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Validation of KASP Markers Associated with Hydrogen Cyanide in Fresh Cassava Roots in Uganda Cassava Germplasm

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Abstract: Low hydrogen cyanide (HCN) concentration is a high-priority trait for cassava varieties targeting their fresh and dry product profiles. To be acceptable, varieties bred and developed for these market segments must meet international safety standards for maximum acceptable residual levels of cyanide in cassava food and food products. The discovery of molecular markers that co-segregate with low HCN has not yet resulted in widespread usage in marker-assisted selection (MAS) in breeding programs. To deploy these HCN markers in regular MAS, assessing their reliability in various genetic backgrounds is crucial. The aim of this study was to assess the predictive accuracy of trait specific markers for HCN. The study used six HCN kompetitive allele-specific polymerase chain reaction (KASP) markers that had previously been developed in a Brazilian population and verified in segregating West African cassava populations. For most markers used in the study, the average call rate was more than 91.7%. Three markers—snpME00404, snpME00405 and snpME00406—showed a significant co-segregation of genotypes with the HCN phenotype. On average, genotypes that carried at least one copy of the favourable allele had lower HCN scores. The proportion of phenotypic variance accounted for by the three most important markers was 14% (snpME00406), 17% (snpME00405) and 27% (snpME00404). The validation of identified HCN SNP-markers marks a significant step in their deployment to support selection and advancement decisions in cassava breeding programs.

Keywords: marker-assisted selection (MAS); hydrogen cyanide (HCN); KASP markers; genetic gain

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1. Introduction

Cassava is a major staple food crop for more than 600 million people in Africa. Originating from South America [1], the crop is said to have been introduced in Africa via the western and eastern coastlines between the 15th and 17th centuries [2]. The crop's tolerance to drought and ability to survive in marginal soils makes it an important food security crop [3]. Mainly grown for its starchy roots, the crop has many uses for food, feed and industry [4–6]. However, the presence of the cyanogenic glucosides (CGs), linamarin and lotaustralin, prevents the crop's full food potential from being exploited [7]. Linamarin, the most abundant of the CGS, is broken down by linamarase to release cyanide (HCN) when

the plant cells are damaged [8]. The formation of free cyanide in the root is prevented by the compartmentalisation of linamarase in the cell wall and linamarin in the vacuole [9].

Dietary exposure to high amounts of HCN can result in acute poisoning and death [10,11]. Moreover, chronic dietary exposure to even low doses of HCN has been associated with the debilitating, irreversible neurological disorders konzo and tropical ataxic neuropathy (TAN) [10,12,13]. The CGs are synthesised in the leaves and translocated to the roots [9]. Depending on the fresh root HCN content, cassava varieties are classified as bitter (HCN > 100 ppm) or sweet (HCN < 100 ppm) [14]. Bitter cassava varieties are not safe for human consumption and must be processed before consumption [15,16]. The World Health Organization recommends an upper limit of 10 ppm for flour and 2 ppm for Gari [15] given that traditional processing methods like milling, washing and fermentation may not successfully reduce HCN content to safe levels [11,16]. Therefore, the most sustainable way to protect cassava consumers from HCN poisoning is through breeding for low HCN accessions. Most cassava grown in Africa is for food consumption. Therefore, consumers greatly emphasise food quality [17] and end-user preferences significantly impact variety adoption [18,19]. In Uganda, cassava consumers prefer non-bitter cassava roots, characterised by low HCN content [6]. In fact, low fresh cassava root HCN content is among the top four must have quality traits for the Ugandan boiled cassava product profile [20].

Cassava has a long breeding cycle, taking up to 8 years to develop a new variety via traditional recurrent selection [21] mainly due to its clonal nature, high heterozygosity, difficulty in making crosses, poor flowering and poor seed set [21–23]. For accelerated genetic gains, breeding programs must deploy modern tools like marker-assisted selection (MAS) [22,24]. A major step in the adoption and application of MAS is the identification of major genes or genomic regions linked to the trait of interest, followed by their validation in independent populations [25,26]. In cassava breeding, the potential of MAS has been successfully demonstrated for cassava mosaic disease resistance, root dry matter content and carotenoids [26–29]. For root HCN content, [30] used an S1 population derived from a cross between the varieties MCOL 1684 and Rayong 1 to identify quantitative trait loci linked to cyanide potential (CNP) and root dry matter content (DMC) and reported two QTL for CNP on linkage groups 10 and 23. Similarly, they found six QTL on four linkage groups controlling the variation in DMC. Later, [31] conducted a genome scan for QTL affecting the cyanogenic potential of cassava roots in a bi-parental population in the environments 2008 Rayong, 2009 Rayong and 2009 Lop Buri in Thailand. They reported five QTLs; CN08R1 for Rayong 2008, CN09R1 and CN09R2 for Rayong 2009 and CN09L1 and CN09L2 for Lop Buri 2009. What was striking is the fact that none of the QTLS were cross cutting for all the environments. More recently, a large-scale genome wide association study (GWAS) using historical data from Brazil characterised the genetic architecture and gene mapping for HCN [32]. They reported two major loci on chromosome 14 and 16 controlling the natural variation in HCN, showing dominance and additive patterns, respectively (Table 1). Furthermore, they developed six kompetitive allele-specific PCR (KASP) markers (Table 2) that could be used in MAS. However, before deploying these markers in Uganda or the East African region, they must first be validated for their efficacy under the prevailing environmental conditions [33]. Thus, this study sought to assess the predictive accuracy of the six KASP markers [32] for HCN in fresh cassava roots using a breeding population developed at the National Crops Resources Research Institute (NaCRRI). The integration of HCN KASP markers will mark a milestone in low HCN variety breeding at NaCRRI as it will facilitate early selection and/or identification of low HCN parents. This will enable the screening of larger populations at increased accuracy, which will accelerate the rate of genetic gain in low HCN cassava variety breeding. Given the recent deaths reported in Uganda due to dietary consumption of HCN from cassava [11,34], breeding for low HCN cassava is a matter of urgency.

Table 1. Genes associated with variation in HCN in fresh cassava roots from a genome wide association study [32].

Chromosome	SNP	Position, bp	Allele	*p*-Value	Gene	Name	Function
16	S16_773999	773999	G/A	7.53×10^{-22}	Manes.16G007900	MATE efflux family protein.	Multi-drug and Toxic Compound Extrusion or Multi-Antimicrobial Extrusion.
16	S16_795990	795990	A/T	2.41×10^{-10}	Manes.16G008000	MATE efflux family protein.	Multi-drug and Toxic Compound Extrusion or Multi-Antimicrobial Extrusion.
16	S16_796041	796041	T/A	1.36×10^{-20}	Manes.16G008100		
14	S14_6050078	6050078	G/A	1.09×10^{-8}	Manes.14G074300	HPP family protein.	Integral membrane HPP family protein involved in Nitrite Transport Activity.
14	S14_5775892	5775892	G/T	1.63×10^{-8}	Manes.14G071000	K03355—anaphase-promoting complex subunit 8 (APC8, CDC23).	Interacting selectively and non-covalently with any protein or protein complex (a complex of two or more proteins that may include other non-protein molecules).
14	S14_6021712	6021712	A/T	7.32×10^{-8}	Manes.14G073900	H(+)-ATPase	The plasma membrane H+-ATPase mediated H+influx may be associated with the plasma membrane gradients as well as Al-induced citrate efflux mediated by a H+-ATPase-coupled MATE co-transport system.

Table 2. KASP markers and their primer sequences as reported by [32].

SNP	Intertek SNP ID	SNPNum	AlleleY	AlleleX	Sequence
S16_773999	snpME00402	140060	A (Low)	G	TTCACTGATGGTGAA[G/A]CTGTTTCCAAAGCA
S16_795990	snpME00403	140061	A (Low)	T	GGCTGCCAAATCTGG[T/A]GGACTAATGACATG
S16_796041	snpME00404	140062	T (Low)	A	TGGATCTCAGCAGCA[A/T]TTTAACCCACTGAT
S14_5775892	snpME00405	140063	G (Low)	T	TTTATCTGCCTGGAC[T/G]CTTATGGGTCATGA
S14_6050078	snpME00406	140064	A	G (Low)	CGGAAAGATGGACCA[G/A]TTACTTGCGCCTAA
S14_6021712	snpME00407	140065	G (Low)	C	TGATTTAGCGAAGAA[C/G]AAAAGCTGCGCGAG

2. Materials and Methods

2.1. Description of the Validation Population

Two populations were used in this study. A pre-breeding population (124 clones) and an advanced breeding population at yield trial stage (64 clones). Where the advanced population was genotyped and phenotyped, the pre-breeding population was only genotyped. The advanced breeding population was drawn from the genomic selection cycle two (C2) clonal population [35]. For the advanced trial, clones were established in field trials in a randomised complete block design across four locations; The National Crops Resources Research Institute (NaCRRI) in central Uganda, the National Semi-arid Resources Research Institute (NaSARRI) located in Serere in eastern Uganda, Abi Zonal Agricultural Research Institute (Abi ZARDI) located in Arua in northern Uganda and Tororo in eastern Uganda for one growing season. For the pre-breeding trial, clones were established in the field at NaCRRI in an augmented design.

2.2. Phenotyping

At harvest (12 months after planting), the three middle rows of each plot were uprooted, and all roots were pooled together. Three uniformly sized roots were then selected and taken to the laboratory for analysis. At the laboratory, roots were washed under running water to remove soil and debris and then dried with a towel. For each root, the distal and proximal portions were sliced off with a kitchen knife and discarded, leaving a 10-centimetre middle portion. Data on fresh root HCN content were collected using the picrate method [36] as described by [37]. Briefly, a cross-sectional sample (1 cm^3) was taken between the peel and the center of the parenchyma. The cut tube and five drops of toluene were added to a glass tube, and the tube was tightly sealed with a stopper. A qualitative score of the HCN was determined based on a one to nine scale with one and nine representing extremes of low and high HCN, respectively. To achieve this, a strip of Whatman filter paper (1 cm × 3 cm) was dipped into a freshly prepared picrate solution

(picric acid (0.5% *w*/*v*) in 2.5% (*w*/*v*) sodium carbonate) until saturation, allowing the paper to dry at room temperature. The saturated paper was then placed above the cut root tube in a glass tube and tightly sealed for 16–24 h before reading the colour intensity.

2.3. HCN Marker Discovery and Genotyping

The KASP assay is a robust, high throughput and cost-effective polymerase chain reaction (PCR) based marker technology [38]. In addition, the KASP data are easier to interpret as they do not require complex bioinformatics pipelines and strong computational resources to interpret, making them ideal for resource limited breeding programs. The markers validated in this study were derived from [32]. A large-scale genome wide association study (GWAS) was conducted using a population of 3354 accessions, which consisted of landraces and breeding lines from 26 Brazilian states. Of the 3354 accessions, 1389 had been phenotyped for HCN across multi-year trials. The population was genotyped following genotyping by sequencing (GBS) and phenotyped for HCN, according to [37]. Two major loci were identified on chromosomes 14 and 16, encoding for ATPase and a MATE protein, contributing up to 7 and 30% of the HCN concentration in roots. The markers tagged the major peak on chromosome 14; S14_5775892, S14_6050078 and S14_6070331 and chromosome 16; S16_773999, S16_796041 and S16_800090. A summary of the genes on chromosomes 14 and 16 associated with variation in HCN in Brazilian germplasm extracted from [32] is presented in Table 1.

Furthermore, [32] used a KASP assay to develop diagnostic markers for HCN based on association peaks, local linkage disequilibrium (LD) and allelic effects. A summary of the kompetitive allele-specific PCR markers and their primer sequences is presented in Table 2.

2.4. Genotyping

Leaf samples were collected from vigorously growing plants 3 months after planting from the trial in NaCRRI. From each plant, four leaf discs measuring 5 mm were picked from young, tender leaves, put into a 96-well genotyping plate, dried and preserved over silica gel. For quality control, two wells were left blank (non-template controls). The samples were shipped to Intertek Australia for genotyping with the KASP assay using six KASP markers (Table 2). This is the first set of KASP markers for HCN available to the global cassava breeding community. The protocol for genotyping with the KASP assay is detailed in the KASP manual. Briefly, the KASP genotyping assay is based on competitive allele-specific PCR and enables bi-allelic scoring of SNPs and insertions and deletions (indels) at specific loci. A SNP assay mix consisting of two allele-specific forward primers and one common reverse primer, and a universal KASP Master mix consisting of universal fluorescent resonant energy transfer (FRET) cassettes, ROX^{TM} passive reference dye, free nucleotides, and $MgCl_2$ in an optimised buffer were used. Each of the allele-specific primers used had a unique tail sequence corresponding to a universal FRET cassette, one labelled with a FAM^{TM} and the other with a HEX^{TM} dye that were added to the DNA template. During cycling, the relevant allele-specific primer was bound to the DNA template and elongated, thus attaching the tail sequence to the newly synthesised strand. The compliment of the allele-specific tail sequence was then generated in subsequent PCR rounds, enabling the FRET cassette to bind to DNA. Bi-allelic discrimination was achieved by competitive binding of the 2-allele-specific forward primers. If the genotype at a given locus was homozygous (either homozygous for high HCN or low HCN content), one fluorescent signal was generated. A mixed signal was generated if the genotype was heterozygous. A non-template control (NTC) was included for quality control. The NTC contained all the reagents except the sample DNA. The NTC helps to identify potential contamination or non-specific amplification because it does not generate a fluorescence signal. The signals were converted into allele calls using KRAKEN^{TM} software v2.1.3. Once the SNP calls were plotted on a cartesian plot, the NTC was expected to plot close to zero, giving assurance that observed signals were due to amplification of the DNA sample.

2.5. Marker Robustness, Segregation Ability and Marker Effects

The SNP's call rate and clarity were used as technical metrics to assess their robustness in identifying useful marker–trait relationships. In computing frequency of genotypes per marker, genotype calls returned as "unused", "?" or "uncalled" were all coded as NA. After filtering out all clones that had genotype but no phenotype data, 62 clones were retained and used in the downstream analyses. Marker effects were evaluated by regressing marker genotypes on to corresponding phenotypes to estimate the amount of phenotypic variance accounted for by each marker. The *lm* function in R (version 4.4.1) was used with marker genotypes and their corresponding phenotypes treated as independent and response variables, respectively. Furthermore, we conducted the Kruskal–Wallis test to identify marker genotypes that were associated with significant differences in HCN phenotype and visualised the results on boxplots using the *ggpubr* package in R. We used a confusion matrix to further evaluate the predictive ability of the markers using the metrics; accuracy, false positive rate (FPR) and false negative rate (FNR). Accuracy denotes the proportion of correctly predicted clones as low or high in HCN. On the other hand, false positive rate is the proportion of high HCN samples that are wrongly predicted as low HCN while false negative rate is the proportion of low HCN clones that are wrongly predicted as high HCN. Positive (low HCN) clones were those with HCN less than six while negative (high HCN) clones were those scoring six and above on a one to nine scale [39]. The metrics were computed according to the following formula:

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN}$$

$$FPR = \frac{FP}{FP + TN}$$

$$FNR = \frac{FN}{FN + TP}$$

where TP is true positive, TN is true negative, FP is false positive and FN is false negative.

The lmer function of R statistical package was used to fit the mixed linear model

$$y = X\beta + Zu + \varepsilon$$

where y is the response vector of HCN, β is the vector of fixed effects and u is the vector of random genetic effects with design matrix Z (relating trait values to genotype, environment and genotype by environment interaction) and ε is the error (residual). Variance components were then extracted and used for the estimation of broad sense heritability (H^2).

Within locations (single trial), broad sense heritability was estimated as

$$H^2 = \frac{V_g}{V_g + V_{ge} + V_e}$$

where V_g is the genotypic variance, V_e is the error (residual) variance and V_{ge} is variance due to genotype by environment interaction.

3. Results

3.1. Phenotypic Variation for HCN

The cyanide (HCN) scores in fresh cassava roots ranged from 2.5 to 7.5 with an overall mean of 5.4 and standard deviation of 1.5. The lowest HCN score was recorded in the clone UG16F293P066 while the highest was recorded in the bitter landraces Tongolo and Quinine and the breeding lines UG16F290P332, UG16F290P047, UG16F303P009 and UG16F318P035 (Figure 1). The distribution of HCN scores was bi-modal with peaks at 4 and 6.5. The first peak was associated with low HCN clones while the second peak was associated with high HCN clones. The distribution of HCN was skewed at the low HCN scores as indicated by the long tail on the left of the distribution curve.

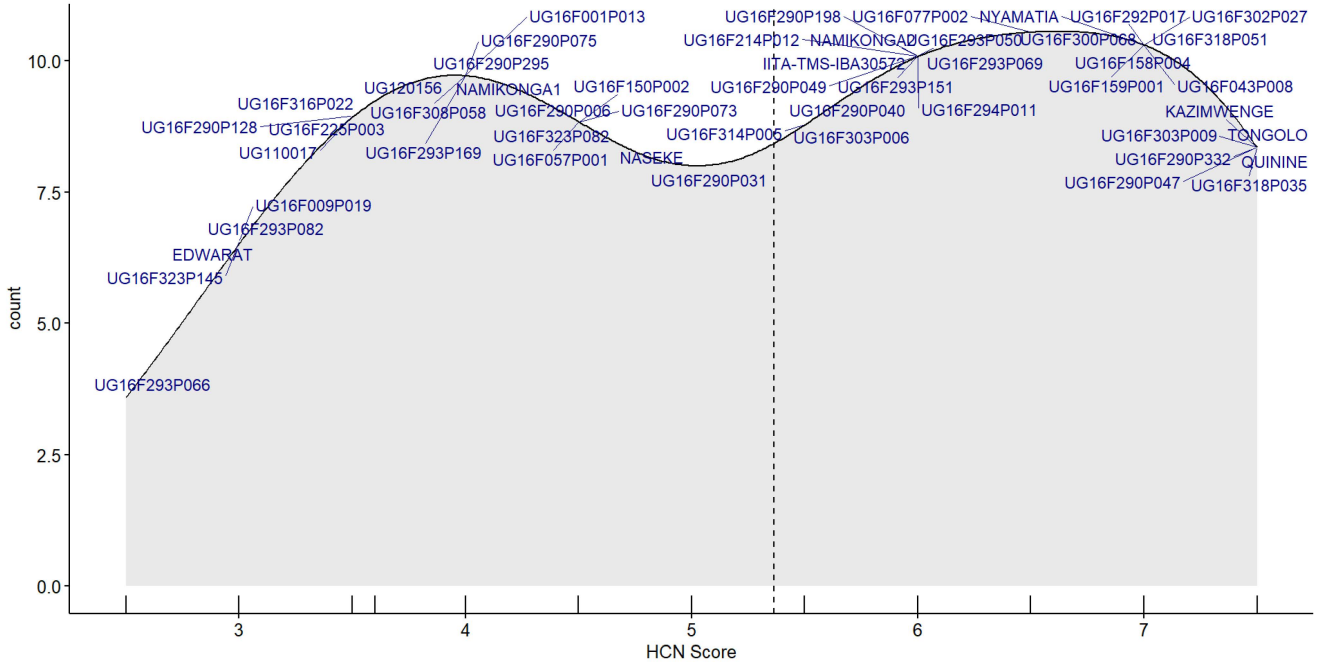

Figure 1. Density distribution of fresh cassava root HCN content based on plot mean from four locations. The x-axis shows the HCN scores on a one to nine scale, while the y-axis shows that data points are distributed over the range of HCN scores. The dotted vertical line shows the mean HCN score of the population while the peaks indicate a higher density of observations at the corresponding HCN score.

Analysis of variance revealed highly significant differences ($p < 0.0001$) among clones and among locations. The clone by environment interaction (Clone:Location) was also significant ($p < 0.05$) (Table 3). The genotypic component of the variation (V_g) was 0.49, variance due to the clone by environment interaction (V_{ge}) was 0.35, while residual variance (V_e) was 1.05. Broad sense heritability was 0.26 (Table 3).

Table 3. Analysis of variance, partitioning of variance components and heritability of phenotypic data from 62 clones grown across four locations.

SOV	DF	MS
Clone	61	5.20 ***
Location	3	127.55 ***
Block	1	
Clone:Location	149	1.57 *
Residuals	144	
V_g	0.49	
V_{ge}	0.35	
V_e	1.05	
H^2	0.26	

*** = significantly different at alpha = 0.0001, * = significantly different alpha = 0.05. SOV = source of variation, DF = degrees of freedom, MS = mean square, H^2 = broad sense heritability.

3.2. Performance of HCN KASP Markers

SNP Call Rate and Frequency of Marker Genotypes

The SNP call rate ranged from 73.4% to 100% with a mean of 91.7%. The marker snpME00405 had the lowest call rate (73.4%) followed by marker snpME00404 (77.4%). The marker snpME00402 had a call rate of 98.9%, while snpME00403 and snpME00406 had a call rate of 100% (Figure 2). For markers snpME00404, snpME00405 and snpME00407, genotype calls reading "unused", "uncallable" or "?" were all coded as NA. The percentage of NA

genotypes was 1% for marker snpME00407, 27% for snpME00405 and 28% for snpME00406. Minor allele frequency (MAF) was low for snpME00403 (0.02) and snpME00404 (0.03), while snpME00402 had an MAF of 0.05. The other three SNPs all had an MAF > 0.05. The percentage of favourable (low HCN) alleles was lowest in snpME00405 (23%), followed by snpME00406 (29%). Marker snpME00403 had the highest percentage of favourable alleles (96%). The percentage of heterozygous genotypes ranged from 0% in snpME00403 to 43% in snpME00406, while the percentage of the unfavourable alleles ranged from 4% in snpME00404 to 29% in snpME00406 (Figure 2).

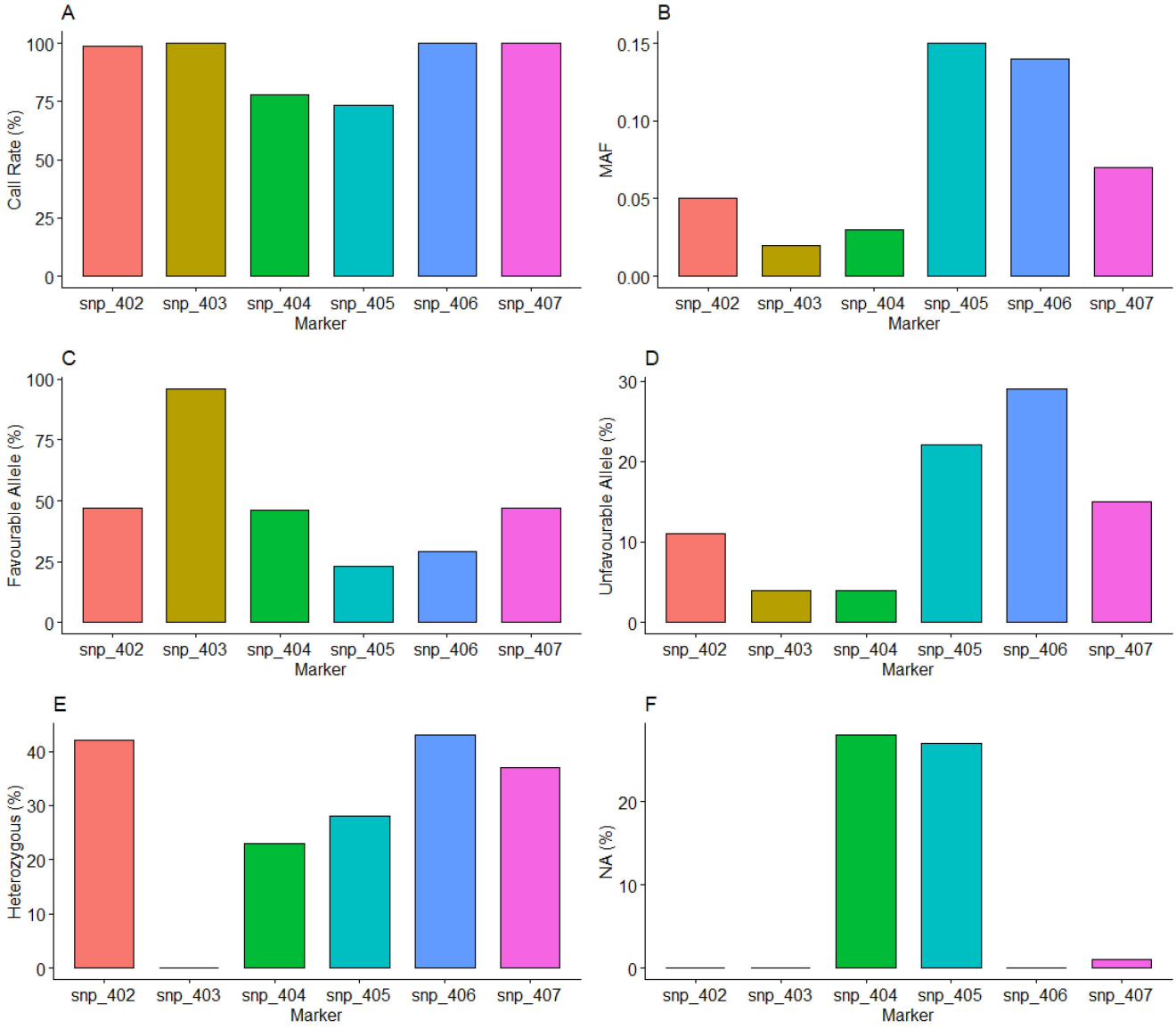

Figure 2. Call rate, minor allele frequency (MAF) and frequency of marker genotypes for six KASP markers segregating for HCN in a Ugandan breeding population. Variation in SNP call rate is shown in (**A**), minor allele frequency (**B**), percentage of favourable alleles (**C**), percentage of unfavourable alleles (**D**), percentage of heterozygous alleles (**E**) and percentage of non-callable genotypes (NA) (**F**). snp_402 = snpME00402, snp_403 = snpME00403, snp_404 = snpME00404, snp_405 = snpME00405, snp_406 = snpME00406, snp_407 = snpME00407, % = percentage.

With the exception snpME00403, all the other markers were polymorphic, showing three distinct clusters which corresponded to different genotypes. Clones homozygous for the X allele clustered close to the x-axis while those homozygous for the Y allele clustered close to the y-axis. Heterozygous genotypes constituted the middle cluster for

each marker (Figure 3). For snpME00403, only four clones were homozygous for the alternate allele (T:T). The rest of the clones were homozygous for the favourable (low HCN) allele (A:A). However, two distinct clusters are visible for clones homozygous for the Y allele (Figure 3). Generally, clusters close to the x-axis represent genotypes with FAM type allele (homozygotes), clusters close to the y-axis represent genotypes with HEX type allele (homozygotes), while clusters in the middle of each plot represent the heterozygotes. The blue dots at the bottom corners of the plots represent the non-template controls (NTC) (Figure 3).

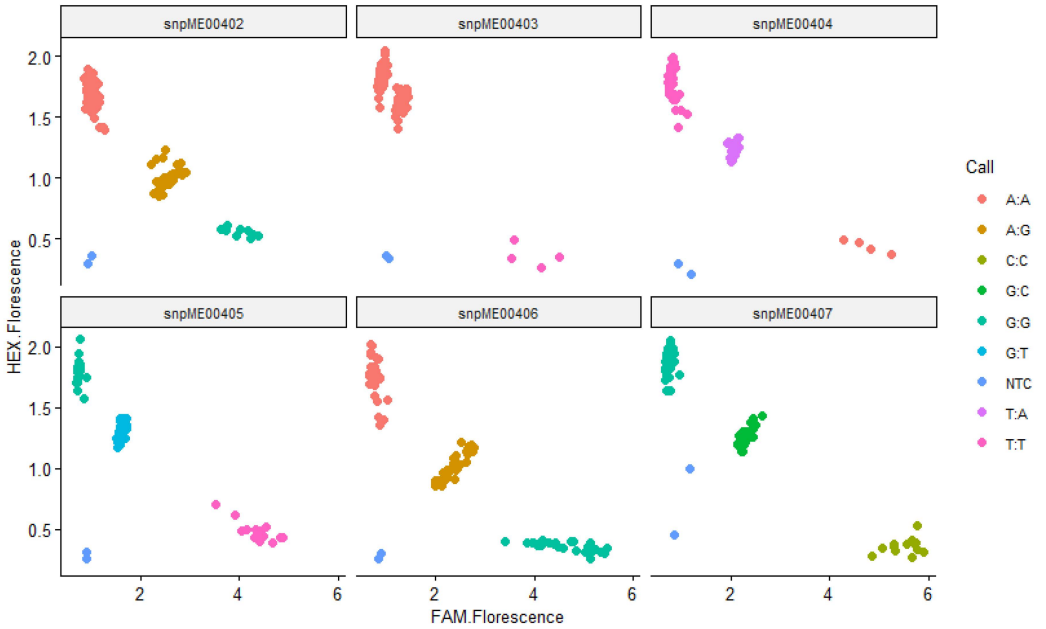

Figure 3. Scatter plot of KASP assays showing clustering of genotypes with FAM florescence (*x*-axis) and HEX florescence (*y*-axis).

3.3. Biological Validation

Only three marker genotypes showed significant association with the phenotype. The strongest phenotype–genotype association was between the marker ME00404 ($p < 0.001$) followed by ME00405 and ME00406 ($p < 0.05$). However, the proportion of phenotypic variance explained by these markers was small, ranging from 27% (snpME00404) to 17% (snpME00405) and 14% (snpME00406). The other markers each accounted for less than 1% of the observed phenotypic variance (Table 4).

Table 4. Regression coefficients for six kompetitive allele-specific PCR markers evaluated for HCN in Ugandan cassava germplasm.

Marker	Degrees of Freedom	Mean Square	R^2
snpME00402	2	1.024 ^{ns}	0.09
snpME00403	2	1.225 ^{ns}	0.05
snpME00404	2	2.39 **	0.27
snpME00405	2	1.475 *	0.17
snpME00406	2	1.587 *	0.14
snpME00407	2	0.439 ^{ns}	0.04

* = significantly different at alpha = 0.05, ** = significantly different at alpha = 0.001, ns = not significant, R^2 = coefficient of determination.

3.4. Marker Effects on Fresh Cassava Root HCN Levels

Three markers showed significant co-segregation with the HCN phenotype. For marker ME00404 (T/A), the favourable allele (T) was associated with low HCN. The

homozygous genotype (T:T) was associated with the lowest HCN content and was significantly different ($p < 0.05$) from the heterozygote (T:A), which was associated with higher HCN content; the homozygous genotype (A:A) was associated with high HCN. However, the A:A genotype (high HCN) was not significantly different from the heterozygous genotype (Figure 4). For ME00405 (G/T), the favourable allele (G) was associated with a low HCN phenotype and was significantly different ($p < 0.01$) from the heterozygous (G:T) and the homozygous (TT) phenotypes. The T allele was associated with an increase in the HCN phenotype; although, there was no significant difference between the heterozygous (G:T) and the homozygous genotype (T:T). A similar trend was observed for snpME00406, where the favourable allele (G) in the homozygous state was associated with a low HCN phenotype and was significantly different ($p < 0.01$) from the heterozygous (G:A) and homozygous phenotype (A:A). Allele A was associated with increased HCN levels but there was no significant difference between the heterozygous (G:A) and homozygous (A:A) genotype. For snpME00402 and snpME00407, there were no significant differences in association of genotypes with HCN phenotypes (Figure 4).

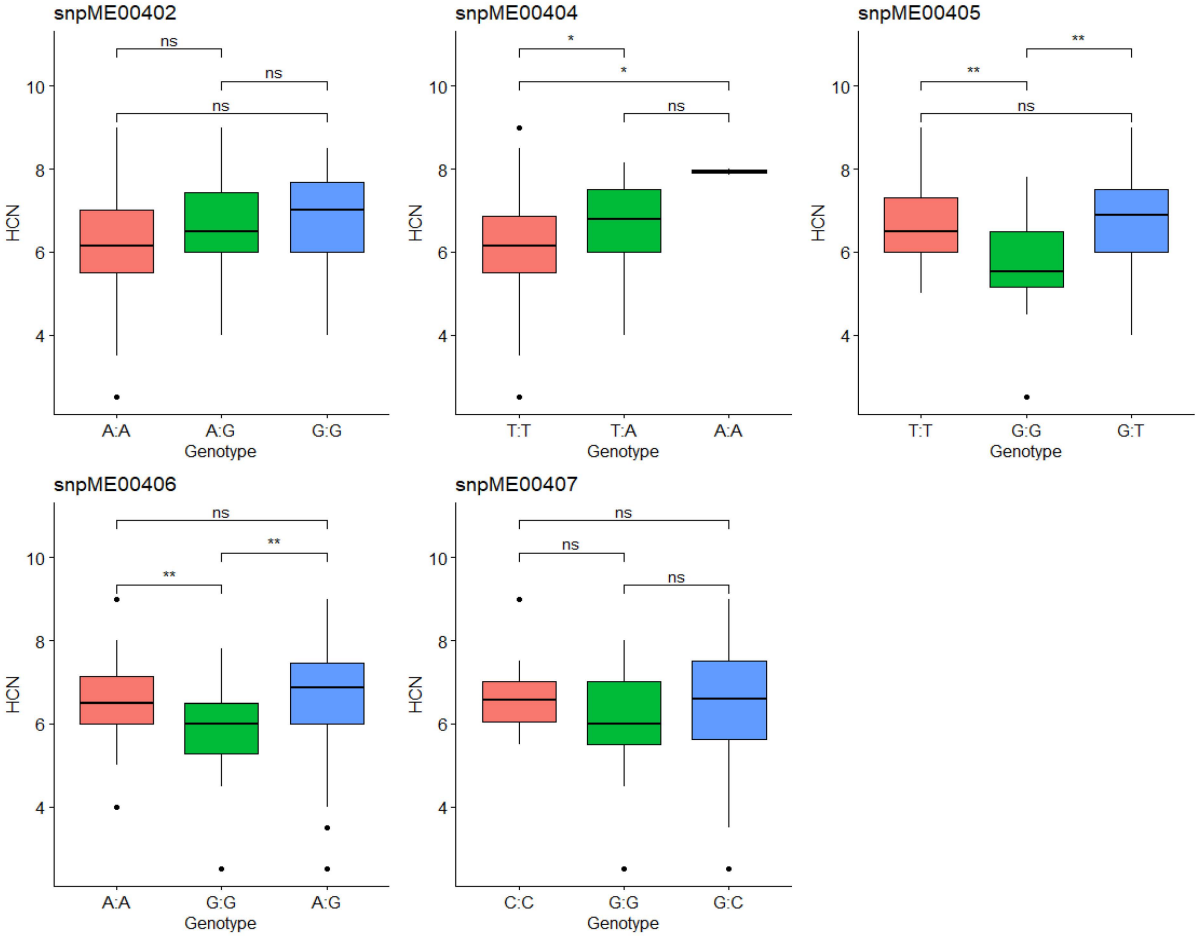

Figure 4. Box plots showing the co-segregation of five KASP markers with fresh cassava root HCN content. ** = significant at $p < 0.01$, * = significant at $p < 0.05$, ns = not significant.

For the three markers with significant segregation for the HCN phenotype, we computed a confusion matrix. Generally, the accuracy of the markers was low to moderate. Marker snpME00404 had the highest accuracy (73%) and lowest misclassification rate (27%). This was followed by snME00406 with an accuracy of 65% and a misclassification rate of 35%. snpME00405 had the least accuracy (59%) and highest misclassification rate (41%) (Table 4). However, despite having the highest accuracy, snpME00404 had the highest false positive rate (FPR) at 47%. snpME00405 and snpME00406 had lower FPR at 26% and 25%,

respectively. The false negative rate (FNR) was highest for snpME00405 at 56%, distantly followed by snME00404 (6%) and snpME00406 (5%) (Table 5).

Table 5. Confusion matrix showing predictive ability for three markers with genotypes significantly co-segregating for the HCN phenotype.

		Count					
Marker		**Low**	**High**	**Total**	**FPR (%)**	**FNR (%)**	**Accuracy (%)**
snpME00404	Low	17	9	26	47	6	73
	High	1	10	11			
	Total	18	19	37			
snpME00405	Low	8	5	13	26	56	59
	High	10	14	24			
	Total	18	19	37			
snpME00406	Low	13	5	18	25	5	65
	High	13	20	33			
	Total	26	25	51			

FPR = false positive rate, FNR = false negative rate, % = percentage.

4. Discussion

For breeding programs to accelerate genetic gains, they must optimise their operations according to the breeder's equation. This involves either increasing the selection intensity (*i*) or accuracy (*r*) or reducing the cycle time (L) [40,41]. Marker-assisted selection (MAS) offers an opportunity for fast tracking genetic gains by increasing efficiency of selection as selection decisions are based on genotype, which is effective for traits that are expensive and/or difficult or time consuming to the phenotype [42]. Furthermore, MAS can reduce cycle time by facilitating early selection and identification of parents, especially for traits that are usually phenotyped late in the crop growth cycle and can increase selection intensity as breeders are able to screen large populations [43]. The potential of MAS to transform cassava breeding was demonstrated by [44] when they conducted marker-assisted introgression of resistance to cassava mosaic disease (CMD) into Latin American germplasm for the genetic improvement of Nigerian cassava germplasm. First, a population of over 20,000 seedlings introduced between 1990 and 1994 was of no use as they succumbed to CMD despite their high yield potential. However, a second population consisting of invitro cultures where the dominant *CMD2* resistance gene was introgressed survived under Nigerian field conditions. This gave the country hope of producing 14 high-yielding, CMD resistance breeding lines. However, to date, no African cassava breeding program has successfully deployed MAS for routine CMD resistance breeding. Recent efforts are focused on the validation and deployment of KASP markers for CMD resistance breeding in African cassava populations. Other traits of focus include root dry matter content and total carotenoids [26,28,29,45]. The deployment of molecular markers is essential for enhancing the rate of genetic gain in cassava breeding.

However, before markers can be deployed for routine selection, they must first be validated in a population different from the one they were developed in. Markers developed in one population may not be transferable to another because of lack of polymorphism or the absence of marker–trait association [46]. Thus, marker validation involves technical validation to assess the robustness of the marker assay with respect to genotype call rate and clarity of the genotype classes. Furthermore, biological validation should be performed to assess the accuracy of markers in predicting the phenotype [33] and is key for the success of MAS [33,40]. Thus, the objective of this study was to validate six KASP markers for their predictive ability for HCN in a Ugandan breeding population.

The intrinsic difficulty in HCN phenotyping, the phenotypic variability observed in the population, coupled with the fact that HCN can only be reliably phenotyped on physiologically mature roots justifies the use of molecular markers. The KASP assay

is a robust, high throughput and cost-effective PCR-based marker technology [47,48]. The population used had variability for HCN (Figure 1), so it was suitable for the study. Moreover, the field trials were established across four geographical locations, which enabled capture of the effect of environmental variability on HCN phenotypic expression [7,14]. The geographical locations NaCRRI and Tororo are associated with lower temperatures and low HCN phenotypes while NaSARRI and Abi ZARDI are associated with high temperatures and high HCN phenotypes. This phenotypic plasticity of HCN across test environments in Uganda [49] makes it absolutely important to capture multi-location data for biological marker validation because the Ugandan cassava breeding program seeks to develop varieties that are widely adapted across the country. Otherwise, a marker significant in one environment may fail to show significance in another [31]. All the markers had clear calls with three distinct clusters, indicating that they were polymorphic. However, marker snpME00403 formed two large clusters, homozygous for the Y allele; thus, it was not polymorphic. It also had a low MAF, less than 0.05, and was not used in other analyses. The snpME00404 and snpME00405 markers had low call rates given the high percentage of NA calls, a major indictment on their quality in this study. However, this could be due the concentration of DNA used in running the KASP assay. Some of the HCN KASP markers require a lower concentration of DNA to achieve better call rates [32].

Markers snpME00404 (S16_800090), snpME00405 (S14_5775892) and snpME00406 (S16_6050078) showed co-segregation patterns with the HCN phenotype. Homozygotes with alternative alleles for both loci showed higher HCN content than heterozygotes whereas homozygotes with reference alleles showed lower HCN content. This is consistent with observations made by [32] for marker S14_6050078 (snpME00406). Marker snpME00404 showed a dominant gene effect as genotypes with at least one copy of the favourable allele had lower HCN (Figure 4). Cyanogen-free (acyanogenic) cassava plants are thus expected to be homozygous although these have only been produced through genetic transformation [50]. Marker S14_6050078 is located on gene Manes.14G074300, which codes for the integral membrane HPP family protein involved in nitrite transport activity [51]. On the other hand, S14_577892 (snpME00405) is located on gene Manes.14G081700, which codes for an aquaporin protein, a family of integral membrane proteins that facilitates the transport of water and other solutes across cell membranes [52]. Marker S16_80090 (snpME00404) is located on Manes.16G00790, and codes for a multi-drug and toxic compound extrusion (MATE) protein, which is involved in the transportation of substances across membranes [53]. The role of these genes in transportation supports their association with cassava root HCN content given that HCN is manufactured in the leaves and transported to the roots [9].

Despite having the most significant association with the phenotype from the GWA study [32] and despite showing a co-segregation pattern consistent with marker snpME00402 (S16_773999)—where clones with two copies of the favourable allele (A:A) had low HCN and those with at least one copy of the alternative allele (A:G) had higher HCN and those with two copies of the alternative allele had the highest HCN—the segregation of marker genotypes with phenotypes was not statistically significant at the 5% alpha level and the marker explained less than 1% of the variation. This could be due to the phenotypic plasticity of HCN since the phenotypic expression of the trait is affected by changes in soil chemical composition and environmental conditions [7,14,49,54] as demonstrated by [31] who conducted a genome scan for QTL affecting cyanogenic potential (CNP) of cassava roots and reported five QTLs from three different environments, with no QTL cutting across all the environments. Furthermore, this discrepancy might be due to epistatic interactions on chromosome 16. Previously, analysis of inter chromosomal epistasis interaction on chromosome 16 revealed 242 significant interactions with three of them being separated by only 1 Mb between each SNP pair [32]. Epistasis denotes a gene interaction in which the expression of one gene is modified by another gene or genes. This could take the form of the gene being masked, inhibited or suppressed by the expression of another gene or genes thus not producing the desired effect. Due to epistatic interactions, markers linked

to HCN content and other traits may not consistently predict the traits across genetic backgrounds [42]. The lack of strong phenotype–genotype association renders the marker snpME00402 unsuitable for MAS in the genetic background studied.

In this study, accuracy was the proportion of low HCN clones correctly predicted as low HCN by the markers and high HCN clones correctly predicted as high HCN. The false positive rate (FPR) was the proportion of high HCN clones wrongly predicted by the markers as low HCN while the false negative rate (FNR) was the proportion of low HCN clones that were wrongly predicted by the markers as being high HCN clones. From the confusion matrix, the predictive accuracy of the markers was moderate where snpME00404 displayed 73% prediction accuracy but had a high false negative rate (FNR) (47%). This implies that 47% of the samples predicted as having low HCN content actually had high HCN content. This marker better predicts high HCN content samples given its low false negative rate (6%). The markers snpME00405 and snpME00406 had lower FNR at 26% and 25%, respectively, and thus predicted lower HCN content samples better. The wide variability in the predictive ability of the markers suggests a case-by-case application of the markers or their deployment in combination. Markers snpME00404 and snpME00405 have a low false negative rate of 5% and 6%, respectively, implying that they identify high HCN clones with high accuracy. Additionally, marker snpME00406 has a low false positive rate (25%), implying that it correctly identifies low HCN clones 75% of the time. Used in combination, these two markers can identify the worst performers (high HCN clones) especially in large early-stage trials for the breeder to select against them. However, the large proportion of variation not explained by the HCN markers indicates the need for furthering efforts to identify markers that explain more variation within Ugandan populations. The failure to obtain strong genotype–phenotype association for some KASP markers has recently been reported in cassava breeding [29,45]). To overcome challenges posed by MAS in cassava, ref. [55] suggested the use of MAS alongside genomic selection. Where MAS relies on a few HCN trait-specific markers, genomic selection uses genome wide markers to capture both additive and non-additive effects, potentially improving the prediction accuracy and subsequently accelerating the rate of genetic gain.

5. Conclusions

For the MAS of HCN in Ugandan cassava populations, the markers snpME00404 and snpME00406 can be used in combination to identify high HCN clones and select against them in early-stage breeding trials. Taken collectively, this study sheds light on the prospects of deploying MAS for the early selection of low HCN accessions. However, we recommend that further validation studies be conducted using the three markers snpME00404, snpME00405 and snpME00406 on diverse breeding trials at different stages of the breeding cycle. Furthermore, given the moderate to low predictive abilities of the markers, genome wide association studies (GWAS) should be conducted to explore the prospects of finding markers that account for more variation for HCN within Ugandan cassava populations.

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