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Molecular diversity and association of simple sequence repeat markers with bud necrosis disease in interspecific breeding lines and cultivars of peanut (*Arachis hypogaea* L.)

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Abstract

Molecular markers are useful tools for assaying genetic variation and provide an efficient means for early and reliable selection of genotypes having resistance to peanut bud necrosis disease (PBND) in peanut breeding programs. Molecular diversity and association of simple sequence repeat (SSR) markers with resistance to PBND was detected in 21 interspecific pre-breeding lines and three cultivars of peanut differing in degree of resistance to PBND. Forty-five primer pairs yielded a total of 531 fragments, of which 337 were polymorphic, with an average of 7.5 polymorphic fragments per primer. Polymorphism ranged from 0 - 100% with an average of 60.2%. Cluster analysis (UPGMA) revealed two main clusters separated at 77% Jaccard's similarity coefficient based on resistance to PBND. All 14 susceptible lines were grouped into a single cluster, while 11 resistant lines grouped into a separate cluster. AMOVA among 24 lines detected 43% ($P < 0.001$) of total variation associated with resistance to PBND. Kruskal-Wallis ANOVA detected the significant association of 16 primers with resistance to PBND. Nine out of 16 primers explained more than 10% of phenotypic variation due to resistance to PBND. It appears that these loci are associated with the resistance to PBND in peanut and major QTLs with regression coefficient value (r^2) ranging from 10.1% to 77.5%. Of which $PM15_{190,}$ $PM188_{165}$ and $PM201_{130}$ loci effectively differentiated most of the resistant lines from the susceptible lines.

Keywords: bulk segregant analysis; molecular marker; peanut; peanut bud necrosis disease; simple sequence repeat; thrips.
Abbreviations: AFLP_Amplified fragment length polymorphism, AMOVA_Analysis of molecular variance, ANOVA_Analysis of variance, BSA_Bulked segregant analysis, CTAB_Cetyl Trimethyle ammonium bromide, DAS_Days after sowing, HR_Highly resistant, HS_Highly susceptible, ISSR_Inter-simple sequence repeat, MAS_Marker assisted selection, MI_Marker index, MR_Moderately resistant, MS_Moderately susceptible, NRCGCS_National Research Center for Groundnut Cytogenetics Selection, PCR_Polymerase chain reaction, PIC_Polymorphic information content, PBND_Peanut Bud Necrosis Disease, PBNV_Peanut Bud Necrosis Virus, QTL_Quantitative trait loci, R_Resistant, RAPD_Random amplified polymorphic DNA, RCBD_Randomized Complete Block Design, S_Susceptible SSR-Simple sequence repeat, UPGMA_Unweighted pair group method with arithmetic mean.

Introduction

Peanut (*Arachis hypogaea* L.) is an important oilseed crop grown in approximately 24 m ha in SAT region of the world (FAO statistical database, 2010). In peanut, and other plant species, the majority of economically important agronomic characteristics are controlled in a quantitative fashion. Until recently, plant breeders have relied on phenotypic selection methods to improve specific quantitative traits based on type of gene actions they observed in various set of cross combinations. Due to effects of the environment on these traits, such methods can be expensive, time consuming, labour intensive and moreover may be some times ended with no deliverables. Breeding efforts to improve these traits could be more efficient and successful with the use of molecular marker and well saturated genomic map (Samizadeh et al., 2003; Varshney et al., 2005a; 2005b; Holbrook et al., 2011). The Peanut bud necrosis disease (PBND) caused by Peanut bud necrosis virus (PBNV), vectored by *Thrips palmi*, has emerged as a serious yield constraint which was also reported earlier as important virus disease of peanut in South Asia (Satyanarayana et al., 1996) and in parts of China, Nepal, Sri Lanka and Thailand (Reddy et al., 1995). It can cause yield losses of over 50% in peanut (Dwivedi et al., 1995) and many other crops including chilli, potato, tomato, tobacco, jute and early-maturing legumes such as mung bean and urd bean (Dwivedi et al., 1993; Basu 1995; Singh and Srivastava 1995; Sivaprasad et al., 2011). The disease incidence of peanut genotypes differs considerably in the field based on time of infection. Infection in plants that are less than 50 days after sowing (DAS) result in no pod development while those > 70 DAS are less susceptible to the disease (Buiel 1993). Economic losses due to PBND still remain a cause of concern to the peanut breeder world wide. Over the years progress has been made in breeding peanut for resistance to PBND and several PBND-resistant peanut genotypes have been generated (Reddy et al., 1995; Bera et al., 2010a; 2010b; Gopal et al., 2010). However, complete host plant resistance to PBNV in peanut is scarce. Pensuk et al. (2002a) found that the disease could be best differentiated at 50 and 60 DAP and the type of gene action governing resistance to PBND was mainly additive (Pensuk et al., 2002b). The reciprocal effect in this study was in favour of using resistant lines as female parents. Pensuk et al. (2004) in a separate study reported that the type of gene action governing resistance to PBND was non-additive and controlled by multiple genes. Information on the association between genetic markers and resistance to PBND should help breeders construct beneficial allelic combinations

and accelerate the development of peanut resistant to PBND and enhance pod yield in peanut. Cultivated peanut has been characterized with narrow genetic base and exhibits a low level of variation at the DNA level as revealed by using RAPD (Halward et al., 1991; Subramanian et al., 2000), ISSR (Raina et al., 2001), AFLP (Herselman 2003; Gimenes et al., 2002), and SSR markers (Halward et al., 1991; Paik-Ro et al., 1992; Kochert et al., 1996; He et al., 2005). Of the major DNA marker types, SSR marker has been the most successful at identifying molecular variation within the cultivated peanut species (Hopkins and Casa, 1999; Ferguson et al., 2004; Mace et al., 2006) and good progress has been made in tagging economically-important traits in peanut using RAPD, ISSR, SSR and SCAR markers (Burow et al., 2008; Selvaraj et al., 2009; Khedikar et al., 2010; Gautami et al., 2011). In addition to, few genetic linkage maps have been developed using wild species (Burow et al., 2001; Garcia et al., 2005; Moretzsohn et al., 2005) as well as cultivated peanut (Varshney et al., 2009; Ravi et al., 2010; Sujay et al., 2012). Recent advances in molecular genetic technology have enabled the development of low density genetic maps for *A. hypogaea* and the identification of molecular marker or QTL's for several economically significant traits (Holbrook et al., 2011). However, report on genetic diversity for resistance to PBND and molecular marker linked with PBND is very scanty. Recently, Srinivasaraghavan et al. (2012) first reported molecular diversity in a set of 15 peanut genotypes resistant to PBND using SSR primers. Nipaporn et al. (2008) first time reported a RAPD maker $OPG16_{850}$ linked to resistance to PBND in peanut which is the only literature available till date on molecular marker linked to resistance to PBND irrespective of crops vulnerable to PBND. In this direction, 435 interspecific breeding lines were tested in natural field conditions for PBND incidence over two seasons. Selected genotypes ranging from highly susceptible to resistant were subjected to molecular analysis for identification of SSRs linked with resistance to PBND in interspecific peanut.

Results

Phenotyping

Peanut interspecific breeding lines along with known susceptible cultivars were screened for resistance to PBND under natural hot spot over two seasons. More than 70% scoring of disease incidence in susceptible cultivar confirms high level of disease pressure under normal field conditions. Based on pooled PBND incidence over two seasons lines were grouped into highly resistant (0-1% scoring), resistant (1.1-5% scoring), moderately resistant (5.1-10% scoring), moderately susceptible (10.1-25% scoring) susceptible (25.1-50% scoring) and highly susceptible (above 50 scoring). Based on the PBND scoring 13 highly resistant and 8 highly susceptible to PBND along with three susceptible cultivars were selected further for molecular analysis (Table 1). Thus selected breeding lines used in this study represent two distinct groups of highly resistant and highly susceptible interspecific peanut breeding lines. These two distinct groups of peanut breeding lines were used in molecular analysis using Bulk Segregant Analysis.

Genotyping

Primer pairs (Table 2) used in the study yielded a total of 531 fragments, of which 337 were polymorphic, with an average of 7.5 polymorphic fragments per primer. Forty-one out of 45 SSRs were polymorphic, while four were monomorphic (Table 3). Polymorphism ranged from zero to 100% with an average of 60.2%. Higher polymorphism (> 50%) was observed in case of 26 primers. Number of amplified fragments ranged from 5 to 28 per locus. Above average number of alleles per locus was observed in 21 primers. Among polymorphic primers, PM65 produced the highest (28) number of alleles.

Genetic diversity

The PIC value of SSRs ranged from 0.78 to 0.96 with an average of 0.90. The MI value of primers ranged from zero to 95.0 with an average value of 53.9. Out of 26 polymorphic primers, PM137, PM145, PM15, PM188, PM201, PM204, PM210, PM322, PM36, PM402, PM65, PMC99, RNOX602 and TC3E02 had higher number of alleles per locus (> 11.8) along with higher polymorphic per cent (> 50.0), PIC content (> 0.50) and MI value (> 50.0). These 14 primers can be considered as highly informative in revealing the genetic diversity and partitioning genetic variation in cultivated peanut. The dendrogram was constructed through SHAN clustering and UPGMA analysis. Forty-five polymorphic primers discriminated 24 genotypes into two clusters. All resistant genotypes used in this studies were grouped into a single cluster (denoted as cluster-I) on the other hand all susceptible genotypes were grouped into a separate cluster (denoted as cluster-II) (Fig. 1). Cluster-I and II shared 77% of genetic similarity between themselves. Thus, difference in the level of PBND incidence between resistant and susceptible groups is attributed to 25% of the genetic dissimilarity observed between these two clusters. In cluster-I genotype NRCGCS-51 is distantly related to NRCGCS-328 as well as NRCGCS-55 by sharing about 80.5% and 81.5% of genetic variability, while NRCGCS-85 and NRCGCS-86 were closely related to each other by sharing about 91% of genetic variability. On the other hand in cluster-II genotype NRCGCS-313 is closely related to NRCGCS-322 and NRCGCS-362 is closely related to NRCGCS-368 by sharing about 97% of genetic variability, while KRG-1 is distantly related to all the genotypes under study.

Marker trait association

AMOVA between two groups detected 43% ($P < 0.010$) of total variation associated with PBND incidence while 57% variation was within the group (Table 4). Kruskal-Wallis ANOVA detected significant association of 16 primers with the resistance to PBND (Table 5). Of which nine primers RNOX602, PM15, PM53, PM65, PM145, PM188, PM201, PM204 and PM322 explained 76.1% ($p = 0.04$), 25.8% ($P = 0.00$), 77.5% ($P = 0.00$), 35.0% ($P = 0.00$), 29.6% ($P = 0.00$), 10.1% ($P = 0.00$), 31.4% ($P = 0.00$), 62.6% ($P = 0.00$) and 28.4% ($P = 0.00$) phenotypic variations due to resistance to PBND, respectively. It appears that these nine primers are major QTLs associated with resistance to PBND in interspecific genotypes of peanut.

Table 1. Peanut genotypes selected for molecular marker analysis using bulk segregant analysis based on their scoring against peanut bud necrosis disease during rainy 2010 and post-rainy 2010-11.

Breeding lines/ cultivars	PBND Incidence%		Mean PBND Incidence%	Reaction of genotypes for PBND
	rainy 2010	Post- rainy 2010-11		
NRCGCS-51(BL)	Nil	Nil	Nil	HR
NRCGCS-55 (BL)	Nil	Nil	Nil	HR
NRCGCS-75 (BL)	Nil	Nil	Nil	HR
NRCGCS-85 (BL)	Nil	Nil	Nil	HR
NRCGCS-86 (BL)	Nil	Nil	Nil	HR
NRCGCS-103(BL)	Nil	Nil	Nil	HR
NRCGCS-108 (BL)	Nil	Nil	Nil	HR
NRCGCS-159 (BL)	Nil	Nil	Nil	HR
NRCGCS-161 (BL)	Nil	Nil	Nil	HR
NRCGCS-244 (BL)	Nil	Nil	Nil	HR
NRCGCS-319 (BL)	Nil	Nil	Nil	HR
NRCGCS-327 (BL)	Nil	Nil	Nil	HR
NRCGCS-328 (BL)	Nil	Nil	Nil	HR
NRCGCS-313 (BL)	34.1	29.8	31.9	S
NRCGCS-322 (BL)	42.9	38.0	40.5	S
NRCGCS-345 (BL)	36.7	32.4	34.5	S
NRCGCS-362 (BL)	36.4	30.4	33.4	S
NRCGCS-368 (BL)	27.3	24.5	25.9	S
NRCGCS-371 (BL)	36.7	32.7	34.7	S
NRCGCS-427 (BL)	28.8	26.0	27.4	S
NRCGCS-426 (BL)	36.8	31.6	34.2	S
JL-24 (CV)	86.0	69.0	77.5	S
KRG-1(CV)	61.0	60.0	60.5	S
TMV-2 (CV)	67	64	65.5	S

HR-highly resistant (PBND incidence < 1%), S-susceptible (PBND incidence > 20%), Nil- Zero incidence of PBND, BL-Breeding lines, CV-Cultivar

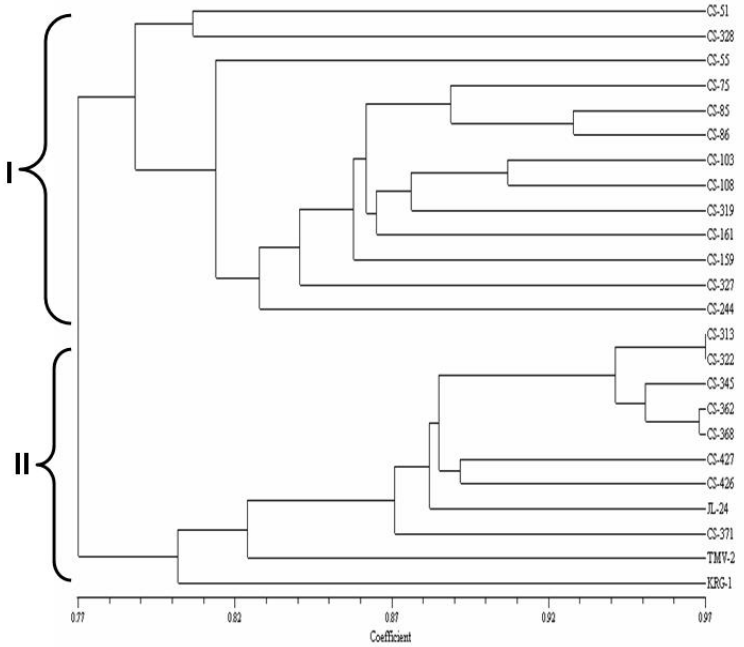

Fig 1. UPGMA tree showing relationship among 21 interspecific breeding lines and three cultivars of peanut based on 45 SSR loci. Cluster I includes all 13 genotypes resistant to PBND and cluster II includes all 11 genotypes susceptible to PBND. Name of interspecific genotypes are presented with CS number instead of NRCGCS number to avoid clumsiness and three cultivars JL-24, TMV-2 and KRG-1 with complete name.

Validation of marker trait association

Out of nine associated primers PM15, PM188 and PM201 could able to discriminate majority of the genotypes of resistant group from genotypes of susceptible groups. The $PM15_{190}$ allele was amplified in all the 11 genotypes of susceptible group which was absent in nine genotypes of resistant group (Fig. 2a). Similarly, $PM188_{165}$ allele was amplified in 11 genotypes of resistant group (Fig. 2b) and absent in 10 genotypes of susceptible group. Besides, $PM201_{130}$ allele was amplified in eight genotypes of

susceptible group and was absent in eight genotypes of resistant group (Fig. 2c).

Discussion

Use of pre-breeding genotypes in molecular marker analysis:
Peanut has lagged other crops on use of molecular genetic technology for cultivar development because of low levels of molecular polymorphism among cultivated varieties. However, advances in technology has enabled the identification of molecular markers associated with quantitative trait loci (QTLs) for several economically significant traits (Holbrook et al., 2011) although many of these QTLs are not major which account for <10% of the phenotypic variation explained. Wild diploid Arachis species, which are native to South America, are genetically diverse and rich in sources of disease resistance (Halward et al., 1992; Galgaro et al., 1997; Upadhya et al., 2011). Since much higher levels of molecular polymorphism occurs in diploid Arachis in comparison to A. hypogaea, the use of interspecific pre-breeding genotypes could be of immense help to mine QTLs/wild alleles for disease resistance. Hence, genetic variation, available in the pre-breeding genotypes, developed through interspecific hybridization, was used to identify molecular markers associated with resistance to PBND. Previously major QTLs for rust and late leaf spot have been identified from recombinant populations developed using at least one germplasm source may be of wild species origin (Sujay et al., 2012; Khedikar et al., 2010; Gowda et al., 2002; Nagy et al., 2010; Simson, 2001).

SSRs and genetic diversity

DNA markers have been used to evaluate genetic diversity in different crops (Cooke, 1995; Azzam et al., 2007). Recently co-dominant markers, such as SSR and EST-SSR available in peanut has greatly aided in diversity and genome studies in this crop. Majority of the primers, used in the study, were highly polymorphic producing higher number of alleles per locus. Primers amplified more than one locus in peanut genotypes indicating loci duplication. This may be attributed to the presence of A-genome as well as B-genome in the allotertraploid cultivated peanut. Amplification of more than one fragment by one pair of primer in tetraploid peanut accessions has also been reported in earlier studies (Gimenes et al., 2007; Varshney et al., 2009; Hopkins and Casa, 1999). The PIC values derived from allelic diversity and frequency among the genotypes were not uniform among the SSR loci tested. The higher PIC value of primers could reveal maximum genetic information among genotypes under investigation. Majority of the primers, used in the study, had higher PIC value (> 0.5). Such higher PIC value could be due to marker pre-selection with higher GC/CT repeats. Quantitative estimation of marker utility and detection of polymorphism have been depicted in terms of mean heterozygosity and MI (Powel et al., 1996). Hence, diversity revealed, based on PIC values, needs to be verified by additional measures, like polymorphic per cent, MI value and number of alleles amplified per locus prior assessing their informativeness. Thus, 14 out of 45 primers used in the study, were highly informative in revealing the genetic diversity and partitioning of genetic variation due to their higher number of alleles per locus as well as higher PIC and MI values. The dendrogram grouped all genotypes into two clusters. All resistant genotypes used in this study were grouped into Cluster-I while cluster-II contains all 11 susceptible genotypes indicating distinct distribution of allele(s) responsible for resistance to PBND only in resistant genotypes which may have been missing in genotypes susceptible to PBND or vice versa. Moreover, all resistant genotypes used in this study may contain either same or limited number of major/minor alleles associated with the resistance to PBND, might have introgressed from either same wild pedigree or closely related wild accessions. Nevertheless, low level of polymorphism has also been reported in cultivated peanut by previous workers (Kochert et al., 1991; He and Prakash, 1997; Moretzsohn et al., 2004; Mace et al., 2006).

Association of SSRs with resistance to PBND

The diversity revealed in this study was further used to identify SSR associated with resistance to PBND and to use in marker assisted selection. MAS has been proved to be a more efficient, accurate, and simpler strategy for selection of desired genotype (Kwon and kim, 2001). In this study, nine SSRs (RNOX536, PM15, PM36, PM65, PM145, PM188, PM201, PM204 and PM322) were found to be associated with major QTLs for resistance to PBND in interspecific peanut. SSRs linked with a trait and explaining more than 10% of total phenotypic variation (r^2) are considered to be major QTLs (Collard et al., 2005). This is the first report on QTLs linked with resistance to PBND in peanut. These QTLs would be of help in linkage mapping and improving resistance to PBND in peanut more precisely through MAS. BSA has been used as an alternative method to the traditional QTL analysis using biparental segregation populations for identifying markers linked to traits of interest (Sun et al., 2003; Selvaraj et al., 2009; Mondal and Badigannavar, 2010; Goswami et al., 2013). Though BSA is generally used to tag genes controlling simple traits, but the method may also be used to identify markers linked to major QTLs (Wang and Peterson, 1994). In the present study, BSA permitted identification of the QTLs for resistance to PBND in peanut both by permitting analysis in absence of a linkage map and by reducing the degree of effort needed to identify associations between markers and phenotypes.

Materials and Methods

Plant materials

Directorate of Groundnut Research (DGR), Junagadh, India has developed a large number of interspecific breeding lines over a period of time to introgress desirable genes from wild Arachis species to cultivated peanut using cultivated peanut as female parent and wild Arachis species, A. diogoi, A. correntina, A. helodes, A. pusilla, A. cardenasii, A. duranensis, A. batizocoi, A. stenosperma, A. monticola, A. villosa, A. kempff-mercadoi, A. pintoi, A. Kretschmeri, A. oteroi and A. villosulicarpa as male parent. Among them a set of 435 interspecific peanut breeding lines were screened for resistance to PBND during rainy 2010 (June to October) and also during post-rainy 2011 (January to May). Selected 13 breeding lines highly resistant (0-1% disease scoring) to PBND and eight breeding lines as well as three cultivars susceptible (25-77% disease scoring) to PBND were further used in molecular analysis (Table 1).

Table 2. Sequences and Tm of 45 SSR primer pairs used for bulk segregant analysis in 21 interspecific breeding lines and three cultivars of peanut

Sr. No.	Primers	Sequence (5´-3´)	bp	Tm °C
1	PM-137	F-AACCAATTCAACAAACCCAGT	42	53.1
		R-GAAGATGGATGAAAACGGATG		51.6
2	PM-145	F-GCTGTAATTAGGATCATTCCACA	41	52.7
		R-CAACGGTTGGATCGATGA		52.3
3	PM-15	F-CCTTTTCTAACACATTCACACATGA	44	53.7
		R-GGCTCCCTTCGATGATGAC		54.8
4	PM-188	F- GGGCTTCACTGCTTTTGATT	40	55.8
		R-TGCGACTTCTGAGAGGACAA		53.8
5	PM-200	F-GCTATGTGGGAAAAATACTGCTT	45	53.6
		R-CAGATGTGTGTGTGTGTGTGTG		56.5
6	PM-201	F-CCTTTATAGAGGACCTTCCCTCTC	44	55.7
		R-GCCTATTTGGTATCGGCTCA		54.6
7	PM-204	F-TGGGCCTAAACCCAACCTAT	40	56.0
		R-CCACAAACAGTGCAGCAATC		55.0
8	PM-210	F-CCGCAGATCTTCTCCTGTGT	43	65.8
		R-CCTCCTCATCCTCTAAACTCTGC		56.5
9	PM-238	F-CTCTCCTCTGCTCTGCACTG	41	57.3
		R-ACAAGAACATGGGGATGAAGA		53.9
10	PM-3	F-GAAAGAAATTATACACTCCAATTATGC	47	51.0
		R-CGGCATGACAGCTCTATGTT		55.0
11	PM-305	F-GCGCTGGAACACAGTAAGAG	40	55.9
		R-GGCAGAAAGGAAAGTTGCAG		54.5
12	PM-322	F-AGTGTTGGGTGTGAAAGTGGGGGACT	42	63.9
		R-CGGAACAGTGTTTATC		43.9
13	PM-325	F-CCTAACAAGGACGGGTGAAC	40	55.5
		R-CAGAGGCCTCACTTTCCTTC		55.3
14	PM-343	F-AGAAACGAGGAGCTCGACAA	42	56.0
		R-GCTCATTTTGATGGAATGAGAG		51.8
15	PM-346	F-AAAGGCGCACTCGATTCTAA	40	54.4
		R-CGCACAGAAACATCAAGCAT		54.0
16	PM-35	F-TGTGAAACCAAATCACTTTCATTC	44	52.3
		R-TGGTGAAAAGAAAGGGGAAA		52.1
17	PM-350	F-CACATTTTCCCAGATCAGCA	42	53.0
		R-GGTGGCAAAGAACTTATTGAGG		54.0
18	PM-36	F-ACTCGCCATAGCCAACAAAC	40	55.8
		R-CATTCCCACAACTCCCACAT		55.1
19	PM-375	F-CGGCAACAGTTTTGATGGTT	39	54.2
		R-GAAAAATATGCCGCCGTTG		52.7
20	PM-402	F-CCGCCCTAAAAACTGTATTCG	41	53.9
		R-CCTAAGAGTACACGCGACGA		56.2
21	PM-42	F-ACGGGCCAAGTGAAGTGAT	41	56.9
		R-TCTTGCTTCTTTGGTGATTAGC		53.3
22	PM-45	F-TGAGTTGTGACGGCTTGTGT	42	57.1
		R-GATGCATGTTTAGCACACTTGA		53.7
23	PM-50	F-CAATTCATGATAGTATTTTATTGGACA	47	50.2
		R-CTTTCTCCTCCCCAATTTGA		52.2
24	PM-53	F-CCTATCCTATGGGTCACTAGCC	44	56.0
		R-GCTTGTGCTCATCTTGAGTTTT		53.9
25	PM-65	F-GGACGTCTGGCTGCTAGAGA	40	58.5
		R-TCGGCATCAAAACAGTGAGA		54.3
26	PMC-478	F-GTCGTGCAGGTCAAAGTGC	39	57.0
		R-TTAAGATGGGTGCCTGCAAT		54.6
27	PMC-99	F-GCATAAGCAGTTTCCAACGA	40	53.3
		R-TGTTGCCTTCACCTTGACAG		55.4
28	RNOX536	F-TGCCATCATTCTGTTCCTCTC	43	54.6
		R-GATTCTGCTGCTTCTTCTGGAT		55.0
29	RNOX602	F-CCCTTGCTAATCGCTCATC	39	53.3
		R-GGG GGCTTGTAATAATCTGC		53.5
30	TC0A01	F-CAGCTCATTTTTCACCTCCA	40	52.9
		R-CCATAACCCCAAAAATGCAG		52.2
31	TC1A01	F-TCAACGCGACACAAGAAGTC	40	55.5
		R-GTCGGTAAATCCGACGAAAA		52.8

32	TC1D01	F-TGCCAATCTCCTCTTCAACC	40	54.8
		R-TCAGGCAAGGGTTCCTACTG		56.6
33	TC1D02	F-GATCCAAAATCTCGCCTTGA	40	52.8
		R-GCTGCTCTGCACAACAAGAA		56.2
34	TC1D12	F-CCCTTTCATTCTCCCTTTCC	41	53.1
		R-TTCTCCTGCACTAGGTTTCCA		55.7
35	TC1E05	F-GAAGGA TAA GCA ATC GTC CA	40	51.7
		R-GGATGGGATTGAACATTTGG		52.2
36	TC1E06	F-ACCGTTACGAACGCTTTGTC	40	55.7
		R-TCCCTCTCATACGACACCCT		57.1
37	TC2D06	F-AGGGGGAGTCAAAGGAAAGA	40	55.6
		R-TCACGATCCCTTCTCCTTCA		55.2
38	TC3A10	F-GCATGGGGTAAATCTTCCAA	40	52.8
		R-ATGTGCCTATCAGGGGTTTG		54.9
39	TC3E02	F-TGAAAGATAGGTTTCGGTGGA	41	53.5
		R-CAAACCGAAGGAGGAACTTG		53.7
40	TC3H07	F-CAATGGGAGGCAAATCAAGT	40	53.3
		R-GCCAAATGGTTCCTTCTCAA		53.3
41	TC4C11	F-TCCTGACTGGGTCCTTTGTC	40	56.5
		R-CCAAAGGGGAGTACGAACAT		54.5
42	TC4E10	F-ACGTCATCTTCCCTCCTCCT	40	52.7
		R-CCATTTTCTCCTCGAACCAA		57.4
43	TC9B12	F-GGCTGGGCTATGTTGATGT	42	55.2
		R-TGCAGTACCTAAACCACCACTAC		56.4
44	TC9B07	F-CCATCTCCTTCTTGACTTTAGCC	45	55.2
		R-GTTCTCCAACCTCCTCCTTTTC		55.3
45	TC9C12	F-GCCTCTATTGCTGAGATTATTGC	43	53.8
		R-CAAAATCAGTAGCAGCATTC		49.6

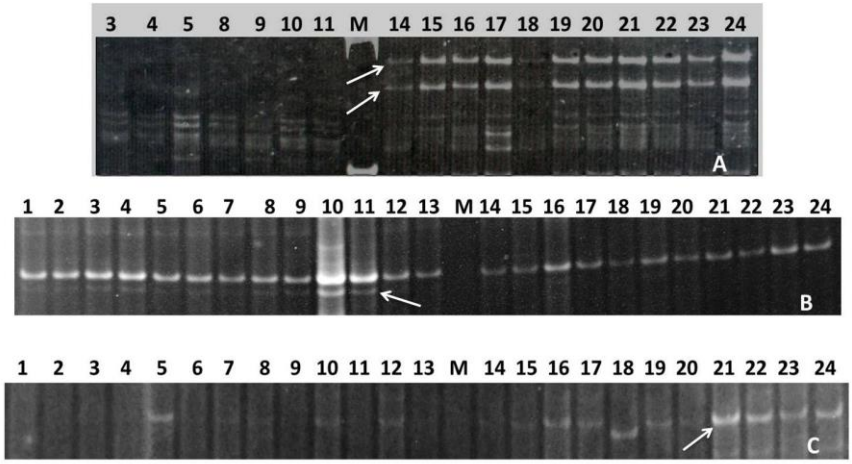

Fig 2. Amplification of alleles associated with resistance to PBND in 21 interspecific breeding lines and three cultivars of peanut and highlighted with arrow. A. $PM15_{190}$ and $PM15_{185}$ alleles amplified in all the 11 susceptible genotypes and absent in nine resistant genotypes. B. $PM188_{165}$ allele amplified in 11 resistant genotypes and absent in 10 susceptible genotypes. C. $PM201_{130}$ allele amplified in eight susceptible genotypes and absent in eight resistant genotypes.

Sampling site

The genotypes were screened under field conditions in the farm of University of Agricultural Sciences (UAS), Raichur, Karnataka, a hot spot for PBND. Raichur is situated between 16°15'N latitude and 77°20'E longitude at an elevation of 389 meters above mean sea level with an average rainfall of 621.33 mm. The monthly mean maximum and minimum temperature of 38.0 °C and 16.2 °C were recorded in the month of April and December, respectively. The mean relative humidity varies between 52.96 per cent in April and 83.86 per cent in August (http://www.uasraichur.edu.in). The screening was done under normal conditions. Genotypes were sown in Randomised Complete Block Design (RCBD) with 3 replications. The crop was raised as per the recommended package of practices except for the plant protection measures against PBND. Each genotype was sown in 2 rows of 5 metre length and at every

Table 3. Polymorphism detected by the use of 45 SSRs on 21 interspecific breeding lines and three cultivars of peanut.

Sr. No.	Primers	Total alleles amplified	Polymorphic Per cent	PIC Value	MI value
1	PM-137	12	100	0.90	90.0
2	PM-145	16	93.8	0.94	88.1
3	PM-15	15	86.7	0.91	78.9
4	PM-188	20	100.0	0.95	95.0
5	PM-200	13	15.4	0.92	14.2
6	PM-201	15	86.7	0.93	80.6
7	PM-204	20	60.0	0.94	56.4
8	PM-210	18	72.2	0.93	67.2
9	PM-238	11	100.0	0.89	89.0
10	PM-3	16	50.0	0.93	46.5
11	PM-305	13	38.5	0.92	35.4
12	PM-322	13	84.6	0.88	74.5
13	PM-325	7	100.0	0.86	86.0
14	PM-343	8	87.5	0.84	73.5
15	PM-346	9	55.6	0.89	49.4
16	PM-35	5	40.0	0.78	31.2
17	PM-350	18	44.4	0.94	41.8
18	PM-36	12	100.0	0.90	90.0
19	PM-375	12	25.0	0.92	23.0
20	PM-402	12	58.3	0.92	53.7
21	PM-42	8	12.5	0.87	10.9
22	PM-45	11	45.5	0.89	40.5
23	PM-50	9	100.0	0.82	82.0
24	PM-53	12	50.0	0.91	45.5
25	PM-65	28	67.9	0.96	65.1
26	PMc-478	8	62.5	0.85	53.1
27	PMc-99	13	100.0	0.90	90.0
28	RNOX536	9	77.8	0.87	67.7
29	RNOX602	12	83.3	0.90	75.0
30	TC0A01	10	40.0	0.88	35.2
31	TC1A01	7	14.3	0.85	12.1
32	TC1D01	8	50.0	0.83	41.5
33	TC1D02	7	14.3	0.84	12.0
34	TC1D12	11	63.6	0.86	54.7
35	TC1E05	11	100.0	0.90	90.0
36	TC1E06	8	0.0	0.88	0.0
37	TC2D06	9	11.1	0.88	9.8
38	TC3A10	9	100.0	0.89	89.0
39	TC3E02	16	100.0	0.94	94.0
40	TC3H07	10	100.0	0.90	90.0
41	TC4C11	6	16.7	0.83	13.8
42	TC4E10	7	0.0	0.86	0.0
43	TC9B07	11	100.0	0.89	89.0
44	TC9B12	10	0.0	0.90	0.0
45	TC9C12	16	0.0	0.94	0.0
	Total	**531**	**2708**	**40.13**	**2425.2**
	Mean	**11.8**	**60.2**	**0.90**	**53.9**

4th row, a susceptible check KRG-1 was planted with a spacing of 45 cm between rows and 10 cm between plants. Crop grown during post- rainy season was irrigated at regular interval whereas life saving irrigation was provided to rainy season crop to maintain healthy growth of the crop.

PBND incidence

Initial plant count was recorded in all genotypes at 20 DAS while the number of healthy and diseased plants were recorded one week before harvest of the crop and expressed in terms of per cent disease incidence. The per cent PBND incidence was calculated by using the formula "Per cent disease = (Number of PBND infected plants/ Total number of plants) X 100" and was pooled over two seasons. Based on pooled disease incidence genotypes were grouped into different groups following standard (0-5) disease rating scale (Sunkad, 2012).

Isolation of DNA

Genomic DNA was extracted from the leaf samples collected

Table 4. Summary of the AMOVA within and among 21 interspecific breeding lines and three cultivars of peanut.

Source	df	SS	MS	Est. Var.	%	Stat	Value	P Value
Among Populations	1	84.51	84.510	6.380	43			
Within Populationss	22	186.49	8.477	8.477	57	PhiPT	0.429	0.010
Total	23	271.00		14.857	100			

Table 5. Association of SSR markers with resistance to peanut bud necrosis disease based on Kruskal-Wallis one way ANOVA.

Sr. No.	Primers	HC	R SQUARE
1	RNoX536	35.57(0.045)	0.761
2	RNoX602	36.41(0.037)	0.039
3	PM-15	51.29(0.000)	0.258
4	PM-36	69.73(0.000)	0.009
5	PM-53	61.43(0.000)	0.775
6	PM-65	75.1(0.000)	0.35
7	pm-145	141.3(0.000)	0.296
8	pm-188	454.1(0.000)	0.101
9	pm-201	58.5(0.000)	0.314
10	pm-204	55.53(0.000)	0.626
11	pm-210	45.76(0.003)	0.001
12	pm-238	52.92(0.000)	0.071
13	pm-322	69.83(0.000)	0.284
14	TC9B07	85.76(0.000)	0.065
15	PM-346	91.43(4.078)	0.015
16	PM-402	164.4(2.415)	0.028

Values mentioned in parenthesis indicates p value

from field grown plants following Cetyle trimethyl ammonium bromide (CTAB) method with modifications (Doyle and Doyle, 1987). The concentration of DNA was checked in Nanodrop spectrophotometer model-ND1000 and the DNA samples were diluted to 100 ng / µl prior to polymerise chain reaction (PCR). The quality of DNA was checked in 0.8% (W / V) Agarose gel electrophoresis. The DNA samples were stored at -20 ºC for downstream use.

PCR amplification and gel electrophoresis

The PCR mixtures (15 µl) contained 0.5 µl (50 ng) genomic DNA, 0.5 µl Taq DNA polymerase, 1.5 µl of Taq Buffer (Genei, Banglore, India), 1 µl dNTPS (Genei, Bangalore, India), 9.5 µl Mili-Q water, 1.0 µl forward primer, 1.0 µl reverse primer (25 pmoles) (IDT, USA). PCR amplification was performed in C1000 thermal cycler (BIO-RAD, USA). Thirty cycles of 30 seconds at 94 ºC for denaturation of template, 1 minute at 54 ºC for primer annealing followed by 30 seconds at 72 ºC for primer extension. The amplified DNA fragments along with 100 bp DNA marker were size separated on 8% Polyacrylamide gel stained in Ethidium bromide and run in 1X TBE buffer at 200 V for 1-2 h (0.1%). The resolved amplification of bands was scanned using laser scanner (Fujifilm FLA 5100, Japan).

SSR analysis

Polymophism of breeding lines and cultivars was done using BSA method. DNA of 13 breeding lines resistant to PBND were bulked together in equal quantity and treated as sample-'A'. While DNA of eight breeding lines as well as three cultivars susceptible to PBND were bulked togather in equal quantity and treated as sample 'B'. DNA samples 'A' and 'B' were screened initially with 450 SSRs reported earlier (Hopkins et al., 1999; He et al., 2005; Moretzsohn et al., 2005). Out of these 45 SSRs were found polymorphic in two bulked DNA samples. These 45 SSRs were further used for screening 21 breeding lines and three culativars individually.

Statistical analysis

Polymorphism per cent was calculated using following formula. Polymorphism % = (number of polymorphic bands/total number of bands in that assay unit) x 100.

PIC was determined using following formula as described by Powell et al. (1996)

PIC = $[1-\sum fi^2]$, where f is the frequency of it allele averaged across loci. Marker index (MI) was calculated by applying following formula given by Powell et al. (1996) and Smith et al. (1997). MI = polymorphism % x PIC value.

BSA analysis was done by pooling separately the DNA samples of breeding lines highly resistant to PBND together and breeding lines as well as cultivars susceptible to PBND together. Genetic similarity analyses were performed using SIMQUAL program in NTSYS (Rohlf, 2000). Cluster analysis was performed using UPGMA based on Jaccard's similarity coefficient. Principal coordinate analysis (PCoA), AMOVA and regression co-efficient were calculated using GenALEx v. 6.5 (Peakall and Smouse, 2012) software and Kruskal-Wallis one way ANOVA was calculated using PAST version 2.07 software (Hammer et al., 2001).

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