Fluorescence In Situ Hybridization (FISH)-Based Karyotyping Reveals Rapid Evolution of Centromeric and Subtelomeric Repeats in Common Bean (Phaseolus vulgaris) and Relatives

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ABSTRACT  Fluorescence in situ hybridization (FISH)-based karyotyping is a powerful cytogenetics tool to study chromosome organization, behavior, and chromosome evolution. Here, we developed a FISH-based karyotyping system using a probe mixture comprised of centromeric and subtelomeric satellite repeats, 5S rDNA, and chromosome-specific BAC clones in common bean, which enables one to unambiguously distinguish all 11 chromosome pairs. Furthermore, we applied the karyotyping system to several wild relatives and landraces of common bean from two distinct gene pools, as well as other related Phaseolus species, to investigate repeat evolution in the genus Phaseolus. Comparison of karyotype maps within common bean indicates that chromosomal distribution of the centromeric and subtelomeric satellite repeats is stable, whereas the copy number of the repeats was variable, indicating rapid amplification/reduction of the repeats in specific genomic regions. In Phaseolus species that diverged approximately 2–4 million yr ago, copy numbers of centromeric repeats were largely reduced or diverged, and chromosomal distributions have changed, suggesting rapid evolution of centromeric repeats. We also detected variation in the distribution pattern of subtelomeric repeats in Phaseolus species. The FISH-based karyotyping system revealed that satellite repeats are actively and rapidly evolving, forming genomic features unique to individual common bean accessions and Phaseolus species.

KEYWORDS  common bean karyotyping fluorescence in situ hybridization satellite repeats chromosome evolution

Common bean (Phaseolus vulgaris L., 2n = 2x = 22) is a very important edible legume, with more than 23 million tons of annual production

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(Broughton et al. 2003). It is a major source of protein in developing countries in Africa, Asia and Latin America. Common bean belongs to the genus Phaseolus, which consist of approximately 50 species, classified into eight clades (Delgado-Salinas et al. 2006). The genus Phaseolus includes other agronomically important crops, such as runner bean (P. coccineus), lima bean (P. lunatus), tepary bean (P. acutifolius), and year bean (P. dumosus). All cultivated species except P. lunatus are phylogenetically close, belonging to the Vulgaris group (Delgado-Salinas et al. 2006). Most Phaseolus species posses 2n = 2x = 22 chromosomes, indicating stability of the chromosome number; there are, however, reports of chromosomal rearrangements between common bean and other Phaseolus species (Broughton et al. 2003; Singh 2004; Bonifacio et al. 2012).

Common bean is a geographically and phenotypically diverse species. Wild common bean is distributed from northern Mexico to northwestern Argentina (Toro Chica et al. 1990). Domesticated common bean consists
of two major gene pools (Andean and Mesoamerican) that are distinguished by partial reproductive isolating barriers (Shii et al. 1980; Gepts and Bliss 1985; Koinange and Gepts 1992), as well as other characteristics, such as morphology, agronomic traits, and seed proteins (Singh et al. 1991). The divergence of the gene pools (> 100,000 yr ago [ya]) predates the domestication events within the individual gene pools (8000 ya), a rather unique scenario among crops (Mamidi et al. 2013; Schmutz et al. 2014). Following independent domestication events, local adaptation has generated diverse landraces.

To date, numerous molecular cytogenetic studies have been undertaken to investigate the chromosomal structure of common bean and other Phaseolus species, and has included rDNA distributions, mapping of single and repetitive BAC clones, and development of cytogenetic maps (Moscone et al. 1999; Pedrosa-Harand et al. 2006, 2009; Fonseca et al. 2010; Altrock et al. 2011; Bonì ficcio et al. 2012). The comparison of cytogenetic maps between Andean (G19833) and Mesoamerican (BAT93) landraces revealed the overall stability of karyotypes between the two gene pools (Bonì ficcio et al. 2012). The major cytological difference between the two gene pools is the distribution of the seven 45S rDNA loci, and three 45S rDNA loci in Andean and Mesoamerican varieties, respectively, suggesting Andean-specific amplification of the rDNA loci after the divergence of the two gene pools (Pedrosa-Harand et al. 2006).

We previously reported that there are two different centromere-specific satellite repeats, CentPv1 and CentPv2, that have evolved independently within the genome of Andean landrace G19833 (Iwata et al. 2013). Unlike most plant species, which have one species-specific centromeric satellite (Jiang et al. 2003), fluorescence in situ hybridization (FISH) analysis revealed that CentPv1 dominates eight centromeres; whereas CentPv2 mainly dominates the other three centromeres. Furthermore, we found that both CentPv1 and CentPv2 satellite repeats are homogenized within the chromosomes and, in some cases, significant amounts of chromosome-specific variants occupy specific centromeres, as determined by FISH (Iwata et al. 2013). However, it is still uncertain how CentPv1 and CentPv2 have selectively dominated subsets of centromeres within the genome of common bean during the evolution of the genus Phaseolus, and how quickly they are evolving.

Based on Southern blot analysis, CentPv1 and CentPv2 are highly conserved in wild and cultivated common bean of the two gene pools, but less so in other Phaseolus species, including P. coccineus, P. acutifolius, and P. dumosus (Iwata et al. 2013). This indicates that contraction, expansion, or divergence of centromeric satellites occurred after common bean and the Phaseolus species diverged—a short evolutionary frame. However, Southern blot analysis does not address whether the contraction or expansion involves alteration of centromeric distribution of the repeats.

In addition to centromeric satellite repeats, David et al. (2009) reported a subtelomeric satellite repeat, khipu, located at cytologically visible knobs at most chromosomal termini of common bean. Khipu units in subtelomers appear to be frequently exchanged between nonhomologous chromosomes, and expansion of khipu elements via local duplication has been suggested (Richard et al. 2013). This indicates that khipu sequences are actively and rapidly evolving by changing their copy numbers and distribution. If this is the case, we hypothesize there is likely variation in copy number and chromosomal distribution of khipu among common bean accessions and between species. The khipu sequence is extensively conserved among Phaseolus species and especially conserved with high copy numbers among species in the Vulgaris group (David et al. 2009).

In order to address how satellite repeats, including centromeric and subtelomeric repeats evolve, we used FISH-based karyotyping approaches to explore their distribution in common bean accessions, and other Phaseolus species. FISH-based karyotyping using repetitive DNA probes has been developed in maize, soybean, and Brassica species (Kato et al. 2004; Albert et al. 2010; Findley et al. 2010; Xiong and Fries 2011), and has been a powerful tool to distinguish individual chromosomes, and to identify variation in chromosome structure and repeat distributions, both between and within species. In this study, we developed a FISH-based karyotype map based on common bean Andean accession G19833 using centromeric satellite repeats CentPv1 and CentPv2, subtelomeric repeat khipu, rDNAs, and chromosome-specific BAC clones. Using this FISH-based karyotyping system, we detected variation in satellite repeats in common bean and other Phaseolus species, and discuss the evolution of these chromosomal structures.

**MATERIALS AND METHODS**

**Plant materials**

All common bean accessions and other Phaseolus species used in this study are listed in Table 1. Plants were grown in a greenhouse to obtain root tips for mitotic chromosome preparations.

**Cloning of 5S rRNA genes (rDNA)**

The 5S rDNA was amplified from genomic DNA using PCR with the following primers: 5’- GGTGCCATCATACGCCACT-3’ and 5’-AAGTCACACGAGGACTTC-3’. A dimer form of 5S rRNA gene was gel-purified using a kit (Qiagen), ligated to a TOPO TA cloning vector, and transformed into the Escherichia coli strain top10 (Invitrogen). The insert was sequenced using Big Dye Terminator chemistry (Applied Biosystems) and run on an ABI3730 sequencer.

**Selection of chromosome-specific single- or low-copy BAC clones**

Single- or low-copy BAC clones were selected from the G19833 PVGBa BAC library (Schlueter et al. 2008). Bng markers (GenBank KM061153–KM061375), located near the end of linkage groups, were used to select corresponding BAC clones using Southern blotting (Table 2; Vallejo et al. 1992 unpublished data; Bhakta et al. 2015). For chromosomes without Bng markers near the ends of the linkage groups, or for which we could not find single- or low-copy BACs using Bng markers, we performed in silico analysis to select chromosome-specific BAC clones. BAC end sequences were used to pull out entire BAC sequences from 11 common bean pseudo-chromosomes (http://www.phytozome.org) using BLASTN with the following criteria: > 99% identity and > 90% query coverage, the BAC end pairs oriented in opposite directions, and putative pairs separated by 50–200 kb (slightly modified from Findley et al. 2010). The repetitive portion of BAC sequences were masked using RepeatMasker (http://www.repeatmasker.org/) with low complexity repeats, simple repeats, identified tandem repeats (rDNAs, CentPv1, CentPv2, and khipu), and the common bean transposon database (http://www.phytozome.org). We selected BAC clones for FISH probes that had a relatively low repeat content (< 20%), no tandem repeats, and that were located near the ends of pseudo-chromosomes (Table 2).

**Searching for potential chromosome-specific variants**

Multiple alignments of CentPv1 monomers from eight pseudo-chromosomes corresponding to chromosomes 1, 2, 3, 4, 7, 8, 9, and 10 were used to find chromosome-specific variants (Iwata et al. 2013).
The following fluorochrome-labeled oligonucleotides were used as FISH probes (Integrated DNA Technologies). CentPv1: CACATGAAATT GTTGTTCAGGAGATA labeled with cyanine 5 (Cy5), CentPv2: CAA TAAAAATCGCACTACAAAAA labeled with TEX615, CentPv1_A: GTTGTTCAGGAGATA labeled with TAMRA (fluorescein), CentPv2_A: CAAATGCTATCAGCTTTTCA labeled with FITC, and CentPv2_B: TCAAGGATATTCACAAGTGTTCGA labeled with TAMRA and Cy5. rDNA, and BAC DNA were extracted using a standard Alkali lysis method (Sambrook et al., 1989). BAC DNA was extracted using a standard Alkali lysis method (Sambrook et al., 1989). BAC DNA was extracted using a standard Alkali lysis method (Sambrook et al., 1989).

**Oligonucleotide FISH probes used in this study**

Table 1 List of Phaseolus species used in this study

| Species          | Common name          | Gene pool     | Accession number      | Gene Pool Type | Source                        |
|------------------|----------------------|---------------|-----------------------|----------------|-------------------------------|
| *P. vulgaris*    | Common bean          | Andean        | G19833                 | Andean         | Landrace Schmutz et al. 2014  |
| *P. vulgaris*    | Common bean          | Mesoamerican  | PI 633451/BAT93        | Mesoamerican    | USDA ARS*                     |
| *P. vulgaris*    | Common bean          | Andean        | G23580                 | Andean         | Wild CIATb                    |
| *P. vulgaris*    | Common bean          | Mesoamerican  | PI535416               | Mesoamerican    | USDA ARS                      |
| *P. coccineus*   | Scarlet runner bean  | PI 226594     |                       | Cultivated     | USDA ARS                      |
| *P. dumosus*     | Year bean            | PI 317574     |                       | Landrace       | USDA ARS                      |
| *P. acutifolius* | Tepary bean          | PI 319443     |                       | Cultivated     | USDA ARS                      |

*United States Department Of Agriculture, Agricultural Research Service (http://www.ars-grin.gov/)

b International Center for Tropical Agriculture (http://ciat.cgiar.org/)

**Fluorescence in situ hybridization**

Mitotic chromosome preparations and FISH using oligonucleotide probes were conducted as described in Gill et al. (2009) and Findley et al. (2010), with the following modifications. Root tips from potted plants were treated with pressurized nitrogen oxide for 90 min, fixed in Carnoy’s solution composed of 3:1 ethanol and glacial acetic acid for a day at room temperature, and then stored at 4°C. After rinsing the fixed root tips in distilled water, the root tips were digested with enzyme solution containing 1% Pectolyase (MP Biomedicals, LLC), and 2% Cellulase (MP Biomedicals, LLC) in citric buffer (10 mM sodium citrate, 10 mM sodium EDTA, pH 5.5) for 80 min at 37°C. The following procedure is the same as reported in Gill et al. (2009) and Findley et al. (2010). FISH using biotin- or digoxigenin-labeled probes were performed as described in Walling et al. 2005. When slides were reprobed with chromosome-specific BAC clones, the slides used for oligonucleotide FISH were soaked in 1x PBS to remove cover slips, and washed three times in 1x PBS for 5 min. each, followed by FISH protocol using biotin- or digoxigenin-labeled probes.

**Microscopy and image processing**

Images were collected with a Zeiss Axio Imager M2 microscope, equipped with AxioCam MRm, controlled by Axio Vision 4.8.2.0. Adobe Photoshop CS5 (Adobe Systems Incorporated) was used to produce publication images as described in Findley et al. (2010) with slight modification. Aligned chromosome images were produced with the “lasso” tool to trace individual chromosomes, and then orient and align them with the “copy and paste” and “rotation” tools. At least three images were analyzed to confirm the intensity and chromosomal distributions of individual FISH signals.

**Table 2 BAC clones used for chromosome identification of common bean**

| Chromosome | Linkage Group | PV_GBa BAC clone | Bng markers | First Base | Last Base | Insert Length (bp) | Physical Position in Pseudo-Chromosomes (%)a | Total Repeat Content (%)b |
|------------|---------------|------------------|-------------|------------|-----------|-------------------|----------------------------------------------|--------------------------|
| 1          | H             | 0043E17          | Bng 083     | 51,533,991| 51,677,621| 143,631           | 98.85                                        | 15.47                    |
| 2          | D             | 0073I08          |             | 42,399,450| 42,539,858| 140,409           | 86.6                                         | 10.05                    |
| 3          | C             | 0084M03          |             | 50,068,422| 50,162,332| 93,911            | 95.85                                        | 15.14                    |
| 4          | B             | 0095D15          |             | 44,627,451| 44,745,580| 118,130           | 97.23                                        | 14.38                    |
| 5          | E             | 0101A23          | Bng 162     | 40,054,960| 40,179,280| 124,321           | 98.28                                        | 15.26                    |
| 6          | G             | 0043E14          |             | 29,436,487| 29,562,452| 125,966           | 92.25                                        | 9.48                     |
| 7          | A             | 0100C06          | Bng 042     | 3,452,024  | 3,577,941  | 125,918           | 6.79                                         | 12.48                    |
| 8          | F             | 0102L19          | Bng 139     | 172,969    | 320,543    | 147,575           | 0.41                                         | 11.41                    |
| 9          | K             | 0100J23          |             | 21,306,679| 21,417,904| 111,226           | 57.01                                        | 16.62                    |
| 10         | I             | 0103D09          |             | 41,338,907| 41,446,050| 107,144           | 95.65                                        | 12.89                    |
| 11         | J             | 0033F21          | Bng 025     | 6,754,339  | 6,864,292  | 109,954           | 13.33                                        | 19.42                    |

a Physical position in pseudo-chromosomes is calculated from the following formula. The median value of the first and last bases/total length of the pseudo-chromosome.
b Total repeat content contains transposon, low complexity repeats, and simple repeats.
RESULTS

FISH probes targeted to repeats for use in karyotyping

Using common bean centromeric satellite repeats and their variants (Iwata et al. 2013), we first examined the utility of these repeats as FISH probes for a karyotype map using the sequenced Andean accession, G19833 (Schmutz et al. 2014; http://www.phytozome.org). For use as karyotyping probes, we designed 25-bp oligonucleotide probes labeled with fluorophores targeted to centromeric repeats CentPv1 and CentPv2, and their variants CentPv1_A and CentPv2_A. CentPv1_A and CentPv2_A are chromosome-specific variants of CentPv1 and CentPv2 (Iwata et al. 2013). Using these four oligonucleotides with different fluorophore labels (Cy5-CentPv1, TEX615-CentPv2, FAM-CentPv1_A, and FAM-CentPv2_A), we performed FISH to determine how many chromosomes have unambiguous and distinguishable patterns of hybridization signals (Supplemental Material, Figure S1). We pseudo-colored the merged images of DAPI, FAM, TEX615, and Cy5 in transparent gray, green, red, and blue, respectively. In this way, we were able to distinguish four of the 11 chromosome pairs (later identified as chromosomes 5, 6, 8 and 11).

Next, we designed a 25-bp oligonucleotide probe targeted to khipu from the consensus sequences of 92 khipu sequences. We tested the khipu oligonucleotide probe in accession BAT93 (Mesoamerican type) to examine the consistency of hybridization signals with a previous report (Figure S2; David et al. 2009; Richard et al. 2013). The khipu oligonucleotide probe had minor signals at most ends of the chromosomes, with one pair of strong signals (arrows in Figure S2B), and a secondary pair of signals (arrowheads in Figure S2B). Later, we confirmed that the strongest signals are on the short arm of chromosome 4, and the secondary signals on the long arm of chromosome 11, in agreement with David et al. (2009) and Richard et al. (2013).

rDNA is known to be present at high copy number in plant genomes, and is often used as FISH markers. We used oligonucleotide FISH probes targeted to 18S rDNA and 5S rDNA, but found only faint or no signals from these probes for unknown reasons (data not shown). Thus, we decided to use probes labeled by directly the nick translation method to detect rDNA loci for further experiments.

We next performed FISH with a mixture of oligonucleotide probes (Cy5-CentPv1, TEX615-CentPv2, FAM-CentPv1_A, FAM-CentPv2_A, and TEX615-khipu), and nick-translated 5S rDNA probe labeled with fluorescein. We did not add the 18S rDNA probe in the mixture because it showed seven strong hybridization signals in G19833 overlapping with several khipu, CentPv1, and CentPv2 signals, which prevented us from

Figure 1 A karyotype map on G19833 metaphase chromosomes. (A) Chromosomes counter-stained with DAPI. (B) FISH image with a probe mixture of Cy5-CentPv1 (blue), FAM-CentPv1_A (green), TEX615-CentPv2 (red), FAM-CentPv2_A (green), TEX615-khipu (red), and fluorescein-5S rDNA (green). (C) Homologous chromosome pairs aligned according to chromosome numbers based on reprobing with chromosome-specific BAC clones. Bars represent 5 μm.

Figure 2 FISH analysis of CentPv1 variant, CentPv1_B, and CentPv1_D. (A) Multiple alignments of CentPv1 and its variant, CentPv1_B. The 25-bp oligonucleotide CentPv1_B for FISH was designed from the region in the yellow rectangle targeted to polymorphisms shown by asterisks. (B) FISH image with CentPv1_B (green), CentPv1_D (red), and CentPv1 (blue). CentPv1_B signals localized at one pair of centromeres. CentPv2_D signals overlapped with all CentPv1 signals. Bar represents 5 μm.
analyzing the distribution of the satellite repeats (data not shown). Using a mixture of the six probes above, all 11 chromosomes of G19833 could be distinguished with different signal locations and intensities (Figure 1, A and B).

Identification of individual chromosomes of common bean

In order to identify individual chromosomes labeled with four CentPv repeats, khipu, and 5S rDNA, we reprobed the slides with chromosome-specific BAC clones from the G19833 PV_GBa BAC library (Schlueter et al. 2008). We identified chromosome-specific BACs either experimentally or by in silico analysis. Southern blotting was conducted using Bng clones anchored near the ends of individual linkage groups (Vallejos et al. 1992; Bhakta et al. 2015; unpublished data). Based on in silico analysis, 12,151 out of 48,000 PV_GBa BAC clones were anchored to 11 pseudo-chromosomes, and their entire BAC sequences were obtained. Because repetitive sequences in BAC clones can complicate chromosome-specific FISH hybridization, we analyzed the repeat content of these BAC clones, and selected those with relatively low repeat content. Table 2 summarizes the selected BAC clones for individual chromosomes that showed unambiguous FISH signals on their chromosomes.

Chromosome-specific BAC-FISH allowed us to identify all 11 chromosomes as correlated with FISH signal patterns of four CentPv components, khipu, and 5S rDNA. Images of individual homologous chromosome pairs were aligned according to their chromosome numbers (Figure 1C). This revealed that CentPv1 is present at centromeres 1, 2, 3, 4, 7, 8, 9, and 10, with its variant, CentPv1_A present only at centromere 8. The four strongest CentPv1 FISH signals were observed at centromeres 1, 3, 4, and 7, suggesting a higher copy number of CentPv1 in these centromeres. CentPv2 is present at centromeres 5, 6, and 11, with its variant CentPv2_A at centromere 11. Occasionally, weak CentPv2_A signals were also detected on the centromere of chromosome 5. Although these FISH signals and intensities were sufficient to distinguish all 11 chromosomes, three chromosomes pairs (chromosome 1, 3, and 7) had similar signal patterns that occasionally made them difficult to distinguish. Therefore, we added more probes to unambiguously distinguish these three chromosome pairs. In addition, the oligo-based probe of khipu was not always detectable, particularly at chromosomal termini with low copy numbers; therefore, we moved from an oligo-based to a PCR-amplified khipu probe for further experiments.

FISH-based karyotype map established for common bean

In order to identify potential chromosome-specific variants of CentPv1 that could help distinguish chromosome 1, 3, and 7, we compared multiple-aligned CentPv1 monomer sequences from eight pseudo-chromosomes corresponding to chromosome 1, 2, 3, 4, 7, 8, 9, and 10. We found three different repeat variants from three pseudo-chromosomes: 1, 3, and 7; 25-bp oligonucleotides targeted to polymorphic regions of each repeat variant were used as a FISH probe to examine their distribution. Only the oligonucleotide targeted to a repeat variant found in pseudo-chromosome 3 hybridized specifically to chromosome 3 (named as CentPv1_B; Figure 2), thereby helping us to distinguish chromosome 3 from chromosomes 1 and 7. The other two oligonucleotides with complementary sequences found in pseudo-chromosome 1 and 7 (named as CentPv1_C and CentPv1_D, respectively; Figure S3) hybridized to multiple chromosomes with different intensities (CentPv1_D in Figure 2, and CentPv1_C in Figure S3). Because we could not find a CentPv1 variant that helped distinguish chromosome 1 from 7, we instead added a chromosome 7-specific BAC clone, 0100C06, directly labeled with fluorescein, which hybridized to the short arm of chromosome 7. This probe set established the final version of a karyotype cocktail for common bean. This set comprised three probes from CentPv1 (Cy5-CentPv1, FAM-CentPv1_A, and TEX615-CentPv1_B), two from CentPv2 (TEX615-CentPv2 and FAM-CentPv2_A), 5S rDNA labeled with fluorescein, khipu labeled with Texas Red, and a chromosome7-specific BAC clone, 0100C06 labeled with fluorescein (Figure 3).

Comparison of FISH-based karyotype maps within common bean

Repetitive DNA sequences are known to be fast-evolving (reviewed in Mehrotra and Goyal 2014). In maize, there is distinct variation of repeats in their copy number and presence among maize lines that
diverged within the past 6,000–10,000 yr (Kato et al. 2004; Albert et al. 2010). Because the developed karyotype map of common bean uses centromeric and subtelomeric satellite repeats, this karyotyping strategy has the potential to investigate satellite repeat evolution not only within common bean, but also in allied species where satellite repeats are conserved.

We applied the same probe mixture to Mesoamerican accession, BAT93, and compared it with the karyotype map of Andean accession, G19833. With the same probe mixture, all 11 chromosomes were distinguished in BAT93. A detailed comparison between BAT93 and G19833 was made using aligned chromosome images (Figure 4). The overall signal patterns of centromeric and subtelomeric satellite repeats in BAT93 were similar to those of G19833. Five components of centromeric satellite repeats showed the same signal patterns between G19833 and BAT93, suggesting chromosomal distributions of these repeats were fixed before the divergence of Andean and Mesoamerican gene pools, ~100,000 ya. The karyotyping probe mixture was also hybridized to wild common bean from the Andean (G23580) and Mesoamerican (PI535416) gene pools (Figure 4). Overall, the repeat distributions were conserved, and it was possible to clearly distinguish all of the chromosomes, except chromosomes 1 and 4.

In G19833 and BAT93, chromosomes 1 and 4 were the strongest, whereas the signal at the short arm of chromosome 1 is evolving actively within the common bean. We conducted a subsequent FISH on these same slides using chromosomes 1- and 4- specific BAC clones to confirm their identity, and aligned the chromosomes (Figure S4).

While we detected consistency of chromosomal distribution of five components of centromeric satellite repeats in these wild accessions, we did detect copy number variation of CentPv1 and CentPv2. We consistently detected different signal intensities of CentPv1 at chromosome 7 between homologous chromosomes of G23580, indicating heterozygosity of the repeat distribution (open arrowhead in Figure 4). Because in other accessions, chromosome 7 has high copy numbers of CentPv1, it seems that the copy number of CentPv1 of one of the homologous chromosomes 7 is reduced in G23580. In PI535416, we consistently detected stronger signals of CentPv2 at chromosome 5 in comparison to chromosome 5 of other accessions, indicating an increase in copy number specific to the PI535416 (closed arrowhead in Figure 4).

High variation of 18S rDNA loci within common bean was previously reported (Pedrosa-Harand et al. 2006). For example, G19833 has seven loci (chromosomes 1S, 3S, 4L, 5L, 6S, 9S, and 10L), while BAT93 has only three loci (chromosomes 6S, 9S, and 10L) of 18S rDNA (Fonseca et al. 2010; Altrock et al. 2011). Here, we determined the positions of 18S rDNA loci in wild accessions by performing the second FISH using 18S rDNA as a probe after karyotyping (Figure S4). There are seven loci in G23580, and three loci in PI535416, supporting extensive amplification of 18S rDNA in the Andean lineage (Pedrosa-Harand et al. 2006). In Mesoamerican accessions, the chromosomal distributions of 18S rDNA loci were consistent between BAT93 and PI535416. In Andean accessions, while the number of loci was the same between G19833 and G23580, the locations varied, as the 18S rDNA locus on chromosome 4L was instead found on chromosome 11L (Figure S4).

**Conservation and variation of the repeats in related Phaseolus species**

We previously confirmed by Southern blot analysis that CentPv1 and CentPv2 are conserved in the Vulgaris group that diverged 2–4 million ya (Iwata et al. 2013). Weak hybridization signals of CentPv1 and CentPv2 on Southern blots indicated dramatic reductions, or divergence in copy numbers of these repeats, in the other Phaseolus species as compared to common bean. The next question was whether the chromosomal distributions of these repeats were conserved in other Phaseolus species. We tested the centromeric repeats, CentPv1 and CentPv2, on *P. dumosus*, *P. coccineus*, and *P. acutifolius* (Figure 5A). Interestingly, FISH using Cy5-CentPv1 and TEX615-CentPv2 showed variation in their chromosomal distribution, depending on the species: CentPv1 repeats were present only at five centromeres, with strong signals at two centromeres, middle-strength signals at two centromeres, and weak signals at one centromere in *P. coccineus* and *P. dumosus*. CentPv2 was localized to only one centromere, with strong signals in *P. coccineus* and *P. dumosus* (Figure 5, B and C). There were CentPv1 signals on two centromeres, and no CentPv2 signals in *P. acutifolius* (Figure 5D). We also tested probes of CentPv variants, FAM-CentPv1_A, FAM-CentPv2_A, and FAM-CentPv2_B, but detected no signals from these probes, indicating the absence of these variants in these three Phaseolus species. Because oligo-based probes target only...
25-bp, and we wanted to test whether the entire sequences of CentPv1 and CentPv2 were conserved, we also conducted FISH using PCR products covering the entire region of CentPv1 and CentPv2. There were CentPv1 signals on five centromeres in *P. cocineus* and *P. dumosus*, consistent with oligo-based FISH results (Figure S5). However, we could not find CentPv1 signals in *P. acutifolius*, and detected only very weak or no signals of CentPv2 in cells of all three species. This indicates that the entire sequences of CentPv1 and CentPv2 are either not conserved, or are present at very low copy number, in *P. acutifolius*, and the same for CentPv2 in all three species, and the 25-bp sequences of oligonucleotide probes appear to represent a more conserved sequence of the repeat (data not shown).

We also examined *khipu* distribution in the other *Phaseolus* species. Based on Southern analysis, *khipu* was conserved across the *Phaseolus* species with varying copy numbers. *Khipu* was conserved at high copy numbers in *P. dumosus* and *P. parvifolius* in the Vulgaris group (David et al. 2009), although the chromosomal distribution remained unknown. Based on FISH analysis using PCR-amplified *khipu* as a probe, we confirmed that *khipu* is present at subtelomeric regions of most of the chromosomes with different signal intensities in *P. cocineus*, *P. dumosus*, and *P. acutifolius* (Figure 6). We did detect variation in *khipu* distributions as compared to common bean. Based on our observations, *khipu* signals in *P. cocineus* and *P. dumosus* were distributed more uniformly at most chromosomal termini, and we did not detect any distinctively strong signals such as on chromosomes 4S (arrows in Figure 6A) and 11L (arrowheads in Figure 6A) in common bean. In *P. acutifolius*, five pairs of chromosomes have very weak or no *khipu* signals. Together with the FISH analysis of *khipu* on wild common bean, we concluded that *khipu* sequences evolve via changes in copy number and distribution over relatively short evolutionary time frames.

**DISCUSSION**

The establishment of a karyotype map is essential for chromosome studies. It requires high quality chromosome spreads (e.g., no overlap of chromosomes, good chromosome morphology, and little or no cytoplasm), and reliable FISH markers, especially for identification of small chromosomes. In this study, we developed a FISH-based karyotype map of common bean using a set of repetitive sequences: CentPv1, CentPv1_A, CentPv1_B, CentPv2, CentPv2_A, *khipu*, 5S rDNA, and chromosome-specific BAC clones hybridized to mitotic metaphase chromosomes. There are several advantages to our FISH-based karyotype map. First, probes of centromere satellite repeats, CentPv1, and CentPv2 enable one to position centromeres easily and accurately in chromosomes. This study enabled us to characterize the distribution and relative copy number of CentPv1 and CentPv2 in individual chromosomes of the common bean. Second, because we used conserved satellite repeats found at centromeres and subtelomeres of the Vulgaris group, we were able to apply this karyotyping system to wild and domesticated Mesoamerican and Andean common bean, as well as to other closely related *Phaseolus* species. Third, the karyotype map has been integrated with the genetic map using chromosome-specific, genetically anchored, BACs.

Common bean is a geographically and phenotypically diverse species, consisting of two major gene pools: Andean and Mesoamerican. Wild accessions of the Mesoamerican gene pool were found to have higher genetic diversity than their Andean counterparts, evidence of a tighter bottleneck in the Andean gene pool (Koenig and Gepts 1989; Koenig et al. 1990; Velasquez and Gepts 1994; Chacon et al. 2007; Bitocchi et al. 2012). One of our interests in this study was to determine the extent of chromosomal variation for an informative set of repetitive DNA sequences, as a corollary to genetic variation within and between the gene pools. Polymorphisms in 45S rDNA loci among plant species are frequently seen (Chung et al. 2008; Hamon et al. 2009; Robledo and Seijo 2009), even within species (Chung et al. 2008; de Moraes et al. 2007; Hayasaki et al. 2001; Raskina et al. 2004; Pedrosa-Harand et al. 2006). Pedrosa-Harand et al. (2006) showed the extensive variation of 45S rDNA loci within common bean, e.g., the number of rDNA loci was amplified in Andean lineage. Fewer studies have analyzed satellite repeat distributions within and among species. In maize, a very genetically diverse species, variation in copy number and/or positions in knob-related repeats, and the centromeric satellite repeat, CentC, were observed within maize, and among closely related species, using FISH-based karyotype maps (Kato et al. 2004; Albert et al. 2010). Unlike maize, common bean is a mostly autogamous selfing species, but geographically dispersed with two distinct gene pools. We were interested in determining how the geographical distribution may have affected
chromosomal evolution, especially of rapidly evolving repeats, within the genus and species. The comparison of FISH-based karyotypes showed that the overall repeat distribution and chromosome structure were very similar between Andean and Mesoamerica landraces, a pattern that is indicative of the fixation of the repeats before divergence of the two gene pools ~100,000 ya.

The majority of eukaryotes have centromeric satellite repeats; however, the function, emergence, and evolution of centromeric satellite repeats is unclear.

Figure 6 FISH analysis of khipu on G19833 (A and B), P. coccineus (C and D), P. dumosus (E and F) and P. acutifolius (G and H). Mitotic metaphase chromosomes were counter-stained with DAPI (blue) merged with khipu signals. Scale bars indicate 5 μm. Panel (A) shows the strongest signals of khipu on chromosome 4S. Arrowheads show the second strongest signals of khipu on chromosome 11L.

![Diagram of khipu distribution in Phaseolus species](Figure 7)

**Figure 7** Summary of FISH-based karyotyping in common bean and Phaseolus species.

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**Table 1:** Summary of FISH-based karyotyping in common bean and Phaseolus species.

| CentPv1 | CentPv2 | 18S rDNA | khipu |
|---------|---------|----------|-------|
| 2 (0)   | 0 (0)   | 1        | subtelomeric |
| 5 (5)   | 1 (0)   | 2        | subtelomeric |
| 5 (5)   | 1 (0)   | 2        | subtelomeric |
|         |         |          | subtelomeric, the strongest signal on 11L |
| G23580  | 8*      | 7***     | subtelomeric, the strongest signal on 4S |
| L       |         |          | subtelomeric, the strongest signal on 11L |
| M       | 8       | 3        | subtelomeric, the strongest signal on 4S |
| PIS35416| 8       | 3**      | subtelomeric, the strongest signal on 11L |
| BAT93   | 8       | 3        | subtelomeric, the strongest signal on 4S |

a: The number of signals observed in haploid genomes using oligonucleotide probes are shown. The number of signals using PCR-derived probes are shown in parentheses.

b: The number of 18S rDNA signals observed in haploid genomes in *P. acutifolius*, *P. dumosus*, and *P. coccineus* are unpublished data.

c: A: Andean gene pool, M: Mesoamerican gene pool

d: W: Wild; L: Landrace

*: Copy number variation was observed between homologous chromosomes of chromosome 7.

**: Increase in copy number was observed in chromosome 5.

***: 18S rDNA locus on chromosome 11L was observed instead of chromosome 4L.
repeats are still ambiguous. Most neocentromeres (ectopic centromeres formed at previously noncentromeric chromosomal regions) form in nonrepetitive chromosomal regions, indicating that satellite repeats are not necessary to form centromeres de novo (Marshall et al. 2008). In a few cases, such as chicken and potato, different centromere structures (repeat-based vs. repeat-free) coexist within the same genome (Shang et al. 2010; Gong et al. 2012), which may represent a transition stage of repeat-free to repeat-based centromeres. Although all common bean centromeres have satellite repeats, we hypothesize that the two centromeric repeats might not yet be fixed in the genome, and ultimately they will reach a “stable” state where one centromeric repeat will dominate all 11 centromeres. In fact, we observed copy number variation for CentPv1 and CentPv2 in wild common bean accessions, which might indicate that these repeats are still actively and rapidly evolving by increasing/decreasing their copy numbers at specific centromeric locations. In addition, the presence of variants of CentPv1 and CentPv2 limited to common bean indicates the rapid evolution of the repeats after its divergence from other Phaseolus species. The presence of CentPv1 and CentPv2 in a fewer number of chromosomes in other Phaseolus species than in P. vulgaris may also favor the hypothesis that centromeres evolve from repeat-free to repeat-based structures. From this study, it is not clear that the centromeres devoid of CentPv1 and CentPv2 signals are repeat-free, or have been replaced with other satellite repeats. In either case, subsets of centromeres are actively evolving in the genus Phaseolus, while the others are conserved within the genus Phaseolus. Whether this has occurred by selective pressure or by random genetic drift remains to be determined.

Khipu is a subtelomeric repeat originally identified from disease resistance gene (R) clusters (David et al. 2009). The B4 and Co-2 R resistance loci at the end of LG-B4 and LG-B11 contain a Coiled-Coil-Nucleotide-Binding-Site-Leucine-Rich-Repeat (CNL) gene, and Khipu is interspersed between CNL sequences in R gene clusters, and present at adjacent heterochromatic knobs. Interestingly, khipu localization is not only restricted to these two regions, but is also found at most chromosome termini, indicating possible ectopic recombination in subtelomeric regions between nonhomologous chromosomes (David et al. 2009). In this study, we found that the distribution of khipu varies within common bean, and among Phaseolus species, in the Vulgaris group. The strongest khipu signals at chromosome 4S were restricted to common bean landraces, regardless of gene pools. Differences in khipu distribution among species further supports frequent ectopic recombination between nonhomologous chromosomes in subtelomeric regions during the evolution of Phaseolus species, as proposed by David et al. (2009).

In summary, FISH-based karyotyping provides insights into the stability of chromosomal structures and the evolution of satellite repeats, both copy number and distribution, as shown for centromeres and subtelomeres within common bean, and variability among closely related Phaseolus species (Figure 7). Our data indicates that, at the cytological level, satellite repeats are evolving actively and rapidly and contribute to the unique chromosomal structures of individual species. Although we observed changes in these structures, the group as a whole appeared to be much more “stable” than their maize counterparts (Kato et al. 2004; Albert et al. 2010). This stability may be due to differences in breeding systems (outcrossing vs. selfing), and the content and activity of transposons. This karyotyping system can be used to trace chromosomal behavior during cell division, detect specific chromosomes involved in trisomics, transgenes, and structural changes such as inversion and translocation, track chromosomes in interspecific crosses, and facilitate breeding strategies that require chromosome identification (Findley et al. 2010; Xiong and Pires 2011).

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LITERATURE CITED
Albert, P. S., Z. Gao, T. V. Danilova, and J. A. Birchler, 2010 Diversity of chromosomal karyotypes in maize and its relatives. Cytogenet. Genome Res. 129: 6–16.
Altrock, S., A. Fonseca, and A. Pedrosa-Harand, 2011 Chromosome identification in the Andean common bean accession G19833 (Phaseolus vulgaris L., Fabaceae). Genet. Mol. Biol. 34: 459–463.
Bhakta, M. S., V. A. Jones, and C. E. Vallejos, 2015 Punctuated distribution of recombinant hotspots and demarcation of pericentromeric regions in Phaseolus vulgaris L. PLoS One 10: e0116822.
Bitocchi, E., L. Nanni, E. Bellucci, M. Rossi, A. Giardini et al., 2012 Mesoamerican origin of the common bean (Phaseolus vulgaris L.) is revealed by sequence data. Proc. Natl. Acad. Sci. USA 109: 788–796.
Bonifacio, E. M., A. Fonseca, C. Almeida, K. G. B. dos Santos, and A. Pedrosa-Harand, 2012 Comparative cytogenetic mapping between the lima bean (Phaseolus lunatus L.) and the common bean (P. vulgaris L.). Theor. Appl. Genet. 124: 1513–1520.
Broughton, W. J., G. Hernandez, M. Blair, S. Beebe, P. Gepts et al., 2003 Beans (Phaseolus spp.)—model food legumes. Plant Soil 252: 55–128.
Chacon, S. M. I., B. Pickersgill, D. G. Debouck, and J. S. Arias, 2007 Phylogeographic analysis of the chloroplast DNA variation in wild common bean (Phaseolus vulgaris L.) in the Americas. Plant Syst. Evol. 266: 175–195.
Chung, M. C., Y. I. Lee, Y. Y. Cheng, Y. J. Chou, and C. F. Lu, 2008 Chromosomal polymorphism of ribosomal genes in the genus Oryza. Theor. Appl. Genet. 116: 745–753.
David, P., N. W. G. Chen, A. Pedrosa-Harand, V. Thareau, M. Sévignac et al., 2009 A nodamic subtelomeric disease resistance gene cluster in common bean. Plant Physiol. 151: 1048–1065.
Delgado-Salinas, A., R. Bibler, and M. Lavin, 2006 Phylogeny of the genus Phaseolus (Leguminosae): a recent diversification in an ancient landscape. Syst. Bot. 31: 779–791.
de Moraes, A. P., W. dos Santos Soares Filho, and M. Guerra, 2007 Karyotype diversity and the origin of grapefruit. Chr. Res. 15: 115–122.
Findley, S. D., S. Cannon, K. Varala, J. Du, J. Ma et al., 2010 A fluorescence in situ hybridization system for karyotyping soybean. Genetics 185: 727–744.
Fonseca, A., J. Ferreira, T. R. dos Santos, M. Mosiolek, E. Bellucci et al., 2010 Cytogenetic map of common bean (Phaseolus vulgaris L.). Chr. Res. 18: 487–502.
Gepts, P., and F. Bliss, 1985 F1 hybrid weakness in the common bean: Differential geographic origin suggests two gene pools in cultivated bean germplasm. J. Hered. 76: 447–450.
Gill, N., S. Findley, J. G. Walling, C. Hans, J. Ma et al., 2009 Molecular and chromosomal evidence for allopolyploidy in soybean. Plant Physiol. 151: 1167–1174.
Gong, Z., Y. Wu, A. Koblikova, G. A. Torres, K. Wang et al., 2012 Repeatless and repeat-based centromeres in potato: implications for centromere evolution. Plant Cell 24: 3559–3574.
Hamon, P., S. Siljak-Yakovlev, S. Srisuwan, O. Robin, V. Poncet et al., 2009 Physical mapping of rDNA and heterochromatin in chromosomes of 16 Coffea species: a revised view of species differentiation. Chr. Res. 17: 291–304.
Hayasaki, M., T. Morikawa, and J. M. Leggett, 2001 Intraspecific variation of 18S–5.8S–26S rDNA sites revealed by FISH and RFLP in wild oat, Avena agadriana. Genes Genet. Syst. 76: 9–14.
Iwata, A., A. L. Tek, M. M. Richard, B. Abernathy, A. Fonseca et al., 2013 Identification and characterization of functional centromeres of the common bean. Plant J. 76: 47–60.
A. Iwata-Otsubo, J. Jiang, J., J. Birchler, W. Parrott W, and R.K. Dawe, 2003 A molecular view of plant centromeres. Trends Plant Sci. 8: 570–575.

Kato, A., J. C. Lamb, and J. A. Birchler, 2004 Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize. Proc. Natl. Acad. Sci. USA 101: 13554–13559.

Kato, A., P. S. Albert, J. M. Vega, and J. A. Birchler, 2006 Sensitive fluorescence in situ hybridization signal detection in maize using directly labeled probes produced by high concentration DNA polymerase nick translation. Biotech. Histochem. 81: 71–78.

Koenig, R., and P. Gepts, 1989 Allozyme diversity in wild Phaseolus vulgaris—further evidence for two major centers of genetic diversity. Theor. Appl. Genet. 78: 809–817.

Koenig, R. L., S. P. Singh, and P. Gepts, 1990 Novel phaseolin types in wild and cultivated common bean (Phaseolus vulgaris, Fabaceae). Econ. Bot. 44: 50–60.

Koinange, E., and P. Gepts, 1992 Hybrid weakness in wild Phaseolus vulgaris L. Hered. 83: 135–139.

Mamidi, S., M. Rossi, S. M. Moghaddam, D. Annam, R. Lee et al., 2013 Demographic factors shaped diversity in the two gene pools of wild common bean Phaseolus vulgaris L. Heredity 110: 267–276.

Marshall, O. J., A. C. Chueh, L. H. Wong, and K. H. A. Choo, 2008 Neocentromeres: new insights into centromere structure, disease development, and karyotype evolution. Am. J. Hum. Genet. 82: 261–282.

Mehrotra, S., and V. Goyal, 2014 Repetitive sequences in plant nuclear DNA: types, distribution, evolution and function. Genomics Proteomics Bioinformatics 12: 164–171.

Moscone, E. A., F. Klein, M. Lambrou, J. Fuchs, and D. Schweizer, 1999 Quantitative karyotyping and dual-color FISH mapping of 3S and 18S-25S rDNA probes in the cultivated Phaseolus species (Leguminosae). Genome 42: 1224–1233.

Pedrosa-Harand, A., C. C. de Almeida, M. Mosiolek, M. W. Blair, D. Schweizer et al., 2006 Extensive ribosomal DNA amplification during Andean common bean (Phaseolus vulgaris L.) evolution. Theor. Appl. Genet. 112: 924–933.

Pedrosa-Harand, A., J. Kamil, P. Gepts, V. Geffroy, and D. Schweizer, 2009 Cytogenetic mapping of common bean chromosomes reveals a less compartmentalized small-genome plant species. Chr. Res. 17: 405–417.

Raskina, O., A. Belyaev, and E. Nevo, 2004 Quantum speciation in Aegilops: molecular cytogenetic evidence from rDNA cluster variability in natural populations. Proc. Natl. Acad. Sci. USA 101: 14818–14823.

Richard, M. M. S., N. W. G. Chen, V. Thareau, S. Pflieger, S. Blanchet et al., 2013 The subtelomeric khipu satellite repeat from Phaseolus vulgaris: lessons learned from the genome analysis of the Andean genotype G19833. Front. Plant Sci. 4: 109.

Robbledo, G., and G. Seijo, 2009 Species relationships among the wild B genome of Arachis species (section Arachis) based on FISH mapping of rDNA loci and heterochromatin detection: a new proposal for genome arrangement. Theor. Appl. Genet. 121: 1033–1046.

Sambrook, J., and D. W. Russell, 2001 Molecular Cloning: A Laboratory Manual, Ed. 3rd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schlueter, J. A., J. L. Goicoechea, K. Collura, N. Gill, J. Y. Lin et al., 2008 BAC-end sequence analysis and a draft physical map of the common bean (Phaseolus vulgaris L.) genome. Trop. Plant Biol. 1: 40–48.

Schmutz, J., P. McClean, S. Mamidi, G. Wu, S. Cannon, et al., 2014 A reference genome for common bean and genome-wide analysis of dual domestications. Nat. Genet. 46: 707–713.

Shang, W. H., T. Hori, A. Toyoda, J. Kato, K. Popendorf et al., 2010 Chickens possess centromeres with both extended tandem repeats and short non-tandem-repetitive sequences. Genome Res. 20: 1219–1228.

Shii, C. T., M. C. Mok, S. R. Temple, and D. W. S. Mok, 1980 Expression of developmental abnormalities in hybrids of Phaseolus vulgaris L.—interaction between temperature and allelic dosage. J. Hered. 71: 218–222.

Singh, S. P., 2004 Common bean (Phaseolus vulgaris L.), pp 13–57 in Genetic Resources, Chromosome Engineering, and Crop Improvement, edited by R. J. Singh and P. P. Jauhar. CRC Press, Boca Raton.

Singh, S. P., P. Gepts, and D. G. Debouck, 1991 Races of common bean (Phaseolus vulgaris, Fabaceae). Econ. Bot. 45: 379–396.

Toro Chica, O., M. J. Tohme, and D. Debouck, 1990 p. 109 in Wild bean (Phaseolus vulgaris L.): description and distribution. Cent. Int. Agric. Trop. (CIAT), Int. Board Plant Genet. Resour, IBPGR, Cali, CO.

Vallejos, C. E., N. S. Sakiyama, and C. D. Chase, 1992 A molecular marker-based linkage map of Phaseolus vulgaris L. Genetics 131: 733–740.

Velasquez, V. L. B., and P. Gepts, 1994 RFLP diversity of common bean (Phaseolus vulgaris) in its centers of origin. Genome 37: 256–263.

Wallung, J. G., J. C. Pires, and S. A. Jackson, 2005 Preparation of samples for comparative studies of plant chromosomes using in situ hybridization methods. Mol. Evol. Prod. Biochem. Data, Part B 395: 443–460.

Xiong, Z., and J. C. Pires, 2011 Karyotype and identification of all homoeologous chromosomes of allopolyploid Brassica napus and its diploid progenitors. Genetics 187: 37–49.

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