Single Nucleotide Polymorphism Genotyping for Breeding and Genetics Applications in Chickpea and Pigeonpea using the BeadXpress Platform

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Abstract

Single nucleotide polymorphisms (SNPs) are ideal molecular markers due to their higher abundance. Although several types of genotyping platforms for assaying large number of SNPs are available, in cases such as marker-assisted selection, where few markers are required for genotyping a set of potential lines, high-throughput SNP genotyping platforms (e.g., iScan or Infinium) may not be cost effective. In this scenario, GoldenGate assays based on VeraCode technology using Illumina BeadXpress seems to be the most cost-effective platform. The objective of this study was to develop cost-effective SNP genotyping platforms in chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* L.). Two sets of SNPs, one each for chickpea (96 SNPs) and pigeonpea (48 SNPs), were developed and tested by genotyping 288 diverse genotypes from respective reference sets. The SNPs selected for the oligo pool assays had high transferability to crop wild relative species. The mean polymorphism information content value of assayed SNP markers was 0.31 and 0.32 in chickpea and pigeonpea, respectively. No unique pattern was observed in the chickpea reference set whereas two major groups were observed in the case of the pigeonpea reference set. The Illumina BeadXpress platform assays developed for chickpea and pigeonpea are highly informative and cost effective for undertaking genetic studies in these legume species.

LEGUMES play a critical role in food security for the people living in Asian and sub-Saharan African regions of the world. Chickpea and pigeonpea are among the most important legumes supplying protein to human diets, fodder for household animals, and manure to agricultural land. Global production of chickpea is approximately 11 million t and approximately 3.5 million t in case of pigeonpea (http://faostat.fao.org/site/567/default.aspx#ancor; accessed 11 Apr. 2013). As these crops are mainly grown in marginal environments and exposed to several biotic (mainly Fusarium wilt, sterility mosaic disease, *Helicoverpa armigera*, and Ascochyta blight) and abiotic stresses (drought, soil salinity, waterlogging, and cold), the productivity of these legumes is less than 1 t ha^{-1} (http://faostat.fao.org/site/567/default.aspx#ancor; accessed 11 Apr. 2013), which is significantly lower than their potential yield (6 t ha^{-1} for chickpea and 2.5 t ha^{-1} for pigeonpea).

The development of high yielding varieties that can tolerate abiotic and biotic stresses has been the area of focus for many researchers in the past. Plant breeders have been addressing these production constraints using conventional approaches. Genomics-assisted breeding (GAB) approaches have been very successful for developing superior varieties or parental lines of hybrids in many crops (Varshney et al.,

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Abbreviations: ADT, assay design tool; CI, cluster; EST, expressed sequence tag; GAB, genomics-assisted breeding; KASPar, KBiosciences Competitive Allele-Specific polymerase chain reaction; MABC, marker-assisted backcrossing; MARS, marker-assisted recurrent selection; MAS, marker-assisted selection; PIC, polymorphism information content; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; TOG, tentative orthologous gene.

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2005, 2010). In the case of chickpea and pigeonpea, GAB applications have been limited (Varshney et al., 2010), mainly due to meager genomic resources, appropriate mapping populations to understand the genetics of complex traits, and narrow genetic diversity in elite germplasm. A series of evolutionary bottlenecks owning to this narrow genetic diversity have been reported and recommend the use of wild species to increase the genetic diversity in primary gene pool (Abbo et al., 2003). Nevertheless, the last 5 yr have witnessed significant progress in the area of developing genomic resources in these legume crops (Varshney et al., 2013a). For instance, thousands of molecular markers including simple sequence repeats (SSRs) (Nayak et al., 2010; Saxena et al., 2010; Bohra et al., 2011; Thudi et al., 2011), single feature polymorphism (Saxena et al., 2011), single nucleotide polymorphism (SNP) (Hiremath et al., 2012; Saxena et al., 2012), and diversity arrays technology (Yang et al., 2006; Thudi et al., 2011) markers and expressed sequence tags (ESTs) or transcript reads (Varshney et al., 2009; Dubey et al., 2011; Hiremath et al., 2011; Kudapa et al., 2012) were developed. Very recently, draft genome sequences have also become available for both chickpea (Varshney et al., 2013b) and pigeonpea (Varshney et al., 2012). The genomic resources currently available in chickpea and pigeonpea open opportunities to use GAB approaches in these species to complement the conventional breeding efforts.

Although a range of molecular marker systems are available, SSRs and SNPs have been considered as markers of choice for genetics and breeding applications (Gupta and Varshney, 2000; Mir and Varshney, 2013). Simple sequence repeat identification and genotyping is a tedious process and the semiautomated data collection also contributes to error-prone data generation. However, in the case of SNPs, greater abundance throughout the genome and complete automated data generation and collection make SNPs the preferred markers for all molecular breeding applications (Varshney et al., 2010; Mir and Varshney, 2013). Recent advances in next generation sequencing technologies and the availability of appropriate bioinformatics tools enable the cost-effective discovery of SNPs and their subsequent use in genome analysis and crop improvement (Azam et al., 2012; Thudi et al., 2012). As a result, SNP markers have started to be developed in a range of crop species (see Ganal et al., 2012). In the cases of chickpea and pigeonpea, several thousand high confidence SNPs are currently available (Hiremath et al., 2012; Saxena et al., 2012).

A range of SNP genotyping platforms are available for various plant species (see Fan et al., 2003; Varshney, 2011) for undertaking genetic studies; however, the choice of platform depends on the molecular breeding objectives, number of samples, and markers to be analyzed. Cost-effective platforms from medium to high throughput can be selected (Mir and Varshney, 2013). Array-based genotyping systems such as the Illumina GoldenGate (Illumina, Inc.) and Infinium (Illumina, Inc.) are now available, of which Illumina GoldenGate has regularly been used for mid-throughput (few selected markers with variable number of genotypes) applications (Fan et al., 2003). Such platforms have been developed and used in many cereal (Akhunov et al., 2009; Close et al., 2009; Thomson et al., 2012) and legume (Hyten et al., 2008; Deulvot et al., 2010) species.

Single nucleotide polymorphism genotyping has been used either for diversity analysis (Hyten et al., 2008; Akhunov et al., 2009) or for development and/or saturation of genetic maps (Gaur et al., 2012). The above mentioned studies required large number of SNP markers to be analyzed, and therefore high-density SNP genotyping platforms (e.g., Illumina GoldenGate assays) are the best choices. However, for breeding applications such as marker-assisted selection (MAS), marker-assisted backcrossing (MABC), and marker-assisted recurrent selection (MARS), typically a small number of informative markers are required. In this context, Illumina BeadXpress SNP genotyping platform (Illumina, Inc.) seems to be the best option.

The objective of this work was to develop cost-effective Illumina BeadXpress based SNP genotyping assays for chickpea and pigeonpea after selecting informative SNPs from a large number of SNPs available in both species and to test the usefulness of the assays for genotyping diverse chickpea and pigeonpea accessions.

Materials and Methods

Plant Material and Genomic DNA Isolation

Two hundred eighty-eight genotypes from chickpea reference set (Upadhyaya et al., 2008) (Supplemental Table S1) and 256 genotypes from pigeonpea reference set (Upadhyaya et al., 2011a) (Supplemental Table S2) were used in the present study. Two-week-old leaves were used for genomic DNA extraction using a high-throughput mini DNA extraction protocol in 96-well plates as described by Cuc et al. (2008). DNA quantification and quality check was done using NanoVue Plus (GE Healthcare Life Sciences). DNA working stocks (50 ng μL^{-1}) having 260:280 ratio ranging from 1.7 to 1.9 were prepared for each genotype.

Single Nucleotide Polymorphism Discovery and Selection

Four different approaches— Illumina (Solexa) sequencing, mining of Sanger ESTs, allele-specific sequencing of candidate genes, and allele-specific sequencing of tentative orthologous genes (TOGs)—were used for identification of SNPs in chickpea (Hiremath et al., 2012) and pigeonpea (Saxena et al., 2012). Single nucleotide polymorphisms with high assay design tool (ADT) scores (>0.6) and designability scores (between 0.50 and 0.99) and higher polymorphism information content (PIC) value (>0.2 in both chickpea and pigeonpea) were selected for developing VeraCode (Illumina, Inc.) assays. Three primers were designed by Illumina for each SNP locus, using the VeraCode Assay Designer software (Illumina, Inc.). Sequence and primer information for

these 96 (chickpea) and 48 (pigeonpea) SNPs are listed in Supplemental Tables S3 and S4, respectively.

Single Nucleotide Polymorphism Genotyping

The genotyping of SNPs was performed using the Illumina BeadXpress platform according to the manufacturer's protocol (Fan et al., 2006). For each oligo pool assay run genotyping using GoldenGate assay is based on polymerase chain reaction using two allele-specific oligonucleotides and one locus-specific oligonucleotide per SNP locus. Polymerase chain reaction products were labeled with Cy3 or Cy5 depending on the allele and contained an IllumiCode address sequence specific for a locus. Each SNP locus was identified by its IllumiCode address and alleles at the SNP locus were discriminated by their fluorescent signals (Lin et al., 2009). Homozygous genotypes amplified products displaying signal in the Cy3 or Cy5 channels whereas heterozygous genotype products displayed signal in both channels.

All SNP data analyzed using the Illumina GenomeStudio genotyping software (Illumina, Inc.), which can cluster allele calls (threshold of 0.25). The software assigned three different clusters based on the fluorescence obtained. The GenCall software (part of GenomeStudio genotyping software) provided several different indexes: (i) the number of SNPs that could be successfully genotyped was called Call Rate for that sample, (ii) the GenTrain score (distance between clusters and fluorescence intensity) determined the quality of the genotyping for one SNP on all samples, and (iii) intensity of fluorescence and distance from the centre of cluster was used to calculate the GenCall score for that point indicating the quality of genotyping for that point. Quality scores (0–4) were given based on the quality of genotyping, where 0 indicates reaction failed, 1 indicates no polymorphism across germplasm detected, 2 indicates polymorphism with low fluorescence intensity, 3 indicates clear genotyping and good cluster separation with some missing (>10%) data, and 4 indicates excellent genotyping. Each SNP was rechecked manually and rescored if any error was observed in the clustering of homozygous and heterozygous groups.

Data Analysis

Single nucleotide polymorphism genotyping data was generated on the reference sets of chickpea and pigeonpea. Marker attributes such as PIC value and gene diversity were estimated using PowerMarker V3.0 (Liu and Muse, 2005). Simple matching allele frequency-based distance matrix was used in DARwin 5.0 program (Perrier et al., 2003) to dissect the genetic diversity of the reference sets (288 accessions with 94 SNPs in chickpea and 256 accessions with 48 SNPs in pigeonpea). Unweighted neighbor joining method in DARwin 5.0 was used for tree construction and used to compare the genetic diversity revealed by SSR markers with SNP markers in both chickpea and pigeonpea.

RESULTS

VeraCode Assays for Single Nucleotide Polymorphism Genotyping

Large sets of SNPs, 2005 in chickpea (Hiremath et al., 2012) and 1616 in pigeonpea (Saxena et al., 2012), constituted the starting point for this study. These large sets of SNPs were developed by using four different approaches, namely Illumina (Solexa) sequencing, mining of Sanger ESTs, allele-specific sequencing of candidate genes, and allele-specific sequencing of TOGs (Hiremath et al., 2012; Saxena et al., 2012). Designability scores for these SNPs were calculated using the Illumina ADT and selected SNPs were called by same name as per our earlier studies in chickpea (Hiremath et al., 2012) and pigeonpea (Saxena et al., 2012).

To select the most informative SNPs to be included in the Illumina BeadXpress platform assay, the following parameters were taken in consideration: (i) presence of polymorphism among the cultivated accessions of chickpea and pigeonpea, (ii) high PIC value, and (iii) high ADT score. By using the indicated criteria, 96 SNPs were selected in chickpea and 48 SNPs in pigeonpea. The selected SNPs showed polymorphism between the parental genotypes of 14 mapping populations of chickpea and two populations of pigeonpea (Table 1). In the case of chickpea, a maximum of 29 SNPs were common between any two populations, while 19 and 14 SNPs were common when comparing any three or four mapping populations respectively and six SNPs were common across two mapping populations of pigeonpea.

Polymorphism Features of VeraCode Assays Based Single Nucleotide Polymorphisms

Developed VeraCode assays for BeadXpress were tested by genotyping diverse germplasm (reference sets of

Table 1. Informative single nucleotide polymorphism (SNP) marker on different crosses.

Parental cross	Population†	Traits	No. of SNPs
Chickpea			
Arerti × ICC 4958	MABC	Drought tolerance	40
C 214 × ILC 3279	MABC	Disease resistance	36
DCP 92-3 × ICC 4958	MABC	Drought tolerance	48
Ejere × ICC 4958	MABC	Drought tolerance	40
ICC 283 × ICC 8261	RIL	Root traits	57
ICC 3137 × IG 72953	RIL	Salinity tolerance	37
ICC 3137 × IG 72933	RIL	*Helicoverpa* resistance	57
ICC 4958 × ICC 1882	RIL	Root traits	56
ICC 4958 × PI 489777	RIL	*Helicoverpa* resistance	39
ICC 6263 × ICC 1431	RIL	Salinity tolerance	44
ICC 97105 × ICC 4958	MABC	Drought tolerance	37
ICCV 10 × ICC 4958	MABC	Drought tolerance	39
ICCV 92318 × ICC 8261	MABC	Drought tolerance	36
KAK 2 × ICC 8261	MABC	Drought tolerance	38
Pigeonpea			
ICPL 20096 × ICPL 332	RIL	Fusarium wilt (FW)	28
ICP 8863 × ICPL 20097	RIL	FW and sterility mosaic disease	14

†MABC, marker-assisted backcrossing; RIL, recombinant inbred line.

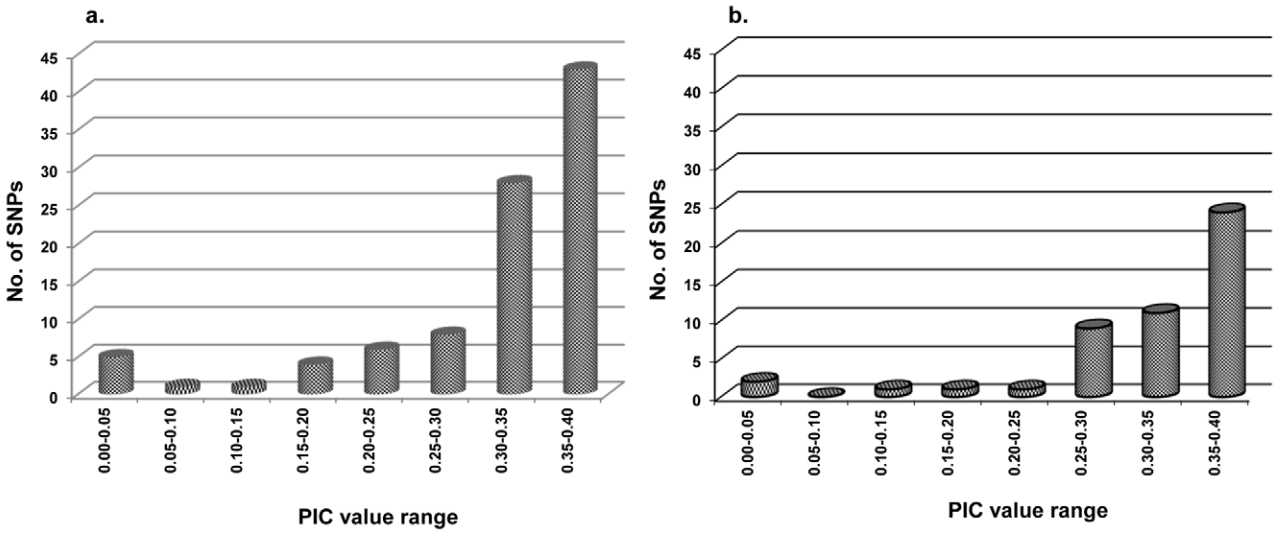

Figure 1. Polymorphism information content (PIC) value of single nucleotide polymorphisms (SNPs). (a) Polymorphism information content value of 96 SNPs across chickpea reference set. (b) Polymorphism information content value of 48 SNPs across pigeonpea reference set.

chickpea and pigeonpea) and conducting diversity studies. Genotyping data obtained were used for allele calling based on quality score and allele detection. It is important to mention here that genotyping data were obtained for all (100%) SNPs assayed.

After analyzing 288 chickpea genotypes with 96 SNPs, polymorphism was obtained for 94 (97.9%) SNPs. The PIC value for SNPs ranged from 0.01 to 0.38, with a mean of 0.31. The majority of SNPs (70 SNPs from a total of 96 SNPs) were highly polymorphic with PIC value ranging from 0.30 to 0.40 whereas less than 5% of the SNPs were found to have PIC value <0.10 (Fig. 1a). Gene diversity (probability of two randomly chosen alleles) across chickpea reference set varied from 0.01 to 0.50, with an average of 0.40 (Table 2).

In the case of pigeonpea, although 48 SNPs were assayed on 288 genotypes, high quality data were obtained for only 256 genotypes. All (100%) SNPs assayed showed polymorphism in the genotypes analyzed. The PIC values varied from 0.01 to 0.37 with an average 0.32 per SNP (Fig. 1b) whereas average gene diversity was 0.41 across the pigeonpea reference set (Table 3). The chickpea and pigeonpea reference sets contain cultivated accessions and crop wild relatives; this has not affected the utility of the SNPs. The transferability (applicability) of the SNPs to their wild relatives was high; more than 95% of the SNPs were useful to genotype crop wild relatives in both chickpea and pigeonpea.

Genetic Relationships in Reference Sets

Single nucleotide polymorphism genotyping data generated on 288 genotypes of the chickpea reference set and 256 genotypes of pigeonpea reference set for 94 and 48 polymorphic SNPs respectively were used to establish the genetic relationships among genotypes of the reference sets (Fig. 2a and 3a). The neighbor joining tree grouped chickpea genotypes into four major clusters (Cl). The Cl I contained 74 genotypes of Indian origin, which include four wild, 20 kabuli, and three pea-shaped genotypes. The Cl II, Cl III, and Cl IV contained 52, 38, and 124 genotypes, respectively (Fig. 2a). However, no specific grouping of the genotypes was observed either on the market class (desi, kabuli, and pea shaped) or origin and biological status. The desi, kabuli, and pea-shaped genotypes were interspersed in all the clusters.

In the case of the phylogenetic tree of pigeonpea genotypes, two main clusters (Cl I and Cl II) were observed. The Cl I includes all the cultivated genotypes except one accession of *Cajanus cajanifolius* (Haines) Maesen (ICP 15629) while the Cl II contained the remaining cultivated lines and seven wild species accessions representing two accessions (ICP 15925 and ICP 15926) from *Cajanus albicans* (Wight & Arn.) Maesen, two accessions (ICP 15630 and ICP 15632) from *Cajanus cajanifolius*, and three accessions (ICP 15692, ICP 15696, and ICP 15701) from *Cajanus scarabaeoides* (L.) Thouars (Fig. 3a).

Comparison of Single Nucleotide Polymorphisms and Simple Sequence Repeats for Phylogenetic Analysis

Simple sequence repeat–based dendrograms of chickpea and pigeonpea reference sets obtained in earlier studies (Upadhyaya et al., 2008, 2011a) were compared with the SNP-based dendrograms developed in this study. In the case of chickpea, 36 SSR markers grouped the reference set into two major clusters (Fig. 2b) while SNP markers demarcated the genotypes into four (Fig. 2a). Despite having enough diversity, no clear group based on biological, geographical, or seed type could be evident when comparing the dendrograms (Fig. 2a and 2b).

In the case of pigeonpea, 48 SNP markers classified the reference set in two clusters (Fig. 3a) while 20 SSR

Table 2. Single nucleotide polymorphism genotyping marker attributes across chickpea reference set.

Marker	Major allele frequency	Gene diversity	PIC† value
CKAM0003	0.55	0.50	0.37
CKAM0005	0.68	0.43	0.34
CKAM0006	0.52	0.50	0.37
CKAM0008	0.69	0.43	0.34
CKAM0997	0.81	0.31	0.26
CKAM2003	0.82	0.30	0.25
CKAM0017	0.73	0.39	0.31
CKAM1001	0.85	0.26	0.22
CKAM0448	0.55	0.50	0.37
CKAM1439	0.89	0.19	0.18
CKAM0234	0.61	0.47	0.36
CKAM1256	0.61	0.47	0.36
CKAM0723	0.85	0.26	0.23
CKAM1821	0.70	0.42	0.33
CKAM0993	0.64	0.46	0.35
CKAM1976	0.70	0.42	0.33
CKAM0020	0.66	0.45	0.35
CKAM1003	0.58	0.49	0.37
CKAM0462	0.75	0.38	0.31
CKAM1548	0.69	0.43	0.34
CKAM0280	0.58	0.49	0.37
CKAM1262	0.78	0.35	0.29
CKAM0789	0.56	0.49	0.37
CKAM1848	1.00	0.01	0.01
CKAM0025	0.56	0.49	0.37
CKAM1066	0.68	0.44	0.34
CKAM0493	0.65	0.46	0.35
CKAM1588	0.92	0.15	0.14
CKAM0290	0.70	0.42	0.33
CKAM1276	0.74	0.39	0.31
CKAM0801	0.99	0.01	0.01
CKAM1850	0.76	0.37	0.30
CKAM0028	0.88	0.21	0.19
CKAM1101	0.57	0.49	0.37
CKAM0494	0.57	0.49	0.37
CKAM1604	0.73	0.40	0.32
CKAM0304	0.71	0.41	0.33
CKAM1293	0.57	0.49	0.37
CKAM0804	0.54	0.50	0.37
CKAM1888	0.60	0.48	0.36
CKAM0036	0.70	0.42	0.33
CKAM1117	0.70	0.42	0.33
CKAM0526	0.52	0.50	0.37
CKAM1641	0.54	0.50	0.37
CKAM0317	0.86	0.24	0.21
CKAM1317	0.50	0.50	0.37
CKAM0833	0.79	0.33	0.28
CKAM1894	0.63	0.47	0.36
CKAM0042	0.66	0.45	0.35
CKAM1118	0.95	0.09	0.09
CKAM0536	0.85	0.26	0.22
CKAM1651	0.65	0.46	0.35
CKAM0321	0.53	0.50	0.37
CKAM1337	0.58	0.49	0.37
CKAM0849	0.77	0.35	0.29
CKAM1902	0.75	0.38	0.31
CKAM0043	0.56	0.49	0.37
CKAM1131	1.00	0.00	0.00
CKAM0588	0.58	0.49	0.37
CKAM1709	0.67	0.44	0.35
CKAM0344	0.75	0.37	0.30
CKAM1341	0.83	0.28	0.24
CKAM0925	1.00	0.00	0.00
CKAM1903	0.74	0.38	0.31
CKAM0044	0.57	0.49	0.37
CKAM1170	0.51	0.50	0.37
CKAM0604	0.58	0.49	0.37
CKAM1713	0.78	0.34	0.28
CKAM0348	0.90	0.18	0.16
CKAM1370	0.60	0.48	0.36
CKAM0939	0.69	0.43	0.34
CKAM1925	0.98	0.04	0.04
CKAM0126	0.66	0.45	0.35
CKAM1175	0.50	0.50	0.38
CKAM0612	0.56	0.49	0.37
CKAM1720	0.87	0.22	0.20
CKAM0405	0.72	0.40	0.32
CKAM1387	0.77	0.36	0.29
CKAM0959	0.53	0.50	0.37
CKAM1933	0.56	0.49	0.37
CKAM0165	0.50	0.50	0.37
CKAM1187	0.64	0.46	0.35
CKAM0639	0.57	0.49	0.37
CKAM1757	0.56	0.49	0.37
CKAM0411	0.52	0.50	0.37
CKAM1396	0.86	0.23	0.21
CKAM0964	0.58	0.49	0.37
CKAM1971	0.67	0.44	0.34
CKAM0189	0.51	0.50	0.37
CKAM1190	0.67	0.44	0.34
CKAM0657	0.60	0.48	0.36
CKAM1797	0.71	0.41	0.33
CKAM0216	0.73	0.39	0.32
CKAM1254	0.77	0.35	0.29
CKAM0707	0.57	0.49	0.37
CKAM1802	0.55	0.50	0.37
Mean	0.69	0.39	0.31

†PIC, polymorphism information content.

markers classified the reference set into four main clusters (Cl I, Cl II, Cl III, and Cl IV) (Fig. 3b). In the case of the SSR dendrogram, cultivated genotypes were found to be equally distributed among four clusters while one accession (ICP 15701) of *Cajanus scarabaeoides* grouped in Cl I, two accessions (ICP 15630 and ICP 15629) of *Cajanus cajanifolius* and one accession (ICP 15692) from *Cajanus scarabaeoides* grouped in Cl II, two accessions

Table 3. Single nucleotide polymorphism genotyping marker attributes across pigeonpea reference set.

Marker	Major allele frequency	Gene diversity	PIC† value
PKAM0118	0.57	0.49	0.37
PKAM0200	0.53	0.50	0.37
PKAM0366	0.59	0.48	0.37
PKAM0656	0.94	0.11	0.11
PKAM0659	0.62	0.47	0.36
PKAM0669	0.57	0.49	0.37
PKAM0676	0.59	0.48	0.37
PKAM0689	0.52	0.50	0.37
PKAM0695	0.99	0.01	0.01
PKAM0727	0.75	0.37	0.30
PKAM0745	0.57	0.49	0.37
PKAM0753	0.60	0.48	0.36
PKAM0784	0.81	0.30	0.26
PKAM0817	0.51	0.50	0.37
PKAM0836	0.69	0.43	0.34
PKAM0903	0.79	0.33	0.27
PKAM0914	0.56	0.49	0.37
PKAM0946	0.83	0.28	0.24
PKAM0952	0.75	0.37	0.30
PKAM0967	0.99	0.02	0.02
PKAM0978	0.67	0.44	0.34
PKAM1028	0.61	0.48	0.36
PKAM1067	0.79	0.34	0.28
PKAM1071	0.51	0.50	0.37
PKAM1102	0.70	0.42	0.33
PKAM1113	0.76	0.37	0.30
PKAM1117	0.71	0.41	0.33
PKAM1128	0.54	0.50	0.37
PKAM1129	0.68	0.43	0.34
PKAM1136	0.77	0.35	0.29
PKAM1140	0.61	0.48	0.36
PKAM1167	0.60	0.48	0.36
PKAM1182	0.53	0.50	0.37
PKAM1194	0.51	0.50	0.37
PKAM1198	0.70	0.42	0.33
PKAM1274	0.75	0.38	0.31
PKAM1276	0.51	0.50	0.37
PKAM1285	0.87	0.22	0.20
PKAM1305	0.59	0.48	0.37
PKAM1342	0.71	0.41	0.33
PKAM1356	0.80	0.32	0.27
PKAM1361	0.60	0.48	0.36
PKAM1381	0.66	0.45	0.35
PKAM1429	0.80	0.32	0.27
PKAM1439	0.52	0.50	0.37
PKAM1442	0.52	0.50	0.37
PKAM1467	0.55	0.49	0.37
PKAM1478	0.71	0.41	0.33
Mean	0.67	0.41	0.32

†PIC, polymorphism information content.

(ICP 15925 and ICP 15926) from *Cajanus albicans* grouped in Cl III, and one accession each (ICP 15696 and ICP 15630) from *Cajanus scarabaeoides* and *Cajanus cajanifolius* grouped in Cl IV (Fig. 3a and 3b). No distinctive clustering of pigeonpea accessions was found; however, the crop wild relatives of pigeonpea species accessions were largely clustered together.

Discussion

Molecular markers are gaining importance in breeding applications for enhancing genetic gains and reducing breeding cycles and have proven useful in many crop plants (Varshney et al., 2005, 2010). Simple sequence repeat markers, being genome specific, codominant, highly polymorphic, and evenly distributed throughout the genome, have been the marker of choice for more than a decade. Chickpea and pigeonpea have more than 3000 SSR markers, which have been used for developing genetic maps (Nayak et al., 2010; Gujaria et al., 2011; Bohra et al., 2012; Choudhary et al., 2012; Saxena et al., 2012) and are being used in breeding application (see Upadhyaya et al., 2011b). Although SSR markers are being used in most of the ongoing marker-assisted selection programs across the world, generating marker genotyping data is tedious and expensive compared to SNPs. The occurrence of size homoplasy (alleles of the same size with different lineages) has been another constraint of SSRs (Goldstein and Pollock, 1997), which is not the case with SNPs. The development and application of SNPs in crop genetics and breeding applications in earlier years was slow. However, in recent years the advances in next-generation sequencing technologies have enabled identification of SNPs very fast and cost effectively (Mir and Varshney, 2013). As a result, thousands of SNPs are available in the public domain for a number of plant species including chickpea (Hiremath et al., 2012) and pigeonpea (Saxena et al., 2012). Single nucleotide polymorphism genotyping can be done by using several high throughput genotyping platforms such as Illumina's GoldenGate or Infinium assays and are regularly being used for diversity analysis and other applications (Fan et al., 2006). Alternatively, another approach for genotyping by sequencing is gaining attention, but this suffers the drawback of a large number of missing loci and capacity to store and handle large amounts data generated can be daunting. This approach is also limited by the expertise in handling algorithm that deal with such data (Mir and Varshney, 2013). In the majority of cases, breeding applications such as MAS, MABC, and MARS need few markers for foreground and/or background selection. In such cases, SNP genotyping based on VeraCode assays seems more reasonable (Mir et al., 2013). Therefore, VeraCode assays have been developed for an informative set of SNPs in chickpea and pigeonpea.

Cost-Effective Single Nucleotide Polymorphism Genotyping Platforms

With the goal of making SNP genotyping cost effective in breeding and genetics applications, VeraCode assays have been developed for 96-plex SNPs for chickpea and 48-plex SNPs for pigeonpea. The SNPs in the VeraCode assays were targeted in such a way that these SNPs

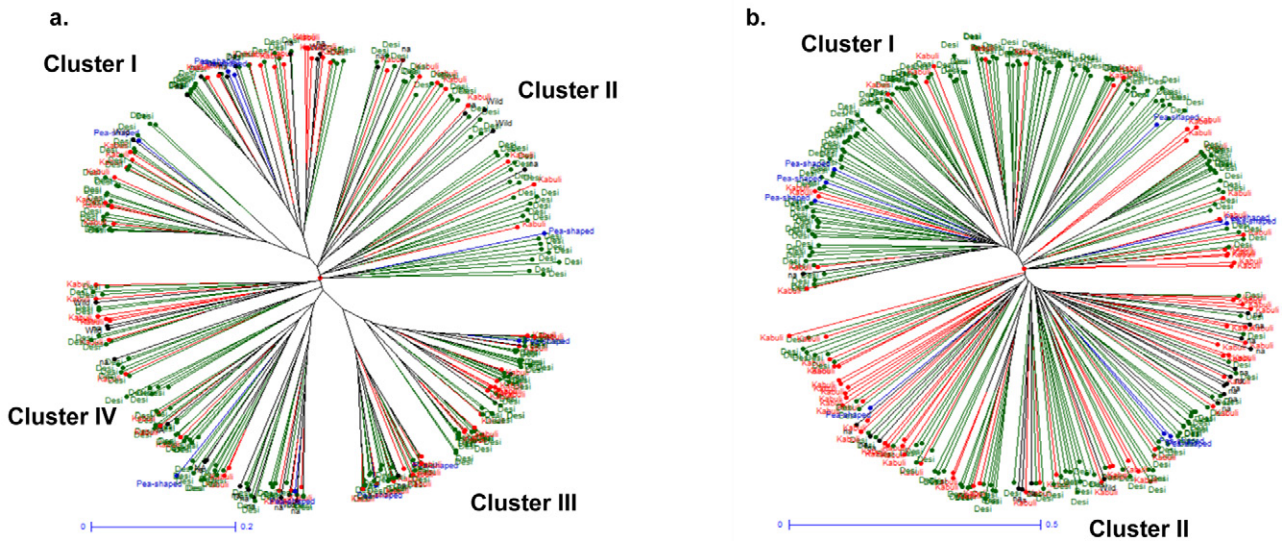

Figure 2. Genetic analysis of chickpea reference set. (a) Analysis by using 94 single nucleotide polymorphism (SNP) markers across 288 chickpea lines. (b) Analysis by using 36 simple sequence repeat (SSR) markers across 288 chickpea lines.

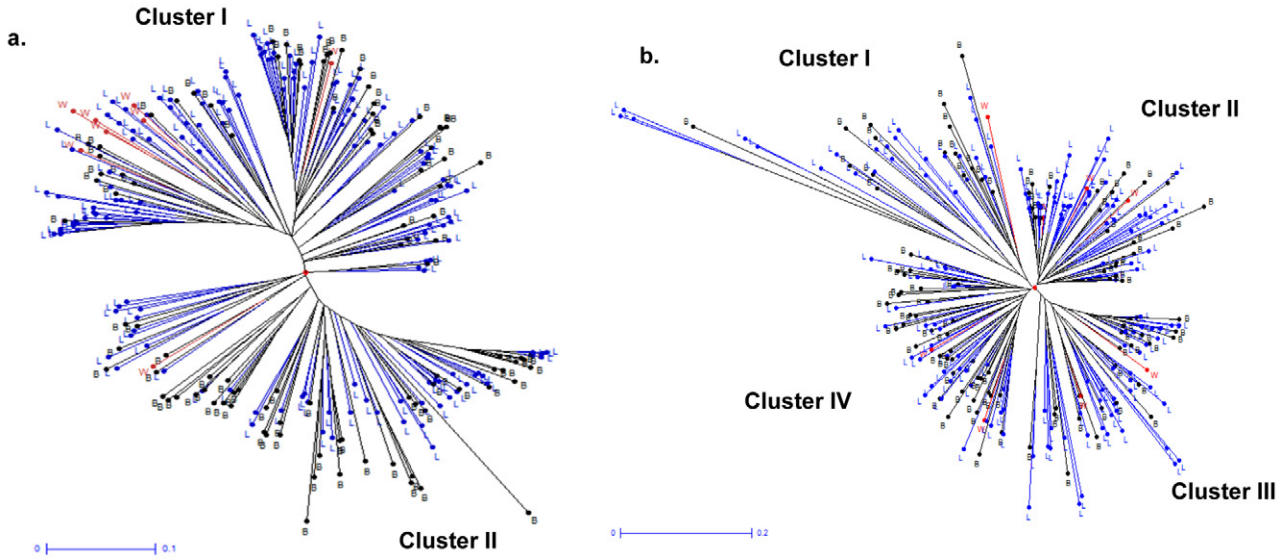

Figure 3. Genetic analysis of pigeonpea reference set (B, breeding line; L, landrace; W, wild relative). (a) Analysis by using 48 single nucleotide polymorphism (SNP) markers across 256 pigeonpea lines. (b) Analysis by using 20 simple sequence repeat (SSR) markers across 256 pigeonpea lines.

represent the common polymorphic SNPs across the different populations. This was also confirmed by PIC values calculated in the reference set. These results also suggest that these VeraCode assays can be used in a wide range of germplasm in chickpea and pigeonpea.

Each set of SNPs was designed with the idea of supporting molecular breeding applications, providing a fast, automatic, and cost-effective method of genotyping. Molecular breeding applications are mainly dependent on the implementation of MAS approaches in an efficient system that can easily automate the genotyping procedure (Collard and Mackill, 2008). Using a multiplexing approach, SNPs are helpful to increase the speed of genotyping in a cost effective manner. Several genotyping platforms with potential to handle any number of samples with low to medium number of markers have regularly been used in modern breeding programs (Thomson et al., 2012). As SNPs present in the VeraCode assays display polymorphism between the parents of several mapping populations (MABC and recombinant inbred lines), these assays are expected to be used extensively in chickpea and pigeonpea.

Applications of VeraCode Assays

The utility of VeraCode assays (SNP plex) was demonstrated by genotyping chickpea and pigeonpea reference sets (Upadhyaya and Ortiz, 2001; Upadhyaya et al., 2006). Since these reference sets represent the diversity available to breeders in the respective species, the SNP assays have broad applicability and are not restricted to

just a limited number of accessions or parental lines. The low to medium throughput SNP platform assays developed provide a cost-effective SNP genotyping option and are expected to enhance the use of SNPs in genetics and breeding applications.

Our results demonstrated the usefulness of SNP assays in diversity analysis in chickpea and pigeonpea. Similar kind of utility of these multiplexes has also been demonstrated in the case of wheat (*Triticum aestivum* L.) (Akhunov et al., 2009) and rice (*Oryza sativa* L.) (Thomson et al., 2012). Similarly, in case of chickpea and pigeonpea, KBiosciences Competitive Allele-Specific polymerase chain reaction (KASPar) SNP genotyping assays have been developed that can be used in genetics research and breeding applications (Hiremath et al., 2012; Saxena et al., 2012). KBiosciences Competitive Allele-Specific polymerase chain reaction assays are cost effective when dealing with large number of accessions needs to be genotyped with large number of markers. In cases where we need to genotype only few lines with very few markers, VeraCode assays are advised. In the case of chickpea, four major groups in the reference set were observed in the SNP dendrogram whereas two major groups are observed in the case of the SNP dendrogram for pigeonpea. The SNP-based dendrograms developed in this study provide similar results when compared with the SSR-based dendrograms. Single nucleotide polymorphism dendrograms provided a complementary view of the genetic diversity present in the reference sets and helped to get a more accurate estimation of genetic distances. Single nucleotide polymorphisms having the possibility to automate and greater abundance across the genome give edge over the SSR in molecular breeding application.

These selected sets of SNP can also be used for genotyping mapping populations to add more markers on the existing genetic map. To check the suitability of these SNP, for mapping we genotyped parental genotypes of 14 mapping populations. These polymorphic SNPs could be used to integrate these polymorphic markers into genetic maps of the respective populations. For instance, 56 SNPs showed polymorphism between parents (ICC 4958 and ICC 1882). Genotyping of these polymorphic markers on the population could integrate 44 novel SNPs on existing SSR based genetic map (Varshney, unpublished data, 2013). This indicates the utility of the available assays in breeding application such as MAS, MARS, and MABC. This set can also be used for background selection in MABC program to estimate the genome recovery of the recurrent parent and select the lines having higher genome coverage.

Conclusions

In summary, the present study reports the development of SNP Illumina BeadXpress assays for two important legume crops (chickpea and pigeonpea), which can be used in the molecular breeding and diversity studies. In the case of chickpea, the assay consists of 96 SNPs, and in pigeonpea, the assay consists of 48 SNPs and are available for genotyping the diverse germplasm to accelerate molecular breeding in a cost-effective manner. Our screening of diverse reference sets and parents of mapping population confirms that such assays can be used to characterize diverse germplasm and also to integrate more markers into existing genetic maps.

Supplemental Information Available

Supplemental material is available at http://www.crops.org/publications/tpg.

Supplemental Table S1. List of accession from chickpea reference set used in the study.

Supplemental Table S2. List of accession from pigeonpea reference set used in the study.

Supplemental Table S3. The single nucleotide polymorphism (SNP) identifications (IDs), variants, and flanking sequences for the chickpea VeraCode SNP set for the BeadXpress platform (Illumina, Inc.)

Supplemental Table S4. The single nucleotide polymorphism (SNP) identifications (IDs), variants, and flanking sequences for the pigeonpea VeraCode SNP set for the BeadXpress platform (Illumina, Inc.).

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