SNP GENOTYPING: THE KASP ASSAY IN *THEOBROMA CACAO*

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ABSTRACT - *The validation of a marker is designing an assay based on the discovered polymorphism and then genotyping a panel of diverse germplasm and segregating the population. The ultimate goal of the study was to develop a reliable, rapid, and inexpensive polymerase chain reaction (PCR)-based method to genotype SNPs previously associated with desirable phenotypes in cocoa. The KASP genotyping assay utilizes a unique form of competitive allele-specific PCR combined with a novel, homogeneous, fluorescence-based reporting system. It is used to identify and measure genetic variation occurring at the nucleotide level to detect single nucleotide polymorphisms (SNPs) or inserts and deletions (InDels). The KASP technology is suitable for use on various equipment platforms and provides flexibility regarding the number of SNPs and the number of samples to be analyzed. Three hundred eighty-four cocoa leaves were collected from Bagan Datuk, Tawau, Madai, PPBK, and KKIP. These samples were collected in three replicates and to be tested with a total of 75 SNPs were used for the KASP assays. These final SNPs were selected for various traits such as; resistance to CPB, VSD and Black Pod, high yielding cocoa, and high cocoa butter. The KASP assays developed in this study were efficient and versatile for determining different traits studied. On average, the method is more straightforward than other methods, fast, and reproducible.*

Key words: Assay, SNPs, cocoa, traits, KASP

INTRODUCTION

Plant breeders usually screen many plants for traits of economic value as determined by the breeding goal, which may include breeding for resistance, bio-fortification to increase some micronutrients, and gene pyramiding (Pérez, 2012). The larger volume of plants screened at the early stages of a breeding program can be laborious, capital intensive, and time-consuming. Germplasm screening is usually an initial step for many breeding programs (Bali,2018). The aim of screening an extensive collection of plants is to narrow those with desired characteristics for advancement to the next stage. It is essential to get it right to meet breeding objectives for crop improvement.

Molecular breeding in recent years has brought about a revolution in plant breeding and has been widely applied in many plant improvement programs (Mochida,2010). Numbers of molecular markers have been developed and successfully used in selection due to their association with a phenotype of interest in various plant species. In conclusion, they have been widely used in plant germplasm screening for desirable traits (Bundock, 2005). As a continuation of the previous project, five traits for cocoa were finalized: resistance to CPB, VSD and Black Pod, high yielding cocoa, and high cocoa butter. Several processes and methods for identifying which SNPs related to which traits have been performed. Finally, KASP genotyping assays were used as a chosen method for SNPs-related markers in cocoa.

KASP Assays

Kompetitive Allele-Specific PCR (KASP) assays are fast and robust to genotype SNPs of interest (Graves,2016). These PCR-based assays use two forward primers in the same reaction: each primer's final (3') base is complementary to one of the alleles at the SNP locus. Assay conditions encourage allele-specific primer binding, so amplification only occurs for the SNP allele present in the template DNA.

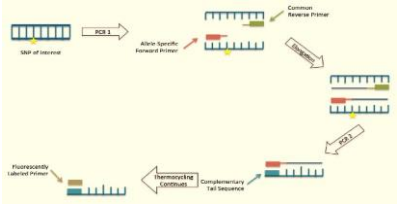


Figure 1: Schematic drawing of the KASP method By JPL - Own work, CC BY-SA 3.0

Amplification is detected as fluorescence, where each SNP allele is associated with unquenching of either the FAM or HEX fluorophore by co-amplifying a tag sequence at the 5' end of each forward primer (Alvarez-Fernandez,2021). The number of PCR cycles required to achieve maximal separation between HEX and FAM fluorescence signals varies depending on genomic DNA concentration and purity.

The appropriate cycle can be easily selected by real-time PCR assays, making the assay highly flexible. The KASP chemistry functions equally well in 96-, 384-, and 1,536-well microtiter plate formats and has been utilized over many years in large and small laboratories by users across the fields of human, animal, and plant genetics.

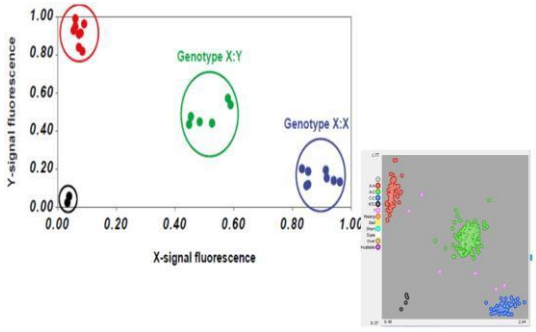


Figure 2: Cluster plot for KASP genotyping assays.

MATERIALS AND METHODS

Plant Materials and Phenotypic Evaluation

Three hundred eighty-four cocoa leaves were collected from Bagan Datuk, Tawau, Madai, PPBK, and KKIP. Each sample will be tested with 75 SNPs finaled from previous works on SNPs identification and validation. Some of the cocoa leaves collected were blind (with known cocoa clones but unknown phenotypic evaluations).

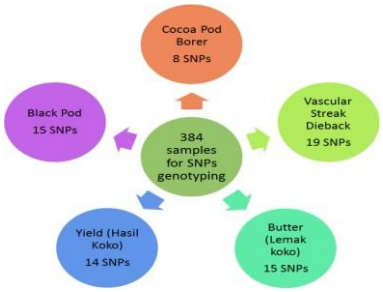


Figure 3: SNP listing used for KASP genotyping assays

Samples of cocoa leaves were prepared using leaves collection samples as instructed in the BioArk Leaf sample collection kit. The BioArk Leaf kits combine DNA extraction with all-inclusive extraction and genotyping services the manufacturer provides.

The DNA concentration for KASP genotyping

The minimum final DNA concentration LGC recommends in KASP genotyping reactions is 2.5 ng / µL. KASP genotyping reactions consist of a universal KASP Master mix, SNP-specific KASP Assay mix, and DNA template. The reactions can be prepared using;

Table 1. The constituent volumes of each component for both 10 and µL reaction volumes per reaction.

KASP genotyping reaction assembly	
Component	10 µL reaction (96-well plate)
DNA*	n/a*
KASP Master mix (2X)	5 µL
KASP Assay mix (72X)	0.14 µL
Water	4.86 µL

Table 2. The standard KASP thermal cycling conditions

Protocol Stage	Temperature	Duration	Number of cycles for each stage
Stage 1 Hot-start Taq activation	94ºC	15 minutes	x 1 cycle
Stage 2 Touchdown	94ºC	20 seconds	x 10 cycle
	61ºC	60 seconds	
Stage 3 Amplification	94ºC	20 seconds	x 26 cycle
	55ºC	60 seconds	
Stage 4 Read stage for qPCR only	30ºC	60 seconds	x 1 cycle

RESULTS AND DISCUSSIONS

All DNA samples were screened with KASP assays for all the traits. A template spreadsheet was designed to import data from multiple plates and SNP assays and automatically assign genotypes. The screening assays that used crude DNA were more variable than high-quality DNA: some samples failed to amplify, which might be due to the collected degraded leaves. Nevertheless, it was possible to assign genotypes to all samples that did amplify. Due to low DNA concentration, problems with sample amplification occurred in only a small number of samples; there is no need for repetition with other DNA extraction.

Table 3: summary of the KASP analysis on SNPs Viewer 2 version 4.0.0

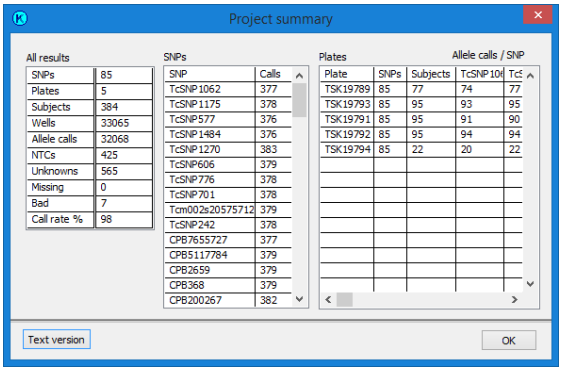
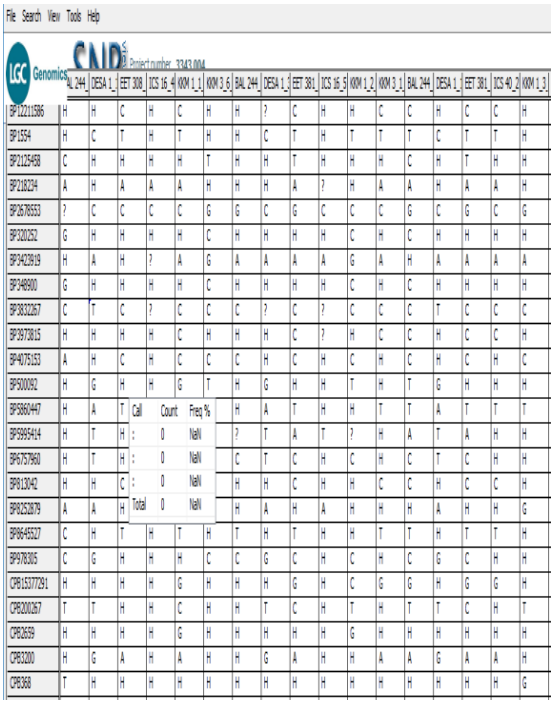


Table 4: Spreadsheet of KASP analysis on each SNP and calling Cluster plots for KASP genotyping.



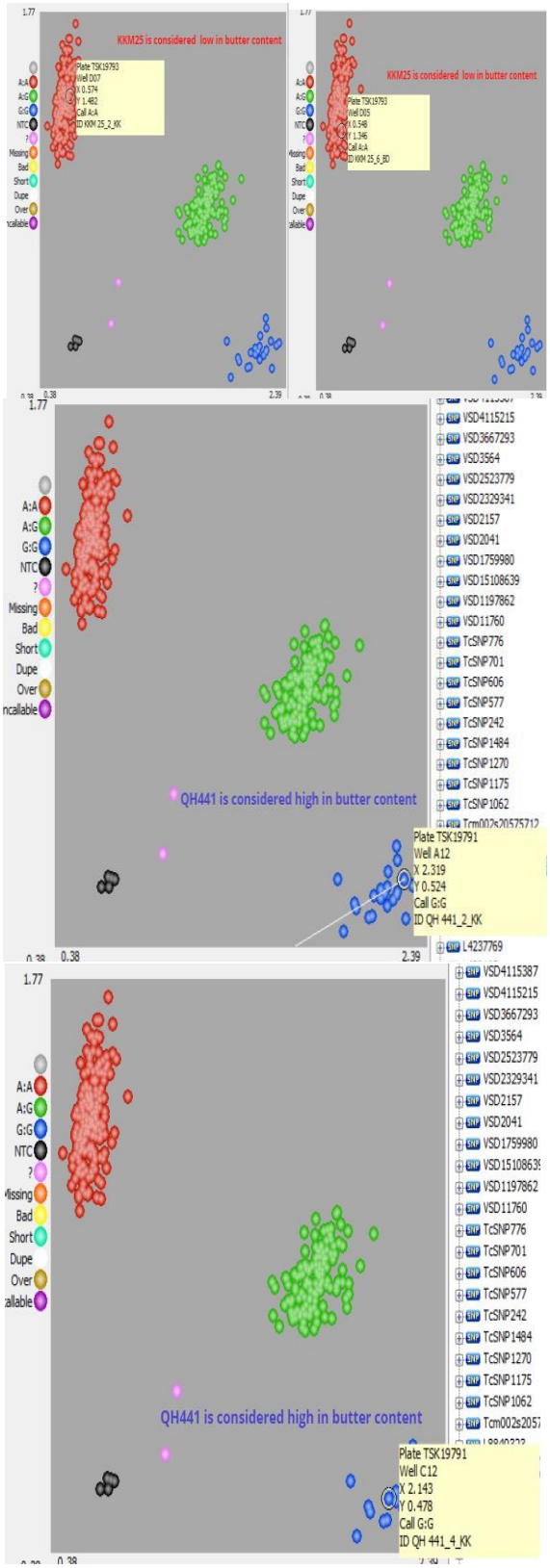
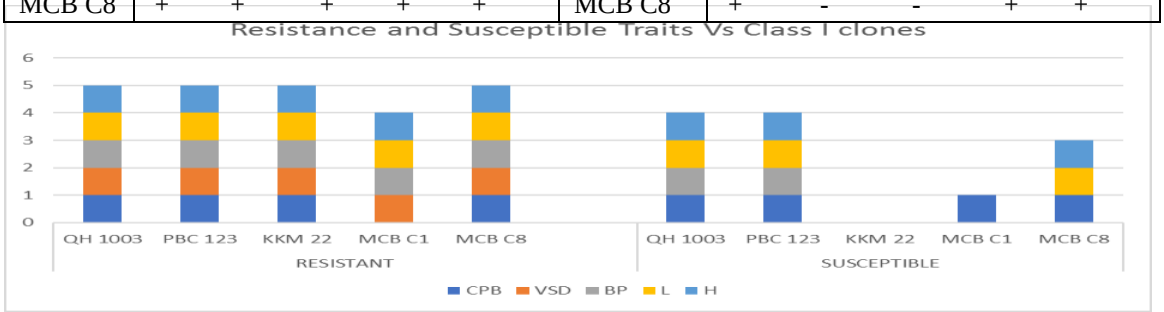


Figure 4: Some of the cluster plots for KASP genotyping assays

Table 5: Comparison of known phenotypic and genotypic analysis for each clone.

Class 1: Malaysian Cocoa Clones (Ver 2013)

RESISTANCE						SUSCEPTIBLE					
CLONE	TRAIT					CLONE	TRAIT				
	CPB	VSD	BP	L	H		CPB	VSD	BP	L	H
QH 1003	+	+	+	+	+	QH 1003	+	-	+	+	+
PBC 123	+	+	+	+	+	PBC 123	+	-	+	+	+
KKM 22	+	+	+	+	+	KKM 22	-	-	-	-	-
MCB C1	-	+	+	+	+	MCB C1	+	-	-	-	-
MCB C8	+	+	+	+	+	MCB C8	+	-	-	+	+



Class 2:

RESISTANCE						SUSCEPTIBLE					
CLONE	TRAIT					CLONE	TRAIT				
	CPB	VSD	BP	L	H		CPB	VSD	BP	L	H
BR 25	+	+	-	+	+	BR 25	+	+	-	+	+
KKM 5	+	+	+	+	+	KKM 5	+	-	-	+	+
MCB C9	-	+	+	+	+	MCB C9	-	-	-	-	-
PBC 139	+	+	+	+	+	PBC 139	+	-	-	-	+
RP 1	+	+	-	-	+	RP 1	+	-	+	-	+
KKM 1	+	+	+	-	+	KKM 1	-	+	+	+	+
KKM 19	+	+	+	+	+	KKM 19	+	-	+	+	+
PBC 112	-	+	-	+	-	PBC 112	+	-	+	+	+
PBC 221	+	+	+	+	+	PBC 221	+	+	-	-	+
KKM 4	+	+	+	+	+	KKM 4	-	-	-	-	-
KKM 25	+	+	+	+	+	KKM 25	+	+	-	+	+
PBC 137	+	+	+	-	+	PBC 137	+	-	+	-	-
QH 22	+	+	+	+	+	QH 22	+	-	-	+	-

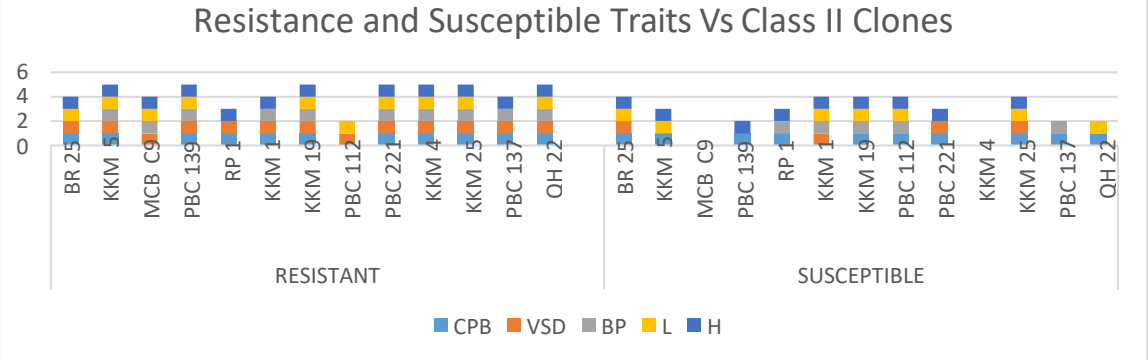


Figure 5: Stacked column of resistance and susceptible traits against Class I and II clones. (Malaysian Cocoa Clone, 2013)

Analysis of KASP genotyping data using cluster plots

In a KASP assay, a homozygous sample for the allele reported by the X-signal oligonucleotide will only generate X-signal fluorescence during the end-point genotyping reaction. This data point will be plotted close to the X-axis, representing a high X-signal and no Y-signal. A homozygous sample for the allele reported by the Y-signal oligonucleotide will only generate Y-signal fluorescence during the end-point genotyping reaction. This data point will be plotted close to the Y-axis, representing a high Y-signal and no X-signal. A heterozygous sample will contain both the allele reported by the X-signal oligonucleotide and the allele reported by the Y-signal oligonucleotide. This sample will generate half as much X-signal fluorescence and half as much Y-signal fluorescence as the homozygous samples for these alleles (Lovina,2021).

The expected graphical KASP results should show three clusters, in addition to the negative controls, corresponding to resistance (favorable), susceptible (non-favorable), and heterozygous samples (moderately expressed good to non-favorable). In the five traits-related SNPs studied, the grouping of the samples according to the expected values is observed.

CONCLUSIONS

This study reports the first research of SNP marker listing for Malaysian cocoa clones. The use of these SNPs for the five traits studied is endless. The future use in genomic selection breeding for cocoa, selection of seedlings with favorable traits, and genotyping of cocoa bud wood gardens are also among recommended use of these SNPs.

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