ISSN: 2287-688X

Annals of
Plant Sciences

Volume 13, Issue 03 (2024) pp. 6199-6211

Research Article

Individual Microspore Genotyping in Sorghum (*S. bicolor*) Cultivars Using a KASP Panel of Single Nucleotide Polymorphism Markers

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Abstract

Sweet sorghum (*Sorghum bicolor* L. Moench) cultivars show promise as a biofuel source, necessitating innovative tools for genetic enhancements. This study employed whole genome amplification (WGA) to amplify genomic DNA from individually isolated haploid microspores. These microspores were genotyped using a panel of novel, locus-specific, single nucleotide polymorphism (SNP) markers. Five sorghum cultivars were grown to the flowering stage, and 30 early developing microspores were individually isolated from each variety. Kompetitive Allele Specific PCR (KASP) based markers' analysis was performed on 20 SNP loci from all varieties using microspore and leaf DNAs. The KASP success rate was 80% for microspore DNAs and 91.3% for leaf samples. Eight SNPs detected 16-42% mono-allelic markers in microspores while the other 12 detected mono-allelism at 10% or less. Microspores that displayed similar profiles were grouped together for each variety. Notably, 13 SNPs were selected for such gametic groups, demonstrating the utility of WGA products from individual gametophytes. This study marks the first application of molecular markers using individually isolated microspores of sorghum varieties.

Keywords: *DNA profiling, gametophyte, haploid, single cell isolation, genetics.*

Introduction

Sorghum bicolor (L.) Moench, a versatile C4 herbaceous annual grass, serves crucial roles as a grain, fodder, and biofuel crop (Ravindra, *et al*., 2022). Certain sorghum varieties, bred for high seed yield, contrast with ancestral sweet sorghums that, while tall and lodging-susceptible, accumulate sugary water (sucrose) in their stems (Ratikant, *et al*., 2021). Globally, sorghum cultivars, particularly grain sorghum, rank as the second most utilized crop for poultry feed in the U.S. covering millions of hectares (USDA, *et al*., 2023). Sweet sorghum, celebrated for its simplicity in syrup extraction and direct fermentability into bioethanol, gains significance for future environmentally conscious agriculture (Cai, *et al*., 2013). Sorghum is a very versatile crop over many geographic regions and can be cultivated in tropical, subtropical, temperate, and semiarid or even poor-soil regions (Hossain, *et al*., 2022). Sweet sorghum, introduced in the U.S. for syrup production around 1850s, experienced a decline in the 20th century but remains a specialty crop for traditional farmers in the southern U.S.A, substituting cane sugar and molasses in regional food culture (Rajvanshi, *et al*., 2020; Reddy, *et al*., 2005). With sweet sorghum's stalks containing up to 25% sugar, significantly surpassing grain sorghum, its potential for biofuel production, particularly ethanol, is noteworthy (Chamarthy, *et al*., 2011).

Sweet sorghum varieties possess stalks capable of accumulating high sugar content, yielding 1.4 to 2.7 times more carbohydrates than grain sorghum cultivars (Tarpley, *et al*., 2007). While the physiological mechanisms

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DOI: http://dx.doi.org/10.21746/aps.2024.13.3.2

governing sugar accumulation may differ between sorghum and sugarcane plants, both sources can be utilized interchangeably by feedstock mills and subsequent processors (Enyew, *et al*., 2021). The genome size of S. *bicolor* is estimated to be 700 Mbp (Ming, *et al*., 2001), with diploid somatic cells (2n = 2x) containing 20 chromosomes, and haploid or gametophytes (1n = 1x) possessing 10 chromosomes (Teingtham, *et al*., 2017). Diploid mega- and micro-spore mother cells give rise to haploid gametes, which then fuse together to produce new embryos, maintaining the species' chromosome number throughout the lifecycle (Mammadov, *et al*., 2012). Pollen grains, serving as gametophytes or male gametes (Younis, *et al*., 2014), are frequently utilized in genomic and plant breeding studies due to being natural haplotypes and abundance in nature (Picard, *et al*., 2011). Despite this, no genetic analysis has yet been conducted on individual microspores of grain or sweet sorghums. Genetic enhancement of sweet sorghum through such methodologies is imperative to fully harness their potential for enhanced production traits such as increased syrup, sucrose, lignocellulosic, and biomass.

Understanding the genetic makeup and variability for microspores in sweet sorghum could provide invaluable insights per species' diversity, aiding in the development of improved cultivars with desirable traits through targeted breeding programs. Single nucleotide polymorphism (SNP) is a cornerstone tool in molecular genetics and genomics, widely applicable in plant studies including gamete analysis. SNPs serve to identify prevalent genetic variations within a population, essential for crop enhancement, genetic diversity evaluation, and various agricultural applications (Enyew, *et al*., 2022). They offer a rich source of DNA information and are favored as molecular markers for rapid cultivar identification and construction of high-density genetic maps (Burow, *et al*., 2019). Understanding the genetic composition and inheritance patterns of gametes (reproductive cells) provides insights crucial for plant breeding per meiotic recombination, contributing to the development of crop varieties with desirable traits (Sprunck, *et al*., 2019). Exploring SNPs for gamete analysis thus can deepen our comprehension of sorghum genetics, facilitating improvements in agricultural products.

Kompetitive Allele Specific PCR (KASP) based SNP assays have been designed to target markers for various agronomically significant traits across several crops, including sorghum cultivars (Gloria, *et al*., 2019). By utilizing KASP assays, specific SNP loci can be accurately identified, offering a time-efficient, high-throughput method for assessing genetic diversity or tracking meiotic recombination events. This approach has significantly contributed to enhancing the nutritional quality of both human and animal products and tailoring crop varieties to exhibit exceptional production traits (Mammadov, *et al*., 2012). This study aims to assess the effectiveness of KASP markers as a novel molecular fingerprinting technology for identifying specific loci and alleles in amplified genomic DNAs extracted from haploid microspores or immature pollen of sorghum varieties. Whole genome amplifications (WGA) were employed to access gametophytic DNAs from individual microspores, which were individually isolated for subsequent marker analyses. Rankings were established for each of the five cultivars to assess the genetic similarities among microspores based on their SNP profiles, thereby determining allelic frequencies.

The utilization of these newly developed marker's techniques holds significant importance for sorghum breeding and finds applications in quality control genotyping for mapping populations of non-parental alleles, as well as marker-assisted selection, which enables accurate and efficient introgression of important agronomic traits (Semagn, *et al*., 2013 and Hussain, *et al*., 2019). The potential to produce sufficient homozygous lines through doubled haploid protocols in a single generation, utilizing allele-specific KASP markers, can expedite the breeding process in

sorghum (Hussain, *et al*., 2019). Employing gametophyte-based assays for SNP genotyping using microspores could lead to reduced KASP-SNP error rates and cost-effectiveness in plant breeding applications (Han, *et al*., 2018). One of the primary objectives of plant breeders is to develop pure lines of highly homozygous organisms (Fortea, *et al*., 2020). Therefore, the utilization of DNAs extracted from microspores followed by WGA, as demonstrated in this study, holds the potential to optimize doubled haploid procedures over conventional methods.

Methods

Plant Materials and Leaf DNA Extractions

In the greenhouse study, five cultivars of *Sorghum bicolor* were grown, each serving distinct purposes:—four sweet sorghum cultivars (Achi Turi, Dale, Dasht Local, and Topper 76-6) and one grain sorghum line (RTx430). Monitoring at least 10 plants of each cultivar per harvesting criteria involving early booting stage, elongated panicles late in the growing season, and specific measurements of flag leaf internode lengths (approximately 10 cm) and boot radius (3.5 to 12 cm, varying by cultivar) ensured the collection of microspores with undeveloped exine (Felts, *et al*., 2022). Upon harvest, panicles were cut two internodes below the crown, stored in labeled plastic bags at 4℃, and later used for individual microspore isolation. For high-quality DNA extraction from leaves of each sorghum cultivar, DNeasy Mini Plant Kit (Qiagen, Valencia, CA) was used. Notably, Johnsongrass (*S. halepense* L.) samples were used for both DNA extraction and as a control in SNP analyses. This comprehensive approach ensured the availability of genetic material for subsequent analyses while maintaining a robust control for comparative assessments.

Microspore Isolations and Whole Genome Amplifications

Sessile spikelets, measuring 3.3 – 3.7 mm in length, were meticulously dissected from the panicles beneath a microscope to access and macerate the anthers, thereby liberating free-floating microspores. A total of 150 microspores (30 per cultivar) from the anthers of the five sorghum cultivars were individually isolated promptly after the tetrad stage of meiosis, marking the commencement of gametophyte DNA extractions. The isolation of individual gametophytes occurred within an aqueous solution of 6% D-sorbitol (Sigma-Aldrich, St. Louis, MO) preserving them as natural protoplasts, which were subsequently lysed to procure genomic DNAs. Genomic DNA amplifications from single gametophytes were executed using REPLI-g Advanced Single Cell Kits (Qiagen, Valencia, CA), employing Multiple Displacement Amplifications. This technique, relying on the annealing of random hexamer primers to the template, followed by DNA synthesis using a high-fidelity enzyme like 29 DNA polymerase (Qiagen), rapidly generated substantial amounts of DNA samples for ensuing genomic analysis and quantification of leaf DNAs as well as gamete WGA samples was performed using the NanoDrop™ One UV-Vis Spectrophotometer (Fisher Scientific, Hampton, NH). The samples were stored at 4°C until required.

Primer Design for SNP Analyses

The SNP sequences utilized for developing KASP genotyping assays were sourced from the reports by Generation Challenge Program (Bruskiewich, *et al*., 2006). A comprehensive selection process involving 20 loci, (Table 1) was based on map locations and the relevance to the production traits, as per quantitative traits' loci (QTL) sequences. Verification of these selected SNP sequences and confirmation of chromosomal locations were conducted using the NCBI (http://www.ncbi.nlm.nih.gov/SNP) and Phytozome (http://www.phytozome.net) databases. Oligo synthesis and validation of primer sequences, using leaf DNA samples, were performed at LGC Genomics (Teddington, Middlesex, U.K). KASP-on-Demand assays, in accordance with LGC Genomics protocols, necessitated the integration of Johnson grass in

primer's design, endowing the assays with a unique capability to transcend species barriers. Such effectiveness of SNP primers through cross-species validation by LGC Genomics, established these as an invaluable resource for this project. The 20 primer sets, tailored to specific SNP loci, comprised KASP Assay mix in quantities sufficient for 2,500 PCR amplification reactions each. The KASP Master mix, available in a ready-to-use 2 mL bar coded tubes (LGC Genomics Beverly, MA), featured the fluorescent reporting dyes Fluorescein amidites (FAM) and Hexachlorofluorescein (HEX), alongside the passive reference background dye Rhodamine X (ROX).

KASP Amplifications SNP Evaluations

A 5 µl aliquot of KASP master mix, paired with the chosen SNP primer, was dispensed into each well of 96-well reaction plates (Fisher Scientific, Hampton, NH). Subsequently, 5 µl of template DNAs from the sorghum samples, each labeled for well identification, were added. Following dispensing, each plate underwent sealing and centrifugation (Eppendorf 5810R, Hamburg, Germany) at 550 g, and then was processed on a Bio-Rad (Hercules, CA) thermal cycler. The amplification reaction was initiated with a denaturation hot-start temperature of 94°C for 15 minutes, succeeded by 10 cycles of denaturation at 94°C for 20 seconds and annealing/elongation for 60 seconds at 55-61°C, incorporating a touch-down of 0.6°C for each cycle. This was followed by 26 cycles of denaturation at 94°C for 20 seconds and annealing/elongation at 55°C for 60 seconds. Post-amplification, the PCR plates for each SNP primer pair-based KASP reaction were read using a FLUOstar Omega plate reader spectrophotometer (BMG LABTECH, Ortenberg, Germany) at a 320 nm excitation wavelength. Alleles detected for each SNP were identified (Fig. 1) following the protocol of Islam and Blair, *et al*., (2018), utilizing KlusterCaller software (LGC genomics, Petaluma, CA). Allele calls were then transferred to a Microsoft Excel worksheet (Redmond, WA) for subsequent analysis.

Table 1: Identification (ID) of the 20 single-nucleotide polymorphic (SNP) loci, which were selected for KASP (Kompetitive Allele Specific PCR) assays, based on chromosome placement and dimorphic allele percentages depicted in the NCBI (http://www.ncbi.nlm.nih.gov/SNP), and Phytozome (http://www.phytozome.net) databases.

SNP ID	Chrom.	X%	Y%	SNP Locus
SB01-002	1	T 54%	C 46%	CCTTGCAAACCAAAG**[T/C]**GCTCCGAATTCAAA
SB01-106	1	T 17%	C 83%	CACATTCTTCTACCT**[T/C]**TGCTGCGGCTGCTT
SB01-109	1	A 64%	C 36%	TATATTATTAACTGT**[A/C]**TTTGTATCTTTGTC
SB01-134	1	T 29%	G 71%	ATGAAGTCATCCGTA**[T/G]**CGTTTGTAGCTATC
SB01-158	1	T 57%	C 43%	ACGCCGACGACAAGA**[T/C]**GGACACCAACCTCT
SB01-169	1	A 83%	G 17%	TGATGCCATGGCATG**[A/G]**TCTATTCCTAGGGT
SB02-112	2	A 75%	G 25%	AAGAAATGGGAGCTC**[A/G]**GTACTCACCACATT
SB02-146	2	T 33%	C 67%	CACAGTGTGCTCTTG**[T/C]**TGTCTCGTTGATGC
SB03-040	3	A 50%	T 50%	GTTCATGCTGTACCA**[A/T]**CATAGGCCGGTGAG
SB03-174	3	A 75%	T 25%	TGTAGCAGGTATATG**[A/T]**GTGTGTAACGATGC
SB04-058	4	T 100%	C 0%	GGTCGCGAACCACGT**[T/C]**CTTCTCAGTTACTT
SB04-074	4	A 33%	G 67%	TAAAATCTGTTGCCG**[A/G]**GAGGTTGGAGAAAC
SB06-063	6	C 35%	G 65%	CATGCATGAGCGCTA**[C/G]**CTTTTGGACGGTAC
SB06-119	6	A 65%	G 35%	TGCTGGCCAGTGAAC**[A/G]**CTCCAGCAAGTCCA
SB07-038	7	C 82%	G 18%	CAGCCAATTGCACGA**[C/G]**TGCACCACGTCACC
SB07-054	7	T 100%	C 0%	TGGCCTCATGAAAAT**[T/C]**GACGAAGAAGGGAG
SB08-010	8	T 29%	C 71%	ATAACCGAACCCAGC**[T/C]**CAAGGGCAACCGTT
SB08-046	8	C 85%	G 15%	CCCGGCCTTTGCCTT**[C/G]**TCAATCTCCAGATT
SB09-125	9	A 48%	G 52%	TTTCCAATTTCAACA**[A/G]**CGGTAAGGATCGAT
SB09-128	9	A 100%	G 0%	CAGGATCTGATCACT**[A/G]**AAACTGTGACTAAC

Results

Single Gametophytes Isolation and DNA Yields

Microspores ranging from the early uni- to mid bi-nucleate developmental stages were pinpointed for individual isolation as natural protoplasts, simplifying the extraction of their DNAs directly into reaction tubes. Due to the notably low DNA concentration from single gametophytes, a WGA step became imperative to prime the samples for subsequent marker analyses. The 50 µl WGA reaction tubes for individual microspores yielded typical DNA concentrations ranging from 1,652 ng/µl to 4,761 ng/µl, with an average of 2,961 ng/µl for the amplified samples. Gel electrophoresis indicated fragment lengths exceeding 2 kbp, and A260/A280 ratios averaged 1.8 based on UV spectrometer (Fisher Scientific) absorbance readings. WGA yields from individual gametophytes provided high-quality PCR templates for subsequent KASP reactions across all sorghum samples. Although slightly higher amplification levels were obtained from the "RTx430" and "Topper 76-6" cultivars, the overall success of the WGA reactions remained consistent. Control plant leaf DNA extractions were carried out for producing typical yields ranging from 15 to 45 ng/µl with an average A260/A280 ratio of 1.78. Both the REPLI-g Advanced Single Cell and DNeasy Mini kit by Qiagen (Valencia, CA) furnished high-quality genomic DNA yields, ensuring successful outcomes for subsequent KASP reactions involving both types of plant samples (microspores and leaves, respectively).

Alleles Observed for SNP Markers

In this study, NCBI Blast (http://www.ncbi.nlm.nih.gov/SNP) was employed to identify DNA homology between SNP sequences and genomic positions, while the Phytozome plant genomics portal (http://www.phytozome.net) helped locate the chromosomal positions of the

SNPs through a quick search function. A total of 124 microspore WGA samples, along with two template controls, 24 parental diploid DNAs, along with two additional diploid Johnson grass leaf DNA samples were evaluated for KASP genotyping using each KASP primer. The allele frequency data based on 20 SNP assays showed the presence of either X or Y gamete alleles (Fig. 1). Distinct separation of microspore amplification profiles, observed through fluorescence imaging, facilitated the identification of the haploid state when compared with the diploid parental DNAs (Fig. 1). Among the 20 SNP assays, 13 markers displayed monomorphic profiles in three or more cultivars, while the remaining seven showed mostly dimorphic profiles among microspores. The assays, through allelic frequency data, allowed to estimate the percentage of mono-allelic gametophytes detected for each cultivar. The results for each cultivar were as follows: Achi Turi: 19% mono-allelic, 81% bi-allelic, Dale: 29% mono-allelic, 71% bi-allelic, Dasht Local: 26% mono-allelic, 74% bi-allelic, RTx430: 26% mono-allelic, 74% bi-allelic, and Achi Turi/Topper 76-6: 18% mono-allelic, 82% bi-allelic.

When comparing all markers, three SNPs (SB01-134, SB03-040, and SB09-128) exhibited 100% mono-allelic genotypes among microspores for two or more cultivars. Three SNPs (SB01-134, SB07-054, SB09-128) exhibited 79 to 94% mono-allelism, whereas two markers (SB03-040, SB04-058) displayed 42-43% mono-allelism. The following three SNPs; (SB02-148, SB02-112, and SB09-125) revealed 16-17% mono-allelism, while the remaining five SNPs; (SB00-169, SB04-058, SB06-119, SB06-063, and SB08-046) showed 7-10% mono-allelism. Another group of five SNPs (SB01-134, SB02-112, SB04-058, SB06-119, and SB07-054) displayed dimorphic alleles across all cultivars, with SB07-054 showing elevated levels (>88%) of mono-allelism in all five cultivars. Four additional markers (SB08-046, SB09-125, SB09-128, and SB00-169) revealed dimorphic alleles among microspores for at least four cultivars, while another four markers (SB02-148, SB03-040, SB06-063, and SB07-038) showed dimorphic alleles among microspores for at least three cultivars.

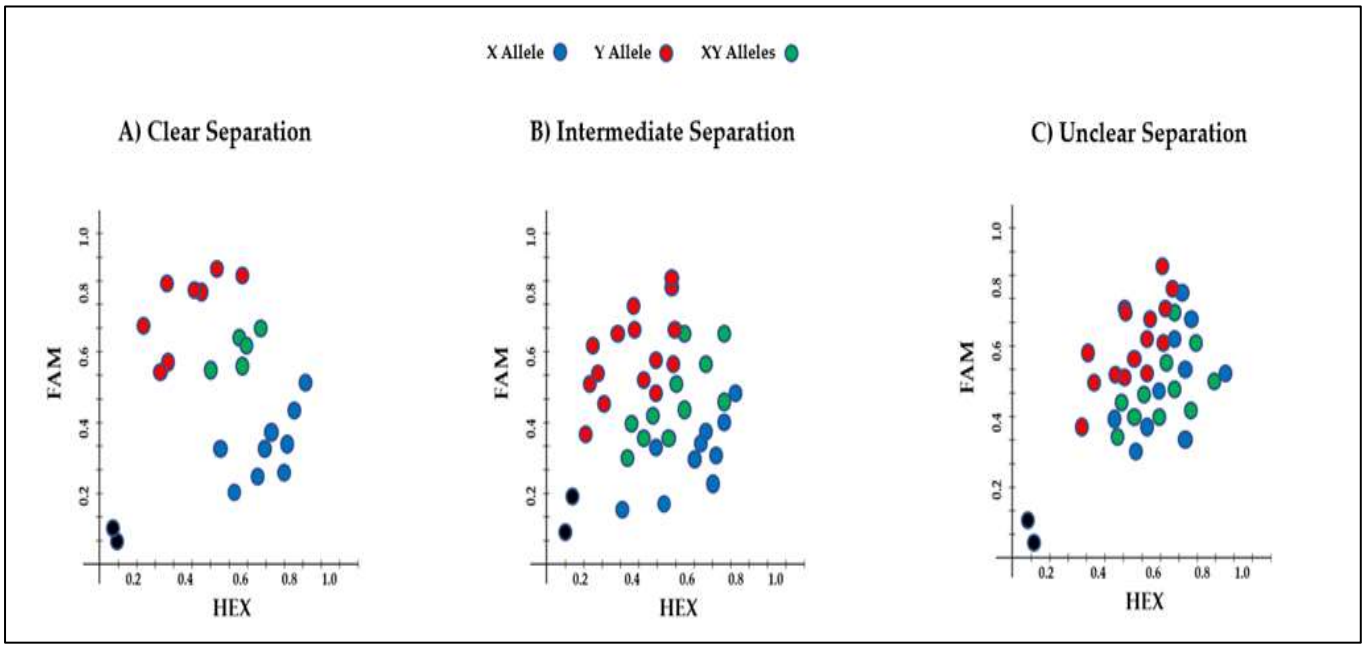

Figure 1. Typical KlusterCaller genotyping results illustrated in a cluster plot following Kompetitive Allele Specific PCR (KASP) analysis. In the plot, blue data points

represent a homozygous state for the alleles detected by Fluorescein amidites (FAM), and green data points depict heterozygous states, while red data points indicate a homozygous state for the alleles detected by Hexachloro-fluorescein (HEX). Black data points represent the no template controls (NTC), while pink data points denote unconfirmed results. The genotyping output of single nucleotide polymorphisms (SNPs) from microspores and leaf tissue DNAs of five sorghum cultivars reveals three distinct clustering patterns: A) Clear Separation, B) Intermediate Separation, and C) Unclear Separation of X and Y alleles, as color-coded in the legend.

KASP Success Rate

The amplification success of KASP reactions for the 124 microspores averaged over 89%, with leaf DNAs showing a slightly higher success rate at 91% (Fig. 2). Two out of the 20 SNP markers (SB00-106 and SB00-109) failed to amplify gametophyte DNAs from any cultivar, while SB00-109 specifically failed during SNP amplification reactions for Achi Turi and Topper 76-6 microspores. However, all other primers successfully amplified and displayed SNP allelic profiles for microspores from all five sorghum cultivars. The success rates for SNP amplification reactions were categorized into four groups (I, II, III, IV) based on both leaf and microspore KASP success rates (Fig. 2). Group I: Comprising of six SNPs (SB00-002, SB00-109, SB00-112, SB00-125, SB00-134, and SB00-148) with <25% success rates. Group II: Comprising of nine SNPs (SB00-134, SB02-112, SB00-109, SB09-125, SB03-174, SB03-040, and SB04-058) with 25-50% success rates. Group III: Comprising of two SNPs (SB03-174 and SB03-040) with a 50-75% success rate. Group IV: Comprising of 11 SNPs (SB04-074, SB00-106, SB06-119, SB06-063, SB07-038, SB07-054, SB08-010, SB08-046, SB09-128, SB00-169, and SB00-158) with 75-100% success rates.

In summary, a total of 13 SNP assays (SB04-074, SB00-106, SB00-109, SB06-119, SB06-063, SB07-038, SB07-054, SB08-010, SB08-046, SB09-125, SB09-128, SB00-169, and SB00-158) showed > 100% amplification success rates, while 7 SNPs (SB01-134, SB01-002, SB02-148, SB02-112, SB03-174, SB03-040, and SB04-058) showed < 50% success rates. The nucleotide differences for the 13 high success markers identified above are provided in Table 2. Analysis of these 13 SNPs revealed variation in detecting mono-allelic profiles for each cultivar: Achi Turi microspores exhibited mono-allelism at 26.38%; Dale gametophytes at 41.77%; Dasht Local immature pollen were at 36.77%; RTx430 mono-allelic microspores displayed at 38.69%; and Topper 76-6 mono-allelic gametophytes revealed at 21.54%. Using these 13 SNPs, microspores of the cultivars were grouped together per their similar genetic profiles (Fig. 2). Achi Turi showed a total of 17 microspores distributed across four haplotype groups (with 10, 3, 2, and 2 gametophytes in each group, respectively). Topper 76-6 microspores could be categorized into three groups (with 10, 3, and 2 members). RTx430 microspores, after analysis with the 13 SNPs, were found in three groups (comprising 7, 5, and 3 gametophytes in each). Dasht Local had a total of 11 microspores categorized into two groups (with 2 and 9 microspores each having similar genetic profiles). Three microspores of Dale with similar genetic profiles were grouped together, while others remained ungrouped after analysis with the above 13 SNPs.

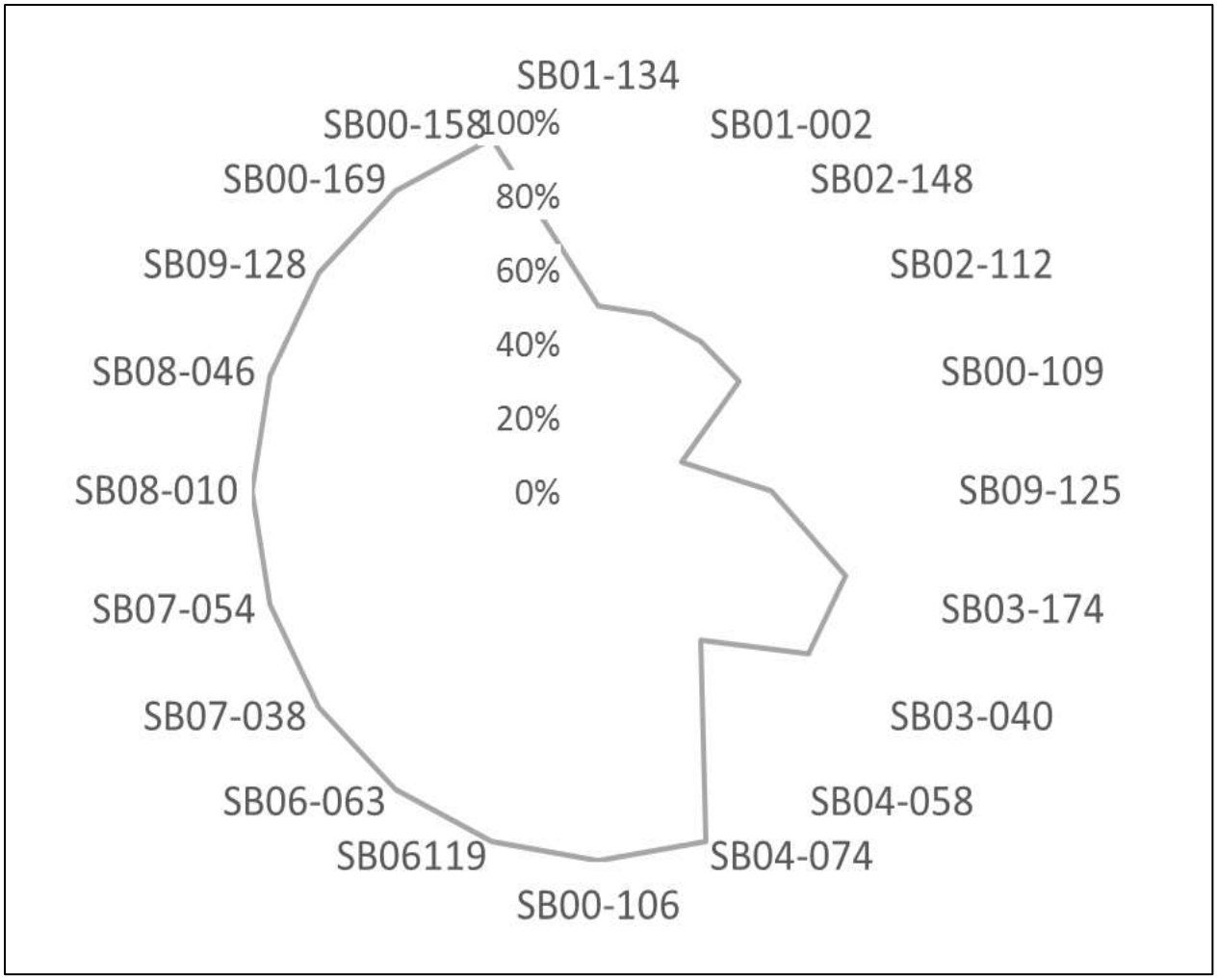

Figure 2: Sorghum (*S. bicolor*) DNA amplification success rates for five cultivars (Achi Turi, Dale, Dasht Local, RTx430, and Topper 76-6) based on Kompetitive Allele Specific PCR (KASP) genotyping while using 20 (production traits related) Single Nucleotide Polymorphism (SNP) markers.

Table 2: Similar groups of *S. bicolor* microspores from five cultivars after Single Nucleotide Polymorphism allele (identified by SNP designations) segregations based on the 13 Kompetitive Allele Specific PCR (KASP) assays and profiling.

Cultivar (Microspore Groups)	No. of gametes	SB01-134	SB02-148	SB02-112	SB03-040	SB04-058	SB06-119	SB06-063	SB07-038	SB07-054	SB08-046	SB09-125	SB09-128	SB00-169
Achi. Turi	10		CT	GA	TA	T	GA	GC	GC	T	GC	GA	A	GA
Achi. Turi	3		CT	GA	TA	T	GA	GC	G	T	GC	GA	A	GA
Achi. Turi	2		CT	G	TA	T	GA	GC	GC	T	GC	GA	A	GA
Achi. Turi	2	GT	CT	G	TA	T	GA	GC	GC	T	GC	GA	A	GA
Dale	3	T	CT	GA	A	CT	GA	GC	GC	T	GC	GA	A	GA
Dasht Local	2	T	CT	GA	A	CT	GA	GC	GC	C	GC	GA	A	GA

Dasht Local	10	T	CT	GA	A	CT	GA	GC	GC	T	GC	GA	A	GA
RTx 430	3	G	CT	GA	A	CT	GA	GC	GC	T	GC	GA	A	GA
RTx 430	2	T	CT	GA	A	CT	GA	GC	GC	T	GC	G	A	GA
RTx 430	7	T			A	CT	GA	GC	GC	T	GC	GA	A	GA
RTx 430	3	T	T	A	A	CT	GA	GC	GC	T	GC	GA	A	GA
Topper 76-6	10	GT	CT	GA	TA	T	GA	GC	GC	T	GC	GA	A	GA
Topper 76-6	3	GT	CT	G	TA	T	GA	GC	GC	T	GC	GA	A	GA
Topper 76-6	2	GT	CT	A	TA	T	GA	GC	GC	T	GC	GA	A	GA

Discussion

Rapid SNP assays are crucial for resolving genetic queries across various disciplines of health sciences to agriculture. KASP markers, as a rapid testing method evaluating one locus at a time, are particularly valuable for single gene diagnoses, marker-assisted selection, and quality control of hybrids in animals or plants (Islam, *et al*., 2018). In medical sciences, KASP assays have been employed for detecting variant nucleotides in pathogen strain testing, while in plant science, they have been utilized for QTL mapping, studying diversity, and germplasm fingerprinting to distinguish cultivars for resolving plant property rights (Islam, *et al*., 2018; Wang, *et al*., 2021). In this study, microspores were used as immature pollen, while their availability as membrane-bound protoplasts facilitated DNA extraction and amplification similar to that of animal cells (Felts, *et al*., 2022). The WGA procedure successfully produced high-quality DNA products, enabling haploid microspore studies, including the assessment of gamete genetic merit. Individual microspore genotyping can identify segregating populations and measure meiotic crossovers in plants (Dreissig, *et al*., 2015). While SNPs are primarily used for diploid plants, their utility has been reported for haploid analysis (Dreissig, *et al*., 2015). Therefore, KASP markers were useful in studying plants at different ploidy levels, especially when cells were individually sorted with a flow cytometer (Tang, *et al*., 2022). Here, a total of 2,400 KASP reactions were performed on individually isolated sorghum microspores, along with 480 control reactions on sorghum leaf tissues.

The novel approach in this study was to score alleles among individually isolated microspores from different sorghum varieties using KASP assays. Leaf-derived diploid DNAs were used for SNP assay validations along with haploid gametophytes. Allele frequencies showed heterozygous SNP profiles among both microspores and parental samples, suggesting allele duplication from the haploid state or DNA polymerase-mediated errors (Poetsch, *et al*., 2020). Some microspore-based KASP amplifications revealed dimorphism for the haploid gametophytes in this study. The presence of dimorphic SNPs across microspore populations could be due to amplification errors made during WGA (Poetsch, *et al*., 2020) through haploid genome copying. However, the use of the WGA procedure was necessary to yield sufficient microspore genomic DNAs for subsequent KASP-based genotyping. An alternative to REPLI-g based WGA could be Ampli-1 WGA from Menarini Silicon Biosystems (Huntingdon Valley, PA), a method used for SNP analysis in haploid cells and prenatal diagnosis (Deleye, *et al*., 2017). Alternatively, the WGA step could be omitted by conducting single-cell DNA sequencing to confirm if such assays still reveal dimorphism. KASP genotyping assays that resulted in the display of double alleles from gametophytes may also be due to nuclei duplication, a natural process of pollen development, reported for diploid and tetraploid plants during the formation of unreduced gametes (Kreiner, *et al*., 2017). Polyploidy tissues are known to be less conducive for KASP-based genotyping than for plants and animals having diploid tissues (Cockram, *et al*., 2012).

KASP reactions for microspores in this study revealed clear heterozygous calls, unlike the barley study where most pollen-based KASP reactions showed homozygous calls (Dreissig, *et al*., 2015). The comparison of plant diploid and haploid DNAs indicated that diploid genetic backgrounds were better for KASP-based assays compared to haploid pollen DNA. However, all KASP-based assays, even when revealing diploid profiles for microspores, were equally useful for grain and sweet sorghum microspores. Among sweet sorghum cultivars, Dasht Local exhibited the highest number of single allele profiles for gametophytes when analyzed with 16 out of 20 SNPs. The assays of sorghum microspores revealed the suitability of certain SNP markers for gametophyte analyses, as indicated by higher variability for detection of mono-allelic states. Thirteen SNP markers (SB01-134, SB02-148, SB02-112, SB03-040, SB04-058, SB06-119, SB06-063, SB07-038, SB07-054, SB08-046, SB09-125, SB09-128, and SB00-169) were then recommended for further genotyping in gametes, with three markers (SB01-134, SB03-040, and SB09-128) exclusively revealing mono-allelic genotypes for microspores of Dasht Local. The development and utilization of SNP markers are essential for genetic studies, and KASP assays are a widely used genotyping method enabling high-throughput analysis in various species. In this study, it was found that most KASP primers designed for S. *bicolor* also amplified the SNP markers from Johnson grass samples, indicating cross-species utility. However, SNP markers specifically developed for Johnson grass have been based on the PCR Allele Competitive Extension platform (Maity, *et al*., 2022).

KASP markers have been found to work well for many agricultural plants and animals (Surbhi, *et al*., 2020), although wheat genomes often have a low KASP success rate due to polyploidy and PCR amplification of homeologous loci (Makhoul, *et al*., 2020) . Low success rates have also been observed when using KASP markers for sheep and goats (Wanjala, *et al*., 2023), due to DNA quality issues, contrasting with the better results obtained using pollen DNA and WGA in this study. For plants, KASP genotyping success rates have been reported to be 78.5 to 88.4% (Cockram, *et al*., 2012), while in animals, these rates were found to be 81 to 94% (Churamani, *et al*., 2019). Compared to animals, DNA extractions from leaf tissues are often challenging due to the presence of rigid cell walls, requiring a mechanical grinding step to disrupt them for DNA release. Here, the KASP-based assays designed for S. *bicolor* were found useful for sorghum microspores as well as for parental leaf DNA genotyping. Overall, the sorghum KASP genotyping system generated an average success rate of 80% for microspores and 91% for leaf DNAs. In conclusion, this study represents the first report on the use of sorghum haploid gametophytes for demonstrating KASP-system-based SNP markers' amplification and segregation. KASP assays have been innovatively used to identify allelic status using microspore samples post-WGA, allowing both methods to be universally used in genotyping haploid gametophytes as per Dreissig, *et al*. (2015). The high levels of allele detection reported here indicate that the newly developed KASP markers can be useful for applications in the breeding of grain and sweet sorghum cultivars. Given their ability to detect alleles in microspores, KASPs can also be used to monitor potential double haploid development in S. *bicolor* for evaluating hybrid production and quality control, something that has been challenging in the past.

Grants to A. N. Aziz and M.W. Blair, as well as funding from the National Institute of Food and Agriculture through a 1890 Capacity Building Grant to A. Aziz.

Acknowledgments

The authors thank LGC staff for marker design and Daniel Demissie for his technical assistance while running the KASP Assays. We acknowledge funding from The United States Department of Agriculture Evans Allen

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Source of support: The United States Department of Agriculture Evans Allen Grants to A. N. Aziz and M.W. Blair, as well as funding from the National Institute of Food and Agriculture through a 1890 Capacity Building Grant to A. Aziz;
Conflict of interest: The authors declare no conflict of interests.

Cite this article as:
Yahya, D.K., Matthew, W. B., Aron, M. F. and Ahmad, N. A. "Individual Microspore Genotyping in Sorghum (*S. bicolor*) Cultivars Using a KASP Panel of Single Nucleotide Polymorphism Markers." *Annals of Plant Sciences.*13.03 (2024): pp. 6199-6211.