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MOLECULAR DIVERSITY PATTERNS OF DIFFERENT SEED COAT COLOURED SOYBEANS (*Glycine max*) DURING SEED LONGEVITY USING SIMPLE SEQUENCE REPEATS MARKERS

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ABSTRACT
Molecular diversity analysis was carried out at the University of Agricultural Sciences, GKVK to elucidate the genetic basis of seed storability and testacolour variation in soybean. Twenty genotypes, five distinct seed coat colour (Black, Yellow, Brown, Variegated and Green) were evaluated using twelve Simple Sequence Repeat (SSR) markers. 3 SSR markers showed highly polymorphism and differentiated genotypes based on storability i.e. Satt281, Satt513, and Satt371 exhibited high polymorphism. The three informative loci collectively amplified fourteen alleles, with two to three alleles per locus and an average of 2.6 alleles, indicating moderate yet biologically meaningful genetic variability. Two markers were bi-allelic, whereas one marker was tri-allelic. Alleles Satt281_{220}, Satt513_{200}, and Satt371_{180} were predominantly observed in good storers, while Satt281_{200}, Satt513_{140}, and Satt371_{120} were characteristic of poor storers. Intermediate genotypes were mainly associated with Satt281_{210}, Satt513_{150}, and Satt371_{160}. The identified SSR loci revealed significant genetic differentiation related to seed longevity and testacolour. These SSR loci demonstrate significant potential as reliable molecular markers for marker-assisted selection, thereby facilitating the development of soybean cultivars with enhanced storage potential, improved seed qualityand greater commercial value.

KEYWORDS: Molecular characterization; Polymorphism; SSR; Seed longevity; Soybean; Storability; Testacolour.

1. INTRODUCTION
Soybean (*Glycine max* (L.) Merrill) is one of the most important economic pulse–cum–oilseed crops globally and ranks among the leading grain legumes in terms of total production and international trade. Popularly referred to as a "miracle crop," soybean seeds contain approximately 40% high-quality protein and 20% oil, of which nearly 85% comprises polyunsaturated fatty acids. In addition, soybean is a rich source of carbohydrates (25–30%), essential minerals, antioxidants, β-carotene, and health-promoting isoflavonoids, making it nutritionally and industrially valuable. Cytogenetically, soybean is a diploid species (2n = 40) belonging to the family Fabaceae and is believed to have originated in China before spreading to major production regions such as the United States, Brazil, Argentina, India, and other Asian countries. Globally, nearly 85% of soybean produce is processed into vegetable oil and soybean meal, reflecting its pivotal role in food, feed, and agro-industrial sectors. Approximately around 85 per cent of the world's soybean processed into vegetable oil and soybean meal. The soybean producers are USA (36 %), Brazil (36 %), Argentina (18 %), China (5.0 %) and India (4.0 %) (Anonymous, 2018).
Seed deterioration is a major constraint in soybean production, as its seeds deteriorate more rapidly than those of most other crops (Finch-Savage and Bassel, 2016) primarily due to high temperature and relative humidity during storage. Soybean is thus classified as a poor storer (Jagadish et al., 2013). Adverse weather conditions during harvest and post-harvest handling further accelerate deterioration and reduce seed quality (Singh et al.,

2008). The rate of quality decline varies among genotypes and is largely influenced by genetic compositionandlipid peroxidation of polyunsaturated fatty acids (Lavanya, 2015). Compared with many other crops, soybean seeds deteriorate rapidly during storage, particularly under conditions of high temperature and relative humidity. Consequently, soybean is categorized as a poor storer. Seed longevity, defined as the capacity of seeds to retain viability during dry storage, is governed by complex physiological and molecular mechanisms. It is a complex biological activity and involves a network of physiological, biochemical, metabolic and molecular processes (McDonald, 1999 and Zaheer et al., 2016). The quality of soybean seed during storage has been reviewed and seed deterioration has been identified as one of the basic reasons for low productivity in soybean (Shelar et al., 2008).The rapid decline in seed viability is primarily attributed to lipid peroxidation of polyunsaturated fatty acids, leading to membrane damage, loss of cellular integrity, and reduced germination potential. Furthermore, adverse environmental conditions during harvest and post-harvest handling exacerbate physiological and biochemical deterioration.

Importantly, the rate of seed quality decline varies significantly among genotypes, indicating a strong genetic basis for storability. However, conventional evaluation of seed longevity through long-term storage trials is time-consuming, labor-intensive, and highly influenced by environmental variability. There remains a need for precise, rapid, and reliable molecular tools to identify genotypes with inherent genetic potential for enhanced storability. Understanding the genetic determinants associated with seed longevity will not only facilitate early selection in breeding programs but also contribute to the development of cultivars suited for diverse storage environments, particularly in tropical and subtropical regions where high temperature and humidity prevail.

In this context, molecular marker technologies, particularly Simple Sequence Repeat (SSR) markers, provide a robust and reliable approach for assessing genetic diversity and establishing marker trait associations. The use of SSR marker technology provides a powerful and reliable approach for assessing genetic diversity and marker–trait association in soybean (Sajib et al., 2021). The present study was therefore undertaken to differentiate and identify good and poor storer genotypes among twenty soybean accessions using seed longevity specific SSR markers. Genotypes exhibiting enhanced storability can serve as valuable parental lines in breeding programs aimed at improving seed longevity, maintaining seed quality during storage, and ensuring sustainable soybean production.

The novelty of the present study lies in seed longevity-associated SSR markers validationin twenty soybean accessions with contrasting storage behaviour and in identifying molecular markers capable of differentiating good and poor storer genotypes. Unlike previous studies that primarily focused on genetic diversity or marker development, the present investigation evaluates the practical applicability of reported longevity-specific SSR markers for the rapid identification of superior storability genotypes.

Therefore, the objectives of the present study were to:(i) evaluate genetic diversity among twenty soybean accessions using seed longevity-associated SSR markers; (ii) identify polymorphic markers capable of distinguishing good and poor storer genotypes; and (iii) identify promising parental lines for soybean breeding programmes aimed at improving seed longevity and maintaining seed quality during storage.

2. MATERIALS AND METHODS

Physical seed traits such as seed coat colour, seed size, and seed weight are important indicators of seed storability and can assist in determining the optimum storage duration for soybean seeds (Mosavi et al.,2011, Tubic et al., 2011). In view of this, prior to molecular characterization, twenty soybean genotypes were evaluated for their physical[Proportion of seed coat to seed (%), Mechanical strength of seed coat (N), Seed density (g/cm3), Seed coat permeability (%)] and physio-biochemical attributes[Total soluble sugars (µg/ml of seed leachate),peroxidase activity**,** amylase content, DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity (%),Total dehydrogenase activity (A520) and Malondialdehyde content (µM/g of fresh weight)]related to seed longevity. Significant genotypic variation was observed for several key parameters, including DPPH (1, 1-diphenyl-2-picrylhydrazyl) radicalscavenging activity (%), peroxidase activity**,** amylase content**,** seed germination percentage**,** and seedling dry weight, indicating considerable diversity in physiological vigor and biochemical stability among the genotypes. These variations reflect differences in oxidative stress tolerance**,** enzyme activity**,** and metabolic efficiency**,** which are known to influence seed viability and ageing behavior. To further substantiate these phenotypic and a biochemical difference, molecular characterization was undertaken using SSR markers to identify genetic polymorphism underlying these traits. This integration of physio-biochemical profiling with molecular analysis provides a more comprehensive understanding of the genetic basis of seed storability and facilitates the identification of genotypes with superior longevity potentialfor soybean improvement programs.

DNA Extraction

The selected 20 genotypes were sown under greenhouse conditions and 21 days old healthy leafs were used for genomic DNA extraction following 8 % cetylTrimethyl Ammonium Bromide (cTAB) extraction method (Doyle and Doyle et al., 1987). The protocol adopted for DNA extraction is outlined briefly as follows:

21 days old fresh and healthy leaves (0.5g) were collected for DNA extraction. The leaves were cut into pieces and ground into fine powder by using liquid nitrogen, then homogenized completely by adding 400µl of extraction buffer.[Extraction buffer consists of Tris-HCl (100 mM, pH 7.5–8.0), NaCl(500 mM),EDTA (10 mM) and β-mercaptoethanol (50 mM)]. From this around 400 µl homogenized solution was taken into an

eppendorf tube. 400 µl of Chloroform was added and the mixture was centrifuged at 14,000 rpm for one minute. The supernatant was transferred into another eppendorf tube. To the supernatant obtained, 800 µl of absolute alcohol was added and centrifuged for another 3 minutes at 14,000 rpm. The supernatant was discarded. The DNA pellet (10ng) was dissolved in 50 µl of TE buffer (10 mMTris-HCl and 1 mM EDTA, pH 8.0)and stored at -20^0 C for further use.A nano drop was used to quantify DNA, and an agarose gel electrophoresis was employed to assess the quality of the samples.The different seed coat coloured soybeans is listed in table 1.

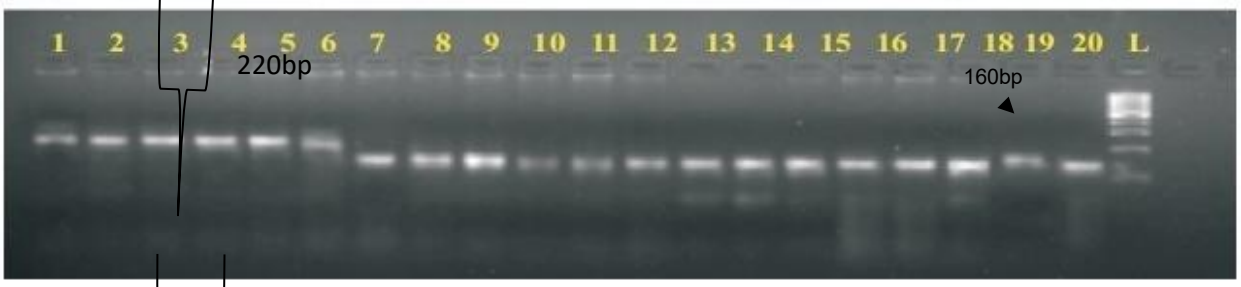

Figure 1a. Amplification pattern of the soybean genotypes obtained using the SSR marker Satt281.

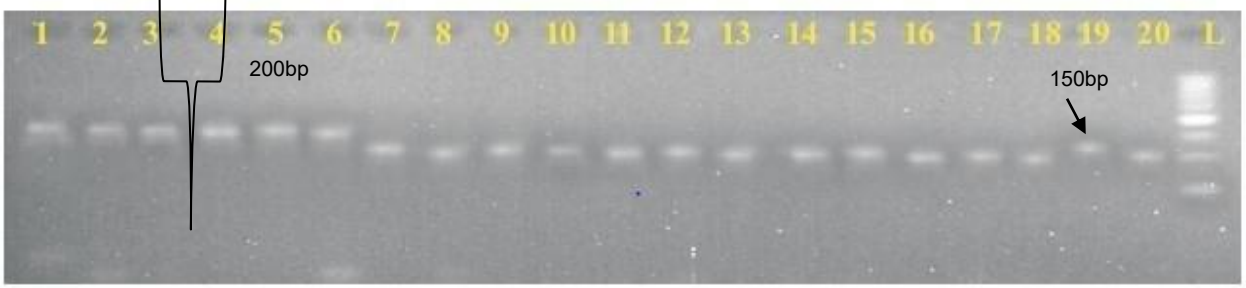

Figure 1b. Amplification pattern of the soybean genotypes obtained using the SSR marker Satt531.

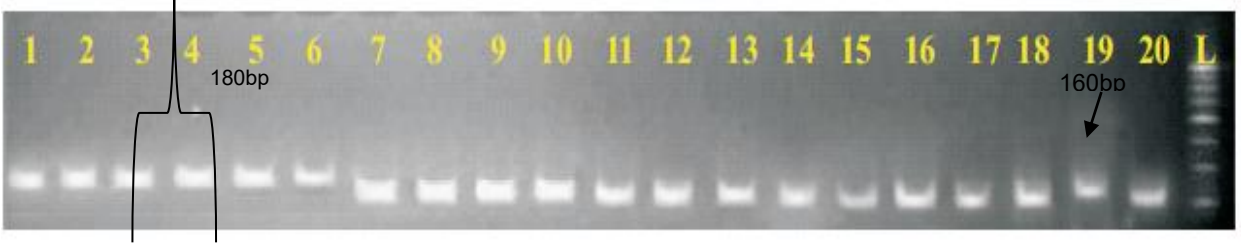

Figure 1c. Amplification pattern of the soybean genotypes obtained using the SSR marker Satt371.

Figure 1 a-c: Amplification pattern of the soybean genotypes obtained using the SSR marker.
Lane 1-6, good storer genotypes, Lane 7-11, 12-15,16-20 poor storers, L-Ladder

Lane No.	Genotypes	Lane No.	Genotypes	Lane No.	Genotypes	Lane No.	Genotypes
1	TR-5	6	DS 72-244	11	NRC-37	16	DSB-21
2	IC-501268	7	RKS-45	12	JS-2069	17	11 5-B
3	EC-76756	8	JS-9041	13	NRC -86	18	AGS 29
4	PB-5	9	JS-335	14	JS-2029	19	RSC 10-71
5	EC-57042	10	JS-9560	15	JS-2034	20	RKS 24

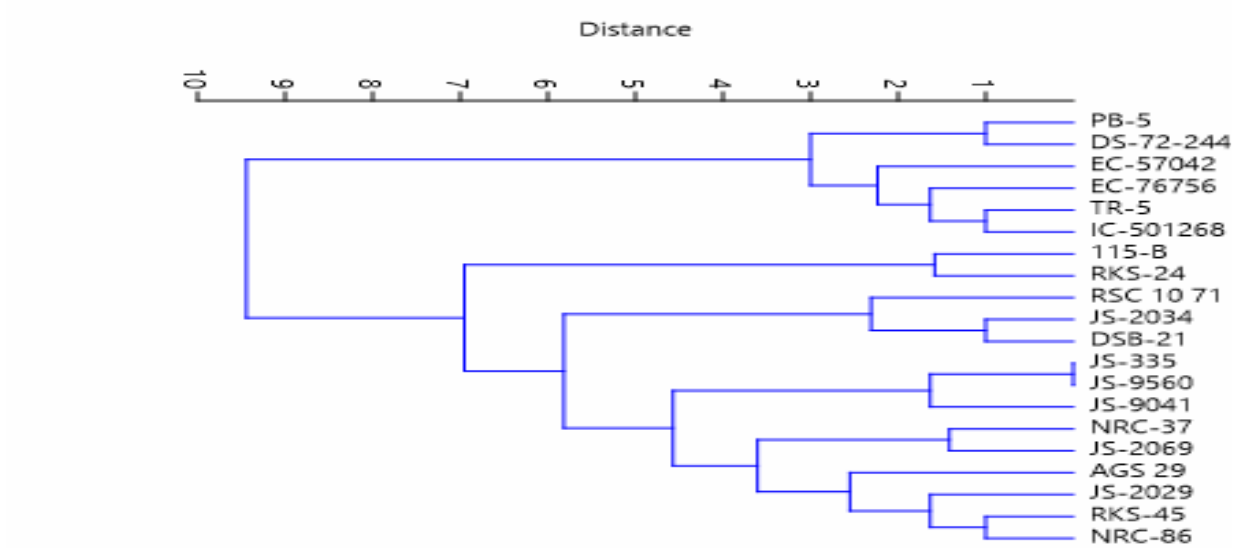

Figure 2: Dendrogram showing relationship among 20 genotypes of soybean based on physio-biochemical changes during seed ageing according to Ward's method (24)

SSR analysis of DNA

List of 12 soybean seed longevity specific SSR primers along with annealing temperature are provided in table 2. Seed longevity specific markers were selected from published literature (Jagadishet al. 2013).The PCR reaction mixture consisted of 3.0 µl of template DNA, 1 µl forward primer, 1 µl reverse primer, 1 µldNTPs, 0.3 µlTaq polymerase, 2.0 µl of 1X PCR buffer, 0.5 µl of $MgCl_2$ and 6.2 µl of sterile water in a volume of 15 µl. PCRamplification was performed using a Bio-Rad T100 Thermal Cycler, initial denaturation at 95°C for 45 sec, followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at the marker-specific temperature for 45 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min. Ethidium Bromide was added at the rate of 0.5µg/ml and the gel was allowed to solidify fully before loading the sample. 5µl of gel loading dye mixed with 15µl of PCR product was loaded into each well of the gel matrix. A 100bp ladder was loaded alongside. Electrophoresis was carried out at 80V, 50mA for 3 hours. The gel was visualized under UV transilluminator and captured in a gel documentation system. Each sample was amplified twice.

The DNA fragments were scored in a binary form with '1' for the presence and '0' for the absence of bands and scored data was used for constructing a dendrogram by NTSYS-Pc software. Clustering was done using Ward module as described by Ward (1963). PIC values were calculated (Botstein, et al., 1980) based on the presence ('1') or absence ('0') of SSR bands, and the data were exported in binary format. SSR markers used for molecular characterization of 20 soybean varieties were listed in table 2.

Table I: Genotypes of different testacolour

Testa colour of genotypes				
Brown	**Variegated**	**Green**	**Black**	**Yellow**
DS-72-244	RSC 10-71	JS - 90 41	EC-57042, TR-5, IC-501268, EC-76756, PB-5.	JS-335, JS - 9560, NRC-86, 115-B, DSB-21, RKS-45, NRC-37, RKS-24, JS - 2034, JS - 2069, AGS 29, JS – 2029

Table II: List of SSR primers used for the study

Sl. No.	**Primer Name**	**5'<-----Sequence----->3'**	**Annealing temperature (^{0}C)**
1	Satt 423	TTCGCTTGGGTTCAGTTACTT	63.0
		GTTGGGGAATTAAAAAAATG	54.4
2	Satt 592	GCGAAGATTGGTCTTTTATGTCAAATG	64.4
		GCGGAGGAATACAAGTCTCTATTCAA	65.3
3	Satt 513	GCGCATCACAAGTTTTATAGATGCTA	64.8
		GAGGTCTAGTGCTTTGTAAGGTT	63.1
4	Satt 600	GCGCAGGAAAAAAAAACGCTTTATT	65.2
		GCGCAATCCACTATGGTGTTAAT	64.1
5	Satt 545	CAATGCCATTCCATATTTGTT	58.4

		CAATTGCCCTAGTTTTGATAG	57.8
6	Satt 434	GCGTTCCGATATACTATAAATCCTAAT	61.7
		GCGGGGTTAGTCTTTTTATTTAACTTAA	63.3
7	Satt162	GGGAAGAAGTATATGCTACATCAA	61.1
		GGGTTAATTTTTATCTTCTAATAGTTT	57.7
8	Satt 281	GCGGCGTTAATTTATGCCGGAAA	67.3
		GCGTTTGGTCTAGAATAGTTCTCA	63.4
9	Satt 143	GTGCCACAAATTTAAAATTACTCA	59.8
		TCCCTCCCTTTTGATTTACAC	60.9
10	Satt 400	TTGGTCATCAAACTGTTA	54.3
		CATAAGGGGTCCCACTCTA	60.6
11	Satt 371	TGCAAACTAACTGGATTCACTCA	63.3
		GAGATCCCGAAATTTTAGTGTAACA	62.2
12	Satt 453	GCGGAAAAAAAAACAATAAACAACA	61.1
		TAGTGGGGAAGGGAAGTTACC	63.9

Table III: Distribution of soybean genotypes based on molecular markers into different clusters

Sl no.	Main cluster	Sub cluster	Number of genotypes	Name of genotypes
1	I	I	2	11 5 –B, RKS-24
		II	12	JS -2069, JS -335, JS -9560, JS -9041, NRC-37, NRC-86, RSC 10-71, AGS 29, DSB-21, RKS-45, JS-2029, JS -2034
2	II	I	2	PB-5, DS-72-244
		II	4	EC-57042, TR-5, IC-501268, EC-76756

3. RESULTS AND DISCUSSION

In the present investigation, twelve SSR primers previously reported to be associated with seed coat pigmentation and seed longevity were employed to characterize twenty soybean genotypes and elucidate the genetic basis of storability.

PCR amplification revealed that three of the twelve SSR loci exhibited significant polymorphism, demonstrating high discriminatory efficiency. Satt281, Satt513, Satt371 produces three alleles.These polymorphic loci Satt281, Satt513, and Satt371 collectively amplified fourteen alleles across the twenty genotypes. The number of alleles per locus ranged from two to three, with a mean of 2.6 alleles per marker, indicating moderate yet functionally relevant genetic diversity for storability-associated traits. Two loci were bi-allelic, while one locus exhibited tri-allelic variation, thereby enhancing genotypic resolution.

The polymorphic SSR markers effectively differentiated genotypes into two distinct storability groups: good storers and poor storers. The locus Satt281 exhibited a highly diagnostic allelic pattern. $Satt281_{220bp}$, $Satt513_{200bp}$ and $Satt371_{180bp}$ for good storer genotypes, $Satt281_{200bp}Satt371_{120bp}$ and $Satt513_{140bp}$ for poor storers and $Satt281_{210bp}$, $Satt513_{150bp}$, $Satt371_{160bp}$ for intermediate storers were exhibited. The allele $Satt281_{220}$ was exclusively detected in genotypes with superior storability, whereas $Satt281_{200}$ was confined to poor-storing genotypes, indicating a strong marker–trait association. Similarly, alleles $Satt513_{200}$ and $Satt371_{180}$ were consistently associated with good storers. In contrast, alleles $Satt513_{140}$ and $Satt371_{120}$ were predominantly observed in poor-storing genotypes, suggesting linkage with reduced seed longevity and increased susceptibility to deterioration (figure1a to 1c).

To validate molecular differentiation, hierarchical cluster analysis was performed using Ward's minimum variance method based on physio-biochemical responses recorded during accelerated ageing. The dendrogram distinctly delineated two principal clusters corresponding to storability performance.Cluster I comprised fourteen genotypes (11-5B, RKS-24, JS-2069, NRC-86, JS-2029, JS-2034, AGS-29, DSB-21, RKS-45, NRC-37, JS-9560, JS-335, and JS-9041), characterized by rapid viability loss within 10 months and classified as poor storers. Cluster II included six genotypes (TR-5, PB-5, EC-57042, DS-72-244, IC-501268, and EC-76756) that maintained germination beyond 10 months, indicating superior storability. The genotype RSC-10-71 occupied an intermediate position, exhibiting moderate resistance to ageing (table 3).

Similar reports were reported by Naik et al., 2019 that SSR marker Satt 281 exhibited distinct banding pattern that could clearly differentiate good and poor seed longevity in soybean genotypes. Markers Satt 162, Satt 523 and Satt 453 which are either linked with seed coat colour or seed permeability exhibited a specific size allelic fragments in soybean genotypes and crosses revealing better seed longevity (Pawar et al., 2018). Our results are in conformity with earlier reports where SSR markers Satt 371, Satt 453 and Satt 618 produced specific allelic bands making them candidate markers for linkage with seed storability and testa colour (PoojaSahu, 2018, Sital et al., 2008). Black-seeded genotypes were better than yellow-seeded genotypes. Traits such as hard seed

coated seeds; small seed size and black seed coat were identified to have better storability in soybean (Sirisha et al., 2024, Kuchlan et al., 2010, Subhash et al., 2017 and Pallavi et al., 2018).

The concordance between SSR-based grouping and physio-biochemical clustering confirms substantial genetic divergence underlying seed longevity traits. Notably, black-seeded genotypes were predominantly represented among good storers, suggesting that seed coat attributes such as reduced permeability, higher phenolic content, enhanced antioxidant activity, and improved oxidative stability contribute to extended storage life (Priyanka et al., 2021; Bhanuprakash et al., 2006). In addition, the superior storability of these genotypes was reflected in their ability to maintain higher germination percentage, seedling vigour, dehydrogenase activity, membrane stability, and lower electrical conductivity following accelerated ageing. Good-storing genotypes also exhibited enhanced activities of antioxidant enzymes, including catalase (CAT) and peroxidase (POD), coupled with lower levels of lipid peroxidation and hydrogen peroxide (H_2O_2) accumulation, indicating a greater capacity to mitigate oxidative stress during storage. These physiological and biochemical characteristics collectively contribute to delayed seed deterioration by preserving membrane integrity, cellular metabolism, and reserve mobilization. The consistent association of specific alleles ($Satt281_{220}$, $Satt513_{200}$, and $Satt371_{180}$) with superior storability indicates the presence of genomic regions potentially governing seed longevity (Priyanka et al., 2020). These loci may exhibit pleiotropic effects or tight linkage with genes regulating seed coat biochemistry, antioxidant defence systems, membrane integrity, phenolic metabolism, lipid peroxidation, reactive oxygen species (ROS) scavenging, and other physiological processes involved in seed ageing. The integration of molecular marker analysis with physiological and biochemical indicators therefore provides stronger evidence for the genetic regulation of seed longevity and enhances the utility of these SSR markers for marker-assisted selection in soybean breeding. Overall, the differential allelic distribution between good and poor storers underscores the strong genetic control of seed longevity in soybean. The identified SSR markers demonstrate significant potential as molecular diagnostic tools for marker-assisted selection (MAS), enabling rapid and reliable identification of genotypes with enhanced storage stability. Seed deterioration during storage is a major limitation in soybean, and the existence of genetic variability for seed longevity provides substantial scope for genetic improvement. The present study confirms that SSR markers associated with seed coat colour and storability effectively discriminate soybean genotypes based on longevity performance. The polymorphic loci Satt281, Satt513, and Satt371 demonstrated strong marker–trait associations, validating their utility in detecting genetic variation governing seed storability. The exclusive presence of the $Satt281_{220}$ allele in good-storing genotypes indicates tight linkage with enhanced seed longevity and black testa pigmentation. Similarly, alleles $Satt513_{200}$ and $Satt371_{180}$ were consistently associated with superior storability, whereas $Satt513_{140}$ and $Satt371_{120}$ were predominant in poor storers, suggesting their association with increased susceptibility to oxidative deterioration. These findings reinforce earlier evidence linking seed coat characteristics, permeability, and antioxidant capacity with storage behaviour.

Hierarchical clustering based on physio-biochemical responses during ageing further corroborated the genetic control of storability, clearly segregating genotypes into good and poor storers, with RSC-10-71 exhibiting intermediate performance. The predominance of black-seeded genotypes among good storers supports the role of seed coat mediated protection against lipid peroxidation. Collectively, the consistent allelic differentiation underscores the strong genetic basis of seed longevity. The identified SSR loci likely correspond to genomic regions or QTLs regulating oxidative stability and seed coat biochemistry. These markers represent valuable tools for marker-assisted selection (MAS) to accelerate the development of soybean cultivars with enhanced storability and quality retention.

The present investigation clearly demonstrates the existence of significant genetic and physio-biochemical variability among soybean genotypes with respect to seed storability and testa colour. The differential response of genotypes to storage and accelerated ageing confirms that seed longevity is under strong genetic control. Among the twelve SSR markers evaluated, Satt281, Satt513, and Satt371 exhibited pronounced polymorphism and effectively discriminated genotypes into good and poor storers. The congruence between SSR-based grouping and hierarchical clustering derived from physio-biochemical parameters further validates the robustness of these markers in distinguishing storability classes. The predominance of black-seeded genotypes among good storers underscores the role of seed coat characteristics in regulating permeability, oxidative stability, and antioxidant defence mechanisms during storage. Collectively, these findings indicate that the identified SSR loci may correspond to genomic regions associated with seed longevity. Therefore, the markers Satt281, Satt513, and Satt371 can be considered promising molecular tools for marker-assisted selection aimed at developing soybean cultivars with enhanced seed longevity, improved quality retention, and greater storage resilience. Further validation through large-scale populations and functional studies would strengthen their deployment in soybean breeding programs.

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5. Authorship and Contribution

Author's contribution: Priyanka, M– Research work and Writing the manuscript draft, Sowmya K J– Organized and prepared the dataset for analysis, Basavaraja B – Correction of Manuscript, Parashivamurthy - Developed the research idea and objectives, Siddaraju R - Designed the research methodology

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Consent to Publish: All authors have read and approved the final manuscript for publication

Authorship Statement

All authors have made substantial contributions to the conception and design of the study, data acquisition, analysis, and interpretation

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