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ORIGINAL PAPER

Comparative cytogenetic mapping between the lima bean (*Phaseolus lunatus* L.) and the common bean (*P. vulgaris* L.)

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Abstract The common bean (*Phaseolus vulgaris*) and lima bean (*P. lunatus*) are among the most important legumes in terms of direct human consumption. The present work establishes a comparative cytogenetic map of *P. lunatus*, using previously mapped markers from *P. vulgaris*, in association with analyses of heterochromatin distribution using the fluorochromes chromomycin A3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI) and localization of the 5S and 45S ribosomal DNA (rDNA) probes. Seven BACs selected from different common bean chromosomes demonstrated a repetitive pericentromeric pattern corresponding to the heterochromatic regions revealed by CMA/DAPI and could not be mapped. The subtelomeric repetitive pattern observed for BAC 63H6 in most of the chromosome ends of common bean was not detected in lima bean, indicating lack of conservation of this subtelomeric repeat. All chromosomes could be identified and 16 single-copy clones were mapped. These results showed a significant conservation of synteny between species, although change in centromere position suggested the occurrence of pericentric inversions on chromosomes 2, 9 and 10. The low number of structural rearrangements reflects the karyotypic stability of the genus.

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Introduction

The genus *Phaseolus* is especially important among the many legumes, because five of its species are cultivated for food: *P. vulgaris* L., *P. lunatus* L., *P. coccineus* L., *P. polyanthus* Greenman, and *P. acutifolius* A. Gray. The common bean (*P. vulgaris*) is the most economically important protein source for large numbers of Latin Americans and Africans (Broughton et al. 2003). Although lima beans (*P. lunatus*) are less widely cultivated, they are a very important alternative source of income and food for local populations in regions such a northeastern Brazil (Oliveira et al. 2004). Both common bean and lima bean are of neotropical origin and can be separated into two major gene pools, in the Andes and in Mesoamerica, that are probably related to their respective independent domestication centers (Chacón et al. 2005; Serrano–Serrano et al. 2010).

Diverse linkage maps based on different molecular markers have been developed for the common bean (Adam-Blondon et al. 1994; Freyre et al. 1998; Nodari et al. 1993; Vallejos et al. 1992) and these have been integrated into a chromosome map using fluorescent in situ hybridization (FISH) (Pedrosa et al. 2003). More recently, using this same technique and genomic clones from a bacterial artificial chromosome (BAC) library, a cytogenetic map was constructed, correlating physical and genetic distances, establishing chromosome-specific markers, as well as characterizing the distribution of the heterochromatic regions in the chromosome complement of this species (Fonsêca et al. 2010; Pedrosa-Harand et al. 2009). No genetic or cytogenetic map is currently available, however, for lima bean.

BACs contain inserts with average sizes of approximately 100 kb (Men et al. 2001; Wu et al. 2004; Yang et al. 2003) and unique sequences that are generally conserved among related

species. The development of comparative cytogenetic maps based on the hybridization of heterologous probes has been performed with success in studies of karyotype evolution and has contributed greatly to our understanding of the mechanisms responsible for karyotype differentiation of closely related species. One example is the comparative mapping of chromosome-6 of the tomato and potato, which revealed a break in colinearity within the short arms of the two species (Iovene et al. 2008). Related studies such as for cucumber and melon (Han et al. 2009), *Brachypodium distachyon* and *Hordeum vulgare* (Ma et al. 2010) and among *Daucus* species (Iovene et al. 2011), demonstrated the importance of this technique in homeology analyses.

In the present work, a comparative analysis of the genome of lima bean using fluorescent in situ hybridization of BAC clones that had been previously mapped in the common bean was performed, establishing chromosome homeologies and the evolutionary mechanisms that have shaped these karyotypes. In addition, we comparatively mapped the 5S and 45S rDNA sites, BACs containing repetitive sequences, and heterochromatic regions rich in GC and AT by CMA/DAPI staining.

Materials and methods

Plant material

Seeds of *P. lunatus* 'Vermelhinha' (GL0135) were obtained from the EMBRAPA Arroz e Feijão (Sto. Antônio de Goiás, Goiás, Brazil). The material was maintained and multiplied in the experimental garden of the Laboratory of Plant Cytogenetics, Department of Botany, UFPE, Recife, Pernambuco, Brazil.

Chromosome preparation and fluorochrome staining

For cytological analyses, root tips were pre-treated with 0.002 M 8-hydroxyquinoline at 8°C for 20 h, fixed in ethanol/glacial acetic acid 3:1 (v/v) for 2 h at room temperature and stored at −20°C. Root tips were digested using a solution containing 2% cellulase and 20% pectinase (w/v) for 90 min at 37°C, and the meristem dissected in 45% (v/v) aqueous acetic acid, squashed under a cover slip (subsequently removed by freezing in liquid nitrogen), air-dried and then aged for 3 days. Selection of slides, CMA/DAPI staining, and destaining for FISH were performed according to Cabral et al. (2006).

DNA probes

The sites of 5S and 45S rDNA were localized, respectively, with the D2 probe, a 500 bp fragment containing the 5S rDNA of *Lotus japonicus* (Pedrosa et al. 2002), and the R2 probe, a 6.5-kb fragment of an 18S–5.8S–25S rDNA repeat unit from *Arabidopsis thaliana* (Wanzenböck et al. 1997). The BACs used belong to a genomic library of *P. vulgaris* 'BAT93' (Kami et al. 2006) and were previously selected and used in the construction of a cytogenetic map of common bean (Pedrosa-Harand et al. 2009; Fonsêca et al. 2010). In addition, the bacteriophage B61, related to a complex of genes for resistance to anthracnose, was used (Geffroy et al. 2008, 2009), selected as described in Ferrier-Cana et al. (2003). DNA was obtained using the Plasmid Mini Kit (Qiagen) or DNA Nucleobond AX columns (Macherey–Nagel), following the manufacturers' instructions. Probes were labeled by nick translation (Invitrogen or Roche Diagnostics) with digoxigenin-11-dUTP (Roche Diagnostics) or Cy3-dUTP (5-amino-propargyl-2′-deoxyuridine 5′-triphosphate coupled to red cyanine fluorescent dye; GE).

Fluorescence in situ hybridization

The FISH procedure applied to mitotic chromosomes was essentially the same as previously described (Fonsêca et al. 2010). Hybridization mixes consisted of 50% (v/v) formamide, 10% (w/v) dextran sulfate, 2× SSC, and 2–5 ng/µl probe. The slides were denatured for 5 min at 75°C and hybridized for up to 2 days at 37°C. The final stringency was 76%. The *P. vulgaris* C_0t − 100 fraction was added in 10- to 100-fold excess to the hybridization mix to block repetitive sequences when necessary. Digoxigenin-labeled probes were detected with 0.4 µl of sheep anti-digoxigenin conjugated with fluorescein isothiocyanate (FITC; Roche Diagnostics) and amplified with 0.7 µl of anti-sheep-FITC (Dako) in 1% (w/v) BSA. Reprobing of slides for localization of different DNA sequences in the same cell was performed following Heslop-Harrison et al. (1992).

Data analysis

Photographs were taken in an epifluorescence Leica DMLB microscope equipped with a COHU 4912-5010 CCD Camera (COHU, San Diego, CA) using the Leica QFISH software. For final processing, images were super-imposed and artificially colored using the Adobe Photoshop software version 10.0 and adjusted for brightness and contrast only. Chromosomes were named and oriented according to the standard common bean nomenclature (Pedrosa-Harand et al. 2008; Fonsêca et al. 2010).

Results

Lima beans have a karyotype composed of 22 predominantly metacentric chromosomes that have pericentromeric

regions rich in $CMA^+/DAPI^-$ heterochromatin forming blocks of different sizes and intensities (Fig. 1a). Terminal bands, also observed in one chromosome pair, were frequently distended and corresponded to the nucleolus organizer region (NOR) (see Fig. 1a).

Twenty-two BACs of *P. vulgaris* selected with genetically mapped, molecular markers in this species, one bacteriophage, and the 5S and 45S rDNA sequences were localized on the mitotic chromosomes of *P. lunatus*. Seven of these BACs (12M3, 36H21, 81A17, 92I7, 103P12, 193O2, and 230M2) could not be mapped as they demonstrated repetitive pericentromeric patterns in *P. lunatus* similar to those observed in *P. vulgaris*, even with the addition of blocking DNA. The remaining 15 BACs, plus the bacteriophage B61, showed single signals that allowed the identification and characterization of each chromosome pair of the species (Table 1).

The BACs containing pericentromeric repeats, in spite of coming from four distinct chromosomes, showed very similar patterns, labeling all chromosomes of the complement and varying only in intensity (depending on the chromosome pair) (Fig. 1b). Comparison of this repetitive pattern to the CMA banding pattern indicated high similarity between them. The only exception was observed in chromosome-6, which had an extra CMA^+ band, in addition to the pericentromeric one, that co-localized with the 45S rDNA in the extremity of the short arm, where no signs of repetitive BACs were observed (Fig. 1a–c).

In situ hybridization with single-copy clones allowed the identification of all of the chromosomes in the complement of *P. lunatus* (Fig. 2a–k), except for chromosome-5 (which could be identified by the absence of any markers). BAC 36H21 (the only BAC mapped on chromosome-5 in *P. vulgaris*) showed a repetitive pericentromeric pattern in *P. lunatus*, even after using the C_0t-100 DNA blocker (Fig. 2e). BAC 63H6, which showed repetitive blocks in most of the chromosome ends in *P. vulgaris*, corresponding to the distribution of *khipu*, a satellite DNA (David et al. 2009; Santos K.G.B., unpublished data), could not be mapped as a single signal in this species. In *P. lunatus,* on the other hand, this BAC gave a single signal on the extremity of the short arm of chromosome pair 10, even without any blocking DNA (Fig. 2j). Similarly, BAC 255F18 showed only a single signal in the interstitial region of the short arm of chromosome-11 in *P. lunatus*, with no additional signal on chromosome-7 (as was seen in *P. vulgaris*) (Fig. 2k). The other BACs showed single-copy signals in both species: 221F15 and 257L12 for chromosome-1; 127F19 and 225P10 for chr. 2; 147K17 for chr. 3; B61 for chr. 4; 18B15 for chr. 6; 22I21 for chr. 7; 177I19 and 169G16 for chr. 8; 163I7 and 224I16 for chr. 9; 173P6 for chr. 10 and 179N14 for chr. 11. Only single sites of 5S and 45S rDNA were seen on chromosomes 10 and 6, respectively, in *P. lunatus*, altering the morphology of these chromosomes when compared to *P. vulgaris*. Except for BAC 163I7, all BACs were located in the same chromosome arms in both species, indicating the conservation of marker position and confirming the karyotype stability between them. However, the results suggest at least three pericentric inversions involving chromosome pairs 2, 9 and 10. These events are supported by a centromeric repositioning (CR), the presence of BACs 163I7 and 224I16 located on opposite arms in *P. lunatus* (while both are on the long arm in *P. vulgaris*) and the presence of 5S rDNA sequences on the short arm in *P. lunatus* (instead of the long arm, as in *P. vulgaris*), respectively. A schematic representation of the chromosomes of *P. lunatus*, compared with the established cytogenetic map of *P. vulgaris* (Fonsêca et al. 2010), is shown in Fig. 3.

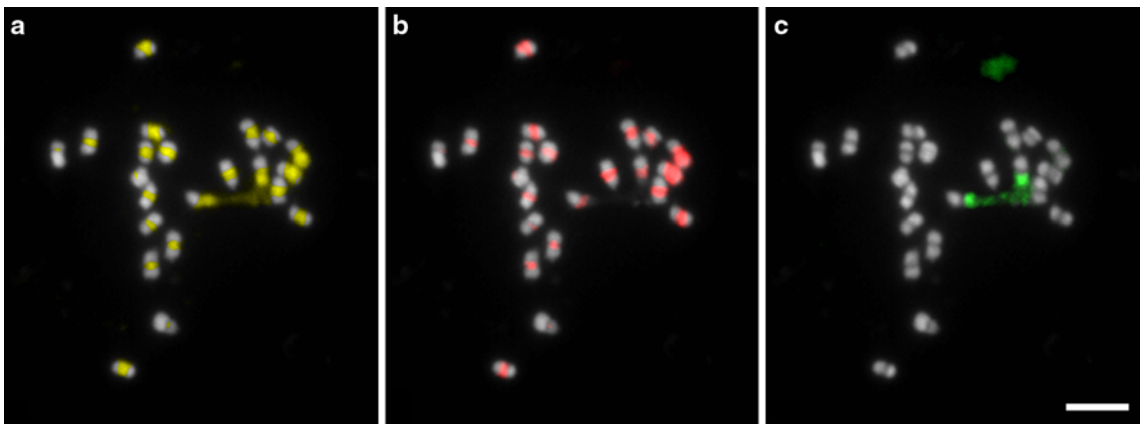


Fig. 1 Distribution of heterochromatin and repetitive sequences in mitotic chromosomes of *P. lunatus*. **a** CMA/DAPI banding pattern; **b** BAC 12M3 (*red*), showing a pericentromeric pattern and **c** 45S rDNA (*green*), colocalized with a single, non pericentromeric CMA^+ band. Chromosomes are counterstained with DAPI and visualized in *gray*. *Bar* in **c** represents 5 μm (color figure online)

Table 1 List of clones used as probes for comparative analysis by FISH between *P. vulgaris* (*P. v.*) and *P. lunatus* (*P. l.*) and distribution and localization in both species

Chromosome	Clone	Distribution		Localization			
				Arm		Position	
		P. v.	*P. l.*	*P. v.*	*P. l.*	*P. v.*	*P. l.*
1	221F15	Unique[a]	Unique[b]	Short	Short	Proximal	Proximal
	257L12	Unique	Unique	Long	Long	Terminal	Terminal
2	127F19	Unique	Unique	Long	Long	Interstitial	Interstitial
	225P10	Unique	Unique	Long	Long	Terminal	Terminal
	92I7	Repetitive	Repetitive	–	–	Pericentromeric	Pericentromeric
3	147K17	Unique	Unique	Short	Short	Interstitial	Interstitial
4	B61	Unique	Unique	Short	Short	Interstitial	Interstitial
5	36H21	Unique	Repetitive	Long	–	Interstitial	Pericentromeric
	103P12	Repetitive	Repetitive	–	–	Pericentromeric	Pericentromeric
	193O2	Repetitive	Repetitive	–	–	Pericentromeric	Pericentromeric
	230M2	Repetitive	Repetitive	–	–	Pericentromeric	Pericentromeric
6	45S rDNA	Repetitive	Repetitive	Short	Short	Terminal	Terminal
	18B15	Unique	Unique	Long	Long	Terminal	Terminal
7	22I21	Unique[a]	Unique[b]	Long	Long	Interstitial	Interstitial
	12M3	Repetitive	Repetitive	–	–	Pericentromeric	Pericentromeric
8	177I19	Unique	Unique	Short	Short	Interstitial	Interstitial
	169G16	Unique[a]	Unique[b]	Long	Long	Terminal	Terminal
9	163I7	Unique[a]	Unique	Long	Short	Interstitial	Interstitial
	224I16	Unique[a]	Unique[b]	Long	Long	Terminal	Terminal
10	63H6	Repetitive	Unique	–	Short	Subtelomeric	Terminal
	5S rDNA	Repetitive	Repetitive	Long	Short	Interstitial	Proximal
	173P6	Unique[a]	Unique[b]	Long	Long	Interstitial	Interstitial
	81A17	Repetitive	Repetitive	–	–	Pericentromeric	Pericentromeric
11	179N14	Unique	Unique	Short	Short	Terminal	Terminal
	255F18	Unique	Unique	Short	Short	Proximal	Proximal

Repetitive signals nonspecific to one of the arms are indicated by (–)

[a] With the use of C_0t-100; disperse when used without blocking

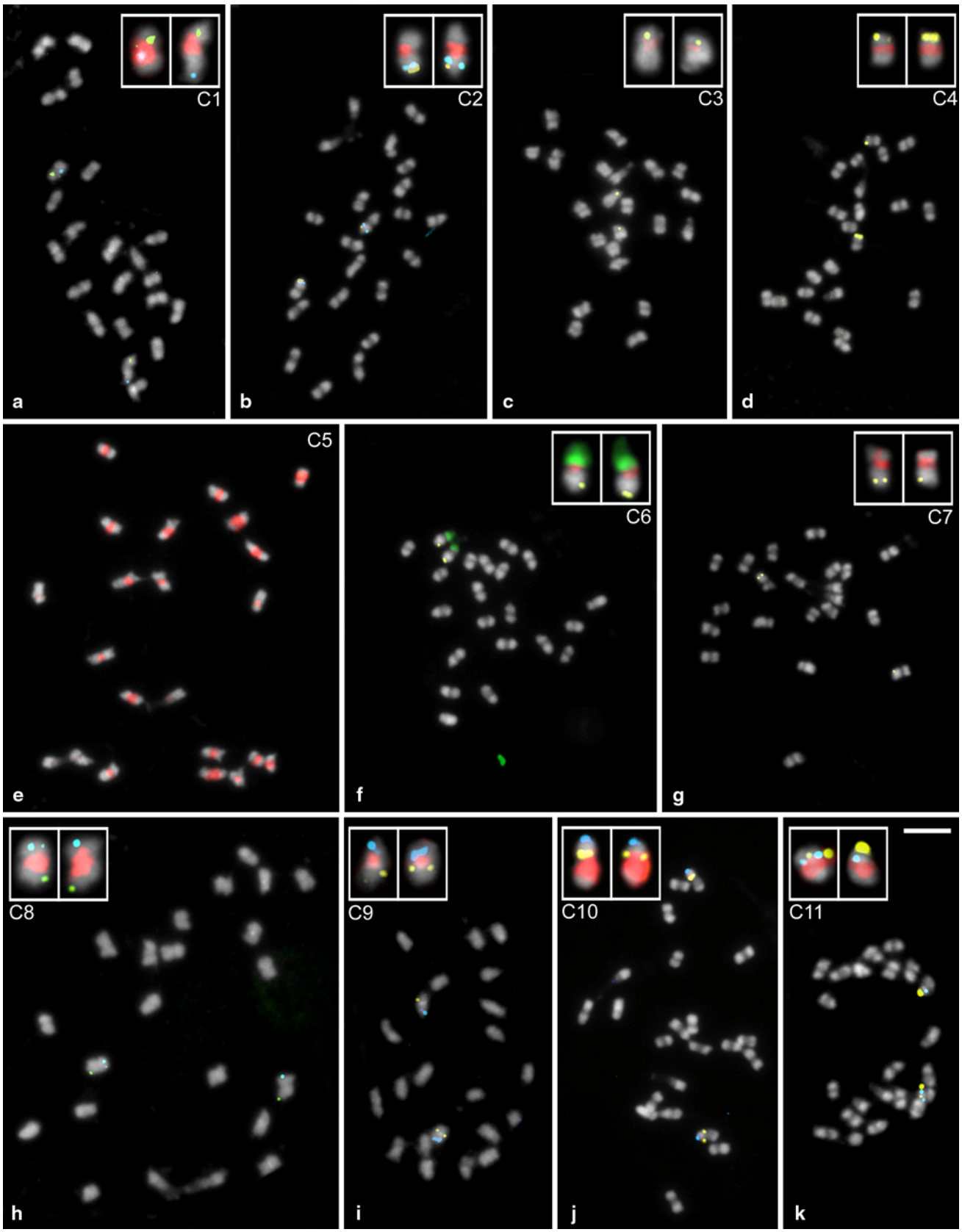
[b] With the use of C_0t-100; unknown pattern without blocking

Discussion

The construction of BAC libraries and their use in comparative cytogenetic studies has been demonstrated to be an excellent strategy for karyotype evolution studies (Lysak et al. 2006; Mandáková and Lysak 2008; Pedrosa et al. 2002). In the present work, we constructed a comparative cytogenetic map for *P. lunatus* by using FISH of BACs that had been previously mapped in the common bean (*P. vulgaris*) (Fonsêca et al. 2010). This allowed an analysis of the synteny between unique sequences and the conservation of repetitive sequence distributions in the genomes of these two species. It was also possible to test the apparent karyotypic stability of the genus *Phaseolus,* which has very similar DNA contents (Arumuganathan and Earle 1991) and karyotypes (Mercado-Ruaro and Delgado-Salinas 1996, 1998; Zheng et al. 1991) among most of its species.

Pericentromeric, CMA^+ bands were observed in all lima bean chromosomes, indicating that this region is composed of GC-rich repetitive sequences, as has been observed in

Fig. 2 In situ hybridization to mitotic chromosomes of *P. lunatus* species with selected clones from the cytogenetic map of *P. vulgaris*. Clones 221F15 (**a** chromosome-1), 225P10 (**b** 2), 147K17 (**c** 3), B61 (**d** 4), 18B15 (**f** 6), 22I21 (**g** 7), 177I19 (**h** 8), 224I16 (**i** 9), 5S rDNA (**j** 10) e 179N14 (**K**, 11) are represented in *yellow*. Clones 257L12 (**a**), 127F19 (**b**), 177I19 (**h**), 163I7 (**i**), 63H6 (**j**) e 255F18 (**k**) are represented in *blue*. BACs 12M3 (**a–d**, **f–k**) and 36H21 (**e**) are represented in *red*. In **f**, site of 45S rDNA (*green*) in the chromosome-6. Chromosomes are counterstained with DAPI and visualized in *gray*. *Bar* in **k** represents 5 μm (color figure online)


C1
C2
C3
C4
a
b
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C5
C6
C7
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g
C8
C9
C10
C11
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i
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k

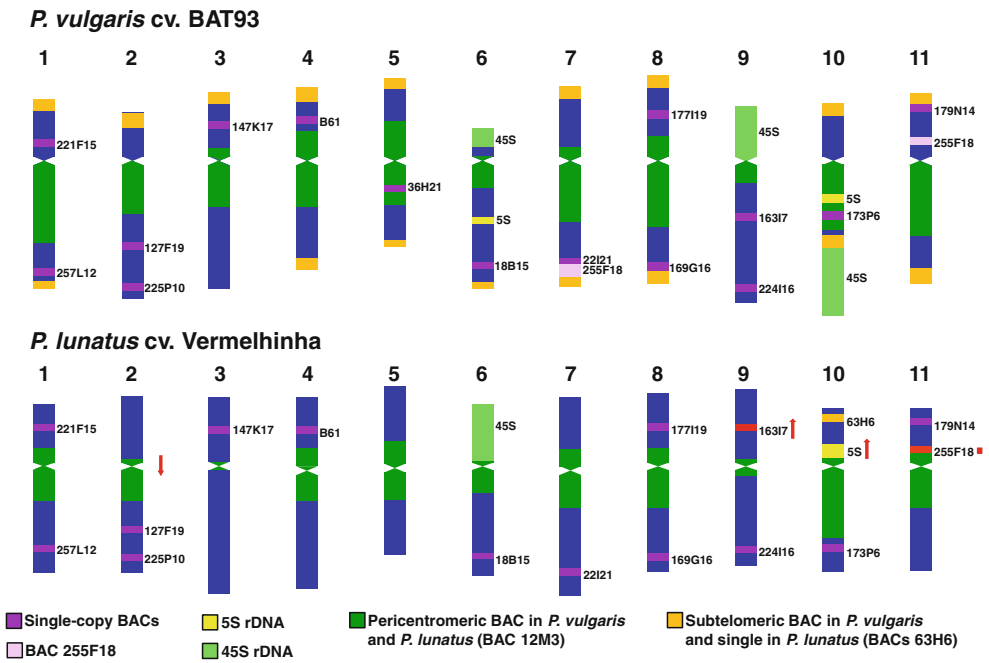

Fig. 3 Schematic representation of *P. lunatus* and *P. vulgaris* (modified from Fonsêca et al. 2010) chromosomes, showing only the clones hybridized to both species. *Arrows in red* indicate changes between species. *The red square* indicates the conservation of BAC 255F18 on the short arm of chromosome-11 and its absence on chromosome 7 (color figure online)

other species (Fonsêca et al. 2010; Silva et al. 2010; Souza and Benko-Iseppon 2004). In addition, only the terminal NOR of chromosome-6 (as confirmed by sequential hybridization with BAC 18B15) was also strongly stained by CMA. The presence of a single terminal CMA^+ site associated with consistent pericentromeric banding makes it essentially impossible to distinguish most of the chromosome pairs of *P. lunatus* based on fluorochrome staining. Furthermore, although the pericentromeric heterochromatin of the common bean also shows CMA^+ staining, the distributions of these blocks among the two species were different, making it impossible to recognize homeologues. Chromosome-9 of the common bean, for example, has the weakest pericentromeric heterochromatic band in the complement (Fonsêca et al. 2010), while in lima bean the weakest band is seen on pair 3. In addition, a significant difference was observed in the length of the pericentromeric band on chromosome-10 of *P. lunatus* in relation to its homeologue, as it occupied almost the entire length of the long arm. Although a number of studies have established relationships among homeologous chromosomes based on their CMA/DAPI patterns, as for the species of *Scilla, Citrus,* and *Pinus* (Carvalho et al. 2005; Deumling and Greilhuber 1982; Hizume et al. 1989), studies of karyotypic evolution in which the chromosomes are not identified using specific markers should be considered with some caution—as the differences observed between the karyotypes may have been underestimated.

The BACs showing repetitive pericentromeric pattern in *P. vulgaris* (Fonsêca et al. 2010) also demonstrated pericentromeric labeling in *P. lunatus* that co-localized with the CMA^+ bands. The pericentromeric pattern shown by BAC 36H21 in *P. lunatus*, however, did not allow it to be mapped—which was different from the situation observed in *P. vulgaris* (where the same BAC could be mapped on chromosome-5 after the addition of $70\times$ $C_0t - 100$). Even after the application of excess of blocking DNA ($100\times$ $C_0t - 100$) to *P. lunatus*, no unique signal was obtained with this BAC, indicating that the pericentromeric repetitive fraction had a distinct composition in this species.

The single signal evidenced by the BAC 63H6 on chromosome-10 of *P. lunatus* confirms the existence of a major divergence between the repetitive subtelomeric fractions of the two species, as well as the usefulness of mapping heterologous BACs probes. Utilizing an opposite approach, Koumbaris and Bass (2003) successfully mapped non-repetitive sorghum BACs on corn chromosomes, which could not be mapped using BACs from corn.

The 5S rDNA had been previously mapped on the short arm of a chromosome pair in lima bean (Moscone et al. 1999), which was later identified as the homeologue of chromosome-10 in the common bean (Almeida and Pedrosa-Harand 2010; and the present work). Interestingly, BAC 63H6 was found to be conserved on the short arm, but the 5S rDNA site in *P. vulgaris* is located on the long arm (Fonsêca et al. 2010), reinforcing the idea of a possible

pericentric inversion in this chromosome, as was suggested by Almeida and Pedrosa-Harand (2010). Alternative hypotheses could be dispersion and amplification of the 5S rDNA repeats mediated by mobile elements or circular extra-chromosomal DNA, as well as segmentary duplication events. Earlier studies pointed to the occurrence of transposition of the 5S rDNA mediated by transposon elements in *Aegilops* L. (Raskina et al. 2004), as well as amplification and de-amplification events of the 5S rDNA without altering overall collinearity (Dubcovsky and Dvorak 1995). Although additional studies will still be necessary to investigate whether there are any relationships between the evolution of the pericentromeric heterochromatin and changes in the position of this pericentromeric 5S rDNA site in *P. lunatus*, evidence for pericentric inversions in pairs 2 and 9 were also observed.

Chromosome-2 demonstrated CR, being metacentric in lima bean and submetacentric in the common bean. This suggested that a pericentric inversion was responsible for this morphological chromosomal alteration (see Schubert and Lysak 2011), even though there was no confirmation of this rearrangement through position changes of single-copy BACs. The establishment of neo-centromeres formed by the accumulation of CENH3 and specific repetitive sequences adjacent to a flanking heterochromatin site cannot, however, be discarded (Han et al. 2009; Topp et al. 2009). Chromosome-9 showed not only a CR but also a change in the position of BAC 163I7, indicating the occurrence of a pericentric inversion in this chromosome.

In spite of the observed differences in the distributions of some repeated sequences and possible pericentric inversions, the synteny between these genomes appears to be conserved. A comparison of the genetic maps of egg plant (*Solanum melongena*) and tomato (*Solanum esculentum*) revealed the existence of 28 structural rearrangements involving their 12 linkage groups. From these, 23 could be explained by paracentric inversions and 5 by translocation events (Doganlar et al. 2002). More recently, cytogenetic studies have demonstrated the utility of the BAC-FISH technique in recognizing homeologues and chromosomal rearrangements (Iovene et al. 2008; Tang et al. 2008). Detailed analyses using chromosomal painting with BACs were undertaken with the model species *A. thaliana* ($2n = 10$) and related plant species, and indicated that numerous chromosomal rearrangements (such as translocations and inversions) had occurred within the Brassicaceae family (Lysak et al. 2006; Mandáková and Lysak 2008). These events appear to have been rarer and simpler in common bean and lima bean, as they resulted in only three detected changes in centromere position. No translocations were observed. This low number of structural rearrangements reinforces the hypothesized karyotypic stability of this genus, with 22 metacentric/submetacentric chromosomes in the large majority of the species (Mercado-Ruaro and Delgado-Salinas 1996, 1998). The results of the present work demonstrated the viability of using BACs libraries in comparative mapping and karyotypic evolutionary studies in the genus *Phaseolus,* and suggest that pericentric inversions were the principal type of structural chromosomal alteration (associated with differences in the repetitive DNA fraction) responsible for the variations observed between the genomes of *P. vulgaris* and *P. lunatus*.

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