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Single Nucleotide Polymorphism Diversity and Hybridity Testing of Peanut F_1 Progenies Derived from Parents with Varying Oleic Acid Content and Late Leaf Spot Resistance using SNP Markers

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ABSTRACT

Background: Hybridity confirmation of F_1S is the key to success in any crop breeding programme. Single nucleotide polymorphism (SNP) genotyping is emerging as a fast and cost-effective tool in crop breeding programs that evaluates large segregating populations to select plants with a combination of desirable traits. SNP markers, with their high specificity and polymorphism, enable precise hybridity verification in F_1 progenies.

Methods: The leaf samples were collected from parents *viz.,* Kadiri Lepakshi, Kadiri 9 (K 9), Narayani and ICGV 201009 and F_1 plants. Polymorphic SNP markers linked to oleic acid content and LLS resistance were used for genotyping. Hybridity was confirmed by analyzing heterozygosity in F_1 progenies at the target loci using high-throughput genotyping platforms.

Result: Fourteen out of twenty-four SNPs were informative and polymorphic between the parents. These markers differentiated the heterozygotes (F_1s) from the homozygotes efficiently. Out of 142 F_1 plants, 126 plants were confirmed as hybrids using 14 highly informative SNP markers. SNP markers, snpAH00110 and snpAH00113, common in all the three crosses for the identification of hybridity, were highly polymorphic among the parents and F_1s. SNP markers were very much useful for the identification of true hybrid seedlings in the early stage of growth and genotyping of the progenies more accurately for gene mapping and accelerating the breeding.

Key words: Heterozygous, Hybrids, Segregating populations, SNP marker.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.), also known as peanut, earthnut, monkey nut, poor man's cashew nut, manila nut and king of oil seeds (Boraiah *et al*., 2012; Ravi *et al*., 2024; Natarajan *et al*., 2024) is an important oilseed, food and cash crop of Africa and Asia. Asia is the global leader with a share of 50% and 60% of the world's groundnut area and production, respectively. In India, more than half of total groundnut production goes for oil, therefore the quantity and quality of the oil is of utmost interest to plant breeders (Sarvamangala *et al*., 2011; Kamalasundari *et al*., 2025). It is a prominent oil seed crop in India sharing about 40% of total oil production (Sunitha *et al*., 2023). The quality of the oil depends on the proportion of fatty acids present in the oil. Groundnut oil contains 75-80 per cent of oleic (monounsaturated fatty acid) and linoleic acids (Polyunsaturated fatty acid), followed by 10 per cent palmitic acid and the remaining 20 per cent are stearic, arachidic, eicosenoic, behenic and lignoseric acids (Sarvamangala *et al*., 2011; Norden *et al*., 1987; Pramanik *et al*., 2022). Saturated fatty acids like palmitic acid have putative detrimental health effects (Carta *et al*., 2017). Though linoleic acid has health benefits and is an essential fatty acid in human diet; very high concentration of this fatty acid is undesirable for cooking purpose owing to its bad flavor and short shelf life due to oxidation properties (Who and Consultation, 2003). From health point of view, monounsaturated fatty acids (oleic acid) have been reported to play a vital role in reducing the risk of cardiovascular diseases by lowering the cholesterol in the blood (Kratz *et al*., 2002). The groundnut oil with a high oleic/linoleic acid (O/L) ratio is considered more stable oil due to its increased shelf life (Dwivedi *et al*., 1996).

Fungal diseases like leaf spots, rust, root rot, stem rot, pod rot and blight causes severe pod yield penalty and quality loss of both pods and fodder. Among the foliar fungal diseases, rust caused by *Puccinia arachidis* Speg., early

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leaf spot by *Cercospora arachidicola* [Hori.] and late leaf spot (LLS) by *Phaeoisariopsis personata* [(Berk. and Curt) Deighton] are widely distributed, most destructive and economically important in India. LLS and rust can cause 50-70% pod yield loss (Subrahmanyam *et al*., 1984 and Janila *et al*., 2016) under favourable conditions. Host-resistance to LLS and rust have been reported in cultivated and wild species of *Arachis* spp. (Subrahmanyam *et al*., 1983; Pande and Rao, 2001; Sudini *et al*., 2015).

SNP markers were proven to be very powerful in the genetic analysis of other species and useful in breeding programs. SNP genotyping is emerging as a fast and cost-effective tool in crop breeding programs that evaluates large segregating populations to select plants with a combination of desirable traits. Perhaps most importantly, SNPs can be used in parallel assays and the cost per data point is favorable compared to other marker types when used in large-scale assays (Bertioli *et al*., 2014). This accelerated the identification of breeding lines and development of cultivars resistant to LLS, rust, root-knot nematode, stem rot, along with high oil content, high O/L content, large kernel size and tolerance to drought (Khedikar *et al*., 2009; Shou *et al*., 2010; Ravi *et al*., 2011; Gautami *et al*., 2011; Varshney *et al*., 2009; Pandey *et al*., 2017; Shasidhar *et al*., 2020).

This study was undertaken for the introgression of oleic acid content and late leaf spot resistance in to the popular cultivars Kadiri 9, Kadiri lepakshi and Narayani, well known to farmers for their high yield released from Agricultural Research Stations, Kadiri and Tirupathi, Acharya N. G. Ranga Agricultural University (ANGRAU) of Andhra Pradesh and to identify true F_1S and select informative SNP markers that would help to differentiate homozygous parents and their heterogygous F_1 hybrids for high oleic acid and late leaf spot resistance in peanut.

MATERIALS AND METHODS

Plant material

In the present study, Kadiri Lepakshi, Kadiri 9 (K 9) and Narayani well-known, high yielding and stable groundnut cultivars, were used as recipient parents. Kadiri Lepakshi and Kadiri 9 (K 9) were developed at Agricultural Research Station, Kadiri and released in 2002 and 2020, respectively and Narayani was developed at Regional Agricultural Research Station, Tirupathi and released in 2002 by Acharya N.G. Ranga Agricultural University (ANGRAU) of Andhra Pradesh. ICGV 201009, an elite Valencia and advanced breeding line of groundnut having high oleic acid content and resistance to late leaf spot, which was developed at International Crop Research Institute for the Semi-Arid Tropics, Hyderabad (ICRISAT) was used a as donor parent, (Table 1). A 24-SNP panel containing SNPs for five target traits *viz*., high oleic acid content, late leaf spot, fresh seed dormancy, rust and quality control were used for genotyping to confirm the presence of desirable alleles (genotype level) (Table 2).

Emasculation and pollination

Emasculation of groundnut can be accomplished on warm bright days between afternoon and evening. The emasculated flower bud was pollinated the following morning. At ICRISAT, generally the success rate for crossing was 70 to 80% and this may vary depending on the skill of the person involved and environmental conditions, temperature and humidity in particular. Fig 1A illustrates the steps followed in emasculation and pollination. Three independent crosses were made with Kadiri 9, Kadiri Lepakshi and Narayani serving as the pistillate parents and ICGV 201009 as the donor parent to generate F_1s during rainy season, 2022 in the hybridization block at ICRISAT, Patancheru, Hyderabad as described by Nigam *et al*. (1990) (Table 1). A total of 142 kernels obtained from the crosses were sown in the next season to identify F_1 hybrids (Table 3) in the hybridization block. At ICRISAT, generally the success rate for crossing was 70 to 80% and this may vary depending on the skill of the person involved and environmental conditions, temperature and humidity in particular.

Leaf tissue sampling for DNA analysis

F_1 seeds from each of the three crosses were planted in the hybridization block in the bays. After 25 days of planting, a young tetra foliate leaf from each F_1 plant was sampled for DNA analysis. The F_1 plants to be sampled were labeled and a single hole-puncher (6.0 mm diameter) was used to punch and collect two leaf discs per sample from young and healthy newly developed tetra foliate leaf. The forceps

Table 1: Characteristics of the four groundnut varieties/lines used as parents.

Characteristics	Narayani	Kadiri 9	Kadiri lepakshi	ICGV 201009 (Advanced breeding line)
Pedigree	JL 24 × Ah316/S	Kadiri-4 × Vemana	(ICGV 92069 / ICGV 93184) × ICGV 98300)	ICGV 87846 × Sunoleic 95R
Crop duration (days)	100 days	120 *(Kharif)* 125 (*Rabi)*	112 *(Kharif)*	105 to 110 days
Season	*Kharif* and *Rabi*	*Kharif* and *Rabi*	*Kharif* and *Rabi*	*Kharif* and *Rabi*
Potential yield (q/ha)	20-25 (*Kharif*) and 35-45 (*Rabi*)	20-25 (*Kharif)* 35-40 (*Rabi)*	35 (*Kharif)*	9-14 (*Kharif)*

were cleaned before and after placing each sample in a well to avoid cross contamination. The 96-well sample plates were sealed with sealing mats (AB0674, Thermo Fisher Scientific), wrapped in plastic bags, secured firmly and shipped for DNA isolation (Fig 1B).

Genomic DNA extraction

Young leaves were collected from the F_1 plants and parental lines for DNA isolation. DNA was extracted using the CTAB method with minor modifications (Li *et al*., 2010). DNA concentration was determined using a nucleic acid detector and diluted to 20 ng μL^{-1} for polymerase chain reaction (PCR). SNPs were analyzed using the Fluidigm SNP Genotyping Analysis software to obtain genotype calls (Wang *et al*., 2009).

Statistical analysis

Data quality and estimates of genetic parameters

Genotyping data was converted into a HapMap file format and subjected to Quality Control (QC) analysis in TASSEL 5 (Bradbury *et al*., 2007) by filtering for missingness and minor allele frequency (MnAF). Summary statistics for extent of missing data, overall heterozygosity and average MnAF for the filtered data were generated using the "Geno Summary" function of the data panel in TASSEL 5. Overall, average Polymorphism Information Content (PIC) for markers using data on both F_1 and their parents was estimated from the allele frequencies according to Anderson *et al*. (1993) as:

$$PIC_j = 1 - \sum_{i=1}^{n} pi^2$$

Where;

i = The i^{th} allele of the j^{th} marker.

n = The number of alleles at the j^{th} marker.

p = Allele frequency.

Hybridity assessment

Initially, a general comparison was made between the F_1S and the parents based on the proportion of heterozygosity obtained from TASSEL 5. Determination of the hybrid status of all F_1s were based on the number of polymorphic loci that were heterozygous. Therefore, for each F_1 sample, counts of SNPs detecting it as a true hybrid (heterozygous) were made and expressed as a ratio of the total number of markers that were polymorphic between its parents. Hybridity status of each F_1 plant was computed as:

$$\text{Hybridity} = \frac{L_{het}}{Pm} \times 100$$

Where;

L_{het} = Number of polymorphic SNPs detecting an F_1 as heterozygous (true hybrid).

Pm = Total number of SNPs that are polymorphic between the parents of a particular F_1.

Analysis of genetic relationships

Genetic relationship trees were created using Neighbor-Joining clustering method (Nei and Saitou, 1987) and visualized using Archaeopteryx tree viewer in TASSEL 5.

RESULTS AND DISCUSSION

A total of 126 F_1 out of 142 plants were confirmed as genuine hybrids after 'Fluidigm SNP genotyping' using 24 SNP

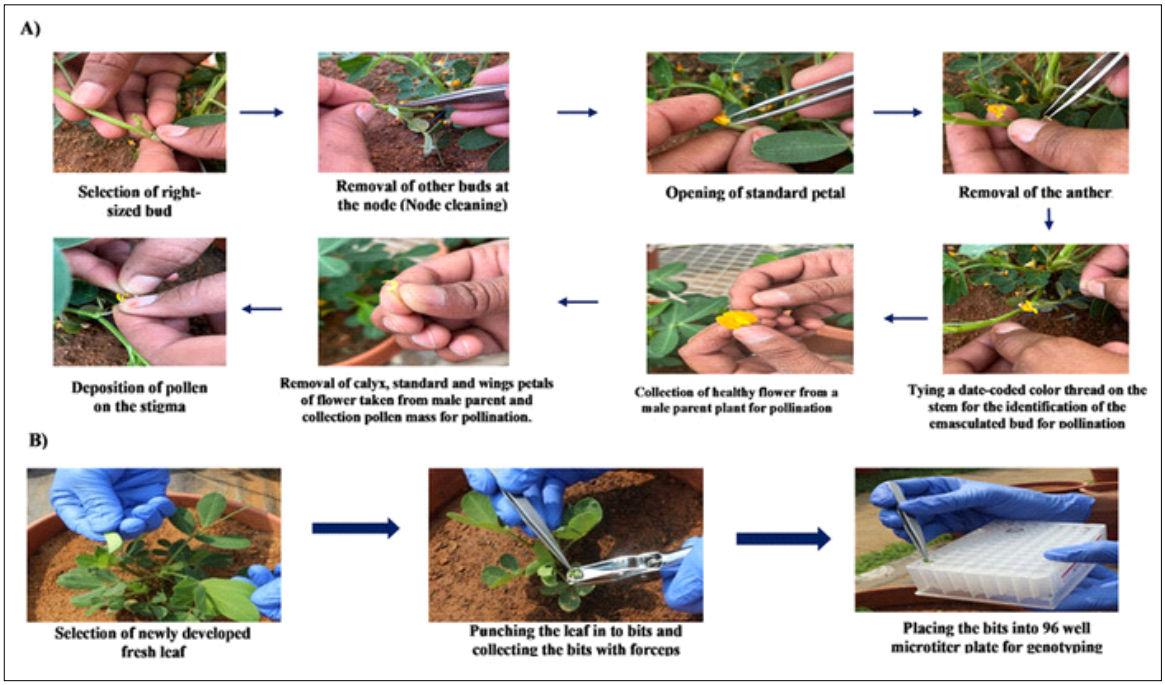


Fig 1: A) Step wise procedure for emasculation and pollination in groundnut B) Leaf tissue sampling for DNA analysis.

markers. Red, blue and green dots indicated XX (fluorescence of the FAM dye), XY (both FAM and HEX dyes) and YY (only HEX dye) types, respectively. The SNP markers and their chromosomal position of 24 SNPs are presented in Table 2. The marker type of each SNP assay was divided into homozygote reference SNP (XX), homozygote alternative SNP (YY) and heterozygote (XY) (Fig 2).

Marker properties and efficiencies

To assess the accuracy of hybridity determination in the progenies, key genetic parameters such as major allele frequency, minor allele frequency, locus heterozygosity and polymorphic information content (PIC) of the SNP markers were evaluated. The results are illustrated in Fig 3.

Analysis of the genetic diversity of SNP markers for three crosses

The summary of the genetic diversity statistics of 9 SNPs for Kadiri 9 × ICGV 201009 is presented in Table 4a. The mean value of the major allele frequency was 0.649, ranging from 0.520 to 0.837. The average heterozygosity and PIC values were 0.599 and 0.327, respectively. Of the 9 polymorphic SNPs, 6 SNPs exhibited the maximum PIC (0.37) and heterozygosity (0.837) value. The SNPs with low PIC value in the present study will not be considered for future studies on genetic diversity. The maximum heterozygosity (0.837) was observed with SNP snpAH0026, snpAH00130, snpAH00113, snpAH00135, while the minimum heterozygosity (0.245) was observed with SNPs marker snpAH0031, snpAH0033 and snpAH0037.

Genetic diversity statistics of 8 SNPs for Kadiri Lepakshi × ICGV 201009 is presented in the Table 4b. The mean value of the major allele frequency was 0.500, ranging from 0.483 to 0.517. The average heterozygosity and PIC values were 0.925 and 0.378, respectively. Almost all SNPs exhibited similar PIC and heterozygosity values with negligible difference.

Genetic diversity statistics of 11 SNPs for Narayani x ICGV 201009 is presented in the Table 4c. The mean value of the major allele frequency was 0.569. The average heterozygosity and PIC values were 0.831 and 0.37, respectively. All SNPs exhibited the same PIC and heterozygosity values. The SNPs with high PIC and heterozygosity values were considered as the informative SNPs for the genetic diversity studies.

Of the 24 SNP markers that were polymorphic only 14 SNPs were able discriminate the four parents and 126 F_1 hybrids (Fig 4). The SNP marker, snpAH00110 showed the highest success rate of 90.3% to distinguish between the parents and hybrids, while SNP marker snpAH0038 showed the lowest success rate of 22.2%. Eight SNP markers comprising snpAH00113, snpAH0005, snpAH00116, snpAH00130, snpAH0026, snpAH00135, snpAH00108

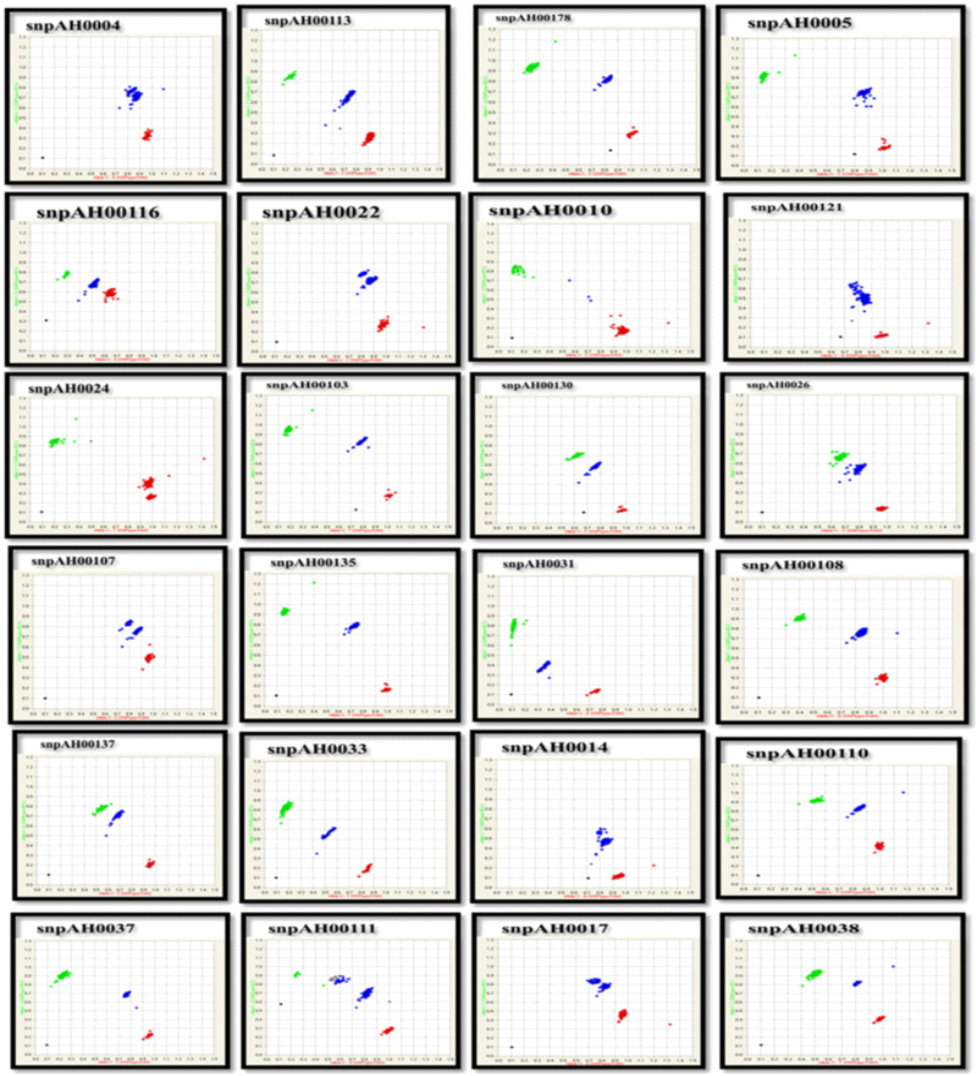
Table 2: List of SNPs used in 'Fluidigm SNP Genotyping' assays.

SNP name	Trait	Variant allele	Chromosome
snpAH0002	High oleic acid	A/-	B09
snpAH0004	Late leaf spot	G/C	A02
snpAH0005	Late leaf spot	G/C	A02
snpAH0010	Late leaf spot	C/A	A02
snpAH0014	Late leaf spot	C/G	A02
snpAH0017	Leaf rust	A/C	A03
snpAH0022	Leaf rust	T/C	A03
snpAH0024	Leaf rust	T/C	A03
snpAH0026	Leaf rust	G/C	A03
snpAH0031	Quality control	G/A	B06
snpAH0033	Quality control	T/C	B06
snpAH0037	Quality control	C/A	B06
snpAH0038	Quality control	T/C	B06
snpAH00103	Fresh seed dormancy	T/G	B05
snpAH00107	Fresh seed dormancy	C/T	A09
snpAH00108	Fresh seed dormancy	G/A	A09
snpAH00110	Fresh seed dormancy	T/C	A09
snpAH00111	Fresh seed dormancy	G/A	A09
snpAH00113	Fresh seed dormancy	C/T	A09
snpAH00116	High oleic acid	A/G	A09
snpAH00121	Late leaf spot	G/C	A02
snpAH00130	Late leaf spot	C/G	13
snpAH00135	Leaf rust+Late leaf spot	T/G	13
snpAH00137	Leaf rust+Late leaf spot	C/G	13

and snpAH00137 had more than 50% success rate in hybrid identification among the parents and F_1S. SNP markers snpAH00110 and snpAH00113 were found to be common for all the three crosses and these two markers can be used as informative markers for hybridity confirmation for particular traits.

Genetic relationships

Neighbor-joining clustering algorithm divided the F_1S into four major groups (Fig 5A). A considerable diversity was shown within sub-clusters and the F_1S. Group I has the highest number *i.e,* 48 F_1S followed by group II, group III and group IV. The group III and IV were again divided into



Red, blue and green dots indicated XX (fluorescence of the FAM dye), XY (both FAM and HEX dyes) and YY (only HEX dye) types, respectively.

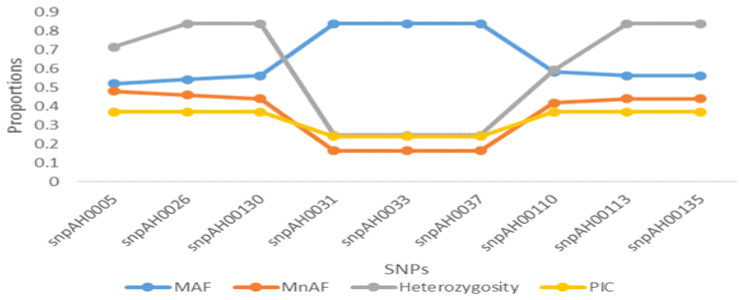
Fig 2: Scatter plots of 24 'Fluidigm SNP genotyping' assays.

Table 3: Details of hybridization made during the rainy season, 2022 in the hybridization block at ICRISAT, Patancheru.

Crosses	Number of pollinations made	Number of putative cross pods harvested	Total number of kernels obtained from putative cross pods	Number of confirmed hybrids	Hybridization success (%)
Kadiri 9 × ICGV 201009	70	39	50	44	88%
Kadiri lepakshi × ICGV 201009	70	37	28	28	100%
Narayani × ICGV 201009	70	48	64	54	85%

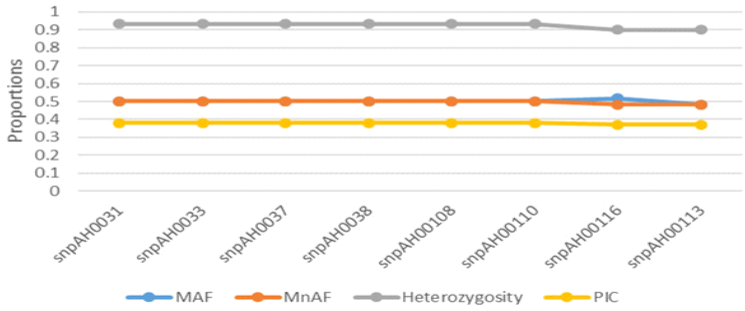
a)

	MAF	MnAF	Heterozygosity	PIC
Min	0.52	0.163	0.245	0.24
Max	0.837	0.48	0.837	0.37
Mean	0.649	0.351	0.599	0.327



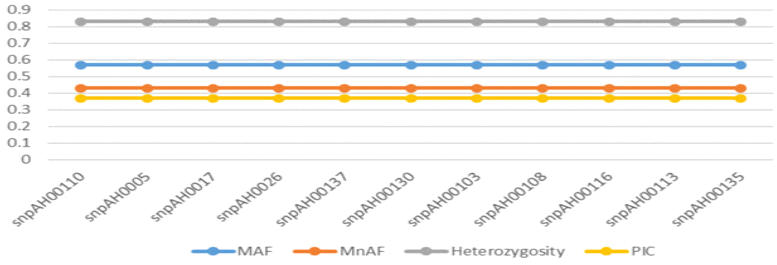
b)

	MAF	MnAF	Heterozygosity	PIC
Min		0.483	0.900	0.370
Max	0.517	0.500	0.933	0.380
Mean	0.500	0.496	0.925	0.378



c)

	MAF	MnAF	Heterozygosity	PIC
Min	0.569	0.431	0.831	0.37
Max	0.569	0.431	0.831	0.37
Mean	0.569	0.431	0.831	0.37



a) Line graph based on combined data of both F_1S and their parents for crosses Kadiri 9 × ICGV 201009. b) Line graph based on combined data of both F_1S and their parents for crosses Kadiri lepakshi × ICGV 201009. C) Line graph based on combined data of both F_1S and their parents for crosses Narayani × ICGV 201009.

Fig 3: Distrubution of major allele frequency (MAF), minor allele frquency (MnAF), proportion of locus heterozygosity and polymorphism information content (PIC).

Table 4: Summary of statistics calculated for genetic diversity based on 14 informative SNP for three different crosses *viz.*, Kadiri 9 × ICGV 201009, Kadiri Lepakshi × ICGV 201009 and Narayani × ICGV 201009, respectively.

Marker	Major allele frequency	Minor allele frequency	Heterozygosity	PIC
		4.a) Kadiri 9 × ICGV 201009		
snpAH0005	0.520	0.480	0.714	0.37
snpAH0026	0.541	0.459	0.837	0.37
snpAH00130	0.561	0.439	0.837	0.37
snpAH0031	0.837	0.163	0.245	0.24
snpAH0033	0.837	0.163	0.245	0.24
snpAH0037	0.837	0.163	0.245	0.24
snpAH00110	0.582	0.418	0.592	0.37
snpAH00113	0.561	0.439	0.837	0.37
snpAH00135	0.561	0.439	0.837	0.37
Mean	0.649	0.351	0.599	0.327
		4.b) Kadiri Lepakshi × ICGV 201009		
snpAH0031	0.500	0.500	0.933	0.38
snpAH0033	0.500	0.500	0.933	0.38
snpAH0037	0.500	0.500	0.933	0.38
snpAH0038	0.500	0.500	0.933	0.38
snpAH00108	0.500	0.500	0.933	0.38
snpAH00110	0.500	0.500	0.933	0.38
snpAH00116	0.517	0.483	0.900	0.37
snpAH00113	0.483	0.483	0.900	0.37
Mean	0.500	0.496	0.925	0.378
		4.c) Narayni × ICGV 201009		
snpA0.569	0.431	0.831	0.37	H00110
snpAH0005	0.569	0.431	0.831	0.37
snpAH0017	0.569	0.431	0.831	0.37
snpAH0026	0.569	0.431	0.831	0.37
snpAH00137	0.569	0.431	0.831	0.37
snpAH00130	0.569	0.431	0.831	0.37
snpAH00103	0.569	0.431	0.831	0.37
snpAH00108	0.569	0.431	0.831	0.37
snpAH00116	0.569	0.431	0.831	0.37
snpAH00113	0.569	0.431	0.831	0.37
snpAH00135	0.569	0.431	0.831	0.37
Mean	0.569	0.431	0.831	0.37

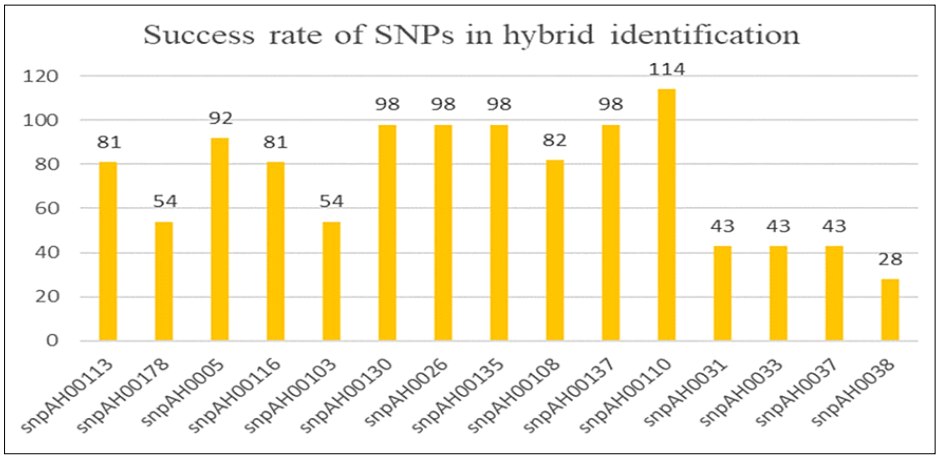
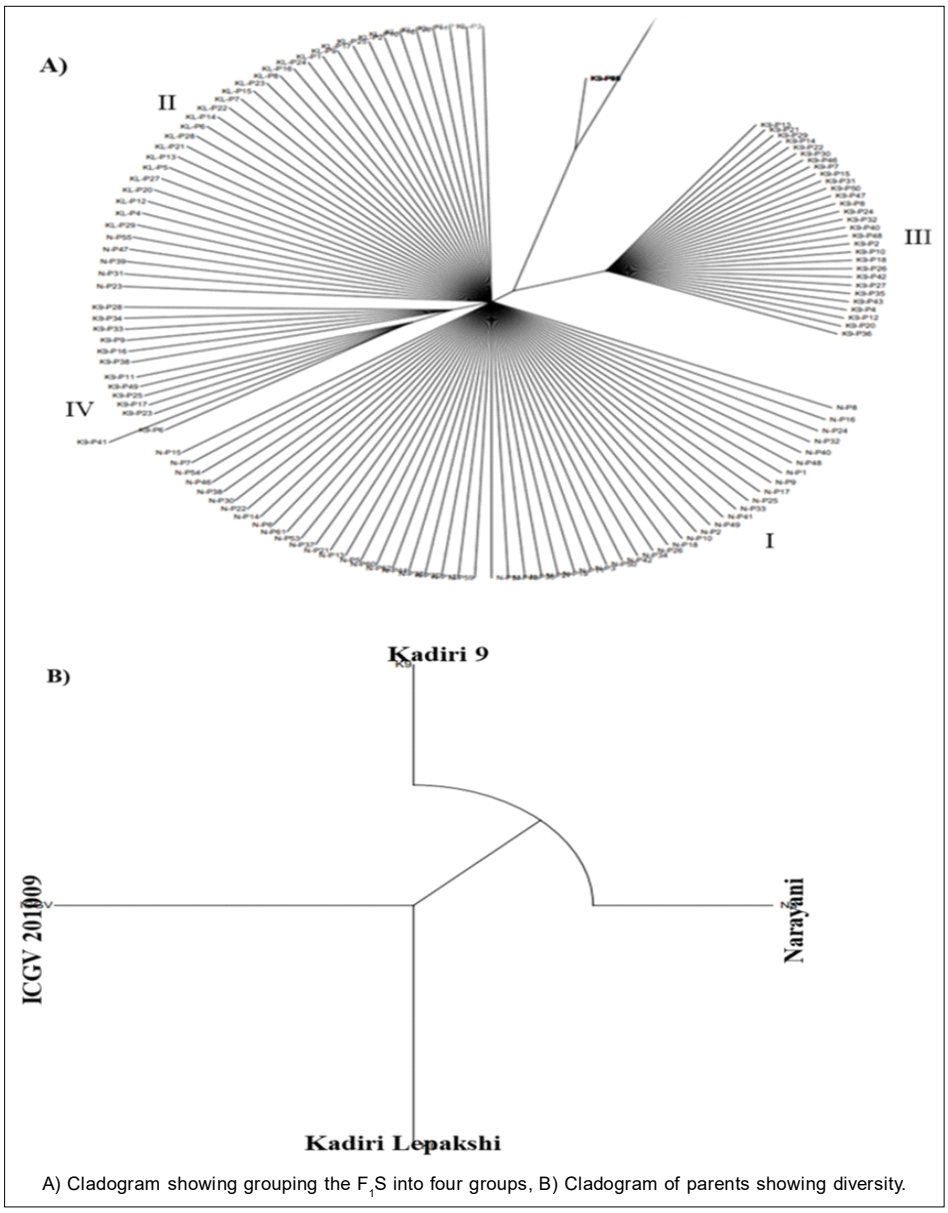


Fig 4: SNP marker's performance in hybrid identification.



A) Cladogram showing grouping the F_1S into four groups, B) Cladogram of parents showing diversity.

Fig 5: Relationship among the parents that were used to derive the F_1S progenies.

sub groups. However, the parents were considered alone, the diversity among them were noticeable (Fig 5B), since the parents formed into four distinct groups and were highly divergent.

SNP markers are used for germplasm identification, cultivar fingerprinting, true hybrid identification, genetic purity testing, parentage confirmation of hybrids and heterotic pattern in hybrids. In the present investigation, the SNP markers that identified true hybrids from the three crosses *viz.,* Kadiri 9 × ICGV 201009, Kadiri Lepakshi × ICGV 201009 and Narayani × ICGV 201009 were specific to each cross combination. For new crosses, similar kind of homologous markers need to be identified. Similar findings were reported for identifying true hybrids in peanut (Gomez *et al*., 2008 and Busisiwe *et al*., 2015).

CONCLUSION

The study was conducted to identify true F_1 hybrids using SNP markers, with the goal of facilitating the development of inbred lines through marker-assisted selection (MAS) and marker-assisted backcrossing (MABC). The study revealed that SNP markers are effective in accurately distinguishing true heterozygous plants. Such markers are highly valuable for early-stage identification of true hybrid seedlings or target traits, as well as precise genotyping of progenies in gene mapping studies.

Conflict of interest

All authors declared that there is no conflict of interest.

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