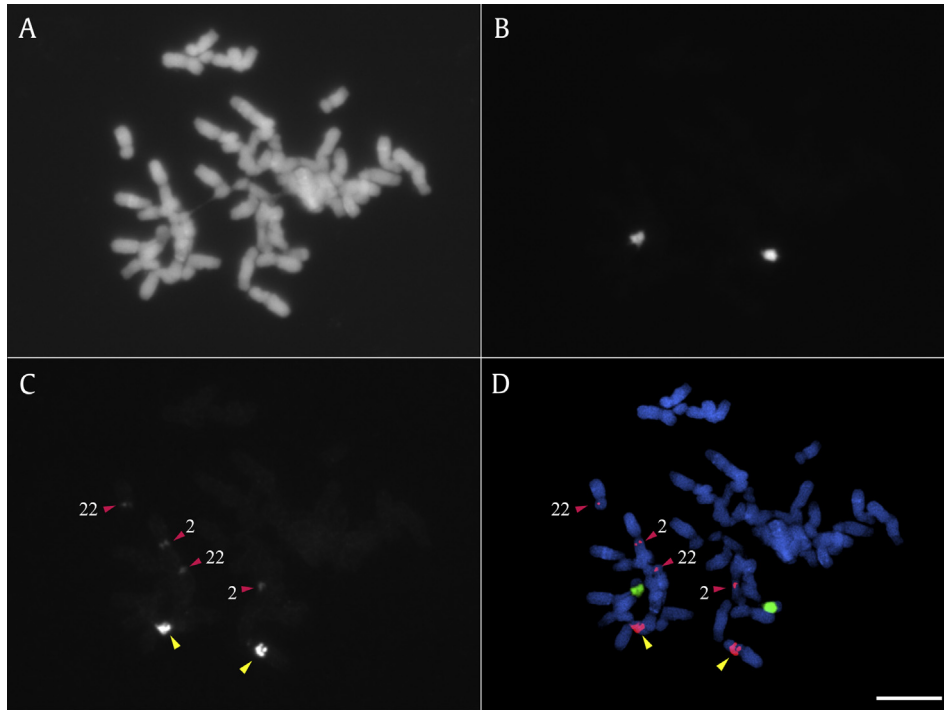


Fig. 5. Minor 5S rDNA loci. Two previously unidentified 5S rDNA loci (arrowheads in C) were observed using direct-labeled probes. These minor loci were localized in the proximal region of 2S and the interstitial region of 22S. (A–D) Raw DAPI, raw 45S rDNA, raw 5S rDNA, and merged signals with 45S rDNA in green. Bar, 10 μ m. DAPI, 4',6-diamidino-2-phenylindole.

3.2. Identification of homologous chromosomes using Pg167TR probe

Efficient identification of homologous chromosomes provides a more robust cytogenetic map of *P. ginseng* for future integration with genetic linkage maps and pseudo-chromosomes from genome assembly scaffolds. It also enables comparative cytogenetics among *Panax* and related species, which will allow a cytogenetic view of the history of *P. ginseng* and related genomes. Cytogenetic mapping of Pg167TR elements enabled the identification of individual *P. ginseng* chromosomes, allowing the refinement of the *P. ginseng* karyogram. We previously prepared a *P. ginseng* karyogram with only 4',6-diamidino-2-phenylindole (DAPI) bands and rDNA as cytogenetic markers, which was challenging considering the fairly small and uniform size of *P. ginseng* chromosomes [24].

With Pg167TR as a cytogenetic marker, all 24 pairs of homologous chromosomes were identified successfully (Figs. 3A and 3B, Table 1). Although certain chromosomes required more detailed analysis, some signals were readily distinguishable. For instance, chromosome 5 was easily distinguished by the presence of three very intense signals, two of which were localized respectively in the pericentromeric and intercalary regions of the long arm (5L), whereas the third was located in the subtelomeric region of the short arm (5S). Another example was chromosome 7, identifiable by a large block of Pg167TR loci at the intercalary region of 7L. Although some chromosomes (e.g., 22–24) bore similar Pg167TR signal patterns, they could be distinguished via other characteristics, such as chromosome length, DAPI bands, and centromeric indices. This Pg167TR-based method thus enabled the identification of individual chromosomes from different chromosome spreads (Figs. 4A–4C).

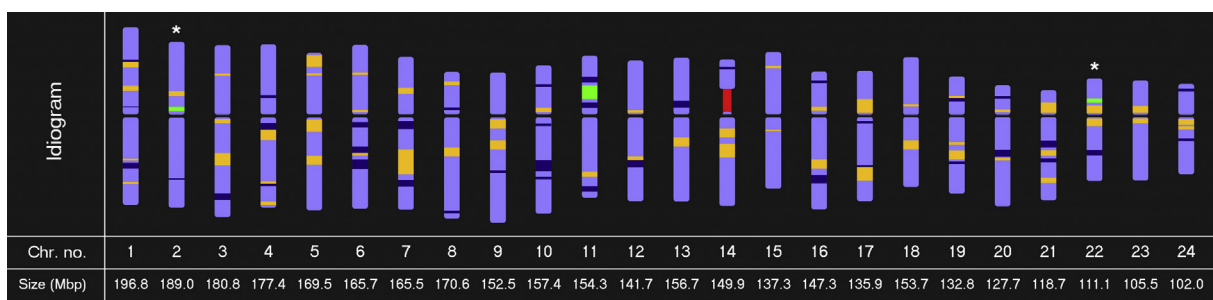


Fig. 6. FISH ideogram of ginseng. 5S and 45S rDNA are indicated by green and red bars, respectively. DAPI bands are indicated by dark blue bars. Pg167TR signals are indicated by orange bars. Asterisks indicate chromosomes in which minor 5S rDNA signals were detected. Chromosome sizes are based on the 3.6-Gbp haploid genome size of ginseng. DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization

3.3. Additional minor 5S rDNA loci

FISH experiments rely on the efficiency of the probe hybridization and signal detection [34,35]. Background noise is often a major problem, and a high signal/noise ratio is difficult to achieve using conventional FISH techniques involving indirectly labeled probes (using haptens such as dig and biotin) because of the nonspecific binding of antibodies. Direct labeling of FISH probes provides an advantage in terms of the signal/background ratio and time required [34]. In addition, a shorter total probe length can be detected with minimal background noise after efficient labeling [34]. With directly labeled probes, we observed two additional minor loci in the proximal region of chromosome 2 and interstitial region of chromosome 22 (Figs. 5A–5D). These 5S loci were not previously detected most likely because of the lower signal/background ratio associated with the indirect FISH used previously. Our previous experiment used 5S rDNA from *Brassica rapa* [24,36] rather than that of *B. oleracea* as probe. However, coding regions of 5S rDNA are conserved across unrelated taxa [37,38], suggesting that the difference in the ability to detect these loci is likely not related to the probes used but rather to the techniques used for labeling and FISH, which considerably influence the end signal [34]. A karyotypic idiogram of the *P. ginseng* chromosomes showing the DAPI bands, rDNA and Pg167TR signals is shown in Fig. 6.

3.4. Summary

The chromosomal distribution of Pg167TR indicates that it accounts for a considerable portion of the *P. ginseng* genome. Molecular cytogenetic analysis of Pg167TR, which is first presented in this study, enabled the identification of each *P. ginseng* chromosome despite their relatively uniform lengths, demonstrating its utility in *P. ginseng* karyotyping. This cytogenetic analysis allowed refining of the *P. ginseng* karyotype, which will promote future integration of *P. ginseng* genetic maps, the validation of pseudo-chromosomes from the *P. ginseng* genome assembly, and comparative analysis with related species to elucidate the evolutionary history of the *P. ginseng* genome.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

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