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RESEARCH

Exploring the Genetic Diversity of the Waxy Gene in Selected Foxtail Millet (*Setaria italica* (L.) P. Beauv.) Accessions from Sri Lanka

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ABSTRACT

Purpose: *Foxtail millet is an ancient cereal crop and a model species used for studying C4 photosynthesis and stress resilience. The composition of its seed starch is primarily determined by the waxy gene, which encodes granule-bound starch synthase1 (GBSS1). Polymorphisms in the first intron of GBSS1 are known to influence allelic diversification in cereals; However, Sri Lankan foxtail millet germplasm has not yet been characterized at the molecular level. This study aimed to evaluate endosperm type and apparent amylose content (AAC) of Sri Lankan accessions, analyze the first intron polymorphism of the GBSS1 gene, and assess their phylogenetic relationships with reported haplotypes.*

Research Method: *Six accessions collected from diverse agro-ecological regions were evaluated using I_2–KI staining, AAC and sequencing the first intron of GBSS1 gene.*

Findings and Values: *All accessions displayed a dark blue color in I_2–KI staining and 17.7–31.5%, AAC values conformed non-waxy phenotypes. Sequence comparison with the reference non-waxy genotype (AB089142.1) revealed 152 polymorphic sites (SNPs and indels). Moreover, a novel 4bp deletion was identified across all local accessions, representing the first report of this mutation in foxtail millet. Phylogenetic analysis confirmed that all accessions belong to the Type 1-1 non-waxy haplotype but exhibit considerable intra-type diversity. These findings provide new insights into the regional diversification of GBSS1 alleles in Sri Lankan foxtail millet and highlight the potential of this germplasm as a resource for molecular marker development and breeding programs aiming at improving grain quality.*

Keywords: *Amylose content, Foxtail millet, Granule-bound starch synthase 1 (GBSS1), Haplotype diversity, Intron polymorphism*

INTRODUCTION

Foxtail millet, (*Setaria italica* (L.) P. Beauv.) belongs to the family Poaceae, subfamily Panicoideae, genus Setaria (Swamy 2023). The genus Setaira comprises more than 125 species, yet *S. Italica* and its wild ancestor *Setaria viridis* (Green foxtail millet), are considered as the most studied millets in this genus (Anand & Vanniarajan 2025). Foxtail millet was domesticated 9000-11000 years ago, near the Yellow River valley in Cishan, China, and 5000 years back in Taiwan (Pant *et al.* 2016, Tsang *et al.* 2017). Based on the inflorescence structure, foxtail millet has been divided into three groups, as maxima, indica and moharia. These races have been subdivided into 10 sub-races solely based on phenotypic characteristics including seed color, bristle length, number of tillers, panicle size and days to maturity

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(Swamy 2023). Over the past decade, studies of genus Setaria have gained popularity, as a model system for the C4 photosynthetic pathway, due to its short life cycle, small plant structure, and small diploid genome (2n=2X=18, 500 Mb). Furthermore, this millet has gained wide popularity due to its high resilience nature towards biotic and abiotic stresses (Aggarwal *et al.* 2022). Foxtail Millet is the second most widely cultivated millet type in the world, serving as a staple crop in various parts of the world, and a feed and fodder in many other regions, including Australia, North America and Europe (Hammer & Khoshbakht 2007, Geethanjali & Jegadeeswaran 2016). It is widely cultivated on poor soils in semi-arid regions of Asia and is considered climate-smart crop grown with low inputs, including water and fertilizer (Ramesh *et al.* 2023). However, despite its many favorable agronomic traits, foxtail millet is still classified as a minor or underutilized crop worldwide (Van *et al.* 2008, Sapkota *et al.* 2016).

Two different research groups Bennetzen *et al.* (2012) and Zhang *et al.* (2012) have independently sequenced the complete genome of foxtail millet in 2012. Their findings revealed that the genome is approximately 500Mb, distributed across nine chromosomes, containing 38,801 protein-coding genes, of which 81% are expressed. Evolutionary studies have suggested that several genes, including the waxy gene, the polyphenol oxidase gene, the phenol color reaction genes, and the C gene responsible for leaf sheath pigmentation, played major roles in the adaptation, diversification, and domestication of foxtail millet (Fukunaga & Kawase 2024).

Starch in the foxtail millet endosperm is composed of amylose and amylopectin. The gene responsible for determining amylose content (AC) is the GBSSI (waxy) gene, which encodes the enzyme granule-bound starch synthase I (GBSSI), a key enzyme responsible for amylose synthesis that directly determines the AC and the amylose-to-amylopectin ratio in endosperm starch. There are two isoforms, GBSSI and GBSS II in the GBSS enzyme family, of which GBSSI plays a crucial role in amylose synthesis in storage tissues such as endosperm, while GBSSII functions in non-storage plant tissues (Wang *et al.* 2023). The functional (non-mutated) waxy gene encodes GBSSI enzyme, responsible for producing a non-waxy endosperm phenotype (Fukunaga 2019). Non-mutants are commonly known as wild genotypes because, their wild ancestor (Green foxtail) also carries a non-waxy endosperm. AC negatively correlates with stickiness and digestibility of endosperm starch (Li *et al.* 2017a). The texture of starch produced by waxy genotypes is stickier due to its high amylopectin content, a trait conferred by a recessive allele (wx). In contrast, the non-waxy type is governed by a dominant allele (Wx) (Nakayama *et al.* 1998, Fukunaga *et al.* 2002). It has been proposed that during evolution, the loss of GBSS I function and the diversification of the dominant Wx allele resulted from small indels, insertion of transposable elements, and single-nucleotide polymorphisms. These mutations ultimately reduce GBSSI activity and suggest a polyphyletic origin for the waxy phenotype (Hachiken *et al.* 2013).

The waxy gene of foxtail millet, located on 4th chromosome, is 4211 bp in length and comprises 14 exons. Exons 2-14 encode a protein of 605 amino acids (Kawase *et al.* 2005). Due to the previously mentioned evolutionary mutations, this gene has given rise to 13 distinct allele. Among these, types I and II are non-waxy; types III, II/VI (a recombination between type II and type VI), VI, and IX are considered low-amylose genotypes; and types V, VII, VIII, X, IVa, and IVb are categorized as waxy types. Additionally, based on AC, the genotypes are classified as follows: waxy (0–3.5% AC), low-amylose (7.8%–16.0% AC), and non-waxy (17.0%–31.9% AC) (Kuo *et al.* 2018).

According to this classification, phenotypic studies have shown that East and Southeast Asia predominantly cultivate waxy-type landraces, while South Asia, including India, primarily cultivates non-waxy genotypes. Domestication and diversification of these waxy and non-waxy types seem to be mainly based on the culinary preferences of people in specific regions; therefore, this can be considered an ethnobotanical trait (Peng & Zhang 2021,

Makwana *et al.* 2023, Jayalakshmi *et al.* 2024).

The first intron of GBSS1 is a known hotspot, and plays a crucial role in distinguishing between different haplotypes (Biselli *et al.* 2014). In rice, polymorphisms in the first intron of the Wx gene are closely associated with endosperm amylose content and influence splicing efficiency. Single Nucleotide Polymorphisms (SNPs) at the splice donor site are responsible for distinguishing waxy from non-waxy phenotypes (Crofts *et al.* 1999). Similarly, maize exhibits high sequence conservation in waxy alleles, suggesting strong selection during domestication, with much greater variation retained in non-waxy flint types (Zheng *et al.* 2013). In sorghum, allele-specific PCR assays have used intron/exon markers to quantify waxy vs. non-waxy content in mixed-grain samples (Cho *et al.* 2015, Maung *et al.* 2021). Moreover, targeted deletion studies have demonstrated the first intron regulates amylose biosynthesis in rice (Liu *et al.* 2022).

Although the first intron of GBSSI is a known hotspot for polymorphisms, variations in this region have not yet been characterized at the molecular level in Sri Lankan foxtail millet germplasm. Therefore, this study aimed to characterize and compare the first intron sequence variation of the GBSS1 gene in selected Sri Lankan foxtail millet accessions.

In the present study, six accessions of foxtail millet, collected from different parts of the country with diverse morphological traits, were selected to check the domestication pattern and diversity of the waxy gene of Sri Lankan foxtail millet germplasm.

MATERIALS AND METHODS

Location

Laboratory analysis was carried out in the biotechnology laboratory and biology laboratories of the Faculty of Agricultural Sciences, and the Faculty of Technology respectively, at Sabaragamuwa University of Sri Lanka. Plants were established at the Teaching farm of the Faculty of Agricultural Sciences, (6.7056 N, 80.7886 E) at an elevation of 584 m above the mean sea level (msl).

Plant materials

Six foxtail millet accessions were selected to represent the diversity of Sri Lankan accessions based on different geographical origins such as, Tanamalvila (FM8.1), Jaffna (FMJ12), Kilinochchi (KCFM3), and the Plant Genetic Resource Centre (FM15537, FM 15156, FM7778) and inflorescence morphology and seed color (Fig. 1). All accessions were cultivated according to the Department of Agriculture's recommendations and multiplied for three crop seasons under strict self-pollination. Seeds of each accession were sown in plastic pots and grown under greenhouse conditions.

Endosperm type evaluation

The endosperm type (waxy or non-waxy) was determined by the Iodine (I_2–KI) staining assay. Five seeds obtained from self-pollinated plants were split open and soaked in a 3% KI and 1% I_2 solution for 5 minutes. Endosperm types were classified based on color: non-waxy (dark blue), low-amylose (bluish- to reddish-purple), or waxy (reddish-brown) (Nakayama *et al.* 1998).

Analysis of apparent amylose content (AAC)

The Iodine (I_2–KI) staining assay was conducted to determine the AAC of each sample. A standard curve was prepared using the amylose standard solution, which consisted of 40 mg of Potato amylase (Sigma-USA), mixed with 450 μL of 1M NaOH and 50 μL of 95% ethanol, to a final volume of 500 μL. The amylose mixture was gently heated at 50 °C, until the amylose was completely dissolved. This solution (80 mg mL^{-1}) was considered the 100% amylose stock solution, and it was then used to prepare amylose working standards at 4 mg mL^{-1}, 8 mg mL^{-1}, 16 mg mL^{-1} and 24 mg mL^{-1} (5%, 10%, 20% and 30% of the stock concentration, respectively). Each dilution, after mixing with an appropriate amount of 1M NaOH and 95% ethanol solution, had 1.5 mL final volume. Then, 25 μL of each amylose

Figure 1. Inflorescences and seeds of different Foxtail millet accessions used in this study.

sample was mixed with 1.475 mL of freshly prepared staining solution (5 mL H_2O, 0.5 mL 1M acetate, and 0.5 mL iodine solution of 2% KI and 0.2% I_2) and kept for 5 minutes at room temperature to facilitate color development. The absorption was recorded at 620 nm using the UV spectrophotometer (Thermo Scientific GENESYS 10), and a standard curve (y = 0.144x + 0.0209) was plotted as absorbance (620 nm) vs amylose concentration (mg mL^{-1}). Starch samples from each accession were prepared following the same procedure as above. Three replicates of each sample were used to measure the absorbance, and the mean absorbance was used to calculate the AAC. The AAC (Table 1) was then estimated based on the standard curve values (Kuo *et al.* 2018).

Genomic DNA extraction and sequencing of the first intron of Waxy gene

Genomic DNA was isolated from the leaves of 14-day-old seedlings using a modified CTAB extraction protocol (Tharindi *et al.* 2023). Each DNA sample was extracted from two grams of leaf tissue collected from 3 to 4 seedlings of the same accession. The integrity of the extracted DNA was assessed through 0.8% agarose gel electrophoresis, and its concentration was measured at 260 nm using a spectrophotometer (Thermo Scientific GENESYS 10). For bidirectional sequencing of the first intron region (800 bp) of GBSSI gene with ex1 forward primer (5'-TGCAAGCCAGTGACGGATCGACGACGACAC-3') and ex2 reverse primer (5'-TAGCTAGCCCTCC ACGCTGGTCACCGGCAT-3') (Fukunaga *et al.* 2002) (Fig. 2), all the DNA samples were sent to GeneLabs Medical Pvt. Ltd., Colombo, Sri Lanka. The consensus sequences were generated using BioEdit version 7 and deposited in NCBI GenBank under the accession numbers PX532075, PX532076, PX532077, PX532078, PX532079, and PX532080.

Analysis of the sequence data of the first intron of the Waxy gene

Multiple sequence alignment was conducted using BioEdit version 7 and MEGA version 12. The reference DNA sequence for the first intron of the GBSSI gene was retrieved from the NCBI GenBank database for a non-waxy genotype (NC_028453 Region: 1479579-1483789) (Table 1). The SNPs, indels, transitions, and transversions were identified relative to these reference sequences (Table S1). In addition to the six Sri Lankan foxtail millet accessions, these reference sequences were subsequently used to identify genetic relationships among GBSSI intron 1 haplotypes. To examine any possible recombination within aligned region of intron 1 was tested using the Pairwise Homoplasy Index (PHI) test implemented in SplitsTree v4.18.3 (Bruen *et al.* 2006). Then subsequent haplotype analyses were conducted based on the P value obtained from PHI test. DNA polymorphism was estimated in DnaSP v6.12.03 (Rozas *et al.* 2017). The number of segregating sites (S), total mutations (Eta), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), and Watterson's theta (θ_W) were estimated as polymorphism indices in DnaSP v6.12.03. Relationships among haplotypes were visualized through TCS (Templeton–Crandall–Sing) network in PopART v1.7 (Leigh & Bryant 2015). Haplotype clustering based on intron 1 polymorphism was performed using the Neighbor-Joining method (Tamura *et al.* 2004, 2021). The phylogenetic tree based on first intron DNA

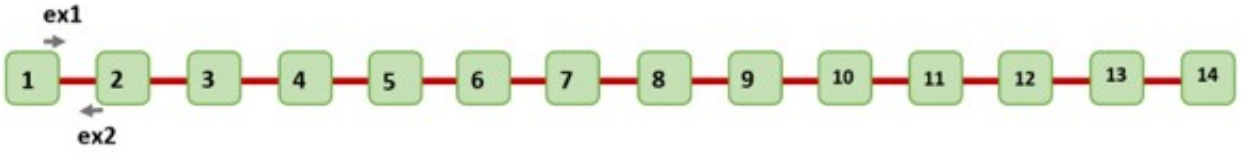

Figure 2. Molecular structure of foxtail millet GBSS1gene. The numbers 1-14 denote the fourteen exons of the gene. Arrows labeled ex1 (forward primer) and ex2 (reverse primer) denote the positions of the primer binding sites used to amplify the first intron.

Table 1. Reference genotypes retrieved from the NCBI GenBank database for comparative analysis.

Accession No.	Geographical origin	Endosperm type	PCR types of ex1/ex2	Reference / Source
AB679633.1	Japan	Non-waxy	Type 1-1	Hachiken *et al.* (2013)
AB682914.1	Japan	Non-waxy	Type 1-2	Hachiken *et al.* (2013)
AB682917.1	China	Non-waxy	Type 4	Hachiken *et al.* (2013)
AB682919.1	Taiwan	Non-waxy	Type 6	Hachiken *et al.* (2013)
AB682920.1	Japan	Non-waxy	Type 7	Hachiken *et al.* (2013)
AB682921.1	China	Non-waxy	Type 8-1	Hachiken *et al.* (2013)
AB682922.1	Thailand	Non-waxy	Type 8-2	Hachiken *et al.* (2013)
AB682923.1	Nepal	Non-waxy	Type 9	Hachiken *et al.* (2013)
AB682924.1	India	Non-waxy	Type 10	Hachiken *et al.* (2013)
AB682925.1	Philippines	Non-waxy	Type 11	Hachiken *et al.* (2013)
AB682926.1	Indonesia	Non-waxy	Type 12	Hachiken *et al.* (2013)

sequences was constructed using the neighbor-joining method with bootstrap value of 1000, and visualized with modifications using tvBOT (Xie *et al.* 2023).

RESULTS AND DISCUSSION

Endosperm type and amylose content

The six accessions used in this study were selected based on their geographical origin and variation in inflorescence morphology (Fig. 1), ensuring representation of the diversity present in local landraces. The I_2–KI staining resulted in a dark blue for all six foxtail millet accessions, indicating the existence of non-waxy, high-amylose phenotypes (Fig. 3). This color change corresponds to the formation of a polyiodide-amylose inclusion complex of non-waxy starch granules. Amylose is a linear polymer of α-1,4-linked glucose units, and when mixed with I_2–KI staining reagent, it produces a polyiodide complex that absorbs light in the 620 nm range resulting in a deep blue color. In contrast, amylopectin contains α-1,6 branch points and binds to iodine only at short branches, resulting in a reddish-brown color (Yang *et al.* 2019). This non-waxy phenotype was further confirmed by Apparent Amylose Content (AAC) values, ranging from 17.7% to 31.5% (Fig. 4). This range aligns well with the typical AAC range reported for non-waxy genotypes with a firm, less glutinous grain texture ranging from 17.0% to 31.9% (Kuo *et al.* 2018). The lowest amylose content was recorded for FM15156 (17.7%), while FMT 8.1 showed the highest amylose content (31.5%) among the selected accessions. This observation provides valuable insights into moderate waxy gene variation and adaptive differentiation across the different accessions selected in this study. The results of the present study are consistent with the reports by Hachiken *et al.* (2013) and Fukunaga *et al.* (2002), who reported that the non-waxy type is more common among many foxtail millet accessions collected from South Asia. Nakayama *et al.* (1998) reported that all seven Sri Lankan foxtail millet accessions in their study were non-waxy, with amylose content ranging from 20–30%. In contrast, previous studies have proven that waxy and low-amylose genotypes are more abundant among the genotypes collected from East and Southeast Asian countries (Hachiken *et al.* 2013). This distribution of waxy and non-waxy genotypes reflects the regional culinary preferences and domestication patterns

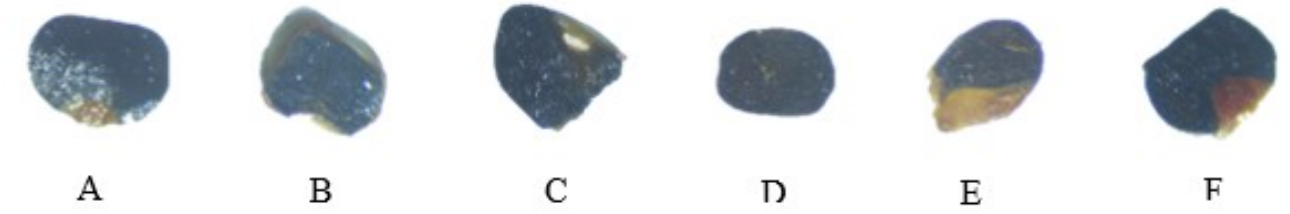

Figure 3. I_2–KI staining of grains from different foxtail millet accessions of Sri Lanka.

of this ancient cereal. Early selection based on food texture and cooking behavior has led to the diversification of the GBSS1 locus in foxtail millet (Fukunaga & Kawase 2024).

The moderately variable amylose levels and non-waxy phenotype observed within the Sri Lankan accessions suggest allelic diversity, which may not be immediately visible in grain characteristics. This variation is important to incorporate into foxtail millet breeding programs that aimed at improving cooking quality, gelatinization temperature, and digestibility (Yang *et al.* 2022).

Sequence Variation in Intron 1 of the GBSSI

The primer pair (ex1/ex2) successfully amplified the intron 1 region, approximately 800 bp of the GBSSI gene, in all six accessions. Several primer sets including ex1/ex2, M2/R4, M5/R7 and ex2int2/ex4r, have been designed to amplify the GBSSI gene in foxtail millet (Kawase *et al.* 2005, Van *et al.* 2008, Hachiken *et al.* 2013). Among these primer combinations, the ex1/ex2 primer pair was selected after confirming the non-waxy phenotype of the six accessions, since the intron 1 region amplified by these primers is a well-known polymorphic marker for distinguishing between waxy and non-waxy alleles (Van *et al.* 2008, Hachiken *et al.* 2013). In non-waxy foxtail millet genotypes, the intron 1 region of the GBSS1 gene amplified with the ex1/ex2 primer pair exhibits high polymorphism (Van *et al.* 2008). This region also consists of specific features, such as SINE- or MITE-like insertions and a 15 bp unique deletion, which are useful in differentiating waxy from non-waxy alleles, as well as distinguishing between Type 1-14 waxy and non-waxy haplotypes as described by Hachiken *et al.* (2013). Therefore, targeting this region enabled the effective assessment of sequence variation and identification of novel polymorphisms specific to Sri Lankan germplasm.

The DNA sequences of the first intron of GBSSI gene of Sri Lankan accessions were aligned with those of eleven reference non-waxy genotypes, AB682914.1, AB682917.1, AB682919.1, AB682920.1, AB682921.1, AB682922.1, AB682923.1, AB682924.1, AB682925.1, AB682926.1 and AB679633.1, retrieved from the NCBI database (Table 1) (Fukunaga *et al.* 2002).

The DNA sequences of the Sri Lankan accessions showed greater similarity to Type 1 reported by Hachiken *et al.* (2013) (Table S1). Based on sequence variation in the ex1/ex2 region, GBSSI gene subtypes were classified. Hachiken *et al.* (2013) has identified 16 sequence types based on nucleotide substitutions and indels. These sequence variants in the ex1/ex2 region served as molecular markers for classifying Waxy allelic diversity. The Sri Lankan accessions exhibited similar nucleotide variation patterns within the ex1/ex2 region, closely resembling the type 1 group particularly the 1-1, 1-2, 8-1, and 8-2 PCR subtypes.

The six Sri Lankan accessions possessed an identical sequence motif, GTTAATTTTGTTTCATGGA, at positions 387–405 (Fig. 5), which corresponds to 19 bp indel from 393 position to 411 position as reported by Hachiken *et al.* (2013). This finding confirmed that all genotypes shared the conserved sequence motif characteristic of type 1, considering the variations in the intron 1 region.

However, compared to reference sequence, 167 polymorphic sites (SNPs and indels) were detected, indicating considerable intra-type diversity (Table S2). A 4 bp micro-deletion was identified in all Sri Lankan accessions at positions 377–380 (Fig. 5), corresponding to the region where a similar deletion was reported at positions 383–386 by Hachiken *et al.* (2013) (Table S1). This 4 bp deletion was uniformly shared among all Sri Lankan accessions but was not reported in any previous publication related to the GBSSI allele in foxtail millet, making this the first report of this 4 bp microdeletion in *Setaria italica* (Van *et al.* 2008, Hachiken *et al.* 2013). This mutation

Table 2. Endosperm phenotype, AAC, shared deletion, transition, transversion, and indel counts in the intron 1 region of the GBSSI (Waxy) gene of six accessions of Sri Lankan foxtail millet.

Accession number	Endosperm phenotype (I_2–KI Reaction)	AAC	Unique Variants	Shared Deletion (377–380) (AT—— TAG)	Transition Count	Transversion Count	Indel Count
FMT8.1	Non-waxy	31.49%	Yes	Yes	6	12	11
FM7778	Non-waxy	24.46%	Yes	Yes	9	9	19
FMJ12	Non-waxy	22.29%	Yes	Yes	7	10	11
KCFM3	Non-waxy	23.87%	Yes	Yes	3	6	9
FM15156	Non-waxy	17.73%	Yes	Yes	1	0	10
FM15537	Non-waxy	18.51%	Yes	Yes	13	21	12

Note: *AAC – Apparent Amylose Content.

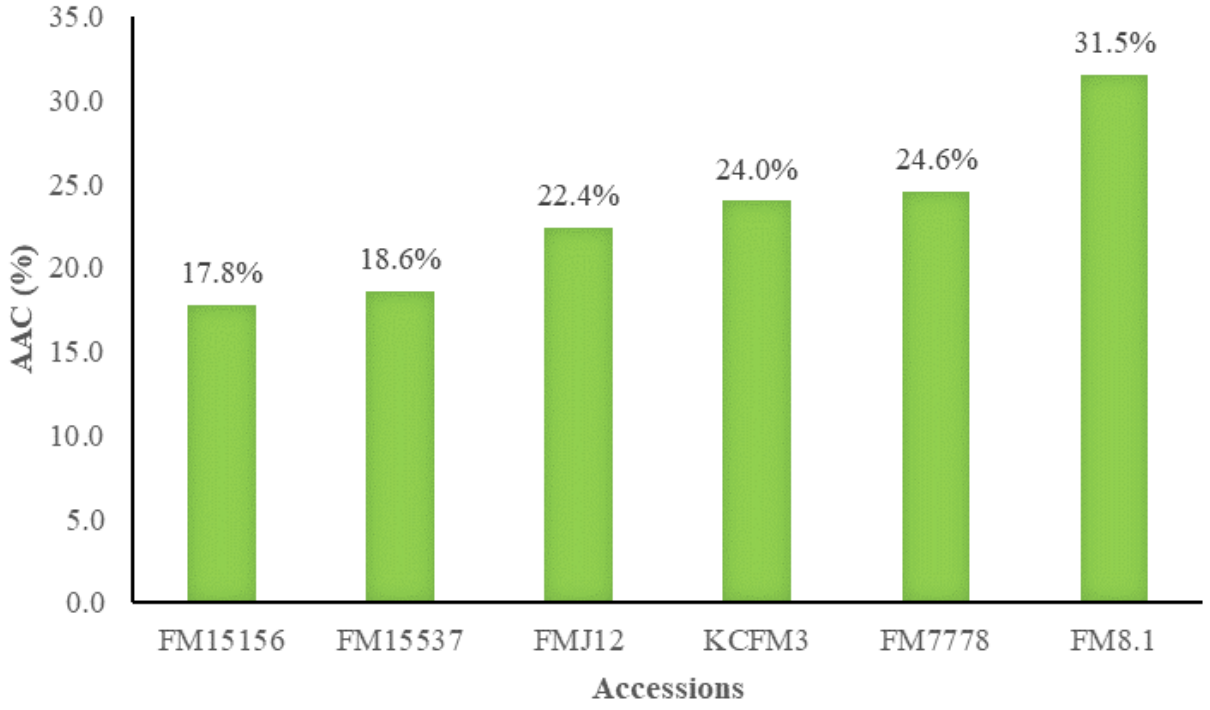

Figure 4. Apparent Amylose Content (AAC) of different Sri Lankan accessions.

might represent a region-specific polymorphism in intron 1, driven by local domestication and selection.

Although located in non-coding regions, such mutations can influence transcriptional regulation through splicing events (Gupta *et al.* 2011). Its uniform distribution among Sri Lankan germplasm indicates that it has been stably inherited within the foxtail millet germplasm. The absence of the well-known 15-bp deletion associated with waxy genotypes further confirmed that all six foxtail millet accessions carried non-waxy GBSS1 alleles. The single-nucleotide polymorphisms (SNPs) identified in this study are likely to be silent mutations that do not express any morphological changes. The observed molecular variation did not correspond to any observable amylose content variation or change in the endosperm type. The lack of correlation between the AAC values and number of SNPs in this study (Table 2) supports this argument, because generally sequences in intron regions evolve faster and are subject to frequent mutations, but these changes rarely influence the protein translation process, and hence, phenotypic changes (Yirgu *et al.* 2023).

The polymorphism observed within intron 1 is summarized in Table 2. The transition and transversion counts ranged from 0–7 and 4–11 respectively per accession. Deletion events were more frequent (0–23) than insertions (0–4) per accession. Accession FM7778 showed the highest number of (23) deletions and one insertion. In contrast, FM15156 exhibited no transitions, but 12 deletions and 2 insertions. Among the accessions, highest number of sequence variation (SNPs + indels) was observed in FM7778. The lowest overall sequence polymorphism was observed in FM15156 and KCFM3 among the other accessions.

These results support the idea that intron regions accumulate a high number of SNPs (73 sites), which suggests the accumulation of silent mutations due to reduced selective pressure, leading to intra-type diversity without phenotypic change (Gelfman *et al.* 2012, Jo & Choi 2015, Li *et al.* 2017b). These findings highlighted that Sri Lankan foxtail millet accessions are closely related to type-1 non-waxy landraces (Hachiken *et al.* 2013). Similarly, some of the SNPs recorded in this study corresponded to those reported by Van *et al.* (2008) for non-waxy Indian accessions. Furthermore, none of these genotypes carried the 15 bp deletions present in the intron 1 region, typically associated with waxy alleles, further confirming their functional GBSS1 gene and non-waxy phenotype, which is consistent with the recorded AAC values and I_2–KI staining results.

The integration of sequence variation with amylose content data strengthens the classification of these genotypes as functional non-waxy types. The most polymorphisms occurred in the non-coding intron 1 region, typically neutral with respect to gene function. Many studies have been conducted to identify SNPs that led to the alteration of existing phenotypes in crops. In particular, changes in non-waxy to waxy genotypes in other cereals, such as a single base pair that change an amino acid due to a point mutation in wheat, give rise to the waxy genotype (Isshiki *et al.* 1998, Yanagisawa *et al.* 2001, Larkin & Park 2003).

This non-waxy phenotype confirmation among Sri Lankan accessions might be directly related to the domestication of the preferred plant type, which complies with the culinary preference of the people living in the country. Amylose variation in foxtail millet is influenced by point mutations, indels, and retrotransposon insertions in the GBSSI (waxy) gene, particularly in intron 1 and exon-intron boundaries (Hariprasanna *et al.* 2017, Prasad 2017). The heterogeneity at the waxy locus explains the diversity among the non-waxy genotypes. These variations are important in improving the grain properties targeting specific food applications during breeding programs (Yang *et al.* 2024). The six accessions analyzed showed SNPs that were not similar to each other, indicating moderate to high diversity in the waxy gene, particularly in the intron 1 region of the Sri Lankan foxtail millet germplasm. The uniform 4 bp deletion and 167 polymorphic sites found in the Sri Lankan accessions suggest that the foxtail millet germplasm has undergone a micro-evolutionary process while maintaining a non-waxy genotype. This process has been observed across Asia, where extensive regional diversification has been observed within haplotypes (Hachiken *et al.* 2013). Variations in environmental conditions across different agro-ecological zones might have led to the development of these silent mutations (Li *et al.* 2021). The distribution pattern and abundance of non-waxy genotypes of foxtail millet in Sri Lanka are similar to the foxtail millet domestication pattern in India (Nakayama *et al.* 1998). Ethnobotanical selections based on cultural and dietary preferences in Sri Lanka have conserved the non-waxy (wild type) allele. However, the main features of starch quality are controlled by exons; hence, frequent mutations within

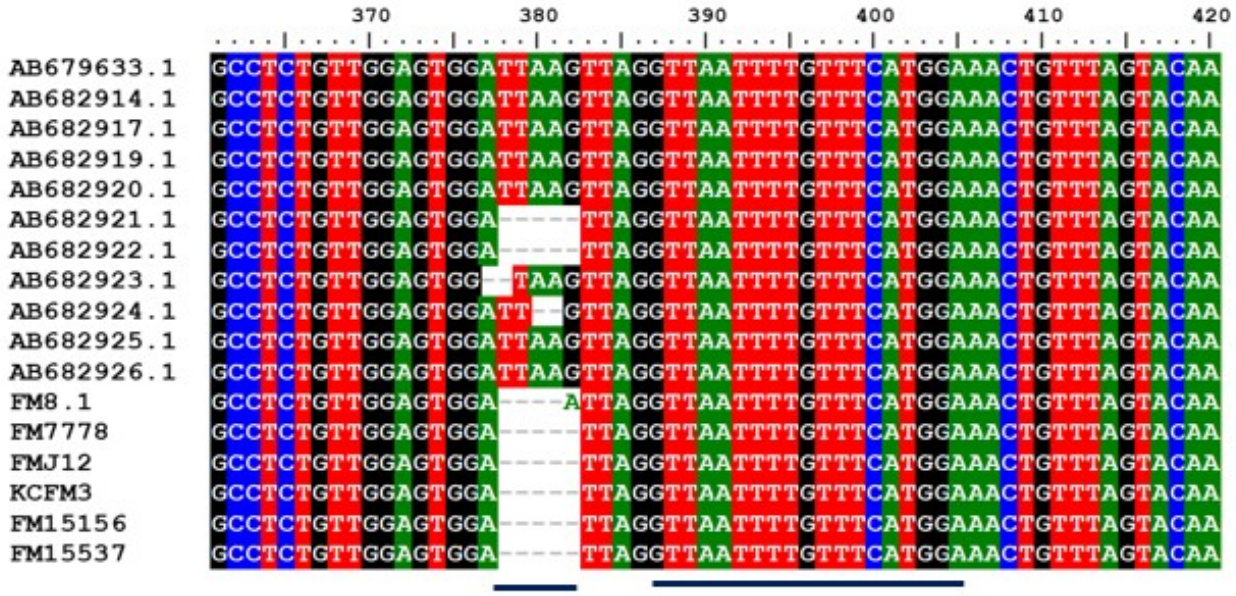

Figure 5. Multiple sequence alignment showing a conserved motif between positions 387–405 bp and a 4 bp microdeletion in the region corresponding to positions 377–380 bp (Underlined) among the Waxy gene first intron sequences of different non-waxy foxtail millet genotypes.

the intron region might not directly be subjected to the selection pressure.

Haplotype clustering based on the intron-1 sequence of GBSSI gene

Polymorphism of intron 1 sequence (654 bp excluding sites with gaps) across both Sri Lankan and reference accessions revealed 57 segregating sites and 62 mutational sites (Table 3). The nucleotide diversity (π = 0.015) and haplotype diversity (Hd = 0.949 $\pm$ 0.044) suggest a relatively high number of different haplotypes (h = 13) (Table 3). The Watterson's θ value (0.02804) denotes the expected level of genetic variation estimated from the number of segregating sites under a neutral model of evolution (Caballero & García-Dorado 2013). This suggest that the variation occurred through random mutation, not because of selection or genetic drift. The mean number of pairwise nucleotide difference (k = 10.188) reflects substantial sequence divergence across the accessions, indicating that these non-waxy lines may have originated from multiple ancestral lineages rather than a single ancestor.

Similar levels of variation in intron 1 have been recorded in previous studies in Asian foxtail millet landraces (Fukunaga *et al.* 2002, Kuo *et al.* 2018). This intron region tends to accumulate mutations, influenced by human selection for starch quality. The high diversity in the intron suggests that the GBSSI gene has evolved mainly through random mutations without affecting its function, as recorded in rice and barley (Domon *et al.* 2002, Yamanaka *et al.* 2004).

Network and haplotype relationships

Haplotype analysis was conducted after performing the PHI test to verify whether the aligned sequences with reference sequences exhibited significant recombination signals (Latinne *et al.* 2012). The PHI test did not find statistically significant evidence for recombination (p = 0.7168), and P > 0.05 confirmed that recombination events were absent and that the observed polymorphism occurred due to point mutations. This evidence suggests that this intron sequence data follow a tree-like evolutionary pattern; hence, they were subjected to clustering and haplotype network analysis to investigate genetic relationships among the accessions (Kosakovsky-Pond *et al.* 2006, Klopfenstein *et al.* 2017).

The constructed NeighborNet network (SplitsTree) revealed a reticulate topology with reference to the Sri Lankan and global reference accessions (Fig. 6). All Sri Lankan accessions were clearly separated from the main cluster of reference sequences, forming long independent branches. The KCFM3 and FM15156 were positioned closer to the central network, while FM8.1, FM7778, FMJ12, and FM15537 occupied peripheral positions, suggesting considerable sequence divergence. The central reticulation pattern suggests that recombination or recurrent mutations occurred within the intron, consistent with the neutral evolutionary pattern

Table 3. Summary of DNA polymorphism and neutrality test statistics for GBSSI intron 1 sequences in *Setaria italica*.

Parameter	Sri Lankan accessions (n = 6)	Sri Lankan + Reference accessions (n = 17)
Analyzed sites (no gaps)	656	654
Segregating sites (S)	46	57
Total mutations (η)	51	62
Singleton mutations (η_s)	39	44
Average pairwise differences (k)	19.2	10.118
Haplotypes (h)	6	13
Haplotype diversity (Hd $\pm$ SD)	1.000 $\pm$ 0.096	0.949 $\pm$ 0.044
Nucleotide diversity (π)	0.02927	0.01547
Watterson's θ (per site)	0.03405	0.02804
Tajima's D (p)	–0.89983 (n.s., P > 0.10)	–1.88474 (*, P < 0.05)
Fu's Fs	–0.045 (n.s.)	–2.013 (n.s.)
Strobeck's S (p)	1.000 (P = 0.489)	0.960 (P = 0.078)
Fu & Li's D*	–0.79224 (n.s., P > 0.10)	–2.14592 (n.s., P > 0.05)
Fu & Li's F*	–0.89383 (n.s., P > 0.10)	–2.39920 (n.s., P > 0.05)
Achaz Y*	–0.09148	–1.42826

Table 4. Haplotype distribution of the GBSSI intron 1 sequences in Sri Lankan and reference *Setaria italica* accessions.

Haplotype ID	No. of sequences	Accession ID
Haplotype 1	4	AB679633.1, AB682914.1, AB682923.1, AB682924.1
Haplotype 2	1	AB682917.1
Haplotype 3	1	AB682919.1
Haplotype 4	1	AB682920.1
Haplotype 5	2	AB682921.1, AB682922.1
Haplotype 6	1	AB682925.1
Haplotype 7	1	AB682926.1
Haplotype 8	1	FM8.1
Haplotype 9	1	FM7778
Haplotype 10	1	FMJ12
Haplotype 11	1	KCFM3
Haplotype 12	1	FM15156
Haplotype 13	1	FM15537

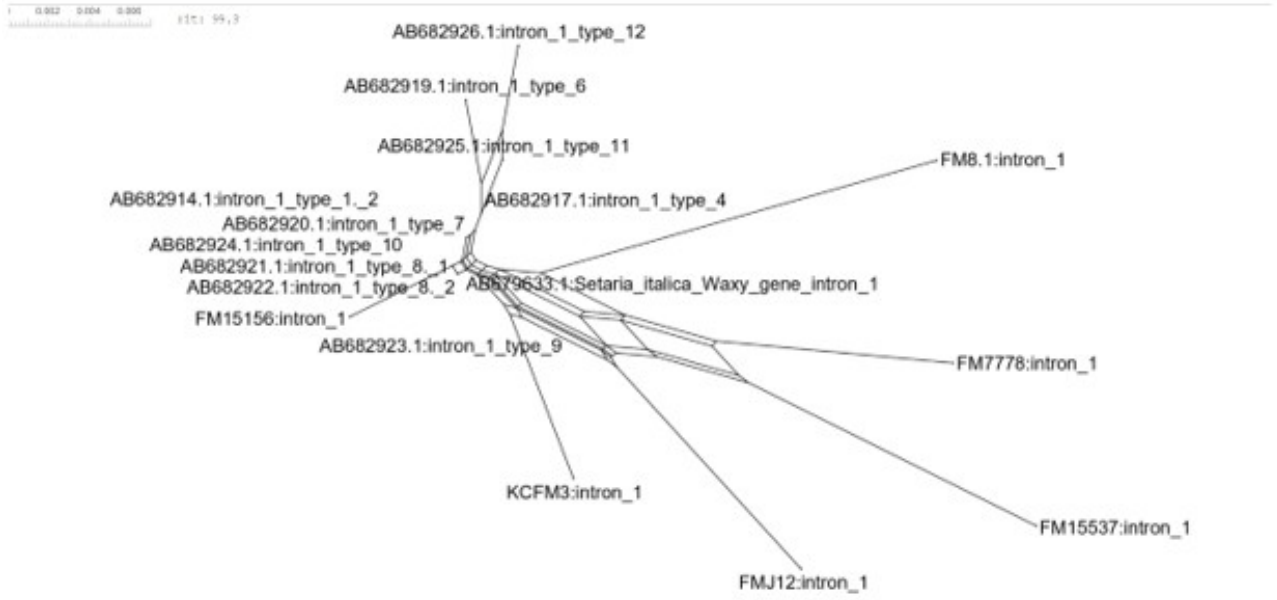

Figure 6. Neighbor-net network for intron 1 sequences of the GBSSI (waxy) gene. Sri Lankan accessions (prefixed with "FM") together with global reference accessions.

Figure 7. TCS haplotype network of Sri Lankan and global S. italica accessions based on intron 1 of the GBSSI gene. Each node represents a unique haplotype, and node size is proportional to the number of sequences sharing that haplotype. Hash marks on connecting branches correspond to single mutational step

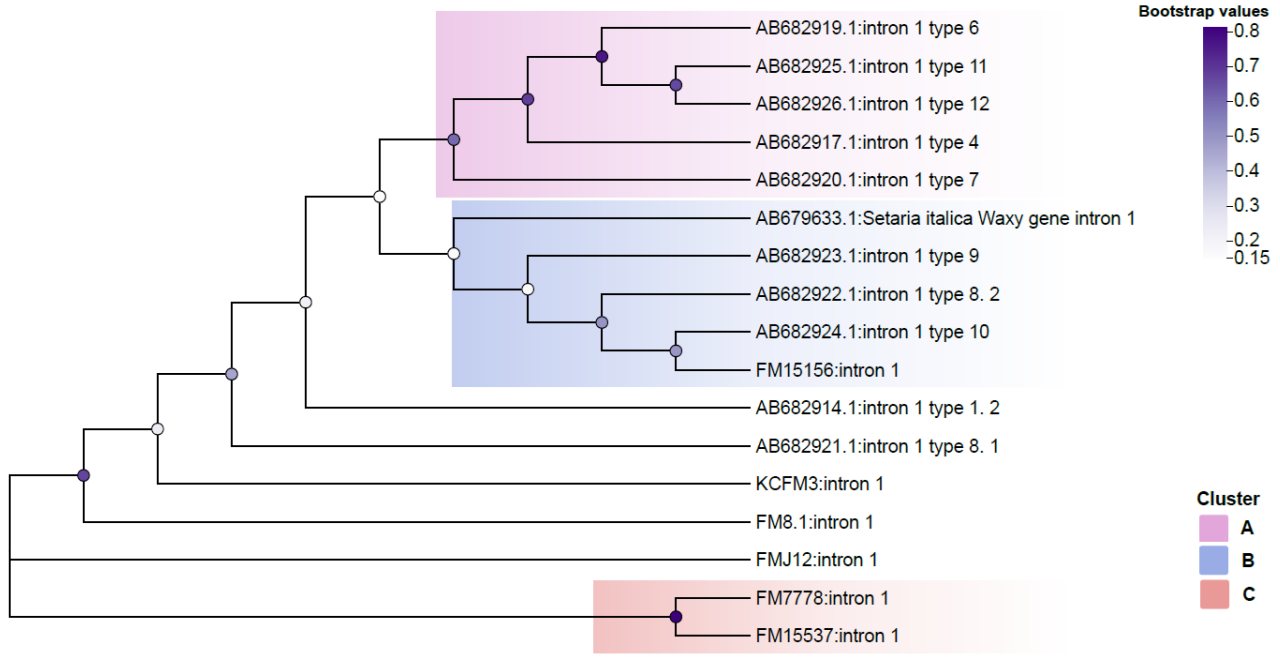

Figure 8. Phylogenetic tree generated based on GBSSI first intron sequences. The tree was constructed using the neighbor-joining method with MEGA 12 and visualized with modifications using tvBOT (Xie *et al*. 2023).

detected in previous high-level allelic diversity (Repar & Warnecke 2017).

Haplotype analysis of the reference sequences and Sri Lankan accession sequences of the GBSSI intron 1 region of *S. italica* identified 13 distinct haplotypes (Hd = 0.9485; Table 3). The high haplotype diversity along with moderate nucleotide diversity (π = 0.01547), indicates considerable allelic variation within the intron region. The central and most frequent haplotype 1 was shared by four reference sequences (Table 4), where these accessions represented the ancestral non-waxy lineage. In contrast, six Sri Lankan accessions have formed individual haplotypes, proving the existing intron variation among the accessions. The TCS haplotype network (Fig. 7) connects haplotypes based on the number of mutational differences between them and shows how haplotypes are related through the fewest possible mutational steps under the assumption of minimal recombination and low divergence (Leigh & Bryant 2015). The analysis identified 13 distinct haplotypes (Hd = 0.9485), showing a high level of haplotype diversity across the accessions. The most frequent central haplotype represented by the reference sequence AB679633.1. This node was connected to the remaining haplotypes by mutational steps forming a star-like topology, which is a typical pattern of populations that have undergone recent diversification from a common ancestor (Chaisiri *et al*. 2021). The network topology showed that each Sri Lankan haplotype occupied the peripheral positions linked to the ancestral node through mutational connections. This suggests independent accumulation of silent mutations with geographic isolation (Marie-Orleach *et al*. 2022). The absence of reticulations among most branches suggests minimal recombination within the intron 1 region; this also agrees with the results obtained from the neutrality-based pattern revealed by Tajima's and Fu's tests (Chaisiri *et al*. 2021). This haplotype network analyses confirm that the Sri Lankan S. italica accessions constitute a genetically distinct subset of the global non-waxy gene pool, representing a regional sublineage (Type 1-SL) within the broader Type 1 haplogroup.

Phylogenetic analysis

Phylogenetic analysis based on the sequences of intron 1 of the GBSSI gene grouped 17 *S. italica* accessions into two major clades (Fig. 8). The clustering revealed clear differentiation between Sri Lankan and reference accessions. Cluster A completely consists of the reference accessions (AB682919.1, AB682925.1, AB682926.1, AB682917.1 and AB682920.1). The high bootstrap support ($\geq$ 0.7) indicates robust evolutionary relatedness within this cluster. Interestingly, one Sri Lankan accession, FM 15156, clustered in cluster B, together with four reference accessions (AB679633, AB682923, AB682922 and AB682924), suggesting the conservation of

specific intron variants across geographically distinct populations. Cluster C comprises the Sri Lankan accessions FM7778 and FM15537, which form a well-supported, distinct lineage (bootstrap > 0.7). The clear separation of this clade from the other clusters indicates a unique evolutionary trajectory, potentially reflecting allelic differentiation or mutation accumulation within intron 1. The remaining accessions including three Sri Lankan accessions (KCFM3, FMT8.1, and FMJ12), did not group within any of the clusters, instead forming independent branches with relatively low bootstrap support. This suggests that these genotypes harbor unique intron-1 haplotypes distinct from those represented in clusters. Such singleton lineages may represent ancestral or derived alleles that have diverged due to mutation accumulation, recombination, or regional isolation. This clustering pattern aligns with the haplotype network and sequence polymorphism results, which together suggest that intron 1 region of GBSS1 contains high variation within Sri Lankan germplasm, while the GBSS1 function remains conserved. Furthermore, the diversity of GBSS1 gene intron 1 among foxtail millet genotypes suggests evolutionary divergence that could underlie variation in amylose content, grain quality, and adaptation to local environments. This is consistent with earlier studies indicating that intron polymorphisms in GBSS1 can influence gene splicing efficiency and starch biosynthetic efficiency in cereals such as rice, maize, and barley.

Evolutionary implications

The results obtained from the neighbor-network, TCS haplotype network and Neighbor-joining tree confirm the phenotypic data observed in I_2–KI staining and apparent amylose content (AAC). The close clustering of local accessions indicates that non-mutant (wild-type) GBSSI has been subjected to ethnobotanical selection for grain texture and agronomic resilience, allowing intronic mutations to accumulate silently while functional exons remain conserved. This study provides evidence for regional adaptation and limited gene flow, confirming the historical isolation of Sri Lankan foxtail millet populations following domestication. These findings provide valuable insights into the evolution and domestication of the waxy gene in South Asian *S. italica* and highlight Sri Lankan germplasm as a genetically conserved reservoir for future breeding and molecular studies.

CONCLUSION

The present study reveals the first molecular characterization of GBSS1 (waxy) gene intron 1 polymorphism in selected Sri Lankan foxtail millet accessions. Based on apparent amylose content and I_2–KI staining, all six haplotypes were phenotypically categorized into the non-waxy group; these results were consistent with the molecular analysis and the six accessions in the study were identified as the Type 1-1 non-waxy haplotype. The discovery of a novel 4 bp deletion, together with 167 polymorphic sites, reveals intra-type variation within the germplasm. The variations observed within the haplotypes indicated the influence of human-mediated selection and gene flow. These findings highlight the allelic diversity and the evolutionary stability of the functional GBSS1 allele in Sri Lankan foxtail millet. The intron 1 sequence information can be used to develop molecular markers for improving starch quality in foxtail millet. Moreover, complete sequencing of GBSS1 gene and collaborative molecular and morphological studies will facilitate further investigation of underlying molecular mechanisms of starch biosynthesis in Sri Lankan foxtail millet germplasm.

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REFERENCES

Aggarwal PR, Pramitha L, Choudhary P, Singh RK, Shukla P, Prasad M, Muthamilarasan M (2022) Multi-omics intervention in Setaria to dissect climate-resilient traits: Progress and prospects. *Frontiers in Plant Science*, 13. https://doi.org/10.3389/fpls.2022.892736.

Anand G, Vanniarajan C (2025) Morphological diversity and high-yielding potential of foxtail millet (*Setaria italica*): Insights from germplasm characterization. *Plant Science Today*, 12(3). https://doi.org/10.14719/pst.6239.

Bennetzen JL, Schmutz J, Wang H, Percifield R, Hawkins J, Pontaroli AC, Estep M, Feng L, Vaughn JN, Grimwood J, Jenkins J, Barry K, Lindquist E, Hellsten U, Deshpande S, Wang X, Wu X, Mitros T, Triplett J, Devos KM (2012) Reference genome sequence of the model plant *Setaria*. *Nature Biotechnology*, 30(6), 555–561. https://doi.org/10.1038/nbt.2196.

Biselli C, Cavalluzzo D, Perrini R, Gianinetti A, Bagnaresi P, Urso S, Orasen G, Desiderio F, Lupotto E, Cattivelli L, Valè G (2014) Improvement of marker-based predictability of Apparent Amylose Content in japonica rice through GBSSI allele mining. *Rice*, 7(1), 1. https://doi.org/10.1186/1939-8433-7-1.

Bruen TC, Philippe H, Bryant D (2006) A simple and robust statistical test for detecting the presence of recombination. *Genetics*, 172(4), 2665–2681. https://doi.org/10.1534/genetics.105.048975.

Caballero A, García-Dorado A (2013) Allelic Diversity and Its Implications for the Rate of Adaptation. *Genetics*, 195(4), 1373–1384. https://doi.org/10.1534/genetics.113.158410.

Chaisiri C, Liu X, Lin Y, Fu Y, Zhu F, Luo C (2021) Phylogenetic and Haplotype Network Analyses of Diaporthe eres Species in China Based on Sequences of Multiple Loci. *Biology*, 10(3), 179. https://doi.org/10.3390/biology10030179.

Cho J, Jung T, Kim J, Song S, Ko J, Woo K, Lee J, Choe M, Oh I (2015) Development of a waxy gene real-time PCR assay for the quantification of sorghum waxy grain in mixed cereal products. *BMC Biotechnology*, 15(1), 20. https://doi.org/10.1186/s12896-015-0134-z.

Crofts N, Itoh A, Abe M, Miura S, Oitome NF, Bao J, Fujita N (1999) Three Major Nucleotide Polymorphisms in the Waxy Gene Correlated with the Amounts of Extra-long Chains of Amylopectin in Rice Cultivars with S or L-type Amylopectin. *Journal of Applied Glycoscience*, 66(1), 37–46. https://doi.org/10.5458/jag.jag.JAG-2018_005.

Domon E, Fuijita M, Ishikawa N (2002) The insertion/deletion polymorphisms in the waxy gene of barley genetic resources from East Asia. *TAG. Theoretical and Applied Genetics*, 104(1), 132–138. https://doi.org/10.1007/s001220200016.

Fukunaga K (2019) Origin of waxy cereals from a genetic point of view: From cultural history to genetic history of waxy cereals. *Breeding Research*, 21(1), 1–10. https://doi.org/10.1270/jsbbr.18j12.

Fukunaga K, Kawase M (2024) Crop Evolution of Foxtail Millet. *Plants*, 13(2), 218. https://doi.org/10.3390/plants13020218.

Fukunaga K, Kawase M, Kato K (2002) Structural variation in the Waxy gene and differentiation in foxtail millet [*Setaria italica* (L.) P. Beauv.]: Implications for multiple origins of the waxy phenotype. *Molecular Genetics and Genomics*, 268(2), 214–222. https://doi.org/10.1007/s00438-002-0728-8.

Geethanjali S, Jegadeeswaran M (2016) Genetic diversity and variability in Foxtail millet [*Setaria italica* (L.)] germplasm based on morphological traits. *Electronic Journal of Plant Breeding*, 7(2), 303. https://doi.org/10.5958/0975-928x.2016.00038.7.

Gelfman S, Burstein D, Penn O, Savchenko A, Amit M, Schwartz S, Pupko T, Ast G (2012) Changes in exon–intron structure during vertebrate evolution affect the splicing pattern of exons. *Genome Research*, 22(1), 35–50. https://doi.org/10.1101/gr.119834.110.

Gupta S, Kumari K, Das J, Lata C, Puranik S, Prasad M (2011) Development and utilization of novel intron length polymorphic markers in foxtail millet (*Setaria italica* (L.) P. Beauv.). *Genome*, 54(7), 586–602. https://doi.org/10.1139/g11-020.

Hachiken T, Sato K, Hasegawa T, Ichitani K, Kawase M, Fukunaga K (2013) Geographic distribution of Waxy gene SNPs and indels in foxtail millet, *Setaria italica* (L.) P. Beauv. *Genetic Resources and Crop Evolution*, 60(4), 1559–1570. https://doi.org/10.1007/s10722-012-9942-3.

Hammer K, Khoshbakht K (2007) Foxtail millet (*Setaria italica* (L.) P. Beauv.) in Mazandaran/Northern Iran. *Genetic Resources and Crop Evolution*, 54(4), 907–911. https://doi.org/10.1007/s10722-007-9211-z.

Hariprasanna K, Jacob J, Patroti P, Visarada KBRS (2017) Breeding Strategies in Foxtail Millet. In: Prasad M (Ed.) *The Foxtail Millet Genome*. Springer International Publishing, 53–61. https://doi.org/10.1007/978-3-319-65617-5_5.

Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K (1998) A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5' splice site of the first intron. *The Plant Journal*, 15(1), 133–138. https://doi.org/10.1046/j.1365-313x.1998.00189.x.

Jayalakshmi M, Babu GP, Kiranmai MJ, Chaithanya BH, Narshimhulu R (2024) On-farm Testing of Foxtail Millet Variety SiA-3223 (Renadu) in Semi-arid Regions of Andhra Pradesh. *Biological Forum - An International Journal*, 16(2), 140–143.

Jo BS, Choi SS (2015) Introns: The Functional Benefits of Introns in Genomes. *Genomics and Informatics*, 13(4), 112–118. https://doi.org/10.5808/GI.2015.13.4.112.

Kawase M, Fukunaga K, Kato K (2005) Diverse origins of waxy foxtail millet crops in East and Southeast Asia mediated by multiple transposable element insertions. *Molecular Genetics and Genomics*, 274(2), 131–140. https://doi.org/10.1007/s00438-005-0013-8.

Klopfenstein NB, Stewart JE, Ota Y, et al. (2017) Insights into the phylogeny of Northern Hemisphere Armillaria: Neighbor-net and Bayesian analyses of translation elongation factor 1- gene sequences. *Mycologia*, 109(1), 75–91. https://doi.org/10.1080/00275514.2017.1286572.

Kosakovsky-Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SDW (2006) Automated Phylogenetic Detection of Recombination Using a Genetic Algorithm. *Molecular Biology and Evolution*, 23(10), 1891–1901. https://doi.org/10.1093/molbev/msl051.

Kuo S, Chen Y, Yin S, Ba Q, Tsai Y, Kuo WHJ, Lin Y (2018) Waxy allele diversification in foxtail millet (*Setaria italica*) landraces of Taiwan. *PLOS ONE*, 13(12), e0210025. https://doi.org/10.1371/journal.pone.0210025.

Larkin PD, Park WD (2003) Association of waxy gene single nucleotide polymorphisms with starch characteristics in rice (*Oryza sativa* L.). *Molecular Breeding*, 12(4), 335–339. https://doi.org/10.1023/B:MOLB.0000006797.51786.92.

Latinne A, Waengsothorn S, Rojanadilok P, Eiamampai K, Sribuarod K, Michaux JR (2012) Combined Mitochondrial and Nuclear Markers Revealed a Deep Vicariant History for Leopoldamys neilli. *PLOS ONE*, 7(10), e47670. https://doi.org/10.1371/journal.pone.0047670.

Leigh JW, Bryant D (2015) popart: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6(9), 1110–1116. https://doi.org/10.1111/2041-210X.12410.

Li C, Wang G, Li H, et al. (2021) High-depth resequencing of 312 accessions reveals the local adaptation of foxtail millet. *Theoretical and Applied Genetics*, 134(5), 1303–1317. https://doi.org/10.1007/s00122-020-03760-4.

Li H, Fitzgerald MA, Prakash S, Nicholson TM, Gilbert RG (2017a) The molecular structural features controlling stickiness in cooked rice. *Scientific Reports*, 7, 43713. https://doi.org/10.1038/srep43713.

Li Y, Xu Y, Ma Z (2017b) Comparative Analysis of the Exon-Intron Structure in Eukaryotic Genomes. *Yangtze Medicine*, 1(1), Article 1. https://doi.org/10.4236/ym.2017.11006.

Liu X, Ding Q, Wang W, et al. (2022) Targeted Deletion of the First Intron of the Wxb Allele via CRISPR/Cas9 Increases Grain Amylose Content in Rice. *Rice*, 15(1), 1. https://doi.org/10.1186/s12284-021-00548-y.

Makwana K, Tiwari S, Tripathi MK, Patel V (2023) Morphological diversity analysis of Foxtail Millet using qualitative and quantitative traits. *Scientist*, 2(2), 319–326. https://doi.org/10.5281/zenodo.7654168.

Maung TZ, Yoo JM, Chu SH, Kim KW, Chung IM, Park YJ (2021) Haplotype Variations and Evolutionary Analysis of the GBSSI Gene in Rice. *Frontiers in Plant Science*, 12, 707237. https://doi.org/10.3389/fpls.2021.707237.

Nakayama H, Afzal M, Okuno K (1998) Intraspecific differentiation and geographical distribution of Wx alleles in foxtail millet. *Euphytica*, 102(3), 289–293. https://doi.org/10.1023/A:1018344819531.

Pant SR, Irigoyen S, Doust AN, Scholthof KBG, Mandadi KK (2016) Setaria: A Food Crop and Translational Research Model for C4 Grasses. *Frontiers in Plant Science*, 7, 1885. https://doi.org/10.3389/fpls.2016.01885.

Peng R, Zhang B (2021) Foxtail Millet: A New Model for C4 Plants. *Trends in Plant Science*, 26(3), 199–201. https://doi.org/10.1016/j.tplants.2020.12.003.

Prasad M (Ed.) (2017) *The Foxtail Millet Genome*. Springer. https://doi.org/10.1007/978-3-319-65617-5.

Ramesh P, et al. (2023) Molecular genetics and phenotypic assessment of foxtail millet landraces. *Frontiers in Genetics*, 14. https://doi.org/10.3389/fgene.2023.1052575.

Repar J, Warnecke T (2017) Mobile Introns Shape the Genetic Diversity of Their Host Genes. *Genetics*, 205(4), 1641–1648. https://doi.org/10.1534/genetics.116.199059.

Rozas J, et al. (2017) DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Molecular Biology and Evolution*, 34(12), 3299–3302. https://doi.org/10.1093/molbev/msx248.

Sapkota M, Pandey MP, Thapa DB (2016) Agromorphological Characterisation of Foxtail Millet (*Setaria italica* L. Beauv) at Rampur, Chitwan, Nepal. *International Journal of Applied Sciences and Biotechnology*, 4(3), 298–307. https://doi.org/10.3126/ijasbt.v4i3.15148.

Swamy KRM (2023) Origin, Domestication, Taxonomy, Botanical Description, Genetics and Cytogenetics, Genetic Diversity, Breeding of Foxtail Millet (*Setaria italica* (L.) Beauv.). *International Journal of Development Research*, 13, 63660–63682. https://doi.org/10.37118/ijdr.27135.09.2023.

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, 101(30), 11030–11035. https://doi.org/10.1073/pnas.0404206101.

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7), 3022–3027. https://doi.org/10.1093/molbev/msab120.

Tharindi PWM, Bandara PKGSS, Karunaratne PMAS, Wimalasiri GEM (2023) Analysis of Molecular Diversity of Selected Foxtail Millet (*Setaria italica*) Germplasm in Sri Lanka Using Microsatellite Markers. Thirteenth Annual Research Session (ARS-2023), Sabaragamuwa University of Sri Lanka, Belihuloya, Sri Lanka, 33p.

Tsang CH, Li KT, Hsu TF, Tsai YC, Fang PH, Hsing YIC (2017) Broomcorn and foxtail millet were cultivated in Taiwan about 5000 years ago. *Botanical Studies*, 58(1). https://doi.org/10.1186/s40529-016-0158-2.

Van K, Onoda S, Kim MY, Kim KD, Lee SH (2008) Allelic variation of the Waxy gene in foxtail millet [*Setaria italica* (L.) P. Beauv.] by single nucleotide polymorphisms. *Molecular Genetics and Genomics*, 279(3), 255–266. https://doi.org/10.1007/s00438-007-0310-5.

Wang L, Liu L, Zhao J, Li C, Wu H, Zhao H, Wu Q (2023) Granule-bound starch synthase in plants: Towards an understanding of their evolution, regulatory mechanisms, applications, and perspectives. *Plant Science*, 336, 111843. https://doi.org/10.1016/j.plantsci.2023.111843.

Xie J, Chen Y, Cai G, Cai R, Hu Z, Wang H (2023) Tree Visualization By One Table (tvBOT): A web application for visualizing, modifying and annotating phylogenetic trees. *Nucleic Acids Research*, 51(W1), W587–W592. https://doi.org/10.1093/nar/gkad359.

Yamanaka S, Nakamura I, Watanabe KN, Sato YI (2004) Identification of SNPs in the waxy gene among glutinous rice cultivars and their evolutionary significance during the domestication process of rice. *Theoretical and Applied Genetics*, 108(7), 1200–1204. https://doi.org/10.1007/s00122-003-1564-x.

Yanagisawa T, Kiribuchi-Otobe C, Yoshida H (2001) An alanine to threonine change in the Wx-D1 protein reduces GBSS I activity in waxy mutant wheat. *Euphytica*, 121(3), 209–214. https://doi.org/10.1023/A:1012051705517.

Yang Q, Zhang W, Li J, Gong X, Feng B (2019) Physicochemical Properties of Starches in Proso (Non-Waxy and Waxy) and Foxtail Millets (Non-Waxy and Waxy). *Molecules*, 24(9), 1743. https://doi.org/10.3390/molecules24091743.

Yang T, Ma S, Liu J, Sun B, Wang X (2022) Influences of four processing methods on main nutritional components of foxtail millet: A review. *Grain & Oil Science and Technology*, 5(3), 156–165. https://doi.org/10.1016/j.gaost.2022.06.005.

Yang Y, Zhou L, Feng L, Jiang J, Huang L, Liu Q, Zhang Y, Zhang C, Liu Q (2024) Deciphering the Role of Waxy Gene Mutations in Enhancing Rice Grain Quality. *Foods*, 13(11), 1624. https://doi.org/10.3390/foods13111624.

Yirgu M, Kebede M, Feyissa T, Lakew B, Woldeyohannes AB, Fikere M (2023) Single nucleotide polymorphism (SNP) markers for genetic diversity and population structure study in Ethiopian barley (*Hordeum vulgare* L.) germplasm. *BMC Genomic Data*, 24(1), 7. https://doi.org/10.1186/s12863-023-01109-6.

Zhang G, Liu X, Quan Z, Cheng S, Xu X, Pan S, et al. (2012) Genome sequence of foxtail millet (*Setaria italica*) provides insights into grass evolution and biofuel potential. *Nature Biotechnology*, 30(6), 549–554. https://doi.org/10.1038/nbt.2195.

Zheng H, Wang H, Yang H, Wu J, Shi B, Cai R, Xu Y, Wu A, Luo L (2013) Genetic Diversity and Molecular Evolution of Chinese Waxy Maize Germplasm. *PLOS ONE*, 8(6), e66606. https://doi.org/10.1371/journal.pone.0066606.