



Genotyping High-oleic Soybean Segregants using Fatty Acid Linked Markers

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ABSTRACT

Background: Improving oil quality is an important mandate in soybean breeding. Marker-assisted selection for the desired fatty acid composition is easier and technically convenient.

Methods: In the present investigation, 110 segregants from parents with contrasting oleic acid content were screened using candidate markers of *Omega-6 fatty acid desaturase 2-1 (Fad2-1A and Fad2-1B)* genes and linked with QTLs for oleic acid content.

Result: Out of the 10 molecular markers studied, 7 were found polymorphic (*IFAD2-1A*, *IFAD2-1B*, *Satt_108*, *Satt294*, *Satt386*, *Satt002*, *Satt001*), while *Satt354* behaved like a null allele. Among them, *Satt386*, *Satt294* and *Satt354* were most informative for screening individual segregants to reveal 38 (F_2 : 14, F_3 : 24); 42 (F_2 : 14, F_3 : 28) and 39 (F_2 : 3, F_3 : 36) segregants, respectively, with markers matching NRC 147, the higher oleate parent. Further, *FAD2-1A/ FAD2-1B* specific primers were developed from sequence analysis, which differentially amplified 401 bp and 378 bp markers simultaneously for those two isoforms. The identified markers may potentially contribute to breeding favorable soybean oil characteristics.

Key words: Fatty acyl desaturase, Markers, Polyunsaturated fatty acids, Soybean Oil.

Abbreviations: FAD: Fatty acid desaturase, PCR: Polymerase chain reaction, QTL: Quantitative trait loci, SSR: Simple sequence repeats.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is the world's leading oilseed crop valued for food and oil extraction, containing 40% protein, 20% oil, 35% carbohydrates and 5% ash (Lui, 1997; Alam *et al.*, 2023). Soybean oil (18-21%) mainly comprises palmitic (10-12%), stearic (4%), oleic (20-23%), linoleic (53-55%) and linolenic (8-11%) acids (Wilson, 2004; Rathod *et al.*, 2021). High oleic acid improves oxidative stability, extends shelf life, reduces hydrogenation and benefits health, while high polyunsaturated fatty acids reduce stability and favor trans-fat formation (Hu and Willett, 2002; Jerish *et al.*, 2025).

Oil biosynthesis starts in chloroplasts with stearic acid, followed by desaturation in cytoplasm and endoplasmic reticulum through Δ^9 desaturase, FAD2 (oleate to linoleate) and FAD3 (linoleate to linolenate) (Shanklin and Cahoon, 1998; Ohlrogge and Browse, 1995; Buchanan *et al.*, 2015). Soybean has multiple *FAD2* gene copies owing to the repeated rounds of genome duplications (Okuley *et al.*, 1994). Seed-specific isozymes GmFAD2-1A/B regulate oleic acid during seed development, while GmFAD2-1C and GmFAD2-2 variants are expressed in cool climates and vegetative tissues (Schlueter *et al.*, 2007).

Improving oil quality requires lowering polyunsaturated and enhancing monounsaturated fatty acids (Liu and White, 1992). Linolenic acid imparts off-flavors, while oleic acid reduces hydrogenation and trans-fat risk (Willett and Ascherio, 1994). Genetic modifications, especially *FAD2-1a/b* mutations, significantly increase oleic acid (Clemente and Cahoon, 2009; Richardson, 2016). While both the *FAD2-1A* and *FAD2-1B* mutant alleles, together are responsible for the elevated oleic acid levels (Kulkarni *et al.*,

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2018). Mutation and molecular breeding approaches, allow large-scale screening (Patil *et al.*, 2010) whereas Near-Infrared Transmittance Spectroscopy facilities are accessible to limited researchers only and it is not perfect, as it does not guarantee the consistency (Gao *et al.*, 2024) while, conventional Gas-Liquid Chromatography is seed destructive method.

NRC 147 (IISR, Indore) with 42% oleic acid (Singh, 2024), was crossed with Phule Agrani (KDS 344), Phule Sangam (KDS 726), Phule Kimaya (KDS 753) and genotypes KDS 869 and KDS 980 (20% oleic acid). Oleic acid levels are strongly affected by environmental conditions such as temperature, rainfall and photothermal interactions (Pham *et al.*, 2012), making phenotype-based selection unreliable in early generations. Marker-assisted selection enables rapid, cost-effective identification of desirable alleles. Thus, F_2 and F_3 segregants were used to fix high-oleate traits early and accelerate breeding progress.

Instead of conventional gas-liquid chromatography is destructive, marker-assisted selection offers a non-destructive and convenient alternative. Hence, this study aimed to screen soybean segregants for oleic acid using molecular markers.

MATERIALS AND METHODS

The F_2/F_3 seeds of five different crosses of soybean were used with NRC147 being the common donor parent for high oleate content and the details are listed in Table 1.

Molecular marker-based analysis of oleic acid trait in soybean

Healthy seeds of crosses involving six parents viz NRC 147 (donor) possess about 42% oleic acid content (Singh, 2024), Phule Agrani (KDS 344), Phule Sangam (KDS 726), Phule Kimaya (KDS 753), KDS 869 and KDS 980 were grown for DNA extraction along with 110 segregants derived from their crosses. Genomic DNA was isolated from tender leaves using a modified CTAB method (Doyle and Doyle, 1990) and the final DNA concentration was adjusted to 30 ng/μl for PCR amplification.

Designing of primers for simultaneous amplification of both *FAD2-1A*/*FAD2-1B* gene

1. Full-length nucleotide sequences of *FAD2-1A* (AB188250.1) and *FAD2-1B* (AB188251.1) were retrieved using "Somewhat Similar Nucleotide BLAST", selecting hits with >97% query coverage and >99% identity. InDel/SNPs in the intron region were identified and primers were designed to be polymorphic between the two genes while conserved at annealing sites.
2. Cluster Alignment with Weights (ClustalW) was used for Multiple Sequence Alignment (MSA) selecting conserved flanking regions around insertion-deletion polymorphisms.
3. Primer BLAST was used for designing the *FAD2-1A* gene-specific primers.
4. *In silico* screening of primer pairs was carried out to confirm specificity, expected product size and to check possible amplification of *FAD2-1B*.

FAD2-1 isoform specific and SSR markers used to determine oleic acid gene in soybean

SSR markers linked to unsaturated fatty acid composition (Bachlava *et al.*, 2009; Priolli *et al.*, 2015) were used to analyze segregants. Isoform-specific primers for *FAD2-1A* and *FAD2-1B* (Bachlava *et al.*, 2008) were employed to amplify the coding regions of respective isoforms. Primer sequences as mentioned in Table 2 were custom-synthesized by BioServe Biotechnologies (India) Pvt. Ltd., supplied in lyophilized form and were diluted before use for PCR.

PCR amplification

PCR reactions were set up in 0.2 ml tubes with 30 ng template DNA, 1 U *Taq* DNA polymerase, 1X *Taq* buffer-B, 1.0 mM $MgCl_2$, 1 μM dNTP mix and 20 pmol of each primer. The amplification regime comprised of initial denaturation (95°C, 5 min), 30 cycles of denaturation (94°C, 1 min),

annealing (41-54°C, depending on primer) and elongation (72°C, 90 s), followed by a final elongation (72°C, 10 min). Amplification was performed in an Eppendorf Thermal Cycler.

PCR products were resolved by agarose gel electrophoresis: 2.5% for SSR primers and 1.6% for *FAD2*-specific primers and run at 80 V for 1.5-2 h followed by gel image analysis.

RESULTS AND DISCUSSION

The study evaluated 110 soybean segregants from five crosses: 40 F_2 segregants (30 from KDS 980 × NRC 147; 10 from KDS 344 × NRC 147) and 70 F_3 segregants (35 from KDS 869 × NRC 147; 30 from KDS 726 × NRC 147; 5 from KDS 753 × NRC 147). *FAD2* gene-specific primers and SSR markers linked to seed oleic acid QTLs were used to assess functional (*FAD2-1*) and dysfunctional (*fad2-1*) alleles in individual segregants (Table 3).

FAD2-1A / *FAD2-1B* primers were designed during this study by comparing the *FAD2-1A* gene (accessions AY954300.1, ACUP0300731.1) with *FAD2-1B* gene (accession AB188251.1). Out of 2662 bases were studied in multiple sequence alignment, 1801 bases aligned with each other of which 1564 (86.84%) were identical (Fig 1). Out of 406 bases in intron region, only 312 base sequences showed similarity. Due to high variation in the intron region, this region was selected for designing primers specific to *FAD2-1A*/*FAD2-1B* isoforms for their simultaneous amplification.

A parental polymorphism study was conducted using ten primer pairs on six parental cultivars: NRC 147 (high oleate donor), Phule Agrani (KDS 344), Phule Sangam (KDS 726), Phule Kimaya (KDS 753) and genotypes under field trials KDS 869 and KDS 980. PCR amplification showed that seven primers (*IFAD2-1A*, *IFAD2-1B*, *Sat_108*, *Satt294*, *Satt386*, *Satt002* and *Satt001*) were polymorphic (Table 3). The detailed parental polymorphism results are as follows.

• *IFAD2-1A*

This isoform-specific primer (GenBank accessions AB188250; Benson *et al.*, 2002) amplified 155 bp in KDS 980, 162 bp in KDS 869 and KDS 753, whereas 140 bp in KDS 726, KDS 344 and NRC 147 (Table 3). As it did not amplify a unique NRC 147-specific band and hence was not considered for individual plant analysis.

• *IFAD2-1B*

This isoform-specific marker (GenBank accessions AB188251; Benson *et al.*, 2002) amplified dual 284 bp and

Table 1: Crosses from which derived F_2/F_3 segregating plants (no. of plants) were used in the present study.

F_2 crosses	F_3 crosses
KDS 980 × NRC 147 (30)	KDS 869 × NRC 147 (35)
Phule agrani (KDS 344) × NRC 147 (10)	Phule sangam (KDS 726) × NRC 147 (30)
	Phule kimaya (KDS 753) × NRC 147 (05)

190 bp bands in low oleate parents, while 163 bp band in NRC 147 (Table 3). Although polymorphic and suitable for oleate trait analysis, due to its low annealing temperature (41°C) it was not considered further.

• *Satt354*

PCR amplification amplified 190 bp band in NRC 147, while it did not amplify in rest five parents in repeated attempts (Table 3; Fig 2a-2h, 3a-3h). Thus, *Satt354*

Table 2: List of primers used in the present study.

Primer name	Primer sequence (5'-3')	Reference	Annealing temp. (°C)	QTL	Chromosome	LG
<i>IFAD2-1A</i> F	GTGTGGCCAAAGTGGAAGTT	1	41	Isoform 1Aspecific	10	O
<i>IFAD2-1A</i> R	ACTGGCTGAAGGGAGCTTTG					
<i>IFAD2-1B</i> F	GTGTGGCCAAAGTTGAAAT	1	47	Isoform 1Bspecific	20	I
				-		
<i>IFAD2-1B</i> R	ATTGGCTGAGGGGTGCTT					
<i>Satt354</i> F	GCGAAAATGGACACCAAAAGTAGTTA	2	53	-	20	I
<i>Satt354</i> R	GCGATGCACATCAATTAGAATATACAA					
<i>Sat_108</i> F	AAAAATCTATTCACTTTGAGTCTA	2	53	-	10	O
<i>Sat_108</i> R	TTGAAAGAGTCACGTCTATTCTAT					
<i>Satt294</i> F	GCGGGTCAAATGCAAATTATTTTT	2	53	Seed oleic 1-g5	4	C1
<i>Satt294</i> R	GCGCTCAGTGTGAAAGTTGTTTCTAT					
<i>Satt386</i> F	GCGGATGATTTTATAGAATAGATAAT	21	53	Seed oleic 1-g11	17	D2
<i>Satt386</i> R	CTTCGTTGATACCTCAGTAGAGTACAAA					
<i>Satt002</i> F	TGTGGGTAAAATAGATAAAAAT	21	54	Seed oleic 1-g10	17	D2
<i>Satt002</i> R	TCATTTTGAATCGTTGAA					
<i>Satt487</i> F	ATCACGGACCAGTTCATTGA	21	41	Seed oleic 1-g33	10	O
<i>Satt487</i> R	TGAACCGCGTATTCTTTTAATCT					
<i>Satt001</i> F	TGTGCAATGATAGTACATAGATAT	21	53	Seed oleic 1-g26	9	K
<i>Satt001</i> R	GTGCTGATTGAACTATTTGTAGT					
<i>FAD2-1B</i>	GGATATTTTCATGTGGGGTTCA	<i>In silico</i> designed in present study	53	-	10 and 20	O and I
<i>InDel</i> F						
<i>FAD2-1B</i>	CTAGACCCTGTGTCAAAGTA					
<i>InDel</i> R						

Table 3: Size of PCR products obtained for different parents by primers in parental polymorphism study.

Primers	Size of PCR products (bp)					
	NRC 147	KDS 344	KDS 726	KDS 753	KDS 869	KDS 980
<i>IFAD2-1A</i>	140	140	140	162	162	155
<i>IFAD2-1B</i>	-	284	284	284	284	284
	163	190	190	190	190	190
<i>Satt354</i>	190	-	-	-	-	-
<i>Sat_108</i>	234	226	220	213	213	207
<i>Satt294</i>	280	290	290	290	296	296
<i>Satt386</i>	190	200	200	200	200	200
<i>Satt002</i>	127	127	127	127	140	127
<i>Satt487</i>	200	200	200	200	200	200
<i>Satt001</i>	215	207	203	203	203	203
<i>FAD2-1A/FAD2-1B</i> specific	401/ 378	401/ 378	401/ 378	401/ 378	401/ 378	401/ 378

behaved as a null allele in low-oleate parents and was selected as a candidate marker for individual plant studies. Both *Satt354* and the *GmFAD2-1B* gene isoforms are reported to be located on chromosome number 20.

• *Sat108*

Positioned on Chromosome 10(O), *Sat_108* amplified product sizes from 207-234 bp: 207 bp (KDS 980), 213 bp (KDS 869, KDS 753), 220 bp (KDS 726), 226 bp (KDS 344) and 234 bp (NRC 147) (Table 3). The 234 bp allele specific to NRC 147 makes *Sat_108* informative for individual plant studies of the oleate trait.

• *Satt294*

Positioned on Chromosome 4(C1) at the *Seed oleic 1-g5* locus, *Satt294* amplified 280 bp in NRC 147, 290 bp in KDS 344, KDS 726 and KDS 753 and 296 bp in KDS 869

and KDS 980 (Table 3 and Fig3a-3h). The distinct 280 bp allele of NRC 147 makes *Satt294* an informative marker for individual plant studies of the oleate trait.

• *Satt386*

Linked to *Seed oleic 1-g11* QTL on Chromosome 17(D2), *Satt386* amplified 200 bp in NRC 147 and 190 bp in the other five parents (Table 3; Fig2a-2h). This NRC 147-specific polymorphism makes *Satt386* suitable for individual plant analysis of the oleate trait.

• *Satt002*

Associated with QTL *Seed oleic 1-g10* on Chromosome 17 (D2), *Satt002* parents amplified a 127 bp band, while KDS 869 produced a 140 bp band (Table 3). It was non-informative for studies of the oleate trait.

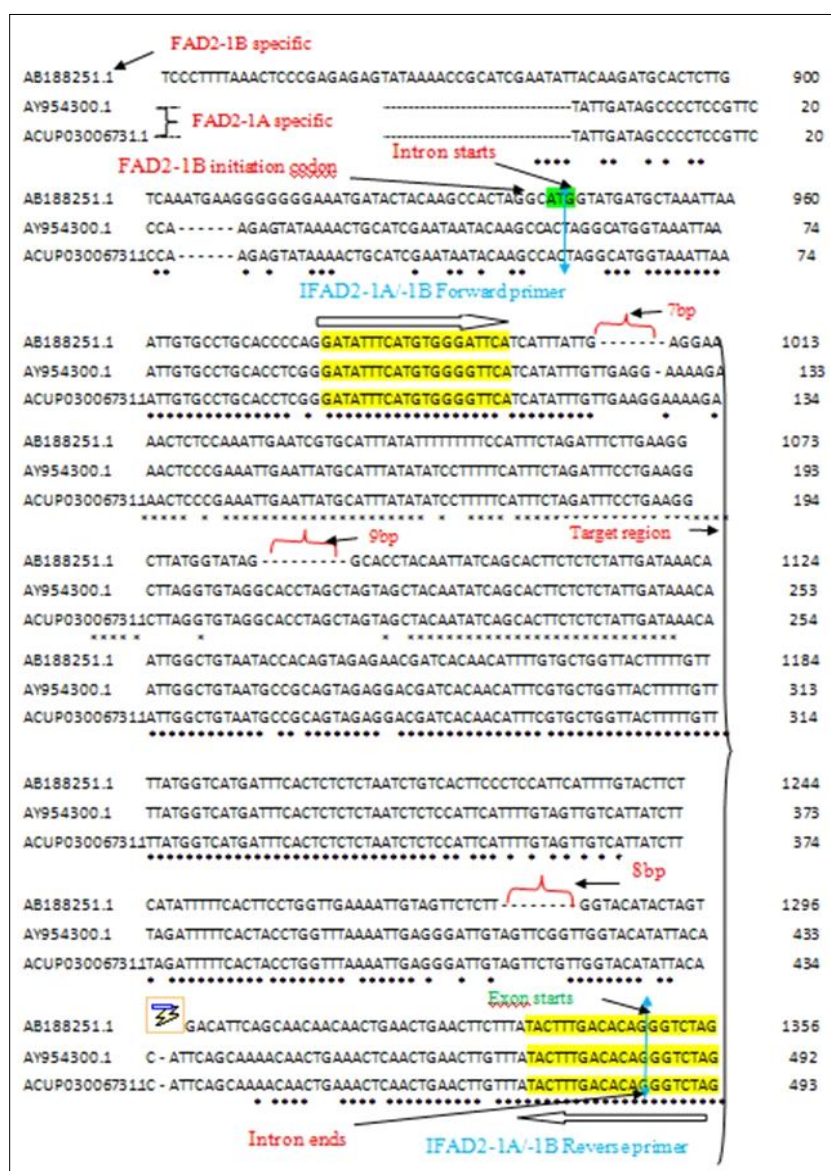


Fig 1: Multiple sequence alignments of partial *FAD2-1A* (AY954300.1, ACUP03006731.1) and *FAD2-1B* (AB188251.1) sp. accessions.

• *Satt487*

Associated with QTL *Seed oleic 1-g33* on Chromosome 10 (O), *Satt487* produced a monomorphic 200 bp band across all parents (Table 3).

• *Satt001*

This marker is linked to *Seed oleic 1-g26* QTL on Chromosome 9 (K). It amplified product sizes of 215 bp and 207 bp, in NRC 147 and KDS respectively, while rest four parents amplified a 203 bp PCR product (Table 3). Thus, *Satt001* exhibited polymorphism among the parents and is informative for individual segregant analysis.

• *FAD2-1A/FAD2-1B* Specific

The *FAD2-1A/FAD2-1B* primers designed in this study specifically amplified both *FAD2-1A* and *FAD2-1B* genes, producing expected twin PCR products of 401 bp and 378 bp, respectively. Amplification in all six parents was monomorphic, with the twin products corresponding to the isoforms, distinguished by three InDel sites (7, 9 and 8 bp) in *FAD2-1B*. These primers allow tracking of *fad* mutant alleles. Earlier studies have shown that Single mutants (*FAD2-1 aaBB*, 34.4%; *FAD2-1 AAbb*, 26.2%) showed higher oleic acid than the wild type (*FAD2-1 AABB*,

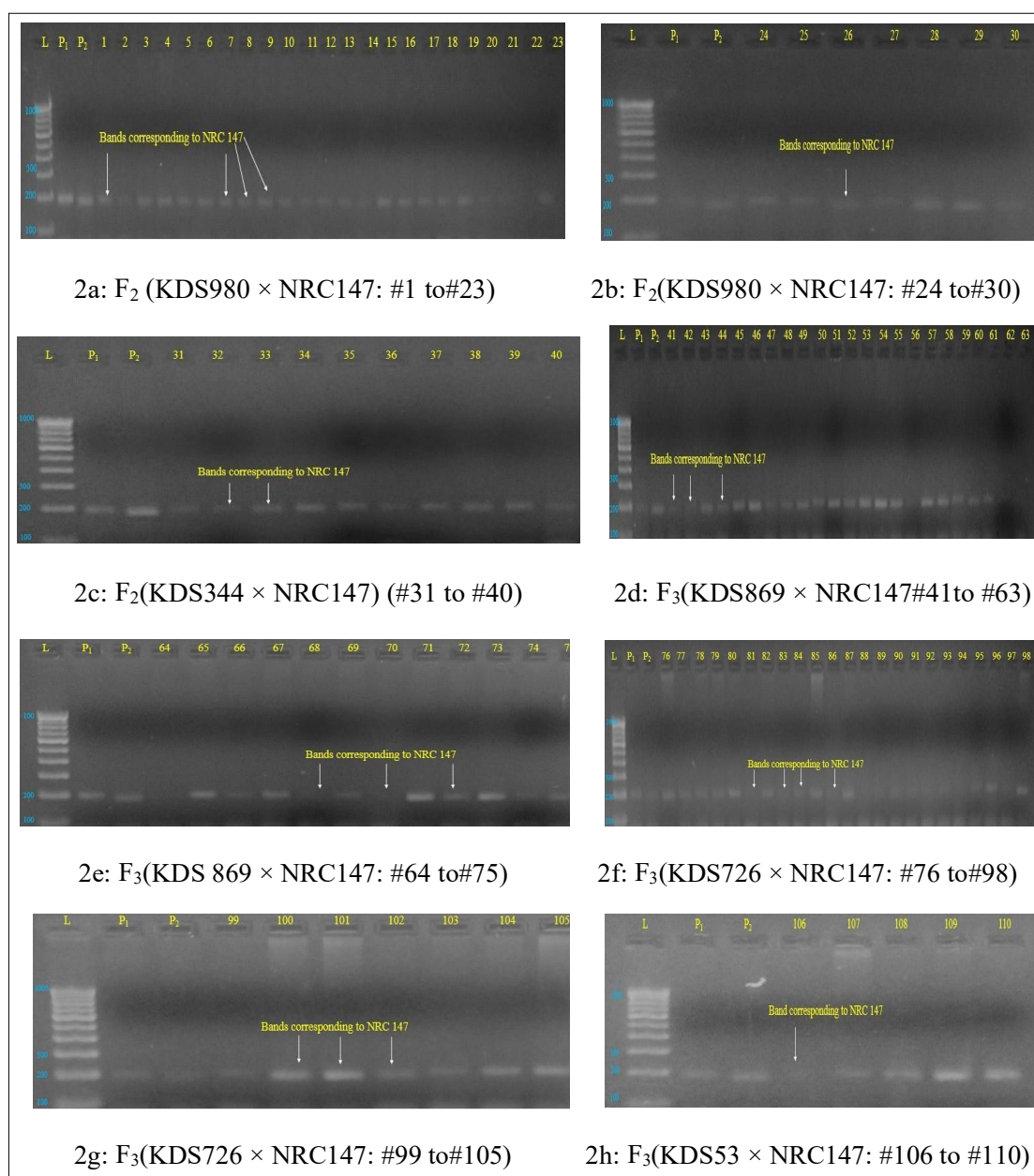


Fig 2a-2h: Multiplex PCR amplification profile of segregants with *Satt386* and *Satt354* primers.

21.3%), while the double mutant (*FAD2-1aabb*) had the highest oleic content (77.1-81.8%) (Richardson, 2016). Molecular analysis revealed that variations in *FAD2-1A* affected conserved histidine residues required for enzymatic activity, whereas *FAD2-1B* mutations introduced a premature stop codon, terminating translation (Lee *et al.*, 2019).

PCR amplification for screening soybean segregants

From parental polymorphism analysis, 4 informative primers viz *Satt354*, *Satt001*, *Satt294* and *Satt386* capable of distinguishing NRC 147 from the rest five parents, with high annealing temperature (53°C), were used to screen individual plants from five crosses. *Satt354* amplified only NRC 147 DNA, while multiplex PCR with *Satt294* and *Satt386* enabled simultaneous detection of multiple alleles

in segregating plants for oleic acid content. Molecular markers linked to QTLs revealed genomic regions controlling soybean oil traits (Priolli *et al.*, 2015). In their study over two years, 33 SSR loci were associated with oleate, followed by linoleate (26), palmitate (24) and linoleate (14). 8 loci with specific alleles contributed to high-performing soybeans, indicating the usefulness of these markers for rapid improvement of oil quality.

Satt386 and *Satt354*

Satt386 and *Satt354* were used to screen 110 individual segregants (Fig 2a-2h). *Satt386* amplified a 190 bp band from NRC 147 and one plant showed dual bands (190 bp from NRC 147 and 200 bp from KDS 344). Among 30 F_2 segregants of KDS 980 × NRC 147, 12 amplified the 190 bp band, while 2 of 10 F_2 plants of KDS 344 × NRC 147

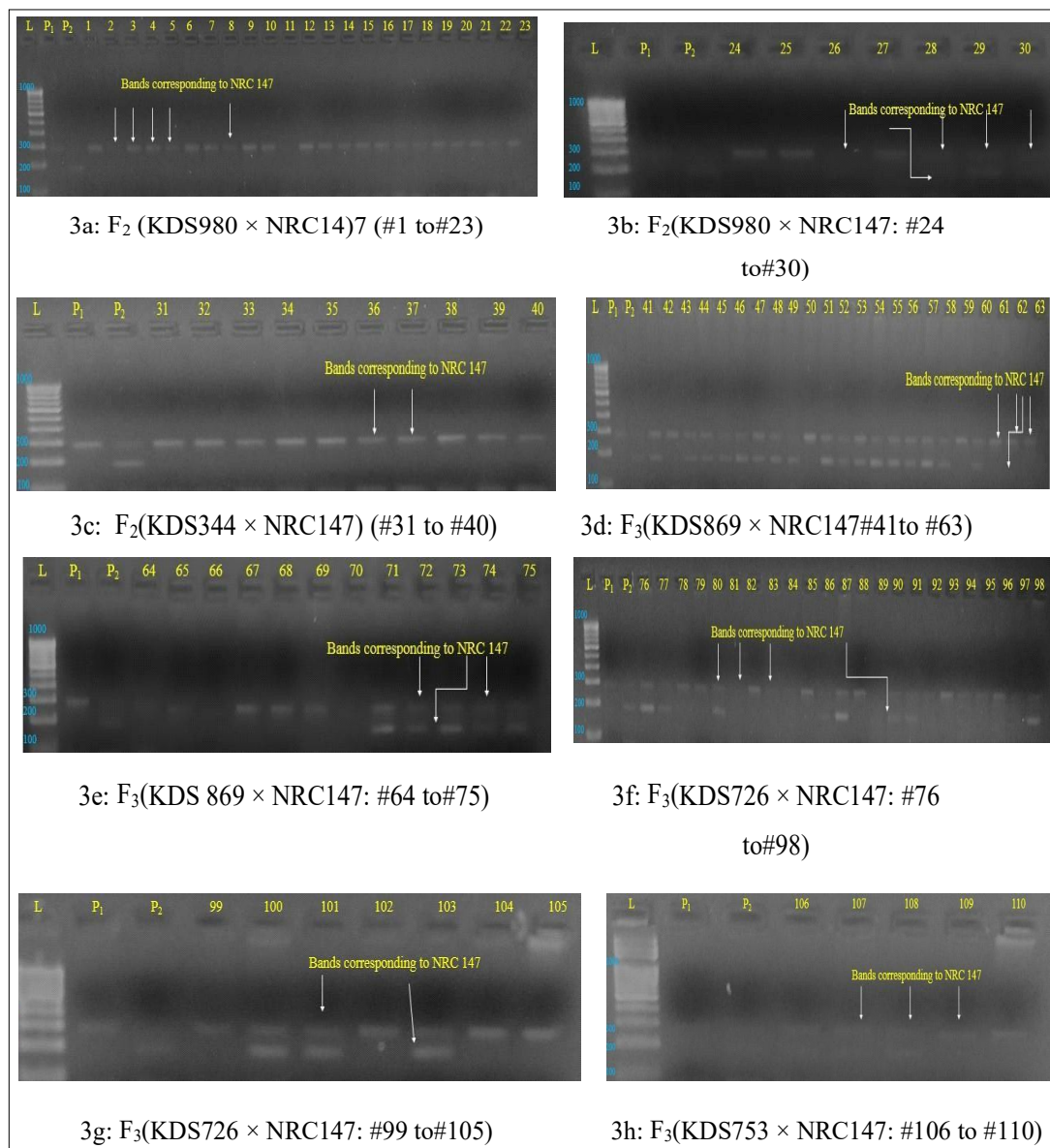


Fig 3-3h: Multiplex PCR amplification profile of segregants with *Satt294* and *Satt354* primers.

also amplified the 190 bp band. In F_3 segregants, 13 out of 30 (KDS 869 $\times$ NRC 147), 10 out of 30 (KDS 726 $\times$ NRC 147) and 1 out of 5 (KDS 753 $\times$ NRC 147) amplified the 190 bp band. As both *Satt386* and *Satt354* amplified this 190 bp fragment in NRC 147 and derived segregants, detailed *Satt354* results were further analyzed using *Satt294* and *Satt354* multiplex PCR to distinguish additional alleles. Similar studies in sunflower revealed that high-oleic individuals (62.49-93.82%) could be effectively identified using SSR markers like *F4-R1*, whereas low-oleic plants (15.24-31.28%) did not amplify the marker (Dimitrijević *et al.*, 2017).

Satt294* and *Satt354

Two SSR primers, *Satt294* and *Satt354*, were used to study individual segregants (Fig 3a-3h). With *Satt294*, 280 bp bands corresponding to NRC 147 were amplified in 11/30 (KDS 980 $\times$ NRC 147), 3/10 (KDS 344 $\times$ NRC 147), 16/35 (KDS 869 $\times$ NRC 147), 11/30 (KDS 726 $\times$ NRC 147) and 1/5 (KDS 753 $\times$ NRC 147) segregants. Earlier, Monteros *et al.* (2008) identified six oleic acid QTLs using SSRs, enabling MAS for high-oleate breeding.

With *Satt354*, a 190 bp band was detected in 3/30 (KDS 980 $\times$ NRC 147), 10/10 (KDS 344 $\times$ NRC 147), 22/35 (KDS 869 $\times$ NRC 147), 11/30 (KDS 726 $\times$ NRC 147) and 3/5 (KDS 753 $\times$ NRC 147) segregants, while acting as a null allele in low oleate parents. This primer maps to Chromosome 20, where *FAD2-1B* gene resides. Bachlava *et al.* (2008) also mapped *FAD2* isoforms to LGs O, I and L, linking them to oleate QTLs.

Overall, *Satt386*, *Satt294* and *Satt354* together identified 38, 42 and 39 segregants, respectively, for NRC 147 alleles. These are being advanced for further correlation study of marker pattern with oleic acid profile.

CONCLUSION

Out of the 10 molecular markers studied, *Satt386*, *Satt294* and *Satt354* were most informative for screening individual segregants to reveal 38 (F_2 : 14, F_3 : 24); 42 (F_2 : 14, F_3 : 28) and 39 (F_2 : 3, F_3 : 36) segregants, respectively, with markers matching NRC 147, the higher oleate parent. Further, *FAD2-1A/ FAD2-1B* specific primers were developed, which differentially amplified 401 bp and 378 bp markers simultaneously for those two isoforms. The identified markers may potentially contribute to breeding favorable soybean oil characteristics.

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Authors' contributions

Author wise contribution to the study's conception and design (VPC), study of the work (AHG), material preparation

(MPD), data collection (AHG), analysis (AHG and VPC) and manuscript (VPC, ONR and ARA). All authors have read and approved the final manuscript.

Compliance with ethical standards

Authors declare that they don't have any conflict of interest and that there are no ethical issues.

Conflict of interest

Authors do not have any conflict of financial or non-financial interests to declare/disclose that are directly or indirectly related to the work submitted for publication.

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