First-generation HapMap in Cajanus spp. reveals untapped variations in parental lines of mapping populations

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Summary

Whole genome re-sequencing (WGRS) was conducted on a panel of 20 Cajanus spp. accessions (crossing parents of recombinant inbred lines, introgression lines, multiparent advanced generation intercross and nested association mapping population) comprising of two wild species and 18 cultivated species accessions. A total of 791.77 million paired-end reads were generated with an effective mapping depth of ~12X per accession. Analysis of WGRS data provided 5 465 676 genome-wide variations including 4 686 422 SNPs and 779 254 InDels across the accessions. Large structural variations in the form of copy number variations (2598) and presence and absence variations (970) were also identified. Additionally, 2 630 904 accession-specific variations comprising of 2 278 571 SNPs (86.6%), 166 243 deletions (6.3%) and 186 090 insertions (7.1%) were also reported. Identified polymorphic sites in this study provide the first-generation HapMap in Cajanus spp. which will be useful in mapping the genomic regions responsible for important traits.

Introduction

Pigeonpea (Cajanus cajan L.) belongs to the genus Cajanus under Fabaceae family. The genus Cajanus is comprised of 32 species including wild and cultivated species. Pigeonpea is one of the most important food legume crops grown in marginal environments of the world. It has been considered a rich source of protein to the vegetarian families. However, the crop productivity has remained stagnant (~750 kg/ha) during last six decades (http://faostat.fao.org/). The low level of yield is due to biotic stresses [fusarium wilt (FW), sterility mosaic disease (SMD), etc.], abiotic stresses (water logging, salinity, etc.) and narrow genetic base in the cultivated gene pool (Saxena et al., 2010). Research efforts have been undertaken to solve the above-mentioned constraints and a number of disease-resistant and high-yielding varieties were released for cultivation. However, the average yield level remains <1 ton per hectare (http://www.iipr.res.in/aicrp.html).

Genomics-assisted breeding (GAB) has been successfully deployed in a number of crops species to tackle the longstanding problems (Varshney et al., 2009). In the case of pigeonpea, GAB could not be adequately deployed to tackle the constraints responsible for low yield. In terms of genomics resources, large number of molecular markers such as simple sequence repeat (SSR), diversity array technology (DArT), single-feature polymorphism (SFP), single-nucleotide polymorphisms (SNPs), etc., and a number of segregating populations have been developed (Bohra et al., 2014; Dutta et al., 2011; Pazhamala et al., 2015). Mapping for economical important traits could not be very successful with above-mentioned resources as only few hundreds of markers were found polymorphic in parental genotypes (Bohra et al., 2014).

Next-generation sequencing (NGS) technologies have provided new avenues to detect genome-wide variations present in gene pools of different species (Bevan and Uauy, 2013; Thudi et al., 2012). After having a draft genome in a particular crop species, deploying NGS becomes more cost effective in assessing the genome-wide variations (Varshney et al., 2009). NGS enables identification of SNPs and InDels in efficient and high-throughput manner (Lam et al., 2010; Xu et al., 2012). At present, draft genome sequences have become available in a number of crop species (Michael and Jackson, 2013) including pigeonpea (Singh et al., 2011; Varshney et al., 2012). Further, in the recent past, NGS has been used in identifying genome-wide variations through WGRS based approaches (Arai-Kichise et al., 2014; Mace et al., 2013; Varshney et al., 2009; Zheng et al., 2011). This will subsequently enable the discovery of SNPs and InDels at the genome-wide scale within both germplasm collections and breeding lines of pigeonpea. SNPs and InDels are of high importance for crop improvement programs (Chen et al., 2014; Tao et al., 2014). Both SNPs and InDels are being used in different trait mapping approaches, haplotype analysis and GAB programs such as marker-assisted selection, marker-assisted back-crossing, genomic selection, varietal identification and hybridity testing. In recent years, SNPs have been detected and analysed in many cultivars and inbred lines of pigeonpea and used for linkage mapping and diversity analysis (Saxena et al., 2012, 2014).

However, these SNPs were identified in limited number of accessions and few SNPs were applicable to other accessions. For implementing modern GAB methodologies, it is highly
imperative to detect genome-wide variations in a larger set of accessions.

In view of above, we have re-sequenced 20 diverse Cajanus spp. accessions representing two distinct gene pools (primary and secondary): the pre-domesticated wild species accessions (C. acutifolius and C. cajanifolius), local landraces (C. cajan) and modern elite cultivars or breeding lines (C. cajan). C. cajanifolius has been considered as the closest wild relative and the progenitor species of cultivated pigeonpea (Kassa et al., 2012; Pazhamalai et al., 2015; Saxena et al., 2014). Selected accessions are crossing parents of six mapping populations segregating for economically important traits. This study not only presents the first generation HapMap in Cajanus spp. but also reports unique molecular signatures for each accession and large-scale variations between crossing parents for high-resolution trait mapping.

Results
Selection and features of Cajanus spp. accessions
A total of 20 Cajanus spp. accessions were selected on the basis of multi-year/locations trait phenotyping data for development of recombinant inbred line (RIL), introgression line (IL), multiparent advanced generation intercross (MAGIC) and nested association mapping (NAM) populations (Saxena et al., 2010; Varshney et al., 2010). For instance, ICP 8863 has been found to be resistant to FW, whereas ICP 7035, HPL 24 and ICPB 2049 were resistant to SMD. Two accessions namely, ICPL 99050 and ICPL 88039, were resistant to FW, whereas ICP 7035, HPL 24 and ICPB 2049 were resistant to SMD. Two accessions namely, ICP 7426 and ICP 14209 produce high number of pods per plant whereas, HPL 24 is known as a high seed protein containing line.

Large-scale data generation and alignment
Illumina paired-end sequencing technology was used to sequence a panel of 20 Cajanus spp. accessions on the MiSeq platform. As a result, a total of 157 Gb raw data have been generated with 791.77 million reads of read length from 150 to 250 bp (Table 1). Paired-end reads were mapped onto the reference genome (Varshney et al., 2012). Across all 20 accessions, 731.28 million reads were mapped onto the reference genome. Around 93% of total reads from 18 cultivated species accessions and 79% from two wild species accessions were mapped onto the reference genome. These variations in mapping may be due to divergence between parental accessions and the incompleteness of the reference genome assembly. Of these 731.28 million mapped reads, 469.18 million reads were mapped uniquely onto the reference genome, while rest of the reads were mapped to the multiple locations in the genome. Remaining 60.49 million reads (7.6% of total reads) could not be mapped onto the reference genome (Table 1, Figure S1). The effective mapping depth ranged from 8X (MN 1) to 16.5X (ICP 14486) with an average of 12X per Cajanus spp. accession. The genome coverage for each Cajanus spp. accession against the reference genome varied from 75% to 91% with an average of 89% in the cultivated spp. accessions and 82% in wild species accessions (Table 1).

Genome-wide variations across Cajanus spp. accessions
Genome-wide SNPs and InDels were identified across Cajanus spp. accessions (Figure 1). Comprehensive data analysis identified

| Genotype | Total reads (million) | Mapped reads (million) | Total mapped reads (%) | Uniquely mapped reads (million) | Genome coverage (%) | Depth (X) |
|----------|-----------------------|------------------------|------------------------|---------------------------------|--------------------|-----------|
| HPL 24   | 48.26                 | 45.92                  | 95.16                  | 29.50                           | 89.19              | 11.32     |
| ICPB 2049| 40.18                 | 37.34                  | 92.94                  | 25.16                           | 89.10              | 14.88     |
| ICPL 20097| 31.57                | 28.99                  | 91.84                  | 18.91                           | 88.66              | 11.51     |
| ICPL 85010| 34.84                | 32.49                  | 93.25                  | 21.19                           | 88.50              | 12.92     |
| ICPL 85063| 40.14                | 37.46                  | 93.31                  | 25.30                           | 89.31              | 14.87     |
| ICPL 87   | 39.36                 | 37.38                  | 94.96                  | 24.50                           | 88.55              | 9.20      |
| ICPL 88039| 25.91                | 24.17                  | 93.29                  | 16.29                           | 86.77              | 9.59      |
| ICPL 99050| 41.41                 | 37.95                  | 91.64                  | 23.73                           | 89.48              | 15.12     |
| MN 1      | 34.03                 | 32.52                  | 95.56                  | 22.37                           | 87.88              | 7.82      |
| ICP 11605| 38.86                 | 35.78                  | 92.06                  | 22.74                           | 88.72              | 14.03     |
| ICP 14209| 35.04                 | 32.50                  | 92.74                  | 20.72                           | 88.52              | 12.83     |
| ICP 14486| 72.22                 | 68.63                  | 95.03                  | 41.74                           | 90.69              | 16.46     |
| ICP 28    | 32.11                 | 29.94                  | 93.24                  | 20.31                           | 88.26              | 11.92     |
| ICP 5529  | 50.10                 | 46.89                  | 93.60                  | 28.04                           | 89.67              | 11.36     |
| ICP 7035  | 36.42                 | 34.52                  | 94.78                  | 23.34                           | 88.19              | 8.50      |
| ICP 7263  | 34.36                 | 32.58                  | 94.82                  | 21.35                           | 87.94              | 8.02      |
| ICP 7426  | 36.31                 | 33.68                  | 92.75                  | 20.53                           | 88.39              | 13.38     |
| ICP 8863  | 43.02                 | 41.04                  | 95.41                  | 25.04                           | 88.96              | 9.97      |
| ICPW 12   | 37.29                 | 24.00                  | 64.37                  | 14.65                           | 74.63              | 9.68      |
| ICPW 29   | 40.34                 | 37.49                  | 92.94                  | 23.76                           | 89.61              | 15.16     |
| Total     | 791.77                | 731.28                 | 469.18                 |                                 |                    |           |
a total of 5,465,676 variations including 4,686,422 SNPs and 779,254 InDels (373,038 insertions and 406,216 deletions) ranged from 1 to 48 bp in length (Figures S2 and S3). In terms of the assembled genome around 47.5% SNPs, 51.1% insertions and 51.3% deletions were present in 11 pseudomolecules (CcLG01 to CcLG11). Rest of the variations were present on unanchored scaffolds (CcLG0) in draft genome (Table S2). The variations across individual pseudomolecules in *Cajanus* spp. accessions were ranged from 47,453 (CcLG05) to 509,422 (CcLG11). SNPs identified in *Cajanus* spp. accessions were classified into two broad categories, homozygous and heterozygous SNPs based on the presence of one or more than one allele at the same position against the reference genome. The percent heterozygosity was ranged from 14% in ICPW 12 to 67% in ICPW 29 (Figure S4, Table S3).

SNPs and InDels densities were calculated per 100 kb along each pseudomolecule. The average density of variations across the whole genome was 879.3 SNPs, 82.7 deletions and 75.8 insertions (Figure 1). Densities of SNPs/InDels varied across pseudomolecules. Highest number of SNPs (976), deletions (97) and insertions (91) per 100 kb were observed on CcLG04. The lowest number of SNPs per 100 kb were found on CcLG05 (770), whereas lowest number of deletions (75) and insertions (69) per 100 kb were present on CcLG10 (Table S2). In terms of individual accessions, maximum variations (SNPs and InDels) were identified in wild species accessions (ICPW 12: 3,868,179; ICPW 29: 1,101,494) in comparison with the reference genome. The per cent heterozygosity was ranged from 14% in ICPW 12 to 67% in ICPW 29 (Figure S4, Table S3).

Identified SNPs and InDels in *Cajanus* spp. accessions were annotated onto the reference genome. Most of the SNPs were located in intergenic regions (83%) followed by intronic regions (10%), exonic regions (4.8%) and 2.2% could not be classified to any category. Exonic SNPs were further classified into synonymous and nonsynonymous SNPs, and the resulting ratio of nonsynonymous to synonymous substitutions was 1.46. Furthermore, the effect of each SNP was also categorized into four classes, viz. modifier, low effect, moderate effect and high effect. About 93% SNPs (presented in intergenic and intronic regions) were classified as modifier. Remaining 4.8% exonic SNPs were classified as low effect (silent-synonymous substitutions and nonsynonymous start), moderate effect (nonsynonymous coding) and high effect (start lost, stop lost and stop gain) (Tables 2 and S10).

The effects of InDels were categorized separately as insertion and deletion effects (Tables S4 and S5). However, majority of InDels were located in intergenic (76%–86%) and intronic region (11%–21%) according to the parental accessions and considered as modifier. Across parental accessions, only 1%–1.5% InDels were located in exonic regions. The moderate effect insertions were classified as codon insertion and codon change plus codon

Figure 1 Distribution of genome-wide variations identified across *Cajanus* spp. accessions against reference genome (the zoom portion of CcLG04 has shown the highest variation densities identified). Different circles ("a" to "i") represent as following: a: outer most circle represent 11 pseudomolecules, b: SNP density, c: lnDel density, d: presence and absence variations (PAVs), e: copy number variations (CNVs), f: synonymous SNP substitutions density, g: nonsynonymous SNP substitutions density, h: intergenic SNP density, i: intronic SNP density; in the 11 *Cajanus* pseudomolecule.
Table 2: Distribution and classification of SNPs in each Cajanus spp. accession

| Genotype | Total SNPs | Intergenic | Intronic | Silent | Start lost | Stop lost | Nonsynonymous start | Nonsynonymous coding | Stop gain | Unknown |
|----------|------------|------------|----------|--------|------------|-----------|---------------------|---------------------|-----------|---------|
| HPL 24  | 346 096    | 280 639    | 42 294   | 7131   | 16         | 64        | 1                   | 9179                | 265       | 6507    |
| ICPB 2049 | 344 666    | 289 482    | 30 652   | 6121   | 18         | 76        | 2                   | 8862                | 274       | 9179    |
| ICP 20097 | 199 761    | 165 235    | 18 201   | 4409   | 14         | 65        | 2                   | 6252                | 200       | 5383    |
| ICPW 85010 | 349 778    | 291 512    | 32 827   | 6608   | 13         | 80        | 2                   | 9747                | 276       | 8713    |
| ICPW 85063 | 304 980    | 255 181    | 27 054   | 5867   | 16         | 69        | 3                   | 8611                | 253       | 7926    |
| ICP 87    | 258 560    | 213 999    | 27 442   | 4932   | 8          | 54        | 2                   | 6907                | 213       | 5003    |
| ICPW 88039 | 287 399    | 238 046    | 27 456   | 5996   | 18         | 67        | 3                   | 8502                | 239       | 7072    |
| ICP 99050 | 360 154    | 303 116    | 32 750   | 6247   | 18         | 81        | 3                   | 8975                | 266       | 8698    |
| MN 1      | 228 688    | 189 834    | 23 634   | 4065   | 6          | 44        | 2                   | 5926                | 172       | 4805    |
| ICP 11605 | 400 435    | 335 043    | 37 427   | 7393   | 16         | 88        | 2                   | 10 638               | 295       | 9533    |
| ICP 14209 | 338 975    | 283 217    | 31 414   | 6275   | 17         | 79        | 3                   | 9066                | 284       | 8620    |
| ICP 14486 | 404 003    | 336 672    | 40 461   | 7530   | 18         | 87        | 1                   | 10 847               | 296       | 8091    |
| ICP 28    | 335 735    | 282 033    | 29 900   | 6138   | 17         | 85        | 3                   | 8915                | 253       | 8391    |
| ICP 5529  | 258 008    | 212 661    | 27 312   | 5 183  | 10         | 58        | 1                   | 7323                | 231       | 5229    |
| ICP 7035  | 265 762    | 218 935    | 28 709   | 5118   | 11         | 50        | 1                   | 7237                | 210       | 5491    |
| ICP 7263  | 220 759    | 182 328    | 23 575   | 4239   | 6          | 34        | 1                   | 6046                | 170       | 4360    |
| ICP 7426  | 360 166    | 298 995    | 33 172   | 7535   | 21         | 99        | 3                   | 11 070               | 287       | 8984    |
| ICP 8863  | 195 546    | 161 342    | 19 415   | 4331   | 10         | 55        | 2                   | 5946                | 195       | 4250    |
| ICPW 12   | 3 357 515  | 2 559 376  | 558 482  | 92 976  | 196        | 386       | 29                  | 99 797              | 1360      | 44 913  |
| ICPW 29   | 917 145    | 776 862    | 85 479   | 14 449  | 54         | 167       | 4                   | 20 006              | 486       | 19 638  |

Candidate variations for trait mapping

To detect parental polymorphism, pairwise SNPs and InDels were identified between each crossing parental (Table S12). In the case of bi-parental mapping populations, maximum SNPs were present between crossing parentals of Introgression libraries-1 (IL-1) (ICPW 12 vs ICPL 87119: 917 145) followed by Introgression libraries-2 (IL-2) (ICPW 29 vs ICPL 87119: 917 145), PRIL_A (ICPB 2049 vs ICPL 99050: 307 593) and PRIL_C (ICPL 20097 vs ICPL 8863: 201 986) (Table S11). In the multiparent mapping populations, pairwise comparisons were made in all the possible combinations. For instance, a set of 28 crossing combinations in MAGIC and 10 crossing combinations in NAM were compared. In MAGIC population, maximum number of SNPs and InDels were found between HPL 24 × ICP 14486 (436 293 and 97 757, respectively) and minimum between ICP 5529 × ICP 8863 (235 332 and 61 819, respectively) (Table S14). In the NAM population, pairwise SNPs and InDels were maximum in ICPW 85010 × ICPL 87119 (349 778 and 71 250, respectively) and minimum in ICP 8863 × ICPL 87119 (195 546 and 41 625, respectively) (Table S12).

Genetic relationships among Cajanus spp. accessions

To explain the genetic divergence among the Cajanus spp. accessions, a phylogenetic tree was constructed with the help of SNPPhylo program (Figure 2). Cajanus spp. accessions ICP 28 and HPL 24 were out-grouped from rest of the accessions. Remaining accessions were broadly categorized into two main clusters (Cl I and Cl II). Cl I contained 11 accessions representing two wild species accessions, five landraces and four breeding lines, whereas Cl II contained seven accessions representing three landraces and four breeding lines. Under these two main clusters, accessions were grouped further into subclusters. For instance, the cluster Cl I contained four subclusters (Ia, Ib, Ic and Id). Wild species accessions were grouped into a small subclusters Cl Ia, four breeding lines with two landraces were grouped in Cl Ib, and ICP 7035 was placed in subcluster Cl Ic, while the landraces ICP 14209 and ICPL 7426 were grouped together in a subcluster Cl Id. Furthermore, the cluster Cl II contained two subclusters (Ia and Ib). Landraces ICP 5529 and ICP 7263 were grouped in Cl IIa, whereas the remaining 4 breeding lines and one landrace were grouped in Cl IIb. To assess divergence among 8 parental accessions of MAGIC population and 11 parental accessions of NAM population, separate dendrograms were also constructed.
Figure S5. Pairwise genetic distances between wild species accessions were greater than those between any pairs of the domestic accessions, that is breeding lines and landraces. Diversity between any pair of the wild species accession with domestic pigeonpea accession is greater than those among all possible domestic pigeonpea accessions pairs (Table S15).

Accession-specific variations

A total of 2,630,904 accession-specific variations (SNPs and InDels) were found in all Cajanus spp. accessions. These accession-specific variations include 2,278,571 (86.6%) SNPs, 166,243 (6.3%) deletions and 186,090 (7.1%) insertions (Table 3). The proportion of accession-specific variants ranged from 0.2% to 65.6% of the total variants. The accession ICPW 12 had the highest number of accession-specific variants 2,537,481 (65.6% of the total variants). However, on the other side, ICPL 85010 had the lowest number of accession-specific variants 791 (0.2% of the total variants). Among the accession-specific variants, wild species accession-specific (97.7%) were more abundant than landrace-specific (1.4%) and breeding linespecific (0.9%) variants (Figure 3). The frequency of accession-specific variants located in exonic regions were ranged from 2.9% in ICPB 2049 to 5.6% in ICP 14486 (Table S16). However, the total number of accession-specific variants (SNPs and InDels) located in exonic region ranged from 24 (ICPB 2049) to 120,336 variants (ICPW 12). Gene ontology annotation was used to assess possible gene functions targeted by accession-specific variants for all 20 accessions, and details have been provided in Table S17. In addition to these accession-specific small variations, some accession-specific large variations in the form CNVs (2,258) and PAVs (288) were also identified (Table 3).

Discussion

Pigeonpea productivity just has not suffered from biotic, abiotic stresses, low polymorphism in cultivated gene pool, but also from less genomics and genetic resources in the past (Pazhamala et al., 2015; Varshney et al., 2010). However, in recent years, NGS technologies were successfully deployed in pigeonpea, and ample genomic resources have been developed such as draft genome assembly (Varshney et al., 2012) and transcriptome assemblies (Dubey et al., 2011; Kudapa et al., 2012). Above-mentioned advances in pigeonpea genomics have provided opportunities to assess genome-wide variations using WGRS approach. WGRS in the present study identified more than 4.5 million SNPs, 779,254 InDels along with 2,258 CNVs and 970 PAVs distributed throughout the Cajanus genome. Prior to this study, only few thousands of SNPs were reported by aligning transcriptome reads from 12 pigeonpea genotypes onto the pigeonpea reference genome (Dubey et al., 2011; Varshney et al., 2012). WGRS efforts have been undertaken to extend the repertoire of polymorphisms in a number of crop species such as rice (Huang et al., 2012; Xu et al., 2012), maize (Hufford et al., 2012), sorghum (Mace et al., 2013), chickpea (Varshney et al., 2013), Medicago (Stanton-Geddes et al., 2013), soybean (Lam et al., 2010; Zhou et al., 2015), tomato (Lin et al., 2014), etc. Notably in Medicago, more than 6 million SNPs were identified through re-sequencing of 236 diverse accessions at ~8X coverage.
In continuation of this work, 384 inbred lines are being re-sequenced under Medicago HapMap project using Illumina NGS technology to discover the SNPs, InDels and CNVs at 5X to 20X coverage (http://www.medicago-hapmap.org). In the case of chickpea, a total of 82,489 genome-wide SNPs (including 38,511 and 43,978 from hapmap.org). In the case of chickpea, a total of 82,489 genome-inDels and CNVs at 5X to 20X coverage (http://www.medicago-project.org) were identified from 93 specific SNPs, insertions and deletions identified in total unique variations represented as 86.6% accession-specific SNPs (green color), 6.3% accession-specific deletions (red color), 7.1% accession-specific insertions (blue color), (b) percentage of accession-specific variations identified within three groups represented as 97.7% in wild species accessions group (red color), 0.9% in landraces group (blue color) and 1.4% in breeding lines group (green color), (c) percentage of accession-specific variations in each accession, individually within a group represented by a specific color (wild species accessions group represented two accessions, landraces and breeding lines groups represented nine accessions in each).

Figure 3 Distribution of accession-specific or unique variations (SNPs, insertions and deletions) across 20 Cajanus accessions: (a) percentage of accession-specific SNPs, insertions and deletions identified in total unique variations represented as 86.6% accession-specific SNPs (green color), 6.3% accession-specific deletions (red color), 7.1% accession-specific insertions (blue color), (b) percentage of accession-specific variations identified within three groups represented as 97.7% in wild species accessions group (red color), 0.9% in landraces group (blue color) and 1.4% in breeding lines group (green color), (c) percentage of accession-specific variations in each accession, individually within a group represented by a specific color (wild species accessions group represented two accessions, landraces and breeding lines groups represented nine accessions in each).
In the present study, we have identified 5.5 million genome-wide variations across parental accessions representing elite breeding lines, landraces and wild species accessions, which are fractionally higher from 1.1, 4.3 and 5.1 million variations identified in parental lines in soybean (Li et al., 2014), tomato (Caussé et al., 2013) and rice (Subbaiyan et al., 2012), respectively. Annotation of identified variations in 20 accessions showed <5% variations in coding regions. This proportion is similar to the ratio of 4.5% of SNP and 1.5% of InDel identified in sorghum (Mace et al., 2013). Among coding regions, the proportion of nonsynonymous SNPs is higher than that of synonymous SNPs. The ratio of nonsynonymous to synonymous substitutions in Cajanus spp. accessions was found within the range of earlier reports in other crop species viz. soybean: 1.37 (1.36 in wild and 1.38 in cultivated; Lam et al., 2010), tomato: 1.41 (1.34 and 1.48 in cherry tomato and cultivated tomato lines, Caussé et al., 2013) and rice: 1.29 (Xu et al., 2012). Additionally, CNVs and PAVs have been identified in Cajanus spp. accessions. Interestingly, results suggested that the frequency of gene lost events is higher in accessions which are under cultivation (landraces and breeding lines) as compared to the wild species accession.

Conclusion

Variants discovered from re-sequencing of 20 Cajanus spp. accessions offer information on sampled loci across the pigeonpea genome harboring high diversity and unique accession signatures. These polymorphic sites will be useful for developing high-density SNP arrays, genotyping of several mapping populations to construct genetic maps and identify the genomic regions responsible for agronomic important traits. Unique accession signatures will be useful in varietal identification or assessment of adoption of varieties in different geographies. This study also re-emphasized that the cultivated pigeonpea genepool has a narrow genetic base and new populations such as IL, MAGIC, NAM, etc. must be used to re-introduce adaptive diversity lost through domestication and human selection in breeding. To use new genetic combinations, it is highly recommended to have high-density genotyping to track the superior haplotypes and avoid linkage drag in breeding programs aimed for pigeonpea improvement.

Experimental procedures

Plant materials

A set of 20 Cajanus spp. accessions including two wild species accessions, nine breeding lines and nine landraces were used in this study. These accessions are crossing parents of six mapping populations, including four bi-parental and two multiparent mapping populations. Among these, ICPW 12 (C. acutifolius) and ICPW 29 (C. cajanifolius) are wild Cajanus spp. accessions and used for the development of two AB populations, namely IL-1 and IL-2, respectively. Accession ICPW 87119 from C. cajan was used as a common crossing parent in both the above-mentioned lines. ICPW 87119 is a leading variety which released as ‘Asha’ in India; subsequently, it was used in developing pigeonpea draft genome (Varshney et al., 2012). Four accessions (ICPB 2049, ICP 99050, ICP 20097 and ICP 8863) were parents of two pigeonpea recombinant inbred lines: PRL_A (ICPB 2049 × ICP 99050) and PRL_C (ICPB 20097 × ICP 8863). Eight accessions (ICP 5529, HPL 24, ICP 7035, ICP 8863, ICP 14486, ICP 11605, ICP 7426 and ICP 14209) representing breeding lines and landraces are being used in developing MAGIC population. 10 accessions (HPL 24, ICP 85010, ICP 85063, ICP 88039, MN 1, ICP 28, ICP 7035, ICP 7263 and ICP 8863) as founder parents and ICPW 87119 as a nested parent are being used in developing NAM population. Details on each accession have been provided in Table S1.

Library preparation and sequencing

Genomic DNA was extracted from young leaves of individual accessions of each accession, using a NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany). DNA quality was checked on 0.8% agarose gel and DNA quantity assessed on Qubit® 2.0 Fluorometer using the dsDNA BR Assay kit (Life Technologies, Thermo Fisher Scientific Corporation, Waltham, MA). Indexed DNA libraries were prepared following Illumina paired-end DNA sample prep protocol (Part # 15026486 Rev.C, July 2012) with minor modifications. Initially, 2 μg of genomic DNA from each sample was sonicated by Bioruptor® NGS (Diogenebio, Liege, Belgium) to get a target insert size of 400–500 bp. After end-repairing and indexed adapter ligation, size selection of 600 bp DNA fragments was performed on E-Gel® SizeSelect™ 2% agarose precast gels (Invitrogen, Life Technologies, Thermo Fisher Scientific Corporation, Waltham, MA). To enrich DNA fragments having adapters on both ends, PCRs were performed using Illumina adapter compatible PCR primers. The size distribution of amplified DNA libraries was checked on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). DNA libraries were sequenced on the MiSeq platform (Illumina Inc., San Diego, CA) with MiSeq reagent kit v2 (300- or 500-cycles) to generate 150 to 250 bp paired-end reads.

Sequence alignment and variant identification

To reduce sequencing errors, paired-end sequencing reads were trimmed and filtered with sickle version 1.200 (https://github.com/najoshi/sickle). Initially, duplicate reads were removed, further low-quality reads (having phred score <30) and sequences shorter than 100 nucleotides, or containing ‘N’, were removed using in-house QC pipeline NGS-QCBox (Katta et al., 2015). After cleaning steps, filtered reads were mapped onto the reference genome with bowtie2 v2.2.4 (Langmead and Salzberg, 2012) using default options. Reads mapped on more than one position or not mapped were filtered to define uniquely mapped reads and unmapped reads. Reads having unique alignment onto the reference genome were retained in the BAM files. BAM files were further processed for variant (SNP and InDel) calling using Genome Analysis Toolkit suite with a minimum depth coverage of five reads per individual accession (McKenna et al., 2010). Using an in-house perl script, the distribution of identified variants was analysed along the entire genome using a contiguous window of 100 kb. Additionally, identified SNPs were classified into homozygous and heterozygous (reads aligned at a position contained reference as well as alternate bases) SNPs, on the basis of mismatch frequencies. InDels were identified within the size range of 1–48 bp. Accession-specific variants (SNPs and InDels) were reported only if the variant call was present in a particular accession and reference allele was present in remaining accessions. For identification of CNVs, CNVnator tool was used with an e-value of 1e-05 (Abyzov et al., 2011). Raw reads from Asha (ICPW 87119) genotype were aligned to draft assembly (Asha) for detecting the false positives in CNVs. Identified CNVs present in genes with length ≥1 kb were then reported. The frequencies of variants (SNPs and InDels) and CNVs were then projected using Circos (Krzywinski et al., 2009) across the categorized genotypes.
Variant annotation and diversity analysis

Identified SNPs and InDels based on their genomic locations were annotated as intergenic, intronic and exonic using SnpEff (Cingolani et al., 2012). The variants were further categorized into synonymous, nonsynonymous, start codon loss, stop codon gain, frame shifts, etc. The effects of variants were classified on the basis of their impacts as high, moderate, low and modifier. Generic feature format files having information on positions of accession-specific variants in various biological pathways were compared with the B73 reference genome. Accession-specific variants (SNPs and InDels) were present in exonic region for each accession were functionally annotated using UniProtKB database, and GO terms were assigned accordingly (Huntley et al., 2014). Further, the impacts of accession-specific variants in various biological pathways were examined using The Biological Networks Gene Ontology tool (Maere et al., 2005).

The phylogenetic tree was constructed using DNASP programs in the PHYLIP package and 1000 bootstraps with other default parameters of SNPPhylo program (Lee et al., 2014). The software MEGA4 was used for visualizing the phylogenetic tree (Tamura et al., 2007).

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Supporting information

Additional Supporting information may be found in the online version of this article.

Figure S1 Classification of the sequencing reads generated through re-sequencing of 20 Cajanus spp. accessions and mapped onto the reference genome.

Figure S2 Genome-wide distribution of InDels with varying length.

Figure S3 Distribution of insertions (blue color) and deletions (red color) identified in 20 Cajanus spp. accessions.

Figure S4 Distribution of homozygous (green color) and heterozygous (red color) SNPs identified between each of the 20 Cajanus spp. accessions and reference genome.

Figure S5 Phylogenetic relationships among: (a) 8 MAGIC parental lines as landraces (triangles shape in blue color) and breeding lines (diamonds shape in green color) (b) 10 NAM founder and 1 nested parental line represented by landraces (triangles shape in blue color), breeding lines (diamonds shape in green color) and nested parent (circle shape in chocolate color).

Table S1 Details of Cajanus spp. accessions included in the present study.

Table S2 Distribution of genome-wide variations in different pseudomolecules.

Table S3 Heterozygosity of SNPs in Cajanus genome.

Table S4 Distribution of insertions and their effects in Cajanus spp. accessions.

Table S5 Distribution of deletions and their effects in Cajanus spp. accessions.

Table S6 Location of genes deleted in Cajanus spp. accessions.

Table S7 Location of genes duplicated in Cajanus spp. accessions.

Table S8 Summary of large deletions (>1 kb) in Cajanus spp. accessions.

Table S9 Summary of large duplications (>1 kb) in Cajanus spp. accessions.

Table S10 Distribution of homozygous SNPs and their effects.

Table S11 Distribution of tandem and interspersed repeats in Cajanus spp. accessions.

Table S12 Pairwise SNPs (below diagonal) and InDels (above diagonal) among Cajanus spp. accessions.

Table S13 Pairwise SNPs and InDels between parental lines of biparental populations.

Table S14 Pairwise SNPs (below diagonal) and InDels (above diagonal) among MAGIC parental lines.

Table S15 Pairwise genetic distances among 20 selected Cajanus spp. accessions.

Table S16 Distribution of accession-specific variants and their effects.

Table S17 Accession-specific variants and their effects on coding sequences and Gene Ontology (GO).