

## Karyotype Analysis of *Lablab purpureus* (L.) Sweet Using Fluorochrome Banding and Fluorescence *in situ* Hybridisation with rDNA Probes

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### Abstract

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The mitotic chromosomes of *Lablab purpureus* (L.) Sweet were characterised using sequential combined propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) (CPD) staining and fluorescence *in situ* hybridisation (FISH) with 5S and 45S rDNA probes. The detailed karyotype of this species was established using prometaphase chromosomes. After CPD staining, CPD and DAPI<sup>+</sup> bands were shown simultaneously. CPD bands occurred in the proximal regions of the long arms of all chromosome pairs and at all 45S rDNA sites, while the DAPI<sup>+</sup> bands appeared in all centromeres. FISH with rDNA probes revealed one 5S locus and eight 45S loci. The single 5S locus was interstitially located on the long arms of the shortest chromosome pair. Among the 45S loci, two large loci were located in the secondary constrictions of the short arms of two chromosome pairs; six small or minimal loci were proximally located on the short or long arms of six chromosome pairs. Each prometaphase chromosome pair could be identified using the CPD and DAPI<sup>+</sup> bands, the rDNA-FISH signals in combination with the chromosome measurements and condensation patterns. The karyotype was formulated as  $2n = 2x = 22 = 14m (2SAT) + 6sm + 2st (2SAT)$ , and the asymmetry indices, CI, A<sub>1</sub>, A<sub>2</sub>, As K%, AI and the Stebbins category were  $38.23 \pm 7.06$ , 0.36, 0.31, 61.99, 5.68 and 2B, respectively.

**Keywords:** combined PI and DAPI staining; FISH; hyacinth bean; karyotyping; ribosomal gene

*Lablab purpureus* (L.) Sweet (formerly *Dolichos lablab* L., hyacinth bean), the only species in the monotypic genus *Lablab*, is native to Africa and cultivated as a food crop throughout the tropics (SMARTT 1990). Hyacinth bean is often grown as vegetables (pods and beans), forages, green manure and cover crops (SMARTT 1990). In addition, it is also cited as medicinal plant (Chinese Pharmacopoeia Commission 2010).

In most crop plants, karyotype analyses have been used to characterize cultivars, to integrate genetic and physical maps, to investigate the relationships among species, etc. (FUCHS *et al.* 1998; MOSCONE

*et al.* 1999; DE MORAES *et al.* 2007; SHE *et al.* 2015). However, karyotyping is usually hampered by the paucity of chromosome markers, which limits the identification of individual chromosomes. To circumvent this shortcoming, chromosome banding techniques such as C-, N-, and fluorochrome banding, as well as fluorescence *in situ* hybridization (FISH) with repetitive sequences as probes were successively applied. Among the fluorochrome banding techniques developed for plant chromosome analysis, the combined propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) staining (called CPD staining) has been proved to be able to reveal

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simultaneously GC-rich and AT-rich regions along chromosomes (PETERSON *et al.* 1999; SHE *et al.* 2006, 2015). The FISH with 5S and 18S-5.8S-25S ribosomal genes (5S and 45S rDNA), the most common application of FISH, can generate effective markers for chromosome identification, and provide valuable information about a genome evolution (MOSCONE *et al.* 1999; HASTEROK *et al.* 2001; DE MORAES *et al.* 2007; SHE *et al.* 2015). Furthermore, the combination of rDNA-FISH with fluorochrome banding has been shown to be a powerful tool for constructing detailed karyotypes displaying chromosome morphological features, heterochromatin distribution, physical locations of repetitive DNAs and providing insights into the evolutionary relationships among related species (e.g. ZOLDOS *et al.* 1999; CHAOWEN *et al.* 2004; DE MORAES *et al.* 2007; DE A BORTOLETI *et al.* 2012; SHE *et al.* 2015).

Although *L. purpureus* is of agricultural importance, its chromosome characterisation at a molecular cytogenetic level has not been conducted until now, in part due to the difficulty of cytogenetic analysis of their small, morphologically homogeneous chromosomes. Previous cytogenetic studies on *L. purpureus* were primarily restricted to the karyotype analysis using conventional staining (LI 1989; CHEN 2003; ALI *et al.* 2011). Very recently, FISH mapping of 5S and 45S rDNA in *L. purpureus* was reported (IWATA *et al.* 2013), but the molecular cytogenetic karyotype of this species is still unavailable.

In plants with small genome, the metaphase chromosomes are usually difficult to be distinguished resulting from their weak morphological differentiation, but the use of prometaphase cells is effective for the identification of individual chromosomes because characteristics caused by the uneven condensation of chromatin fibres along chromosomes are more prominent at this stage (FUKUI & MUKAI 1988). Prometaphase chromosomes have been employed to construct the karyotypes of many plant species (e.g. FUKUI & IJIMA 1991; FUKUI *et al.* 1998; GUIMARÃES *et al.* 2012).

In the present study, the genomic organisation of *L. purpureus* was analysed using sequential CPD staining and FISH with 5S and 45S rDNA probes. A detailed karyotype of this species was established using the combined dataset of prometaphase chromosome measurements, fluorochrome banding, and FISH mapping of rDNA families, providing the primary molecular-cytogenetic characterisation of this species.

## MATERIAL AND METHODS

**Plant material and chromosome preparation.** The seeds of *L. purpureus* were obtained from Chinese Crop Germplasm Resources Information System (CGRIS). The seeds were germinated on filter papers moistened with tap water at 28°C. The root tips were harvested and directly fixed with 3:1 (v/v) methanol/glacial acetic acid. The preparations were made according to the protocol described in detail by SHE *et al.* (2006). The enzyme solution was composed of 1% cellulase RS (Yakult Honsha Co., Tokyo, Japan), 1% pectolyase Y23 (Seishin Co. Tokyo, Japan), and 1% cytohelicase (Sigma, St. Louis, USA) in citric buffer (4mM citric acid, 6mM sodium citrate, pH 4.5).

**CPD staining.** The CPD staining followed the procedure described by SHE *et al.* (2006). Briefly, chromosome preparations were treated with RNase A and pepsin, and then stained with a mixture of PI (0.6 µg/ml) and DAPI (3 µg/ml). Images were acquired with an Olympus BX60 microscope (Olympus, Tokyo, Japan) and a CoolSNAP camera (Photometrics, Tuscon, USA), using MetaMorph software (Molecular Devices, Sunnyvale, USA). PI and DAPI greyscale images of the same plate were merged to produce a CPD image.

**DNA labelling.** The pTa794 clone containing a 410 bp *Bam*HI fragment of the 5S rDNA from wheat (GERLACH & DYER 1980) and the 45S rDNA clone containing a 9.04-kb 45S rDNA insert from tomato (PERRY & PALUKAITIS 1990) were used as probes. Probes were labelled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively, using the Nick Translation Kit (Roche, Mannheim, Germany).

**Fluorescence *in situ* hybridisation.** FISH with the 5S and 45S rDNA probes was sequentially carried out on the same slides previously stained with CPD. The slides previously stained were washed twice in 2× SSC for 10 min each, dehydrated through an ethanol series (70, 90 and 100%, 5 min each) and then used for hybridisation. The FISH methodology followed that described by SHE *et al.* (2015). The 5S probe was detected using Cy3-streptavidin (Amersham Biosciences, Amersham, UK) and amplified using biotinylated anti-avidin (Vector Laboratories, Burlingame, USA) together with Cy3-streptavidin. The 45S probe was detected by anti-digoxigenin fluorescein (Roche, Mannheim Germany) and amplified with anti-sheep fluorescein (Vector Laboratories). Slides were counterstained with DAPI (3 µg/ml). The images were acquired with an Olympus BX60

microscope and a CoolSNAP camera, and adjusted with Adobe Photoshop CS3 for contrast and background optimization.

**Karyotype analysis.** Karyotype analysis was performed according to the methodology proposed by LI and CHEN (1985). Five prometaphase plates at the same condensation state (as determined by the total length of the chromosome complement) which were subjected to CPD staining and rDNA-FISH, were measured using Adobe Photoshop CS3 to obtain the chromosome relative lengths (RL, % of haploid complement), arm ratios (AR = long arm/short arm), size of the fluorochrome band, and percent distance from the centromere to the rDNA site ( $di = d \times 100/a$ ;  $d$  = distance of the centre of the rDNA sites from the centromere;  $a$  = length of the corresponding chromosome arm). The total length of the haploid complement (TCL) was measured using five metaphase cells with the highest degree of chromosome condensation. The arm ratio was used to classify the chromosomes according to the system described by LEVAN *et al.* (1964): 1.0–1.7, metacentric (m); 1.71–3.0, submetacentric (sm); 3.01–7.0, subtelocentric (st), and 7.01– $\infty$ , telocentric (t). Chromosome number assignment was based on the descending order of chromosome lengths. For numerical characterisation of the karyotype, the following parameters were calculated: average centromeric index (CI), intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2), ratio of the length of all long arms in the chromosome set to the total chromosome length in the set (As K%), asymmetry index (AI), and the categories of Stebbins (PASZKO 2006).

## RESULTS AND DISCUSSION

**Detailed karyotype.** The prometaphase chromosomes were exactly identified using the measurements and condensation patterns in combination with the fluorochrome bands and rDNA-FISH signals (Figures 1a, b), while the metaphase chromosomes were not differentiated well morphologically and they were difficult to be completely identified (Figures 1c, d). Data of measurements from five prometaphases are listed in Table 1. The CPD-stained and rDNA-FISH karyotypes are presented in Figures 1f and 1g, respectively. The idiogram displaying the chromosome measurements, position and size of the fluorochrome bands and rDNA-FISH signals is presented in Figure 2.

The chromosome complement of *L. purpureus* was composed of 7 metacentric, 3 submetacentric, and

one subtelocentric homologous pairs. Chromosome pairs 2 and 7 were satellite chromosomes (SAT) with secondary constrictions (SCs) located on the short arms (Figure 1a). In prometaphase cells, the satellites were frequently found separated from the rest of the chromosomes and the SCs were stretched (Figure 1a), while in metaphase cells the SCs of pair 7 were not clear because of the high degree of condensation (Figure 1c). In summary, the karyotype was formulated as  $2n = 2x = 22 = 14m (2SAT) + 6sm + 2st (2SAT)$ . The six asymmetry indices, CI, A1, A2, As K%, AI and Stebbins category were  $38.23 \pm 7.06$ , 0.36, 0.31, 61.99, 5.68 and 2B, respectively. The current karyotype formula differed from the previous formulae described by LI (1989), CHEN (2003) and ALI *et al.* (2011). Such discrepancies were probably due to differences in analysed materials and mitotic stages used, and difficulty in identifying chromosomes using the classical staining technique before.

The metaphase chromosomes were small, 36.65  $\mu\text{m}$  being the total length of the haploid complement (TCL). The lengths of individual metaphase chromosomes ranged from 4.76 to 2.63  $\mu\text{m}$ . The TCL of the prometaphase cell illustrated in Figure 1a was 65.58  $\mu\text{m}$ . The current metaphase TCL of *L. purpureus* is longer than those previously reported (LI 1989; ALI *et al.* 2011). This observed difference could mainly be related to variation in the chromosome condensation levels of measured cells (SHE *et al.* 2015).

**Fluorochrome bands.** After CPD staining, red-fluorescing bands (called CPD bands) and blue-fluorescing bands (called DAPI<sup>+</sup> bands) were shown simultaneously (Figure 1a). CPD bands occurred in the proximal regions of the long arms of all chromosome pairs and in the regions corresponding to the 45S rDNA-FISH hybridisation sites (see below), while DAPI<sup>+</sup> bands appeared in all centromeres. The centromeric DAPI<sup>+</sup> bands became better contrasted after the FISH procedure (Figures 1b, g, h). Both the CPD and DAPI<sup>+</sup> bands in metaphase cells were less clear than those in prometaphase cells (Figures 1c, d). The CPD bands corresponding to the 45S rDNA sites of pairs 3, 4, 5 and 11 were juxtaposed to the respective proximal CPD bands; and those located pericentromerically on the short arms of pairs 1 and 8 were faint, and they could be observed only at prometaphase (Figure 1a). The size of the proximal CPD and centromeric DAPI<sup>+</sup> bands differed more or less among chromosomes (Figures 1f, h; Table 1). The total amount of the proximal CPD bands (including the juxtaposed rDNA CPD bands of pairs 3, 4, 5 and 11)

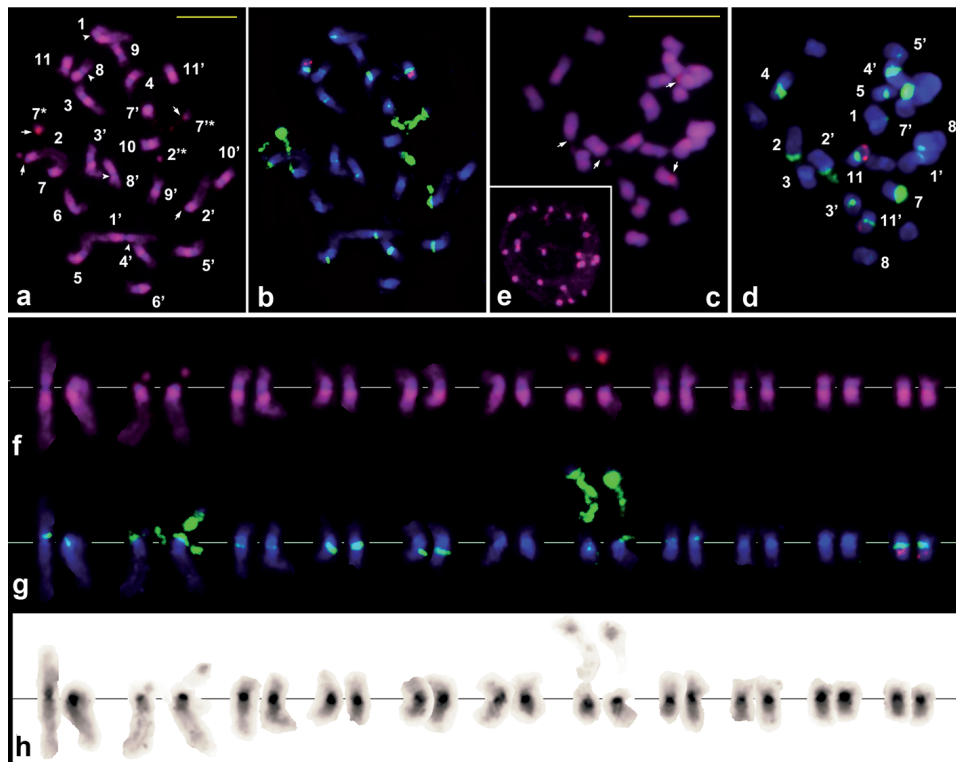


Figure 1. Mitotic chromosomes and interphase nuclei of *Lablab purpureus* after sequential combined propidium iodide and 4',6-diamidino-2-phenylindole (CPD) staining and FISH with 5S and 45S rDNA probes: (a), (c), (e) – CPD banded prometaphase, metaphase and interphase cells, respectively; (b), (d) FISH images that were created using probes as follows: (i) 5S rDNA labelled with biotin and detected by streptavidin conjugated with Cy3 (red) and (ii) 45S rDNA labelled with digoxigenin and detected by anti-digoxigenin conjugated with fluorescein (green); chromosomes were counterstained with DAPI (blue); (f) – the karyotype showing CPD and DAPI<sup>+</sup> bands; (g) – the karyotype showing rDNA-FISH signals and DAPI<sup>+</sup> bands; (h) – the invert of the DAPI image in (g); CPD and FISH images are marked by white arrows indicating the secondary constrictions (SCs) and the respective CPD bands, by white arrowheads indicating the faint CPD bands located on the short arms of pairs 1 and 8, and by the white asterisks indicating the separated satellites of the respective chromosomes; the nomenclature of chromosomes (Arabic numerals) follows the system of LI & CHEN (1985); scale bars represent 10  $\mu$ m

and centromeric DAPI<sup>+</sup> bands accounted for 22.76% and 12.06% of the TCL, respectively (Table 1). In the CPD-stained interphase cells, nineteen to twenty-two heterochromatin blocks within which blue- and red-fluorescing regions were linked together were observed (Figure 1e), corresponding to the proximal CPD banded regions and the centromeric DAPI<sup>+</sup> banded regions of the chromosomes.

The CPD bands have been proved to represent the GC-rich chromosomal regions of plant genomes (PETERSON *et al.* 1999; SHE *et al.* 2006). Our study revealed that, in addition to the 45S rDNA sites, there existed proximal GC-rich heterochromatin in the genome of *L. purpureus*. Centromeric, pericentromeric or proximal GC-rich heterochromatin without colocalization with rDNA sites has also been observed in many Phaseoleae species including the

seven cultivated *Vigna* species (DE A. BORTOLETI *et al.* 2012; SHE *et al.* 2015), the four cultivated *Phaseolus* species (BONIFÁCIO *et al.* 2012), *Psophocarpus tetragonolobus* (CHAOWEN *et al.* 2004), and *Glycine max* (SHE *et al.* unpublished data), suggesting that the presence of such GC-rich heterochromatin was a common genomic characteristic of Phaseoleae species. Our study revealed that *L. purpureus* also had centromeric AT-rich heterochromatin, which has been observed in some chromosome pairs of *Vigna radiata* (DE A. BORTOLETI *et al.* 2012; SHE *et al.* 2015), but not in other Phaseoleae species studied.

**rDNA organisation.** One pair of 5S rDNA signals and eight pairs of 45S rDNA signals were detected by FISH with the rDNA probes (Figures 1b, d, g). The single 5S locus was interstitially located in the long arms of pair 11 (*di* = 51.60%). The eight 45S rDNA



Table 1. Chromosome measurements of *Lablab purpureus*

Chr. No.	Relative length (%)			Arm ratio $\pm$ SD	Type	Proximal CPD band size $\pm$ SD <sup>‡</sup>	Centromeric DAPI <sup>+</sup> band size $\pm$ SD <sup>‡</sup>
	short arm $\pm$ SD	long arm $\pm$ SD	total $\pm$ SD				
1	6.67 $\pm$ 0.91	8.62 $\pm$ 0.70	15.29 $\pm$ 1.50	1.30 $\pm$ 0.14	m	2.58 $\pm$ 0.32	1.29 $\pm$ 0.15
2	2.49 $\pm$ 0.45	9.57 $\pm$ 1.70	12.06 $\pm$ 2.14	3.84 $\pm$ 0.15	st <sup>†</sup>	1.71 $\pm$ 0.32	1.05 $\pm$ 0.37
3	3.54 $\pm$ 0.07	6.84 $\pm$ 0.75	10.38 $\pm$ 0.79	1.93 $\pm$ 0.19	sm	1.98 $\pm$ 0.22	1.05 $\pm$ 0.06
4	3.93 $\pm$ 0.78	5.14 $\pm$ 0.86	9.07 $\pm$ 1.48	1.32 $\pm$ 0.22	m	2.11 $\pm$ 0.29	1.26 $\pm$ 0.16
5	3.76 $\pm$ 0.49	5.15 $\pm$ 0.67	8.91 $\pm$ 1.15	1.37 $\pm$ 0.06	m	2.10 $\pm$ 0.17	1.30 $\pm$ 0.14
6	3.37 $\pm$ 0.37	5.18 $\pm$ 0.55	8.55 $\pm$ 0.74	1.55 $\pm$ 0.21	m	1.99 $\pm$ 0.42	1.14 $\pm$ 0.11
7	3.33 $\pm$ 0.49	4.63 $\pm$ 0.56	7.97 $\pm$ 1.01	1.40 $\pm$ 0.11	m <sup>†</sup>	2.21 $\pm$ 0.53	1.00 $\pm$ 0.19
8	3.60 $\pm$ 0.32	4.31 $\pm$ 0.48	7.90 $\pm$ 0.80	1.20 $\pm$ 0.03	m	2.25 $\pm$ 0.31	1.05 $\pm$ 0.02
9	2.95 $\pm$ 0.56	4.29 $\pm$ 0.22	7.24 $\pm$ 0.72	1.48 $\pm$ 0.25	m	1.88 $\pm$ 0.12	1.06 $\pm$ 0.14
10	2.28 $\pm$ 0.33	4.11 $\pm$ 0.10	6.39 $\pm$ 0.24	1.83 $\pm$ 0.32	sm	1.81 $\pm$ 0.16	1.04 $\pm$ 0.13
11	2.09 $\pm$ 0.06	4.16 $\pm$ 0.33	6.25 $\pm$ 0.27	1.99 $\pm$ 0.21	sm	2.15 $\pm$ 0.31	0.81 $\pm$ 0.06
Total	38.01 $\pm$ 1.73	61.99 $\pm$ 1.17	100			22.76 $\pm$ 1.41	12.06 $\pm$ 1.17

SD – standard deviation; m – metacentric; sm – submetacentric; st – subtelomeric; † satellite chromosome, the length of the satellite was included in the chromosome length but that of the stretched secondary constriction was excluded; ‡ the percentage of the size of the bands of each chromosome pair in relation to the total length of the haploid complement (TCL)

loci varied in position and size. The 45S loci corresponding to the SCs of pairs 2 and 7 were next to the centromeres and displayed strong signals which were highly stretched at prometaphase (Figure 1b). The 45S loci of pairs 4, 5 and 11 were proximally located on the long arms ( $di = 23.67, 26.67$  and  $20.63\%$ , respectively) and showed stronger signals which could usually be detected at both prometaphase and metaphase. The 45S loci situated proximally on the short arms of pairs 1 and 8 ( $di = 14.91, 18.68\%$ , respectively) and on the long arms of pair 3 ( $di = 12.89\%$ ) were minimal, with lower reproductivity of FISH signal especially in metaphase cells (Figure 1d).

In our study, more 45S rDNA loci were revealed in *L. purpureus* compared with the previous study which reported only five 45S loci on metaphase chromosomes (IWATA *et al.* 2013). This difference might mainly be due to the higher sensitivity and resolution of the FISH targeted to prometaphase chromosomes used in our study (CHENG *et al.* 2002). In particular, *L. purpureus* bears a substantial number of proximal 45S rDNA loci, which has not been found in other Phaseoleae species (IWATA *et al.* 2013). Variation in rDNA loci has been postulated to occur by several different mechanisms that can occur alone or in a combination, which include chromosomal rearrangements, unequal crossovers, and/or trans-

position (SCHUBERT & WOBUS 1985; MOSCONE *et al.* 1999; RASKINA *et al.* 2004; DATSON & MURRAY 2006; CHUNG *et al.* 2008). Mobility of rDNA loci does not necessarily result in large-scale changes in chromosome structure (CHUNG *et al.* 2008; SHE *et al.* 2015). The drastic increase of proximal 45S loci in *L. purpureus* might result from transposon-mediated transpositions of the 45S rDNA cluster during or after speciation (RASKINA *et al.* 2004; DATSON & MURRAY 2006; CHUNG *et al.* 2008).

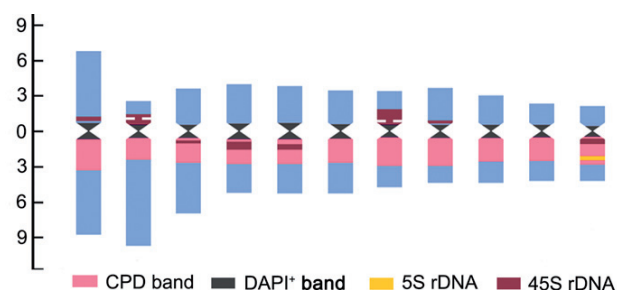


Figure 2. Idiogram of *Lablab purpureus* displaying the chromosome measurements, and the position and size of fluorochrome bands and rDNA FISH signals; the ordinate scale on the left indicates the relative length of the chromosomes

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## CONCLUSIONS

We provide here the first detailed description of the organisation of the *L. purpureus* chromosome complement. The karyotype analysis reveals pronounced GC- and AT-rich heterochromatin differentiation as well as a large number of 45S rDNA loci in *L. purpureus*, differing from the other studied Phaseoleae species (Moscone *et al.* 1999; Chaowen *et al.* 2004; de A. Bortoleti *et al.* 2012; Iwata *et al.* 2013; She *et al.* 2015). Furthermore, the idiogram shown here for this species will be valuable as reference for the development of further cytogenetic maps, integrating genomic markers, and using coding and noncoding DNA sequences for macrosyteny comparison among the Phaseoleae species.

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