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Identification of true hybrid progenies in two different crosses of groundnut (*Arachis hypogaea* L.) involving TMV 2 as the female parent by using SSR markers

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Abstract
In the present experiment hybridization programme was conducted to identify the best possible parents contrasting for late leaf spot disease resistance in groundnut. A collection of 250 minicore germplasm lines were screened for LLS disease resistance, among them four lines were selected as male parent manifested resistance for LLS disease, while TMV-2 and GKVK-4 are agronomically superior varieties but susceptible for LLS disease were used for hybridization programme. Among the crosses carried out in the hybridization best two crosses of groundnut namely TMV 2 × ICGV 86699 (C_1) and TMV 2 × GBFDS 272 (C_2) were selected. These crosses were identified with flanking markers associated with six QTLs and five QTLs respectively. Out of 53 selected SSR primers conferring 38 QTLs were identified from reported research papers. 12 primers and 10 primers were found polymorphic flanking marker for these two crosses. SSR markers provide more discriminate power and faster identification true hybrids. The complementary banding pattern were resolved on 3% (1.5% +1.5%) Metaphor agarose Gel. The cross C_1 and C_2 consisted of 100 and 210 F_1 plants respectively, Out of these F_1's 23 and 46 and plants were confirmed as true hybrids using two highly polymorphic markers namely GM2301 and GM2246 for the cross C_1 and TC6H03 and GM1760 used for cross C_2. True hybrids were used for back cross breeding programme with recurrent parent TMV-2.

Keywords: LLS (Late Leaf Spot), SSR (simple sequence repeats), QTLs (quantitative traits loci), MABC (marker assisted backcross selection)

Introduction
Groundnut or peanut (*Arachis hypogaea* L.) with a genome size of 2891 Mbp is a allotetraploid, cleistogamous, leguminous annual cash crop. Groundnut is an important source of edible oil along with omega-3 fatty acids, proteins, vitamin E and minerals and its stover also rich in nutrient and used as fodder for livestock (Pandey *et al.*, 2012) [11].

Groundnuts manifest low outcrossing range from 0 to 8% because it is a self-pollinating crop. Historically, introgression of existing resistance and other farmer preferred traits is accomplished only through artificial hybridization in targeted breeding from, for example, diploid wild relatives of groundnut with known abiotic and biotic stress resistance and/or tolerance (Knauft *et al.*, 1992) [9].

In addition to biotic stress, foliar diseases such as late leaf spot (LLS) (*Phaeoisariopsis personata*) Berk. & Curt.), early leaf spot (ELS) (*Cercospora arachidicola*), and rust (*Puccinia arachidis*) are generally considered the major constraints for groundnut yield and productivity in semi-arid tropical environments.

Conventional breeding methods has been accomplishment for development of resistant variety. it is laborious, time consuming, resource rigourous and extremely affected by environmental factors, therefore, a technique with rapid development and least affected by environment is required development of resistant variety. So, genetic approach involving introgression of disease resistance into modern and popular cultivars is effective. Thus marker assisted back cross breeding method has been used to improve several biotic and abiotic traits in groundnut. Recently molecular marker-trait association have been done and increasingly adopted in many crops.

Selection of parental lines for use in true to type hybrid development programmes with the help of molecular markers. Peanut genomics is very challenging due to its inherent problem of genetic architecture (allopolyploidy). Blockage of gene flow from diploid wild relatives to the tetraploid; cultivated peanut, recent polyploidization combined with self-pollination, and the

narrow genetic base of the primary genepool have resulted in low genetic diversity that has remained a major bottleneck for genetic improvement of peanut.

Generally, in self-pollinating species, mapping population is developed through crossing of the homozygous parents which are contrasting for the trait that is going to be assessed (Collard *et al*., 2005) [2]. Genetic purity of groundnut seeds is conventionally assessed by the Grow-Out Test. It is time consuming, laborious, restricted to a few characteristics, specific morphological traits which are influenced by environmental condition, and inefficient. True hybrids assessment in groundnut is difficult due to self- fertilization may occur before out crossing preventing the transfer of desired traits in progenies. Plants selection based on such traits may not be true hybrids and it provide inaccurate identification which can be adversely affect all stages of future breeding program (Tamilkumar *et al*., 2009) [16]. These problems can be overcome with the help of molecular markers.

The breeding efficiency for disease resistance can be enhanced by employing new biotechnological tools such as use of DNA markers for mapping and tagging of the markers with desirable traits (Pandey *et al*., 2012) [11]. Several studies have demonstrated that marker assisted backcross breeding has significant advantages over conventional breeding particularly for traits which are difficult to manage through phenotypic selection (Varshney *et al*., 2006) [14] like disease resistance, drought, salinity and nutritional deficiency. Among the molecular markers, microsatellites or simple sequence repeats (SSRs) have received extensive attentions due to their advantages of high reproducibility, co-dominant inheritance and high information content (Gupta *et al*., 2000) [6].

In consideration with the entire prospect for developing LLS disease resistant groundnut genotypes. The present study was undertaken with an objective to identified resistant donor parents for LLS disease through field screening under natural epiphytotic condition. Identification of the flanking markers based on previously reported QTLs for LLS disease resistance while also selection of polymorphic SSR markers for identification of true hybrids from each selected crosses. SSR markers are very useful for identification of true hybrids in early seedling stage. Further these true hybrids will be used in back cross for introgression of LLS disease resistance through marker assisted backcross breeding.

Material and methods

1. Screening of germplasms for identification of LLS disease resistance lines

The experimental material for screening of LLS disease resistant lines comprised of 250 genotypes representing *fastigiata*, *vulgaris*, *peruviana*, *aequatoriana*, *hypogaea runner*, *hypogaea bunch* and other advanced breeding lines. It was conducted in *Kharif* 2017 in Augmented Design at K-block, UAS, GKVK, and Bengaluru. The cultivar TMV 2 was used as spreader row to create natural epiphytotic LLS disease incidence and its spread. Each genotype was grown with the spacing of 30 ×15 cm

2. Selection of the QTLs for LLS disease resistance from previous studies

The major reported QTLs for LLS disease resistance in groundnut were identified from previous reported research papers (Sujay *et al*., 2012, Wang *et al*., 2013, and Kolekar *et al*., 2016) [15, 19, 10] and flanking primers for their respective QTLs were used for checking parental polymorphism for the all cross combinations.

3. Experimental materials

A. Four LLS disease resistance lines ICGV 86699, IVGV 91177, GBFSD 272 and GPBD 4 along with two agronomically superior cultivars *viz*., TMV 2 and GKVK 4 suceptible for LLS disease were used in hybridization programme during summer 2018 at greenhouse of GKVK, Bengaluru to develop eight different cross combinations namely,

1TMV 2 × ICGV 86699	5GKVK 4 × ICGV 86699
2TMV 2 × GBFDS 272	6GKVK4 × GBFDS 272
3TMV 2 × ICGV 91177	7GKVK 4 × ICGV 91177
4TMV 2 × GPBD 4	8GKVK4 × GPBD 4

The F_1 seeds from the selected crosses were sown in pots in greenhouse at K-block, GKVK, Bengaluru during summer 2019 to identify true hybrids.

DNA isolation

Genomic DNA was isolated from fresh, young (15-20 days old) and healthy leaves from selected parents and F_1 individual seedlings of two selected crosses by Cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) [5]. DNA quantification for each sample were assessed on 1 % agarose gel and DNA stored in -20° C for further use.

PCR Amplification

A total 53 SSR primers were used for Polymerase chain reaction (PCR) amplification. PCR reaction were performed in 9µl volume it contains of template DNA (1.0 µl), 10x Hi buffer (without $MgCl_2$), 0.50µl $MgCl_2$ (25 mM), 1.0 µl of dNTPs, 1 µl of forward and 1 µl reverse primers, 0.3 µl of Taq DNA polymerase and sterile distilled water (3.7 µl). PCR was performed in touchdown PCR profile in an Eppendorf Master Cycler Gradient, which was programmed for 35 cycles of 94ºC (5 min.), 50-55 ºC (40 sec.), 72 ºC (30 sec.), then followed by final extension at 72 ºC for 10 min. Electrophoresis of the PCR products were carried out on 3 % gel (1.5 agarose + 1.5 metaphore). Electrophoresis of the amplified products were performed at 90 V (1.5- 2 hrs). After separation the gel was viewed under gel documentation system under UV light.

Result and discussion

A. **Selection of the parents based on LLS disease screening**: 250 genotypes representing *fastigiata*, *vulgaris*, *peruviana*, *aequatoriana*, *hypogaea runner*, *hypogaea bunch* and other advanced breeding lines were screened for LLS disease resistance during kharif 2017. Out of these four lines *viz*., ICGV 86699, ICGV 91177, GBFDS 272 and GPBD 4. Two agronomically superior and susceptible cultivar TMV 2 and GKVK 4 were used as female parents to make eight different crosses as mentioned in table 1.

Table 1: Crossing scheme to develop hybrids from elite male and female parents

Female /Male	ICGV 86699	GBFDS 272	GPBD 4	ICGV 91177
TMV 2	C_1	C_2	C_3	C_4
GKVK 4	C_5	C_6	C_7	C_8

B. Selection of QTLs for LLS disease resistance from previous studies: A total 53 flanking SSR markers conferring 38 QTLs were selected from reported research papers. These SSR flanking markers and QTLs were selected from three different crosses *viz*, TAG 24 × GPBD 4 (Sujay *et al*., 2012) [15], TG 26 × GPBD 4 (Khedikar *et al*., 2010) [8], Tifrunner x GT-C20 (Wang *et al*., 2013) [19] and Tifrunner× GT-C20 (Kolekar *et al*., 2016) [10] all 53 SSR markers are listed in table 2. Genomic DNA was isolated from young (15-20 days old) and healthy leaves from all six parents by CTAB method. Eight combinations from six parents DNA were made to run with 53 SSR flanking markers for parental polymorphism. All SSR primers were not found polymorphic for all cross combination. A maximum number of 25 SSR primers were found polymorphic for TMV 2 and ICGV86699 and 22 SSR primers were found polymorphic for TMV 2 and GBFDS 272. The polymorphic SSR primers for the 2 crosses and the number of QTLs identified based on their polymorphism result were depicted in table 3. The number of QTLs detected for the crosses were quite less as both the flanking marker of a particular QTL was not polymorphic for the cross combination. The resistance donor lines with maximum numbers of QTLs were identified and mentioned in table 3.

C. Confirmation of true F_1 hybrids in two selected crosses: True hybrids identification is important in groundnut because it is a self-pollinated crop and accounts low rate of hybridization 20-30% and very often such crosses results large number of self once. SSR markers were used for identification of true hybrids because SSR markers are enough capable to identified heterozygotes from homozygotes. Total 53 SSR primers were used for parental polymorphism between six parent's combination. Two selected crossed seeds C_1 (TMV 2 × ICGV 86699) and C_2 (TMV 2 × GBFDS 272) were sown in pots and hybridity test was performed by using SSR primers. Genomic DNA was isolated from young (15-20 days old) and healthy leaves from all F_1 individual plants by CTAB method. Individuals was confirmed and proving their heterozygosity (represented by H) at the respective loci for two specific alleles of both parents (Fig. 1) Rest of the individuals were confirmed as off-types as they exhibited only one of the alleles of parents.

Table 2: List of reported major QTLs/ flanking markers linked to LLS disease from reported research papers

	Primer Name	QTL Name	Linkage Group	Flanking Primers (from the reported QTL)		Reference (Cross involved)
				Primer(1)	Primer(2)	
1	pPGPSEQ8D09	QTLR4-LLS01	AhXII*(B10)	GM1009	pPGPseq8D0 9	Sujay *et al*.,2012 (TAG24x GPBD 4) [15]
2	GM1009 & GM1839		AhXII (B10)	GM1839	GM1009	Kolekar *et al*.,2016 (TAG24 xGPBD 4) [10]
3	GM1536 & GM2301	QTLR5-LLS01	AhXV (B3)	GM2009	GM1536	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
4		QTLR4-LLS03	AhXV (B3)	GM1536	GM2301	Khedikar *et al*.,2010 (TAG24x GPBD 4) [8]
5	IPAHM103		AhXV (B3)	IPAHM103	GM2301	Kolekar *et al*.,2016 (TAG24 xGPBD 4) [10]
6		QTLR4-LLS04	AhXV (B3)	IPAHM103	GM1954	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
7	GM1577&IPAHM356	QTLR4-LLS05	AhV*(A5)	IPAHM356	GM1577	Kolekar *et al*.,2010 (TAG24x GPBD 4) [10]
8	TC9H09	QTLLLS03	LG9 (B7)	TC2G05	TC9H09	Khedikar *et al*.,2010 (TAG24x GPBD 4) [8]
9	GM1760 &TC6H03	QTLR4-LLS08	AhVIII*(B8)	TC6H03	GM1760	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
10	GM1867	QTLR5-LLS14	AhVII*(A4)	GM1311	GM1867	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
11	GM2746 & GM2504	QTLR5-LLS02	AhVIII*(B8)	GM2504	GM2746	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
12	GM1988 & RN16F05	QTLR5-LLS04	AhV*(A5)	RN16F05	GM1988	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
13	GM1771	QTLR5-LLS08	Ah1*(B1)	GM1090	GM1771	Sujay *et al*.,2012(TG26x GPBD 4) [15]
14	GM1878	*qF5LS3*	LGT5 (A5)	GM1878	GM637	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
15	PM179	QTLR4-LLS12	AhV*(A5)	PM179	seq11C08	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
16	TC7H11 & IPAHM176	QTLLLS11	LG12 (B10)	TC7H11	IPAHM176	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
17	TC11F12	QTLR4-LLS09	AhXVIII*(B7)	TC11F12	TC2G05	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
18	TC1E06 & PM238	*qF5LS1*	LGT3 (A3)	TC1E06	PM238	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
17	IPAHM229	*qF5LS11*	LGT18 (B8)	IPAHM229	IPAHM219	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
18	PM436 & Lec-1	QTLR4-LLS07	AhIX*(B9)	PM436	Lec-1	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
19	pPGPseq18G1	QTLLLS04	LG10 (A6)	TC1A01	pPGSseq18G 1	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
20	pPGPseq17E03 & GM1911	QTLR4-LLS06	AhXIII*(A9)	GM1911	pPGSseq17E03	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
21	TC1DO2	QTLR5-LLS10	AhXIII*(A9)	GM1911	TC1D02	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
22	GM2444	QTLR5-LLS12	AhX*(B6)	IPAHM407	GM2444	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
23	IPAHM165 & PM137	QTLR5-LLS13	AhX*(B6)	PM137	IPAHM165	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
24	TC1B02 & TC4A02	*qF2LS2*	AhV (B10)	TC1B02	TC4A02	Wang *et al*., 2013 (tifrunner x GT-C20) [19]
25	GM2246 &TC5A07	QTLR4-LLS13	AhVII*(A4)	TC5A07	GM2246	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
26	IPAHM395 & TC5A07	QTLLLS09	LG13 (A4)	TC5A07	IPAHM395	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
27	GM1742	*qF5LS9*	LGT16 (B10)	GM678	GM1742	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
28	GM1097	QTLR4-LLS10	AhVI*(A6)	S108	GM1097	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
29	TC4D09	QTLLLS06	LG2(B6)	IPAHM524	TC4D09	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
30	PM36	*qF2LS4*	AhVIII(B5)	PM36	GM2137	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
31	GM2215 & pPGSseg13E6	QTLR5-LLS15	AhIIIc*	GM2215	pPGSseq13E06	Sujay *et al*.,2012 (TAG24x GPBD4) [15]
32	TC7C06	*qF5LS5*	LGT6 (A6)	TC7C06	seq15D3	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
33	IPAHM108	*qF2LS5*	AhIX (A4)	IPAHM108	AHGS0347	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
34	pPGPSeq7G2	QTLLLS05	LG1 (B9)	gi-1107	pPGSseq7G2	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
35	GM1911	QTLR4-LLS06	AhXIII*(A9)	GM1911	pPGSseq17E 03	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
36	GNB159	*qF2LS12*	AhXVIII (A9)	GNB159	GNB335	Wang *et al*.,2013 (tifrunner x GT-C20) [19]
37	GM1955	QTLR4-LLS11	AhV*(A5)	GM1955	GM1007	Khedikar *et al*., 2010 (TAG24x GPBD 4) [8]
38	GM2407	QTLR5-LLS11	AhXIII*(A9)	GM2407	TC3E02	Sujay *et al*.,2012 (TG26x GPBD 4) [15]

Table 3: List of polymorphic primers & major QTL/ flanking markers linked to LLS disease

S. No.	Parents name	Polymorphic markers	No. of polymorphic marker	QTLs identified	Flanking markers
1	TMV-2 and ICGV 86699	GM2301, GM1536, GM1009, pPGPSeq8D09, TC9HO9, GM1988, TC6HO3, GM1760, GM1867, PM436, IPAHM356, GM1577, GM1839, TC1E06, TC11F12, TC5A07, GM2246, PM137, IPAHM165, GM1955, TC1DO2, TC7H11, GM2215, IPAHM108, GNB159	25	6	GM2301 and GM1536 GM1009 and pPGPSeq8D09 TC6HO3 and GM1760 IPAHM356 and GM1577 TC5A07 and GM2246 PM-137 and IPAHM165
2	TMV-2 and GBFDS 272	GM1009, pPGPSeq8D09, GM2301, GM1536, TC9HO9, TC6HO3, GM1867, PM436, TC11F12, PM137, IPAHM165, IPAHM356, TC5A07,GM2246, GM1839, GM1009, TC1E06, TC1DO2 GM1955, IPAHM108, GNB159, IPAHM103	22	5	GM1009 and pPGPSeq8D09 GM2301 and GM1536 PM137 and IPAHM165 TC5A07 and GM2246 GM1839 and GM1009

1. For cross C_1 (TMV 2 × ICGV86699) among polymorphic primers two highly polymorphic markers GM 2301 and GM 2246 were used for hybrids conformation. Out of 100 crossed seedlings only 23(23%) true hybrids.
2. For cross C_2 (TMV 2 × GBFDS272) among polymorphic primers TC6H03 and GM 1760 were used for hybridity test. Out of 210 crossed seedlings we got 46 (21%) true hybrids. The primers sequence is mentioned in (Table 4).

True hybrids are essential for development of true breeding population. True hybrids were used for further backcross population development or mapping purpose. Polymorphic SSR markers were used in identification of true hybrids in many crops like tomato (Smith and Register 1998) [14], rice (Yashitola *et al*., 2002) [18], maize (Salgada *et al*., 2006) [13], cotton (Dongre and Parkhi 2005) [3] and groundnut (Gomez *et al*., 2008, Busisiwe *et al*., 2015 and Darvhanker *et al*., 2019) [7, 1, 4]. In these all studies SSR markers were used for genetic purity analysis, germplasm identified, genetic diversity, gene mapping, fingerprinting for true cultivars and marker assisted back cross selection.

Table 4: List of primers along with forward and reverse temperature used in this study

S. No.	Primer name	Forward sequence	Reverse sequence	F° Temp	R° Temp
1	GM2301	GTAACCACAGCTGGCATGAAC	TCTTCAAGAACCCACCAACAC	57.9	59.8
2	GM2246	GCAATTTTGTGCACCCTTTT	CGCTTGACACCAATGAAGTCT	57.9	54.0
3	TC6H03	TCACAATCAGAGCTCCAACAA	CAGGTTCACCAGGAACGAGT	59.4	55.9
4	GM1760	TGAAGAGCCATGTCAGATCG	AGGGCCCCAACAAGATAAGT	57.3	57.3

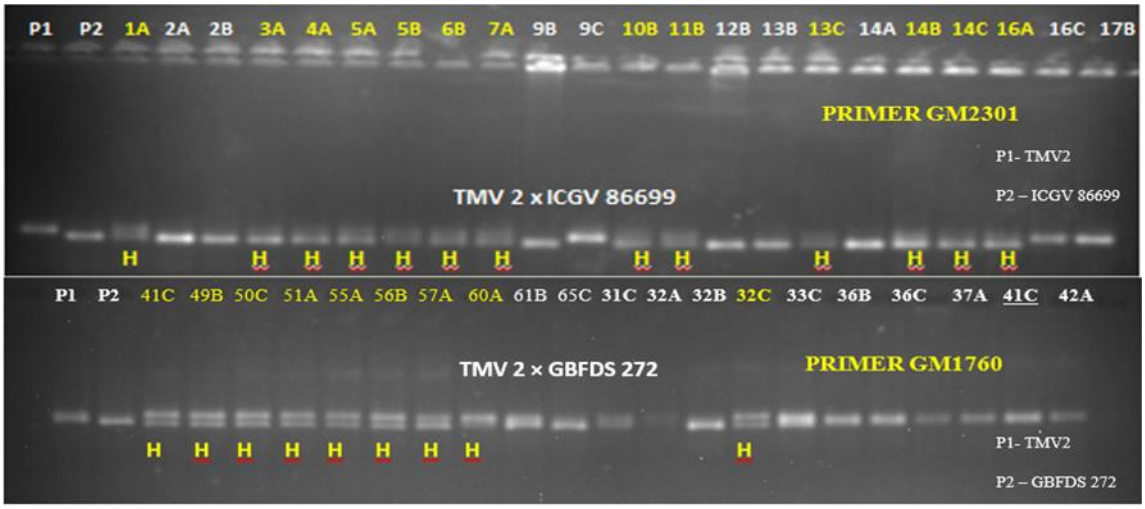

Fig 1: Hybridity test (Double bands hybrid marked as H)

Conclusion

SSR markers are mostly preferred due to its codominance, simplicity, high polymorphism, reproducibility, multi-allelic nature make them very convenient for use. Identifying hybrids in the F_1 generation can be difficult because the F_1 may not be readily distinguishable from the parents, especially in the greenhouse where plants cannot grow to full size due to limited space. In the field, it is often possible to distinguish F_2 plants by segregation for morphological traits. True hybrids identification is important in groundnut because it is a self-pollinated crop and low rate of hybridization 20-30% (Kumar *et al*., 2013) [14]. True hybrids are used for development of mapping populations or gene mapping. In this study we will use true hybrids for marker assisted back cross breeding for development of LLS disease resistance in groundnut.

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