RESEARCH ARTICLE

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SNAP MARKERS DERIVED FROM *CATALASE-1* GENE SEQUENCE USED FOR BLACK POD DISEASE RESISTANCE IN CACAO (*Theobroma cacao* L.)

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SUMMARY

Cacao (*Theobroma cacao* L.) is a small evergreen tree that belongs to the family Malvaceae and is native to the deep tropical regions of Mesoamerica. Cacao black pod (pod rot) is one of the main diseases of cacao that causes 44% global crop loss. It is caused by the fungus *Phytophthora palmivora*. Cacao host resistance to black pod disease is the only way to combat this disease. Single nucleotide amplified polymorphism (SNAP) markers are reliable tools because of their capability to identify cacao resistance levels at the early growth stages without inoculation. In this study, the *catalase-1* (*CAT1*) gene was found to be linked to cacao black pod disease resistance. Its sequence was explored during 2019–2020 at the Molecular Laboratory, Indonesian Industrial and Beverage Crops Research Institute, Indonesia. The *CAT1* gene sequence generated six SNP sites, which were used to design SNAP markers. The testing of the newly designed markers by using six cacao genotypes with different levels of resistance showed that the designed primers for the Cat1-71 marker can differentiate resistant and susceptible genotypes. This marker detected variations [C/T] in the SNP position 71 in the *CAT1* gene on chromosome 8. The nonsynonymous SNP changed leucine into phenylalanine. The visualization of DNA bands through gel electrophoresis revealed that the Cat1-71 marker produced amplifications and has the potential to predict the resistance level of 16 F_1 hybrid cocoa. The phenotypic test of hybrid cocoa resistance can be continued by using the leaf disc assay. In addition, the SNAP marker has the advantage of facile analysis via agarose gel electrophoresis. Hence, the use of SNAP markers will improve the accuracy of the identification of cacao resistance to black pod disease.

Keywords: Cacao *catalase-1* gene, molecular markers, single nucleotide amplified polymorphism, early detection, *Phytophthora palmivora*, *Theobroma cacao* L.

Key findings: Conventional breeding for disease resistance in cacao is difficult and time-consuming. The newly developed primer Cat1-71, which is derived from the *CAT1* gene, classified cacao genotypes on the basis of resistance to black pod disease. The identification of SNAP markers linked to black pod resistance in cacao will increase selection accuracy and efficiency and accelerate the breeding process.

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INTRODUCTION

The demand for cacao beans (*Theobroma cacao* L.) has been increasing continuously due to their usage as a basic ingredient in various food products. Cacao is an evergreen tree, and its seeds are used to produce chocolate liquor, cocoa solids, and cocoa butter. Today, cacao is grown in more than 50 countries around the world. The Ivory Coast, Ghana, and Indonesia are the largest cacao-producing countries; their average cacao production increases by 3.2% per year (ICCO 2019). Cacao also has the potential to be developed further to increase farmers' incomes.

The cacao's main pests and diseases are black pod disease, cacao pod borer, vascular streak dieback (VSD), and cacao mirids (*Helopeltis* spp.). Cacao black pod (pod rot) is caused by the fungus *Phytophthora palmivora*. Some literature, however, have reported that this fungus belongs to the biological kingdom Chromista. Black pod disease caused by *P. palmivora* reduces cacao production by up to 66%. Overall, the loss of cacao production due to pest and disease attacks can reach 26%–36% (Bailey and Meinhardt, 2016). Furthermore, pest and disease control accounts for 40% of the total production cost of cacao (Babin, 2018).

The utilization of resistant varieties is the most effective and efficient way to control pests and diseases. Although conventional breeding has produced several superior cacao varieties, extensive land areas and a long time ranging from 10 years to 15 years are required to complete a single breeding cycle in cacao (Tasma, 2016). In addition, in cacao plants, cross-pollination rates range from 18% to 66%, with 100% incompatibility observed in self-pollination (Vello, 1971, Ahnert and Pires, 2000). In cacao, incompatibility can limit seed production and create difficulties in crossing (Lanaud *et al.,* 2017). DNA marker technology is one of the solutions to the problem of slow progress of cacao conventional breeding (Wickramasuriya and Dunwell, 2018).

Analyses based on molecular markers, including fingerprint analysis, genetic diversity analysis, linkage map construction, and quantitative character mapping, have been widely used in cacao breeding (Santos *et al*., 2015). Single nucleotide amplified polymorphism (SNAP) DNA markers are developed from single nucleotide polymorphism (SNP) sites in a gene. The SNP site is a one-base substitution in genomic sequences. The presence of SNP sites in a gene can influence different amino acids and affect the phenotypic expression of a plant. Disease-regulating genes have the potential to be developed into molecular-based selection markers for marker-assisted selection (MAS) (Lindo *et al*., 2018). Therefore, by using molecular markers, breeders can perform black pod resistance tests at the seedling stage.

The *catalase-1* (*CAT1*) gene regulates black pod disease resistance in cacao plants. The *CAT1* gene induces the activity of the peroxide enzyme, a pathogenesis-related protein (Chelikani and Fita, 2004). On the basis of the above discussion, this study was planned with the aim to a) identify the SNP sites in the *CAT1* gene sequence and to further use them to develop SNAP markers and b) distinguish the black pod resistance levels of cacao genotypes by applying newly developed SNAP markers.

MATERIALS AND METHODS

Genetic material

The genetic material used in this study comprised six cacao clones, 16 cacao F_1 genotypes with unknown resistance levels

Table 1. Cacao clones used to screen markers for resistance to black pod disease.

Clones	Varieties	Productivity (kg/ha/year)	Resistance to black pod disease	References
Sca-6	Forastero	1.540	Resistant	Thevenin et al. (2012)
MCC-02	Forastero	3.132	Resistant	McMahon et al. (2015)
Sul-2	Forastero	1.800–2.750	Resistant	ICCRI (2008)
DR-1	Criollo	1.000–1.500	Susceptible	Iswanto and Winarno (1992)
Sul-1	Forastero	1.800–2.500	Moderate	Suwastika et al. (2019)
DRC-16	Criollo	1.000–1.500	Moderate	Rubiyo et al. (2010)

Sca-6: Scavina-6; MCC-02: Masamba Cocoa Clone-02; Sul-2: Sulawesi-2; DR-1: Djati Runggo-1; Sul-1: Sulawesi-1; and, DRC-16: Djati Runggo Clone-16

Table 2. Cacao F_1 hybrids genotyped by using the selected SNAP primers.

Cross Combinations	Genotyped cacao F_1 hybrids	Genotype codes
ICCRI-03 × Sca-6	4-3 (3), 4-5 (3), 4-11(3), 4-8 (2)	1, 2, 3, 4
TSH-858 × Sca-6	7-10 (3), 7-9 (2),7-8 (3), 7-6 (3)	5, 6, 7, 8
DR-01 × Sca-6	9-7 (2), 9-9 (2), 9-4 (3), 9-11 (3)	9, 10, 11, 12
ICS-13 × Sca-6	10-4 (2), 10-10 (3), 10-9 (2), 10-7 (3)	13,14, 15,16

ICCRI-03: Indonesian Coffee and Cocoa Research Institute-03, TSH-858: Trinidad Selected Hybrid-858; DR-01: Djati Runggo-01; ICS-13: Imperial College Selections-13; and, Sca-6: Scavina-6

to black pod disease, and a *P. palmivora* isolate obtained from the germplasm collection of the Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), Indonesia (Tables 1 and 2). This study was carried out during 2019–2020 at the Molecular Laboratory, IIBCRI, Indonesia.

Leaf inoculation and scoring of *P. palmivora* infection

The *P. palmivora* isolate was inoculated through the leaf disk assay on six cacao clones, namely, Scavina-6, MCC-02, Sulawesi-2 (Sul-2), DR 1, Sulawesi-1 (Sul-1), and DRC-16. For each cacao clone, 10 young leaves were inoculated with the *P. palmivora* fungal isolate. Observations and scoring were conducted in accordance with Nyassé *et al*. (1995) and Tahi *et al*. (2007) (Figure 1). The age of the cocoa plant used is 8 years. The young leaves were 30 cm long and 10 cm wide. The experiment used three replications per clone, with each replication containing four young cocoa leaves. Each leaf was inoculated with *P. palmivora* fungus. Scoring data from each replication was averaged and classified on the basis of resistance level in accordance with Thevenin *et al*. (2012), namely, 0–1 very resistant, >1–2 resistant, >2–2.5 moderately resistant, >2.5–3.5 susceptible, and >3.5–5 very susceptible. This classification was conducted 7 days after infection.

Characterization of the *CAT1* gene

The *CAT1* gene was characterized by using the coding region sequence (CDS) of the *CAT1* gene from cocoa. *CAT1* gene sequences were obtained from the *CAT1* gene from Lanaud *et al*. (2004) and Kurniasih (2012) with the gene ID 1063521531. The similarity analysis of the *CAT1* gene with various catalase genes was carried out by using BLAST pairwise alignment in the National Center for Biotechnology Information (NCBI) database. Each gene sequence was aligned by using Geneious software version 8.1.6 (Biomatters, Ltd.). The alignment results were then exported into MEGA software version 7 (Kumar *et al*. 2016) to reconstruct the phylogenetic tree. The phylogenetic tree was reconstructed by using the maximum likelihood method with 1000 bootstrap

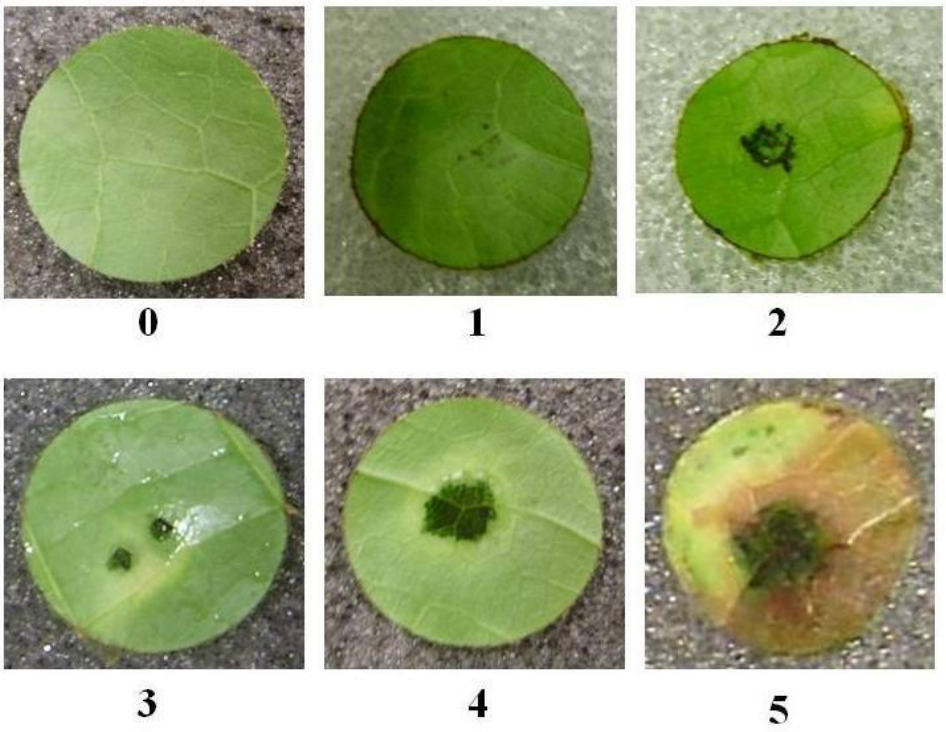


Figure 1. Scoring of cacao resistance to *P. palmivora* infection by using the leaf disk assay. Score 0: no symptoms, score 1: penetration point in the inoculation zone, 2: penetration points interconnected like a net, 3: penetration points that have formed spots, 4: spots that have formed within inoculation zones, and 5: spots that have spread outside the inoculation zone.

times. Identification was carried out to determine the similarity of the *CAT1* gene in cocoa with the catalase genes in other plants.

CAT1 gene isolation and polymerase chain reaction analysis

The *CAT1* gene was isolated with specific primers designed by using the primary3plus program (https://primer3 plus.com/). The reference *CAT1* gene sequence was obtained from Kurniasih (2012). The amplification reaction was conducted on a polymerase chain reaction (PCR) machine with a reaction volume containing 2 µL of DNA samples (10 ng/µL), 7.5 µL of PCR master mix, 2× Bioline MyTaq red mix, 5.2 µL of sterile water, and 0.3 µL of primer mix. The PCR cycle consisted of predenaturation for 2 min at 94 °C, followed by denaturation at 94 °C for 30 s, annealing for 1 min at the temperature specific to the primer, and extension at 72 °C for 30 s. This cycle was repeated 35 times and ended with a final extension at 72 °C for 7 min. The PCR amplification results were electrophoresed with a mixture of 1 µL of cacao DNA samples and 2 µL of loading dye ran on agarose gel 1% (w/v) with 1× TBE buffer at 100 V for 30 min. The gel was visualized on a UV transilluminator. The amplified PCR product was sequenced to determine the sequence of the nucleotide bases.

Identification of SNP sites and SNAP primer designs

The sequence of the PCR-amplified gene was determined through the Sanger sequencing method. The PCR products from cacao DNA amplification were

Table 3. Medians of the scores of cacao genotypes for resistance to *P. palmivora* infection and resistance scores of six cacao clones in the leaf disk assay.

Clones[1]	Days after infection							Resistance score based on leaf disk assay	Resistance level based on leaf disk assay[2]	Resistance level based on References[3]
	1	2	3	4	5	6	7			
DR-1	1.0 [a]	1.8 [a]	2.5 [a]	3.4 [a]	4.0 [a]	4.7 [a]	5.0 [a]	5	Very susceptible	Susceptible[3a]
DRC-16	0.4 [b]	1.0 [b]	1.1 [b]	1.3 [b]	1.7 [b]	2.2 [b]	3.0 [b]	3	Susceptible	Moderate[3b]
MCC-02	0.2 [b]	0.7 [cb]	1.1 [b]	1.1 [b]	1.5 [b]	1.7 [cb]	2.1 [cb]	2.1	Moderately resistant	Resistant[3c]
Sul-1	0.1 [b]	0.5 [cbd]	0.7 [cb]	0.9 [cb]	1.0 [cb]	1.0 [cd]	1.2 [cd]	1.2	Resistant	Moderate[3d]
Sul-2	0.0 [b]	0.3 [d]	0.5 [cb]	0.7 [cb]	0.7 [cb]	0.7 [cd]	1.1 [cd]	1.1	Resistant	Resistant[3e]
Sca-6	0.0 [b]	0.0 [d]	0.1 [c]	0.1 [c]	0.1 [c]	0.1 [d]	0.1 [d]	0.1	Very resistant	Resistant[3f]

Note: Numbers followed by the same letters in the same column are not significantly different based on honestly significant difference test at the 0.5 level.
[1]Clones DR-1: Djati Runggo-1; DRC-16: Djati Runggo Clone-16; MCC-02: Masamba Cocoa Clone-02; Sul-1: Sulawesi-1; Sul-2: Sulawesi-2; and, Sca-6: Scavina-6;
[2]Classification of cacao resistance level scores, namely: 0–1: very resistant, >1–2: resistant, >2–2.5: moderately resistant, >2.5–3.5: susceptible, and >3.5–5: very susceptible (Thevenin et al. 2012)
[3]References: [3a]Iswanto and Winarno (1992), [3b]Rubiyo et al. (2010), [3c]McMahon et al. (2015), [3d]Suwastika et al. (2019), [3e]ICCRI (2008), [3f]Thevenin et al. (2012)

sequenced at the First BASE Laboratory (Singapore). Then, the *CAT1* gene sequence was aligned by using Geneious software version 8.1.6 (Biomatters Ltd.). Analysis was done on site location, nucleotide variation, and amino acid changes on SNP sites. The SNP sites that caused changes in amino acids were recorded as nonsynonymous, whereas those that did not cause changes in amino acids were recorded as synonymous. The SNP sites that had the potential to be developed into SNAP markers were then used for primer design. The SNAP markers were designed by using WebSNAPER software (https://pga.mgh.harvard.edu/cgi-bin/snap3/websnaper3.cgi). The parameters for the design of SNAP primers were a PCR product size range of 325–375 base pair (bp), primer concentration of 10 nM, primer size range of 18–25 bp, and primer melting temperature range of 50 °C–60°C.

SNAP markers used in the genotyping of 16 cacao genotypes

The newly developed SNAP markers were used to genotype six cacao clones (Sca-6, MCC-02, Sul-2, DR-1, Sul-1, and DRC-16) to predict their resistance levels. The marker that could distinguish between resistant and susceptible cacao clones was selected. The selected marker was used to genotype 16 F_1 cacao clones. The selected molecular markers were expected to be capable of predicting resistant and susceptible cacao F_1 genotypes.

RESULTS

Cacao resistance against *P. palmivora* based on the leaf disk assay

The results revealed significant variation among the severity of the symptoms of black pod disease in different clones (Table 3). Sca-6 had the highest resistance level to *P. palmivora*, whereas DR-1 was recorded as most susceptible. The resistance control was the Sca-6 clone, the international reference used in tests involving *P. palmivora* (Thevenin *et al*. 2012). Sca-6 leaves showed no symptoms of *P. palmivora* infection and penetration point in the inoculation zone. The cacao resistance recorded 6 days after infection could be classified on the basis of the scoring scale of symptoms as identified from the results of Tukey's honestly significant difference test, which

were the same as the results obtained 7 days after infection. Furthermore, two cacao clones, Sul-1 and Sul-2, originating from Sulawesi Island had similar resistance levels with scores of 1.2 and 1.1, respectively, on day 7 after infection. In cocoa clones Sul-1 and Sul 2, *P. palmivora* infection showed interconnected, net-like penetration points. The differences in cacao resistance levels were influenced by the mechanisms of prepenetration and postpenetration resistance. These mechanisms determine the development of *P. palmivora* in cacao leaves (Nyadanu *et al*., 2013).

Prepenetration resistance is related to the morphological and physical characteristics of plants, such as water content and wax layers on the surface of leaves and pods. By contrast, postpenetration resistance is related to biochemical mechanisms in plants that involve biochemical compounds, such as the protein chitinase and peroxidase (Lanaud *et al*., 2004). In the leaf disc assay, the MCC 02 clone had a resistance value of 2.1 (moderately resistant). *P. palmivora* penetration points formed spots. Given that clones DR-1 and DRC-16 may have poor post-penetration resistance, the fungus *P. palmivora* grew optimally. The cocoa clones DR-1 and DRC-16 infected with *P. palmivora* had scores of 5 (very susceptible) and 3 (susceptible), and spots formed within and outside the inoculation zones. By contrast, the clone Sca-6 likely had good postpenetration resistance because the fungus grew poorly in this genotype. The Sca-6 clone is a well-known source of resistance to *P. palmivora* and cocoa fungal disease (Rubiyo *et al*. 2010).

Based on the cacao grouping for resistance to *P. palmivora* infection, the clone Sca-6 showed the lowest least score (0.1) 7 days after infection and was classified as very resistant. The cacao clone Sul-1 was classified as resistant, whereas clone DRC-16 was identified as susceptible. However, previous studies classified Sul-1 and DRC-16 as moderately resistant (Thevenin *et al*. 2012). Sul-2 was resistant, MCC-02 was moderately resistant, and DR-1 was highly susceptible.

Characterization of the *CAT1* gene

The *CAT* gene was first identified in tobacco plants, and an increase in its expression induces the activity of the peroxidase enzyme (Chen and Klessig, 1991). According to Chelikani *et al*. (2004), the peroxidase enzyme is included in peroxidase (PR-9) that accelerates the reaction of plants to improve pathogen resistance and actively acts as an antifungal and antibacterial in *Bacillus. CAT1* gene sequences were obtained from the *CAT1* gene deposited by Lanaud *et al.* (2004) and Kurniasih (2012) with the gene ID: 1063521531 (XM_007017972.2) in the NCBI database. In cacao, the *CAT1* gene was detected in a region of chromosome 8. The reconstructed *CAT1* gene phylogenetic tree is shown in Figure 2. The phylogenetic tree was used to group the individuals on the basis of the similarity of their gene sequences with those of various crop plants (Chen *et al*., 2016). The *CAT1* gene from cocoa is closely related to *HuCAT1* (*Herrania umbratica*), *CaCAT1* (*Capsicum annuum*), *DzCAT1* (*Durio zibethinus*), *GhCAT1* (*Gossypium hirsutum*), *GrCAT1* (*Gossypium raimondii*), *GaCAT1* (*Gossypium arboreum*), *DzCAT1* (*Durio zibethinus*), and *IpCAT3* (*Ipomoea nil*). The *T. cacao* and *H. umbratica CAT1* genes had the max identity value of 100% (Table 4). This max identity value reflects the identity percentage and similarity between the sequence of the query and the sequence in the NCBI database (Kumar and Stecher, 2016). This similarity occurred because *H. umbratica* plant is a wild type of the *T. cacao* (Chessman, 1944). The accession of the *CAT1* gene has been published in NCBI and encodes the protein catalase isozyme 1.

Isolation of *CAT1* gene

The *CAT1* gene sequence in the cacao genotypes was isolated with specific primers (Table 5). The position of the

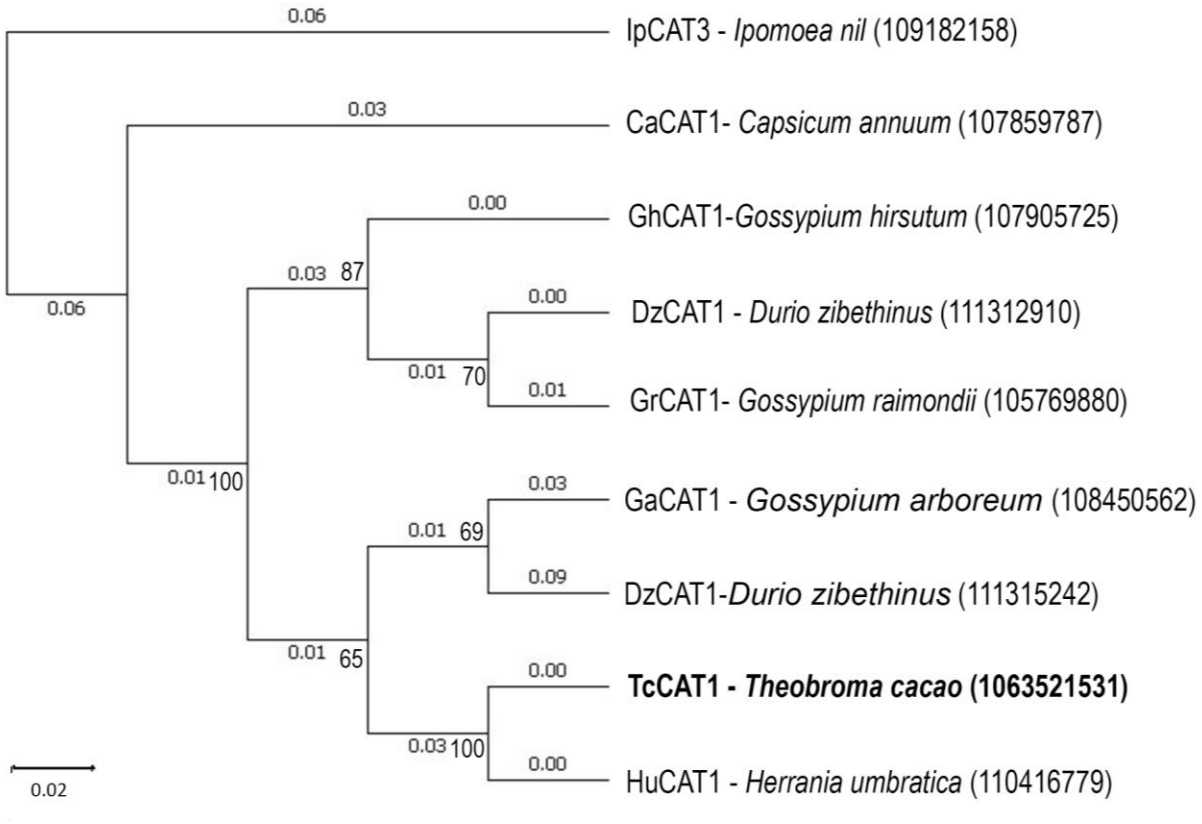


Figure 2. Construction of the *CAT1* gene phylogenetic tree. This tree was produced using BLAST pairwise alignments in the NCBI database.

Table 4. Analysis for similarity of the *CAT1* gene in cocoa with *CAT* genes in different plant accession in NCBI Genbank.

Gene ID	Gene name	Species name	E value	Max identity (%)
110416779	Catalase isozyme 1 (CAT 1)	*Herrania umbratica*	7e−43	100.00
111315242	Catalase isozyme 1-like	*Durio zibethinus*	7e−33	94.06
108450562	Catalase isozyme 1-like	*Gossypium arboreum*	7e−33	94.06
105769880	Catalase isozyme 1	*Gossypium raimondii*	3e−31	93.07
111312910	Catalase isozyme 1	*Durio zibethinus*	2e−29	92.86
107905725	Catalase isozyme 1-like	*Gossypium hirsutum*	2e−29	92.08
107859787	Catalase isozyme 1	*Capsicum annuum*	7e−23	88.12
109182158	Catalase isozyme 3	*Ipomoea nil*	2e−19	86.14

Table 5. Primers used in DNA isolation.

Gene	Sequence	Product length (bp)	Tm (°C)	GC (%)
CAT1	F: TCAAATCTTGCTGAGGTTCGT	885	59.9	42.9
	R: AGAAAATTGTTCCCGCAAAG		59.2	40.0

primer pair for the amplification of the *CAT1* gene in *T. cacao* is shown in Figure 3. The electrophoresis results showed that the *CAT1* primer generated PCR products with sizes ranging from 750 bp to 1000 bp (Figure 4). These results were in accordance with the product length designed for the *CAT1* gene (885 bp). The DNA bands with strong intensity were due to perfect amplification between the forward and reverse primers (Hung and Weng, 2016). The *CAT1* gene in *T. cacao* shared 100 similar DNA bases with that in *Herrania umbratica*. The similarity of gene sequences lies in the sequence of 481 to 581 bases.

Identification of SNP sites in multiple sequence analysis

SNP sites were identified by aligning the DNA fragments from *CAT1* gene sequencing on clones, i.e., Sca-6, MCC-02, Sul-2, DR-1, Sul-1, and DRC-16. The original sequences of the *CAT1 gene* were obtained from Lanaud *et al*. (2004) and Kurniasih (2012) with the gene ID 1063521531. The nucleotide bases that could distinguish the very resistant genotype Sca-6 from other genotypes were selected as the SNP sites, i.e., SNP position 27 in the *CAT1* gene had the base thymine [T] (reference SNP) in the

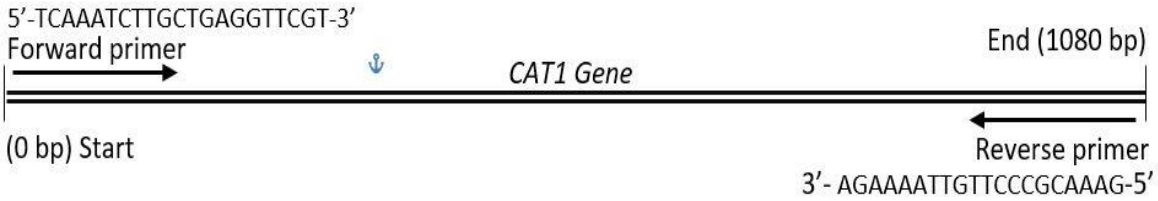


Figure 3. Specific primers used for the isolation of the *CAT1* gene from cocoa

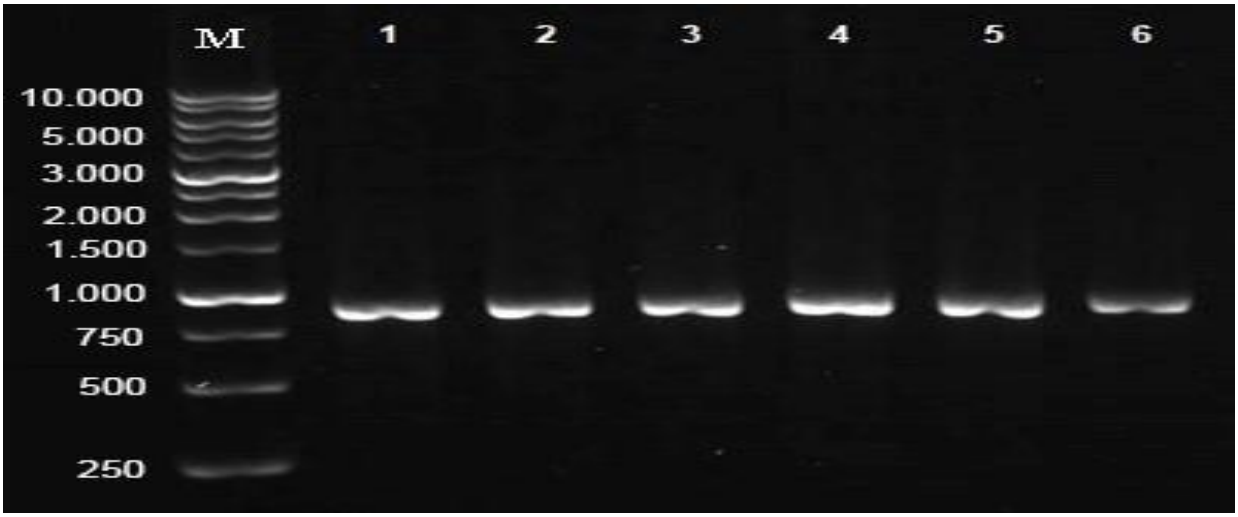
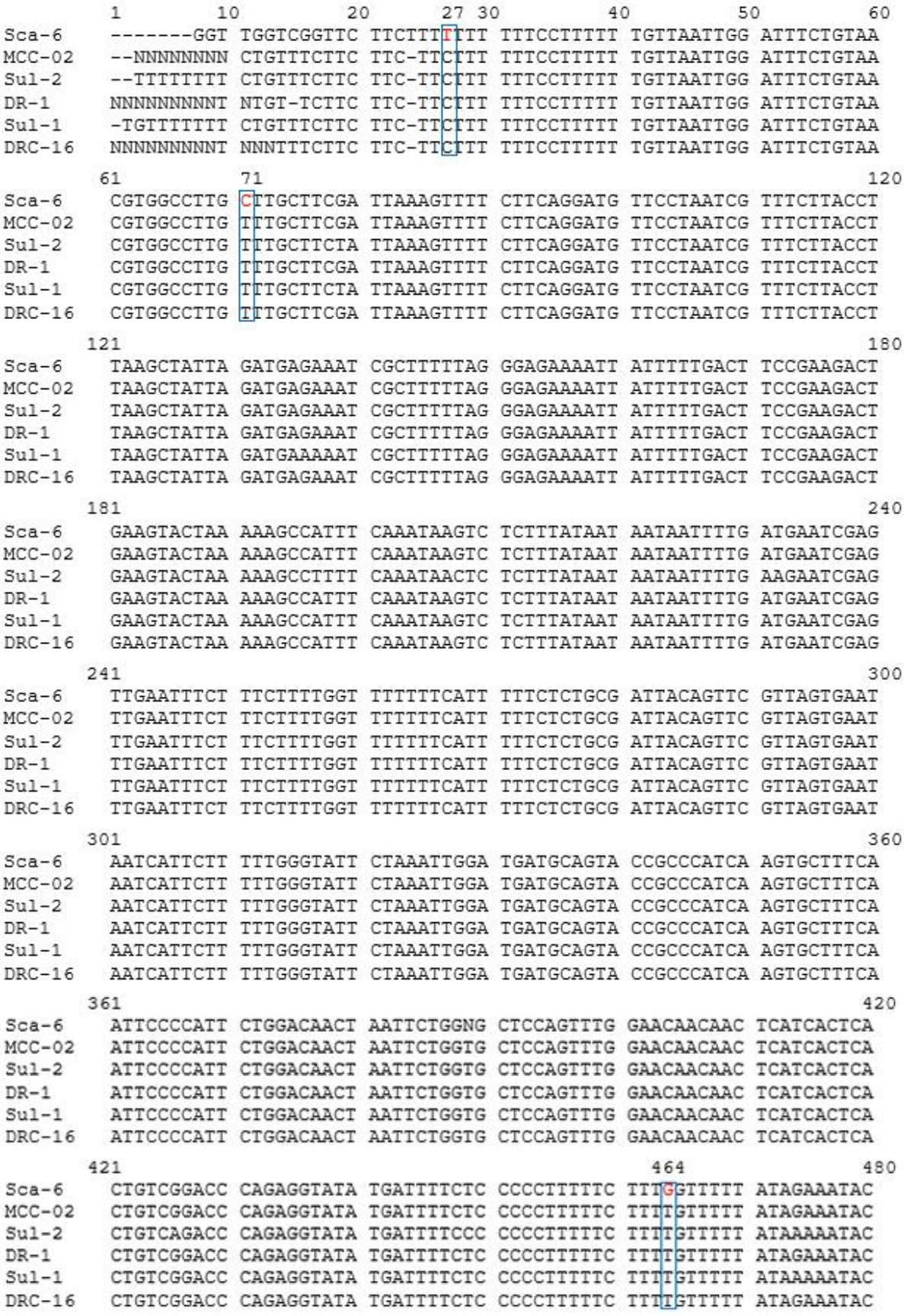


Figure 4. Electropherogram of amplification in six cacao genomes with the *CAT1* primer. Description: M: marker; 1: Sca-6; 2: MCC-02; 3: Sul-2; 4: DR-01; 5: Sul-01; and 6: DRC-16.


1 10 20 27 30 40 50 60
Sca-6 -------GGT TGGTCGGTTC TTCTTTTTTT TTTCCTTTTT TGTTAATTGG ATTTCTGTAA
MCC-02 --NNNNNNNN CTGTTTCTTC TTC-TTCTTT TTTCCTTTTT TGTTAATTGG ATTTCTGTAA
Sul-2 --TTTTTTTT CTGTTTCTTC TTC-TTCTTT TTTCCTTTTT TGTTAATTGG ATTTCTGTAA
DR-1 NNNNNNNNNT NTGT-TCTTC TTC-TTCTTT TTTCCTTTTT TGTTAATTGG ATTTCTGTAA
Sul-1 -TGTTTTTTT CTGTTTCTTC TTC-TTCTTT TTTCCTTTTT TGTTAATTGG ATTTCTGTAA
DRC-16 NNNNNNNNNT NNNTTTCTTC TTC-TTCTTT TTTCCTTTTT TGTTAATTGG ATTTCTGTAA
61 71 120
Sca-6 CGTGGCCTTG CTTGCTTCGA TTAAAGTTTT CTTCAGGATG TTCCTAATCG TTTCTTACCT
MCC-02 CGTGGCCTTG TTTGCTTCGA TTAAAGTTTT CTTCAGGATG TTCCTAATCG TTTCTTACCT
Sul-2 CGTGGCCTTG TTTGCTTCTA TTAAAGTTTT CTTCAGGATG TTCCTAATCG TTTCTTACCT
DR-1 CGTGGCCTTG TTTGCTTCGA TTAAAGTTTT CTTCAGGATG TTCCTAATCG TTTCTTACCT
Sul-1 CGTGGCCTTG TTTGCTTCTA TTAAAGTTTT CTTCAGGATG TTCCTAATCG TTTCTTACCT
DRC-16 CGTGGCCTTG TTTGCTTCGA TTAAAGTTTT CTTCAGGATG TTCCTAATCG TTTCTTACCT
121 180
Sca-6 TAAGCTATTA GATGAGAAAT CGCTTTTTAG GGAGAAAATT ATTTTTGACT TCCGAAGACT
MCC-02 TAAGCTATTA GATGAGAAAT CGCTTTTTAG GGAGAAAATT ATTTTTGACT TCCGAAGACT
Sul-2 TAAGCTATTA GATGAGAAAT CGCTTTTTAG GGAGAAAATT ATTTTTGACT TCCGAAGACT
DR-1 TAAGCTATTA GATGAGAAAT CGCTTTTTAG GGAGAAAATT ATTTTTGACT TCCGAAGACT
Sul-1 TAAGCTATTA GATGAAAAAT CGCTTTTTAG GGAGAAAATT ATTTTTGACT TCCGAAGACT
DRC-16 TAAGCTATTA GATGAGAAAT CGCTTTTTAG GGAGAAAATT ATTTTTGACT TCCGAAGACT
181 240
Sca-6 GAAGTACTAA AAAGCCATTT CAAATAAGTC TCTTTATAAT AATAATTTTG ATGAATCGAG
MCC-02 GAAGTACTAA AAAGCCATTT CAAATAAGTC TCTTTATAAT AATAATTTTG ATGAATCGAG
Sul-2 GAAGTACTAA AAAGCCTTTT CAAATAACTC TCTTTATAAT AATAATTTTG AAGAATCGAG
DR-1 GAAGTACTAA AAAGCCATTT CAAATAAGTC TCTTTATAAT AATAATTTTG ATGAATCGAG
Sul-1 GAAGTACTAA AAAGCCATTT CAAATAAGTC TCTTTATAAT AATAATTTTG ATGAATCGAG
DRC-16 GAAGTACTAA AAAGCCATTT CAAATAAGTC TCTTTATAAT AATAATTTTG ATGAATCGAG
241 300
Sca-6 TTGAATTTCT TTCTTTTGGT TTTTTTCATT TTTCTCTGCG ATTACAGTTC GTTAGTGAAT
MCC-02 TTGAATTTCT TTCTTTTGGT TTTTTTCATT TTTCTCTGCG ATTACAGTTC GTTAGTGAAT
Sul-2 TTGAATTTCT TTCTTTTGGT TTTTTTCATT TTTCTCTGCG ATTACAGTTC GTTAGTGAAT
DR-1 TTGAATTTCT TTCTTTTGGT TTTTTTCATT TTTCTCTGCG ATTACAGTTC GTTAGTGAAT
Sul-1 TTGAATTTCT TTCTTTTGGT TTTTTTCATT TTTCTCTGCG ATTACAGTTC GTTAGTGAAT
DRC-16 TTGAATTTCT TTCTTTTGGT TTTTTTCATT TTTCTCTGCG ATTACAGTTC GTTAGTGAAT
301 360
Sca-6 AATCATTCTT TTTGGGTATT CTAAATTGGA TGATGCAGTA CCGCCCATCA AGTGCTTTCA
MCC-02 AATCATTCTT TTTGGGTATT CTAAATTGGA TGATGCAGTA CCGCCCATCA AGTGCTTTCA
Sul-2 AATCATTCTT TTTGGGTATT CTAAATTGGA TGATGCAGTA CCGCCCATCA AGTGCTTTCA
DR-1 AATCATTCTT TTTGGGTATT CTAAATTGGA TGATGCAGTA CCGCCCATCA AGTGCTTTCA
Sul-1 AATCATTCTT TTTGGGTATT CTAAATTGGA TGATGCAGTA CCGCCCATCA AGTGCTTTCA
DRC-16 AATCATTCTT TTTGGGTATT CTAAATTGGA TGATGCAGTA CCGCCCATCA AGTGCTTTCA
361 420
Sca-6 ATTCCCCATT CTGGACAACT AATTCTGGNG CTCCAGTTTG GAACAACAAC TCATCACTCA
MCC-02 ATTCCCCATT CTGGACAACT AATTCTGGTG CTCCAGTTTG GAACAACAAC TCATCACTCA
Sul-2 ATTCCCCATT CTGGACAACT AATTCTGGTG CTCCAGTTTG GAACAACAAC TCATCACTCA
DR-1 ATTCCCCATT CTGGACAACT AATTCTGGTG CTCCAGTTTG GAACAACAAC TCATCACTCA
Sul-1 ATTCCCCATT CTGGACAACT AATTCTGGTG CTCCAGTTTG GAACAACAAC TCATCACTCA
DRC-16 ATTCCCCATT CTGGACAACT AATTCTGGTG CTCCAGTTTG GAACAACAAC TCATCACTCA
421 464 480
Sca-6 CTGTCGGACC CAGAGGTATA TGATTTTCTC CCCCTTTTTC TTTGGTTTTT ATAGAAATAC
MCC-02 CTGTCGGACC CAGAGGTATA TGATTTTCTC CCCCTTTTTC TTTTGTTTTT ATAGAAATAC
Sul-2 CTGTCAGACC CAGAGGTATA TGATTTTCCC CCCCTTTTTC TTTTGTTTTT ATAAAAATAC
DR-1 CTGTCGGACC CAGAGGTATA TGATTTTCTC CCCCTTTTTC TTTTGTTTTT ATAGAAATAC
Sul-1 CTGTCGGACC CAGAGGTATA TGATTTTCTC CCCCTTTTTC TTTTGTTTTT ATAAAAATAC
DRC-16 CTGTCGGACC CAGAGGTATA TGATTTTCTC CCCCTTTTTC TTTTGTTTTT ATAGAAATAC

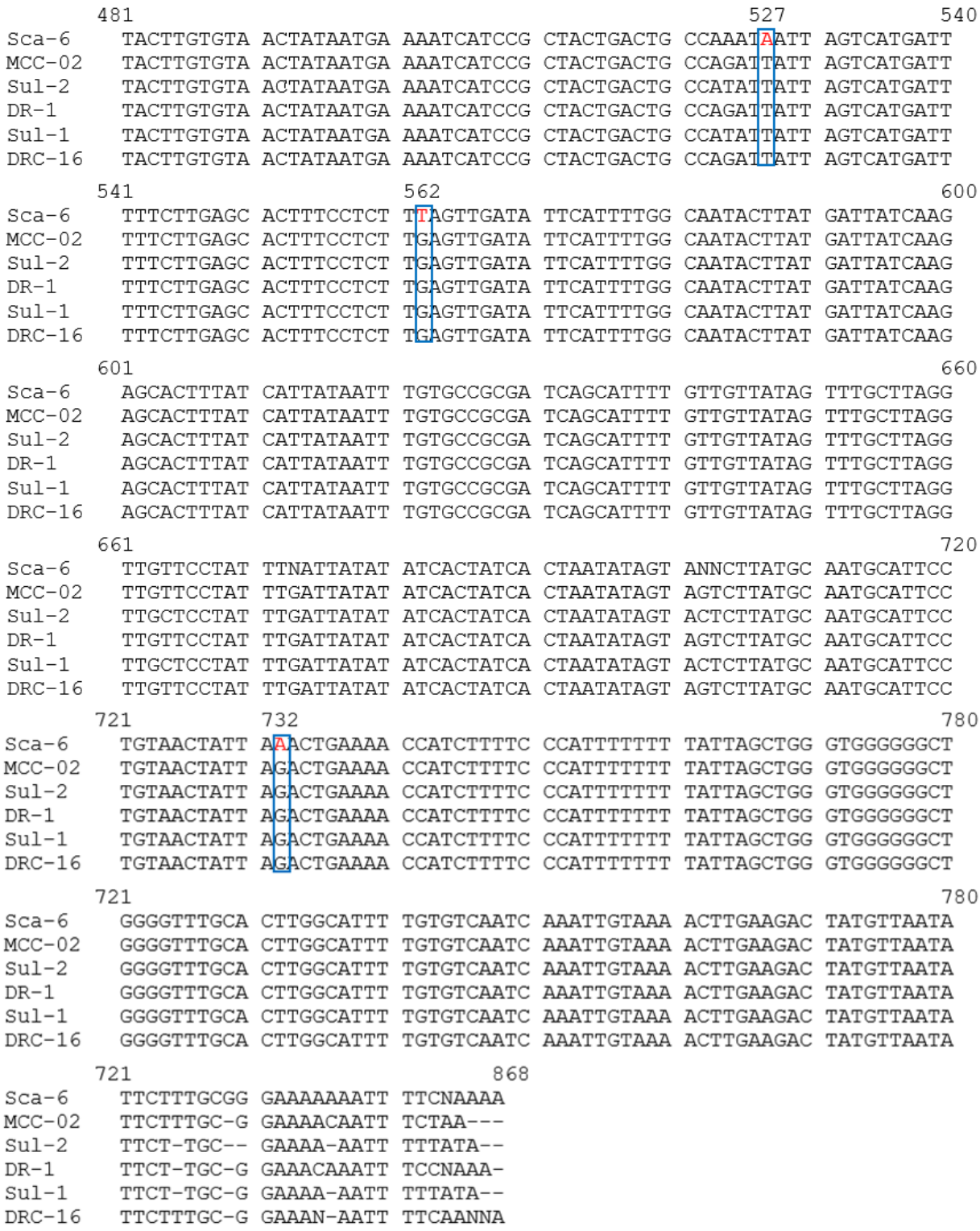


Figure 5. Representation of the SNP site of the *CAT1* gene in six cacao clones by Geneious software version 8.1.6. Boxes indicate SNP sites. Nucleotides were read from the results of the sequencing.

resistant Sca-6 clone, whereas the base cytosine [C] (alternative SNP) was found in other cacao genotypes. The SNP site was read from the results of *CAT1* gene sequencing (Figure 5). The difference in the DNA bases of the sample Sca-6 (black pod resistance) from those in other cocoa clones was used as the location of the SNP. SNP positions and the corresponding variations (reference/alternative SNPs) after sequencing were 27 [T/C], 71 [C/T], 464 [G/T], 527 [A/T], 562 [T/G], and 732 [A/G].

The SNP amino acid changes were divided into two types, namely, synonymous and nonsynonymous. Synonymous SNPs are those that do not change amino acid translations, whereas nonsynonymous SNPs are those that change amino acid translations (Studer and Dessailly, 2013). In this study, all the obtained SNP sites were nonsynonymous (Table 6). The SNP site with a nucleotide variant [T/C] in the SNP position 27 in the *CAT1* gene was a different amino acid residue. The *CAT1* DNA base sequence used was a CDS. Amino acid translation was performed with Geneious software. The base T substitution in the GTC codon was encoded for phenylalanine, whereas base C in the GCC codon substitution encoded serine. Similarly, base substitutions in the other five SNP positions resulted in changes in amino acid residues.

Design and testing of SNAP markers

Six SNP sites were placed one by one on the WebSNAPER page for SNAP markers. Alleles that differentiated the resistant and susceptible cacao clones were used as standards. Resistant alleles were used as references, whereas susceptible alleles were used as alternatives. The SNAP marker designed for the identified SNP positions in the *CAT1* gene is shown in Table 7. Two unique forward primers were designed to detect the reference and alternative SNPs, and a common reverse primer was designed to be used together with either of the two forward primers in PCR.

SNAP markers were used to detect the resistance level of cocoa to black pod disease based on nucleotide base substitution or SNP in the *CAT1* gene. The SNP sites that were identified in the *CAT1* gene were converted into the molecular marker SNAP. Alleles that differentiated resistant (C base/cytosine) and susceptible (T base/thymine) sequences for cocoa black pod disease were used as the basis for the primary design of the Cat1-71 primer. The resistant allele was referred to as the reference, whereas the susceptible allele was referred to as the alternative. The Cat1-71 primer was able to classify cacao genotypes on the basis of their resistance levels (Figure 6). Only the reference primer was amplified in the resistant clone, the reference and alternative primers were amplified in the moderately resistant clones, and only the alternate primer was amplified in the susceptible clones. The other five SNAP markers could not classify the cacao genotypes on the basis of resistance levels.

Application of SNAP markers in cacao F_1 hybrids

F_1 hybrids developed from crosses between the *P. palmivora*-resistant Sca-6 clone and four other clones (ICCRI-03, TSH -58, ICS-13, and DR-01) were genotyped by using the selected SNAP marker. The cocoa clones ICCRI-03, TSH-58, and ICS-13 were grouped into the resistant class (Rubiyo *et al.* 2010), whereas the DR-01 clone was in the susceptible class (Iswanto and Winarno 1992). The resistance to *P. palmivora* fungal infection of the F_1 hybrids has not been determined on the basis of phenotype. The SNAP Cat1-71 marker based on the nucleotide variation [C/T] in the SNP position 71 in the *CAT1* gene between the six initially tested *P. palmivora*-resistant and susceptible cocoa clones was used to predict the resistance level to black spot disease of the cacao F_1 hybrids. The Cat1-71 marker can identify resistant clones on the basis of amplification with the reference primer

Table 6. SNP site analysis on *CAT1* genes in cacao clones Scavina-6, MCC-02, Sulawesi-2, DR-1, Sulawesi-1, and DRC-16.

Gene	SNP position	SNP variations	Type of change in amino acids	Changes in amino acid residues
CAT1	27	[TTT /TCT]	*Nonsynonymous*	Phenylalanine–Serine
	71	[GCC/GTC]	*Nonsynonymous*	Leucine–Phenylalanine
	464	[TGG/TTG]	*Nonsynonymous*	Glycine–Cysteine
	527	[TAA/TTA]	*Nonsynonymous*	Asparagine -Tyrosine
	562	[TTA/TGA]	*Nonsynonymous*	Phenylalanine–Leucine
	732	[AAA /AGA]	*Nonsynonymous*	Lysine–Arginine

Note: The codon with one DNA base in red is the original codon and that with blue is the alternative codon.

Table 7. Primers designed for the six SNAP markers based on the variations in the base sequence of the *CAT1* gene.

Primers Identity	Sequence	Product length (bp)
TcSNAPCat1-27-L-*Ref*-F	5' TTGATTCTGTTTCCTTCTTGTTT 3'	
TcSNAPCat1-27-L-*Ref*-R	3' CATCCAATTTAGAATACCCAAAA 5'	328
TcSNAPCat1-27-L-*Alt*-F	5' TTGATTCTGTTTCCTTCTTGTTC 3'	
TcSNAPCat1-71-L-*Ref*-F	5' TTTCTGTAACGTGGCCTAGC 3'	
TcSNAPCat1-71-L-*Ref*-R	3' GAGTTGTTGTTCCAAACTGGA 5'	361
TcSNAPCat1-71-L-*Alt*-F	5' GATTTCTGTAACGTGGCCTAGT 3'	363
TcSNAPCat1-464-L-*Ref*-F	5' TTTTCTCCCCCTTTTTCTATG 3'	
TcSNAPCat1-464-L-*Ref*-R	3' GATTGACACAAAATGCCAAGT 5'	367
TcSNAPCat1-464-L-*Alt*-F	5' TTTCTCCCCCTTTTTCATTT 3'	366
TcSNAPCat1-527-L-*Ref*-F	5' CGCTACTGACTGCCAAAGA 3'	
TcSNAPCat1-527-L-*Ref*-R	3' GCAAAGAATATTAACATAGTCTTCA 5'	340
TcSNAPCat1-527-L-*Alt*-F	5' CGCTACTGACTGCCAGATT 3'	
TcSNAPCat1-562-L-*Ref*-F	5' TTTTCTTGAGCACTTTCCTATTT 3'	
TcSNAPCat1-562-L-*Ref*-R	3' TCCAAATTATCAACACCATTTG 5'	351
TcSNAPCat1-562-L-*Alt*-F	5' TTCTTGAGCACTTTCCTCATG 3'	349
TcSNAPCat1-732-L-*Ref*-F	5' GCAATGCATTCCTGTAACTACTAA 3'	
TcSNAPCat1-732-L-*Ref*-R	3' CAGAAAATCTGCACATGTAAGG 5'	360
TcSNAPCat1-732-L-*Alt*-F	5' GCAATGCATTCCTGTAACTAATAG 3'	

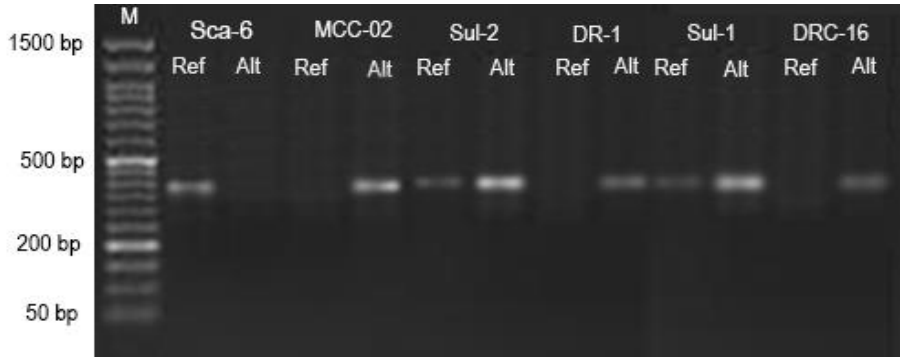


Figure 6. Cat1-71 primer visualization for six cacao clones on 1% agarose gel.
Primers Ref: reference and Alt: alternate; Only the reference primer was amplified in the resistant Sca-6 clone; the reference and alternative primers were amplified in the moderately resistant Sul-2 and Sul-1 clones; and only the alternate primer was amplified in the susceptible DR-1, MCC-02, and DRC-16 clones.

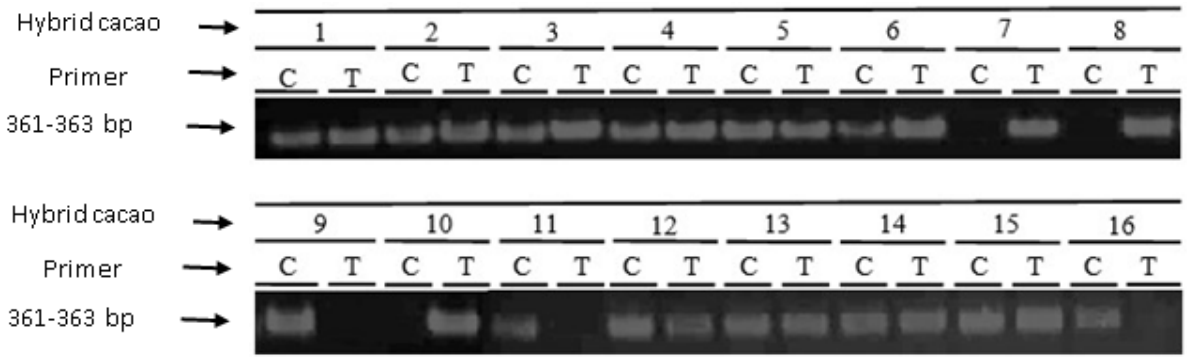


Figure 7. Visualization of Cat1-71 SNAP marker application in 16 cacao hybrid genotypes. Note: Number 1–16 = hybrid and base cacao genotypes; nucleotide variant (C/T) = differentiating nucleotides in SNAP primers; Classified cacao: resistant class (9, 11, and 16), moderate class (1, 2, 3, 4, 5, 6, 12, 13, 14, and 15), and susceptible class (7, 8, and 10)

only, the susceptible clones on the basis of amplification with the alternative primer only, and moderately resistant clones on the basis of amplification with the reference and alternative primers. On the basis of DNA amplification, cacao genotypes 9, 11, and 16 were predicted as resistant clones; cacao genotypes 7, 8, and 10 were predicted as susceptible clones; and the 10 other genotypes were predicted as moderately resistant clones (Figure 7).

DISCUSSION

Information based on host resistance to black pod and pod rot disease is essential for cacao breeding programs and helpful for substantial improvements in disease resistance. The leaf disk assay is a simple identification technique that is used to classify resistance levels in cacao genotypes and predict the morphological responses of plants in the greenhouses (Ali *et al*., 2017). The differences in fungal infection areas on cacao leaves indicated the differences in their resistance levels. Various earlier studies related to the phenotypic resistance of cocoa to black pod disease grouped cacao genotypes into three groups (susceptible, moderate, and resistant). Recent research has classified cocoa resistance into five classes (very susceptible, susceptible, moderately resistant, resistant, and very resistant). The results of this study indicated that the Sca-6 clone was included in the very resistant class with a score of 0.1, whereas Thevenin *et al.* (2012) grouped Sca-6 into the resistant class. The Sul-1 and Sul-2 clones with scores of 1.2 and 1.1, respectively, were grouped into the resistant class, whereas Suwastika *et al.* (2019) reported that Sul-1 belongs to the moderate class and clone Sul-2 belongs to the resistant class (ICCRI, 2008). In this research, the MCC-02 cocoa clones were grouped into the moderate resistance class with a score of 2.1, whereas in the research by McMahon *et al.* (2015), these clones were grouped into the resistant class. The DRC-16 clone was included in the susceptible class with a score of 3, whereas previous studies had classified it into the moderate class (Rubiyo *et al.* 2010). The cocoa clone DR-1 was grouped into a very susceptible class in the present work and in the susceptible class in a previous study (Iswanto and Winarno 1992). The differences in the resistance class of cocoa to black pod disease can be caused by the number of resistance classes used. The scoring based on the visualization of fungal development in the leaves classified the different cacao genotypes (Sca-6, MCC-02, Sul-2, DR-1, Sul-1, and

DRC-16) on the basis of their resistance levels. Fungal development in plants involves several stages, namely, inoculation, germination, penetration, infection, invasion, reproduction, and fungal transmission (Agrios, 2005). The difference in cacao response was caused by the mechanism of prepenetration and postpenetration resistance. Prepenetration resistance is related to the morphological characteristics of cacao plants, whereas postpenetration resistance is related to biochemical regulation with the activation of the resistant gene (R-gene) (Nyadanu *et al*., 2013).

In cacao genotypes, resistance to black pod disease is polygenic and is controlled by several genes (Ahnert and Pires, 2000). One of the regulatory genes of cacao resistance is the *CAT* gene. The *CAT* gene encodes the catalase protein in the homotetrameric form to produce superoxide ions and hydrogen peroxide (H_2O_2), which function to strengthen plant cell walls when attacked by a pathogen (Chelikani and Fita, 2004). Specific genes for disease resistance, such as the *CAT1* gene, can be developed for marker-assisted selection (MAS). The integration of MAS to identify cacao resistance to black pod disease will increase the effectiveness of selection.

The *CAT1* gene in six cacao genotypes with known resistance levels was isolated, and primers were designed to target polymorphic sites. In PCR, the nucleotide primer binds to the target site in the cacao genome in vitro. The primer's attachment to cacao DNA triggers a polymerase enzyme reaction for nucleotide fragment elongation. The capability of a primer to amplify the target genome is influenced by various factors, including primer length, melting temperature, guanine and cytosine content, and primer concentrations (Ozturk and Can, 2017).

Electrophoresis was used to identify DNA visually and observe DNA amplification. The working principle of electrophoresis is based on the movement of negatively charged particles (anions), in this case, DNA, which moves from the negative pole to the positive pole. The results of electrophoresis are given in the form of bands or amplified DNA fragments that indicate the number of base pairs. Electrophoresis involves the use of media in the form of agarose or acrylamide gel with a solvent using a buffet Tris acetate EDTA or Tris borate EDTA. DNA amplification is visualized on 1% agarose gel. Negatively charged DNA is attracted to a positively charged current in the electrophoresis device. Agarose gel separates the DNA bands with sizes of 200 bp to 50 000 bp (Green and Sambrook, 2019). Cat1 primer products have lengths of 885 bp. The intensity of the DNA bands is influenced by purity, DNA template concentration, and variation in nucleotide sequences in primer attachment (Zhuang *et al*., 2019).

The isolated *CAT1* gene fragment was sequenced by using the Sanger method. The Sanger method is used to determine DNA base sequences on the basis of the selective merging of dideoxy terminating chains in DNA polymerization in vitro (Sanger and Nicklen, 1977). The gene alignment of six cacao genotypes revealed six SNP sites that caused changes in amino acids (nonsynonymous). Nonsynonymous SNPs have the potential to change promoter work, mRNA stability, and efficiency during translation (Shastry, 2009). By contrast, synonymous SNPs do not cause changes in the amino acid function of a gene through splicing, mRNA structure, and protein structure (Yamagata *et al*., 2018).

The six SNP sites identified in the *CAT1* gene were designed as SNAP molecular markers. Each marker was amplified in six cacao genotypes that were subjected to the leaf disk assay to identify their resistance levels. The selected Cat1-71 primer visualized the band on the basis of the resistance level to black pod disease. SNAP-based DNA markers are biallelic and have codominant properties and are thus able to distinguish homozygous and heterozygous alleles (Morin and Luikart, 2004). Heterozygous alleles are identified from amplification with reference and alternate primers,

whereas homozygous alleles are amplified from one of the primers (reference or alternate) (Yundaeng *et al*., 2015). The Cat1-71 primer classified the cacao genotype Sca-6 as resistant; genotypes Sul-1 and Sul-2 as moderately resistant; and the cacao clones DR-1, MCC-02, and DRC-16 as susceptible.

The Cat1-71 marker was used to test the resistance of 16 cacao hybrids to black pod disease. The cacao hybrid clones in this study were the derivatives of the genotypes ICCRI-03, TSH-858, DR-01, and ICS-13 crossed with Sca-6 as the male parent. In accordance with DNA amplification in agarose gel, the hybrid cacao genotypes were predicted to be classified as resistant, moderate, and susceptible to black pod disease. Producing new resistant hybrids of cacao is crucial to deal with black pod disease. In cacao, conventional disease resistance breeding is difficult and time-consuming. The identification of SNAP markers related to cacao black pod resistance will increase accuracy and efficiency in the selection process to strengthen cacao breeding programs.

CONCLUSIONS

This study confirmed the identification of different resistance levels of cacao genotypes to black pod disease. SNP sites in the *CAT1* gene that can distinguish highly resistant cocoa clones from other classes were identified. The identified SNP sites were used to design SNAP molecular markers. The Cat1-71 marker may have the potential to predict the resistance levels of cacao genotypes.

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