Plant Disease • 2024 • 108:2615-2624 • https://doi.org/10.1094/PDIS-09-23-1788-SR

Special Report

PCR-Based Detection for the Quarantine Fungus *Colletotrichum kahawae*, a Biosecurity Threat to the Coffee (*Coffea arabica*) Industry Worldwide

Rosa Lilia Ferrucho,[1,†] Gustavo Adolfo Marín-Ramírez,[1] Francisco Ochoa-Corona,[2] and Carlos Ariel Ángel C.[1,†]

[1] Discipline of Plant Pathology, Colombian National Coffee Research Center (Cenicafé) – Colombian Coffee Growers Federation (CCGF), Manizales, Caldas 170009, Colombia
[2] Institute for Biosecurity and Microbial Forensics, Oklahoma State University, Stillwater, OK, U.S.A.

Abstract

Coffee berry disease is caused by *Colletotrichum kahawae*, a quarantine fungus still absent from most coffee-producing countries. Given the potential adverse effects on coffee berry production, it is a severe worldwide threat to farmers and industry. Current biosecurity management focuses on exclusion by applying quarantine measures, including the certification of coffee plants and their products. However, methods for detecting *C. kahawae* by National Plant Protection Organization (NPPO) laboratories still need approval. This research aims to functionally demonstrate, standardize, and validate a method for detecting and discriminating *C. kahawae* from other *Colletotrichum* species that may be present in coffee plant samples. The method proposes to use an end-point PCR marker for the mating type gene (*MAT1-2-1*) and a confirmatory test with a real-time quantitative PCR (qPCR) marker developed on the glutamine synthetase gene. The *C. kahawae* amplicons for the Cen-CkM10 qPCR marker exhibited specific melting temperature values and high-resolution melt profiles that could be readily differentiated from other tested species, including their relatives. Given the fungus's quarantine status, specificity was tested using artificial mixtures of DNA of *C. kahawae* with other *Colletotrichum* species and coffee plant DNA. The described method will enable NPPOs in coffee-producing and exporting countries, especially Colombia, to prevent this pathogen's entry, establishment, and spread.

Keywords: biosecurity, CBD (coffee berry disease), certification, *Colletotrichum kahawae*, etiology, pathogen detection, PCR, quarantine

Quarantine pests are of constant concern for agriculture worldwide, and their threat is high because of the globalization of international markets (Ebbels 2003; Ghelardini et al. 2017; Mathys and Baker 1980; Paini et al. 2016; Ristaino et al. 2021). *Colletotrichum kahawae* J.M. Waller and Bridge is the fungus that causes coffee berry disease (CBD) (Bridge et al. 2008; Cabral et al. 2020), a quarantine pest absent in Colombia (Instituto Colombiano Agropecuario 2015) and many other coffee-producing countries around the world. *C. kahawae* is a severe threat to the Americas, which host the countries that have the highest production of coffee in the world (Brazil, Colombia, Honduras, Perú, and Guatemala) and other important coffee-producing countries in Asia, such as Vietnam, Indonesia, and India (International Coffee Organization 2023), all CBD free (CABI 2024).

At present, the disease is restricted to some African countries (CABI 2024) where it causes coffee bean production losses and increasing management costs between 40 and 100% (Etana 2018; Giddisa 2016; Wubshet and Merga 2020). The prediction of the effects of this fungus in pathogen-free areas is complex, and the risk of damage is high because of the favorable environmental conditions for this disease in coffee-producing regions in Colombia and other countries.

The spreading of *C. kahawae* can occur passively through the legal or illegal movement of contaminated coffee plant material. Disease management in Colombia and other countries free of CBD focuses on excluding, inspecting, and certifying plants and their products (Augustin et al. 2012; Ebbels 2003; International Plant Protection Convention 2018; Kahn 1991). Similarly, periodic field surveillance to detect early introductions of exotic pests is essential for timely management measures (Augustin et al. 2012; Ristaino et al. 2021). Certification of imported material and field surveillance requires laboratory diagnostic methods to support decision-making (Martin et al. 2016; Ristaino et al. 2021). However, there are no validated methodologies for *C. kahawae* detection.

The high diversity of *Colletotrichum* species in coffee plantations (Table 1), wild hosts, and other plant species cultivated near coffee fields creates a risk for misidentification. Wrong identification of *C. kahawae* can potentially compromise the phytosanitary status of the crop and country, as happened with *C. cigarro* in Colombia (Pardo-De la Hoz et al. 2016; Rojas et al. 2018). The morphological discrimination of the *Colletotrichum* species is impossible because of the superposition of characters among species and the high intraspecific variability (Cannon et al. 2012; Nguyen et al. 2009; Weir et al. 2012).

Technical approaches for the discrimination of *C. kahawae* from taxonomically related species include (i) the in vitro growth rate in culture media, (ii) assimilation of carbon sources (Prihastuti et al. 2009; Waller et al. 1993; Weir et al. 2012), (iii) vegetative compatibility groups (Varzea et al. 2002), (iv) multilocus analysis of DNA sequences (Weir et al. 2012), (v) and pathogenicity tests on hypocotyls and green coffee fruits (Cabral et al. 2020; Prihastuti et al. 2009; Waller et al. 1993). However, these approaches are time-consuming and do not allow rapid diagnostic screening.

Methods based on DNA can reduce the laboratory's analysis time and provide fast and reliable results. Previous approaches from

[†]Corresponding authors: R. L. Ferrucho; rosa.ferrucho@cafedecolombia.com, and C. A. Ángel C.; carlosarielangel@gmail.com

Current address of C. A. Ángel C.: Independent Scientist, Villamaría, Colombia.

Funding: Support was provided by the Centro Nacional de Investigaciones de Café (Project ID PAT105004).

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

Accepted for publication 12 April 2024.

2013 were made for *C. kahawae* detection and discrimination by real-time quantitative PCR (qPCR) (Tao et al. 2013) and loop-mediated isothermal amplification (LAMP) (Tao and Cai 2013). These technologies were developed before the taxonomic rearrangement of *C. kahawae* from its closest relative species, *C. cigarro*, in 2020 (Cabral et al. 2020; Weir et al. 2012). Therefore, this relevant taxonomic rearrangement to further diagnostics development was not considered during these detection assay designs. It is essential to highlight that *C. cigarro* has a broad host and geographic range, including wild and cultivated crops (Weir et al. 2012).

Owing to its sensitivity, precision, and speed, the qPCR technique has become a gold standard routinely used in diagnostic laboratories (Grosdidier et al. 2017; Vincelli and Tisserat 2008). qPCR, combined with high-resolution melting (HRM) analysis, allows the discrimination of genotypes based on the melting temperature (Tm) of the amplified DNA fragment (Kanderian et al. 2015; Papavasileiou et al. 2016; Schiwek et al. 2020; Słomka et al. 2017).

This research hypothesizes that qPCR combined with HRM analysis allows the discrimination of *C. kahawae* from other *Colletotrichum* species commonly found on coffee plants. The objective was to develop a method for specific and reliable detection and differentiation of *C. kahawae*. We obtained a set of PCR markers for different genomic regions of *C. kahawae* based on the availability of sequences in the public databases and those recommended as appropriate for diagnosis in *Colletotrichum* spp. (Liu et al. 2015; Sharma et al. 2013; Silva et al. 2012; Vieira et al. 2020; Weir et al. 2012).

The developed method is based on the selective amplification of *C. kahawae* and nearby species using one marker for end-point PCR and a qPCR discriminatory marker. The detection is done using a qPCR DNA intercalating agent, and the discrimination of the *Colletotrichum* species uses the cycle threshold (Ct), the amplicon's Tm, and HRM analysis. Validation of the limit of detection (LOD), analytical specificity, selectivity, reproducibility, and repeatability was performed with the qPCR markers.

Table 1. *Colletotrichum* species reported in coffee crops per species complex

Colletotrichum **species complex**	**Species**
C. gloeosporioides	*C. kahawae*, *C. cigarro*[a], *C. gloeosporioides*, *C. theobromicola* (syn *C. fragariae*), *C. siamense*, *C. asianum*, *C. queenslandicum*, and *C. fructicola* (Cabral et al. 2020; Waller et al. 1993; Weir et al. 2012)
C. acutatum	*C. costaricense*, *C. cuscutae*, *C. walleri*, and *C. acutatum* (Damm et al. 2012a)
C. boninense	*C. boninense* and *C. karsti* (Damm et al. 2012b; Nguyen et al. 2009)
C. gigasporum	*C. gigasporum* and *C. vietnamense* (Liu et al. 2014)
C. orchidearum	*C. plurivorum* (Damm et al. 2019)
No information	*C. coffeophilum* (Jayawardena et al. 2021; Prihastuti et al. 2009)

[a] Not reported in coffee plants but in crops in the same geographic areas where coffee is cultivated in Colombia.

Materials and Methods

Fungal isolates and DNA extraction

Fungal isolates associated with coffee crops were obtained by sampling or donation (Afanador-Kafuri et al. 2014; Cruz et al. 2006) (Supplementary Tables S1 and S2). *C. kahawae* DNA from four reference isolates (International Collection of Plant Microorganisms [ICMP]) was imported to Colombia following biosecurity international procedures. DNA was sourced by the Plant Health and Environment Laboratory-Biosecurity (New Zealand). Native *Colletotrichum* isolates and other fungal species associated with coffee plants in Colombia were obtained by sampling symptomatic coffee plants in four different municipalities. Monosporic cultures were obtained from each Colombian isolate. Isolates were identified by genus morphology and assigned to the *Colletotrichum* species complex by sequence analysis of the internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA gene, ITS2) using the ITS5/ITS4 primers (White et al. 1990).

DNA extraction from the fungal cultures was performed using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The quality and quantity of the genomic DNA were evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and agarose gel electrophoresis. The DNA was adjusted to a 10 ng/µl concentration and stored at 4°C until use.

Owing to the quarantine status of *C. kahawae* in Colombia, the genomic regions selected for this study were cloned and used as positive controls. Each target region was amplified by end-point PCR, with DNA from the *C. kahawae* reference isolates, using high-fidelity Platinum *Taq* DNA polymerase (Invitrogen). Amplification was verified in agarose gels. The PCR fragments were ligated into the plasmid vector pCR 2.1-TOPO TA (Thermo Fisher), and ElectroMAX DH5α-E cells (Invitrogen) were transformed. The presence of the insert was verified by end-point PCR and Sanger sequencing, and sequences were deposited in the NCBI nucleotide database (Supplementary Table S1).

Primer design and qPCR standardization

Primers were designed for six genomic regions. After in silico analysis, markers for qPCR from the *GAPDH* and *GS* genes were selected for standardization and validation, and a marker for end-point PCR was developed for the *MAT 1-2-1* gene.

The qPCR mix consisted of 7.5 µl of Precision Melt Supermix for HRM Analysis (Bio-Rad), 0.1 µM of each primer, and 50 ng of DNA in 15 µl reactions. qPCR reactions were carried out in a CFX96 thermal cycler (Bio-Rad) programmed as follows: initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation (94°C for 30 s), specific annealing temperatures for each primer pair for 20 s, and extension (72°C for 20 s) (Table 2). The emitted fluorescence was measured after the annealing steps. The melting curves of the amplicons were generated by adding a denaturation step at 95°C for 30 s, followed by the formation of the heteroduplex at 60°C for 1 min, and reading from 65°C to 95°C in 0.2°C increments for 10 s. Each DNA sample was analyzed in duplicate. The thermal cycler outputs were analyzed using Bio-Rad CFX Maestro V5.0.021.0616 software. The Ct was determined, and the typical amplification curves were verified. The qPCR amplification products were confirmed in agarose gel electrophoresis to corroborate specificity.

Table 2. Laboratory standardized markers for PCR amplification

Marker label	**Primer name**	**Primer sequence (5′–3′)**	**Primer length (b)**	**Annealing temperature (°C)**	**Amplicon size (bp)**
Cen-CkM22, End-point PCR	Collm-MAT121-F2	CTCAGCCTCAATCCCCTCAA	20	57	428[a]
	Collm-MAT121-R	TACTGGTCAGCACCTGTTCC	20		
Cen-CkM05 real-time quantitative PCR	Collrt-GADPHF1	TGACTCTCATCCACCACCAA	20	56	111[a]
	Collrt-GADPHR	GTCCCGGCCCATGATTTCA	19		
Cen-CkM10 real-time quantitative PCR	Collrt-GSF1	ATTGGAGCTCCCCGCTTGA	19	55	110[a]
	Collm-GSR	GTGGATGTGGCGTTATGATG	20		
Cen-Coff-02 real-time quantitative PCR	Coff-CoxF	CTTCGGGTATCTAGGCATGG	20	55	113[b]
	Coff-CoxR	AGTAGGCACGGGTATCAACG	20		

[a] Expected amplicon size for *Colletotrichum kahawae*.
[b] Expected amplicon size for *Coffea arabica*.

qPCR marker validation

LOD or analytical sensitivity. Serial dilutions of plasmid with the insert and genomic DNA of the reference isolate ICMP17915 were analyzed. For the genomic DNA, nine dilutions were prepared to obtain concentrations from 5×10^4 to 5×10^{-4} pg of DNA per qPCR reaction. Ten serial dilutions were prepared for plasmid DNA to obtain approximately 2×10^9 to two copies of the plasmid per qPCR reaction. The number of plasmid copies was calculated based on Whelan et al. (2003). Three plates were analyzed for each marker and DNA source (Hard-Shell 96-Well PCR Plates, sealed with microfilm 'B' Seals – Bio-Rad). Each plate contained six replicates for a total of 18 PCR reactions for each dilution. The PCR plates were run on different days. The data of Ct and Tm, the standard deviation (SD) of Ct values by each marker, dilution, and the run quality per plate (qPCR efficiency and R^2) were obtained. Finally, the LOD and the cutoff point for Ct were defined based on the individual results of each run, and the results of the joint analysis per marker were performed using the script qPCR_LOD_Calc (Klymus et al. 2020) on the R platform.

Exclusivity and inclusivity

Exclusivity was evaluated with genomic DNA from the *C. kahawae* reference isolates and DNA from 84 nontarget species. Most analyzed species comprised fungal taxa of the *Colletotrichum* species complexes recorded in coffee crops and fungi commonly found as pathogens or natural inhabitants in coffee plants (Supplementary Table S2). DNA from coffee leaves (*Coffea arabica* var. Caturra) was verified by qPCR with the Cen-Coff-02 marker (Table 2) and used as a control. Two dilutions of plasmid DNA were included as positive controls. Two replicates of each sample were run at different times using 50 ng of genomic DNA. The amplification and specificity of the PCR products were verified by agarose gel electrophoresis. The same samples were evaluated using 30 ng of DNA per reaction. For analysis, native *Colletotrichum* spp. samples were grouped as species complexes according to the ITS sequence analysis. The Ct and Tm values range was determined for each taxonomic group and analyzed with the *ggplot* package on the R platform (Wickham 2009).

HRM analysis was accomplished for samples with Ct values lower than 30 cycles, typical curve amplification, and only one Tm peak using the Precision Melt Analysis software (v1.2, Bio-Rad). Melted regions were automatically detected, and clusters were obtained using plasmid standards and *C. kahawae* DNA as the baseline genotypes.

For inclusivity, four *C. kahawae* isolates and the corresponding plasmid DNA were evaluated using three dilutions (50, 5, and 0.5 ng of genomic DNA and 2×10^6, 2×10^4, and 2×10^2 copies of the plasmid per 15 µl qPCR reaction). Three replicates were analyzed for each dilution.

Repeatability

The obtained data in the LOD test were analyzed in three concentrations of genomic DNA (5×10^4, 5×10^3, and 5×10^2 pg/reaction) and three concentrations of plasmid DNA (2×10^6, 2×10^4, and 2×10^2 copies of the plasmid/reaction) at three different times. The results of the six replicates of each concentration were visualized with the *ggplot* package (Wickham 2009). Repeated-measures analysis of variance was performed with the R platform's rstatix package (Kassambara 2022). The dispersion of the Ct values was evaluated with the SD of the mean in the tested times.

Selectivity

Artificial mixtures of DNA were analyzed. Genomic *C. kahawae* DNA from the reference isolate ICMP17915 was mixed with DNA from other *Colletotrichum* species and DNA from coffee leaves. Individual samples and mixtures were prepared for a final DNA concentration of 50 ng per 15 µl qPCR reaction. Decreasing volumes of DNA from *C. kahawae* and increasing volumes of DNA from each nontarget organism were used. For the Cen-CkM05 marker, five *C. kahawae* nontarget DNA proportions were analyzed (50:0, 25:25, 12.5:37.5, 5:45, and 0.5:49.5) using five nontarget species. For the Cen-CkM10 marker, two isolates of *C. kahawae* and 24 *Colletotrichum* spp. isolates were analyzed. Two proportions of DNA were evaluated (25:25 and 12.5:37.5). All sample mixtures were processed in duplicate.

Reproducibility

The consistency of results was evaluated by introducing variations in the method, such as the operator and the brand of the HRM kit. Samples were prepared with low, medium, and high concentrations of genomic DNA and the cloned plasmid controls. Three operators handled the samples with the Precision Melt Supermix (Bio-Rad), and a test was performed using the FastGene 2× Optima PCR HotStart Ready Mix kit (Nippon Genetics).

Marker for end-point PCR

The analytical specificity (inclusivity and exclusivity) was evaluated with DNA from *C. kahawae*, *Colletotrichum* spp., DNA of other fungi associated with coffee plants, and DNA from *C. arabica* leaves. PCR reactions contained 1× PCR buffer, 2.5 mM $MgCl_2$, 0.5 U of *Taq* DNA polymerase (Invitrogen), 0.15 µM of each primer, 0.2 mM of DNTP mixture (Invitrogen), and 40 ng of genomic DNA in a final volume of 15 µl. PCR was performed in a Bio-Rad T100 thermal cycler with the following conditions: 95°C for 5 min, followed by 25 cycles (95°C for 25 s, 57°C for 25 s, and 72°C for 35 s), and a final step at 72°C for 10 min. The amplicons were verified by 2% agarose gel electrophoresis in a 1× TAE buffer with SYBR Safe staining (Thermo Fisher).

Results

The availability of gene sequences for all the *Colletotrichum* species reported in coffee plants and for the close relatives of *C. kahawae* was limited. Genes selected for primer design were based on the diversity of species and previous reports of their utility for *Colletotrichum* taxonomy studies (Liu et al. 2015; Sharma et al. 2013; Silva et al. 2012; Vieira et al. 2020; Weir et al. 2012).

Two qPCR markers (Cen-CkM05 and Cen-CkM10) and one end-point PCR marker (Cen-CkM22) were standardized in the laboratory after in silico specificity and quality analysis (Supplementary Table S3; Table 2). The Cen-CkM05 marker was designed for the *GAPDH* gene. Although this region is conserved for both *C. kahawae* and *C. cigarro,* it is helpful in separating those species from others in the *C. gloeosporioides* complex. Primers for marker Cen-CkM10 were designed on the *GS* gene, a region reported to differentiate *C. kahawae* from *C. cigarro* (Weir et al. 2012) and other relatives, including *C. jiangxiense*. Although a low diversity of sequences was found for the *MAT1-2-1* gene in the public databases, a marker was designed (Cen-CkM22) based on previous studies in the *C. gloeosporioides* complex taxonomy (Sharma et al. 2013).

qPCR markers validation

LOD. The LOD or analytical sensitivity determines the capacity to detect an assay target at low levels with reasonable certainty (95% probability is commonly used) (Bustin et al. 2009; Klymus et al. 2020; OEPP/EPPO 2021). Bustin et al. (2009) proposed a theoretical minimum of three copies per PCR reaction, assuming a Poisson distribution, a 95% chance of including at least one copy in the PCR, and single-copy detection. Both markers detected 20 plasmid copies in 94% of the samples (Table 3; Supplementary Fig. S1) and 5 pg of *C. kahawae* genomic DNA per qPCR reaction for Cen-CkM05 marker and 0.5 pg/reaction for Cen-CkM10 (Table 3; Supplementary Fig. S2). For the final dilutions, the Ct values were higher than 35 cycles, and the curves did not reach the stabilization phase, representing late amplifications. A Ct threshold of 30 cycles was determined for the two markers to avoid false positives in samples amplifying nontarget organisms. Ct values equal to 30 were obtained with approximately 2×10^4 plasmid copies (Supplementary Fig. S1) and 5×10^4 pg of genomic DNA by qPCR reaction (Supplementary Fig. S2).

For the analyzed serial dilutions, quality variables were in the expected range (Table 3). The efficiency of qPCR ranged between 80.9 and 87.2% for plasmid DNA and between 73 and 88.7% for genomic DNA. The linearity (R^2) was between 0.984 and 0.994 for genomic DNA, and it was close to 1 (0.988 to 0.998) for plasmid

DNA, which agrees with the optimal value reported ($R^2 > 0.98$) (Bustin et al. 2009). In most samples, the SD was less than one unit among the 18 replicates. It was higher for the lowest concentrations (2 to 2,000 copies), resulting from the lower target concentration in the analyzed sample, as reported (Forootan et al. 2017; Klymus et al. 2020).

DNA Tm. The Tm value, driven by the amplicon length and the guanine-cytosine content, allows the verification of primer specificity (Słomka et al. 2017; Winder et al. 2011). Additionally, the Tm values can discriminate PCR products from nearby organisms with differences of up to one nucleotide in the sequence (Lipsky et al. 2001; Winder et al. 2011). The predominant Tm of the amplicons of the marker Cen-CkM05 was 82.2°C, whereas for the marker Cen-CkM10 two values were obtained (84.8 and 85°C) with DNA from the ICMP17915 reference isolate (Table 3; Supplementary Fig. S3).

Exclusivity

qPCR amplification. For marker Cen-CkM05, only the *C. kahawae* and *C. cigarro* samples had Ct values lower than 30 cycles when 50 ng of DNA per qPCR reaction was used (Table 4; Supplementary Fig. S4). Ct values are below the threshold for four *Colletotrichum* species for marker Cen-CkM10. Some isolates of the *C. boninense* and *C. acutatum* complexes and other fungi associated with coffee plants also amplified but over the threshold point (30 cycles). In some cases, the amplification and dissociation curves were atypical; similarly, this was observed with DNA extracted from healthy coffee leaves (Table 4).

When using 30 ng of genomic DNA, low Ct values for *C. kahawae* were registered compared with the nontarget species. However, the Ct value increased in all the samples, and the proportion of nonamplified samples decreased for nontarget species (Supplementary Fig. S5). Although this result is acceptable, one sample of *C. kahawae* exceeded the established threshold Ct value of 30 cycles with both markers, causing the threshold Ct criterion of 30 for *C. kahawae* not to be correct, leading to false negatives or uncertainty reports when they are close to the Ct threshold of 30.

Tm and HRM analysis to differentiate *C. kahawae*. The Tm value was variable for samples tested with the Cen-CkM10 marker. Those differences were particularly relevant in discriminating *C. kahawae* from *C. cigarro* and nearby species (Table 4; Fig. 1), showing the pertinence of HRM analysis. HRM was sensitive to distinguishing *C. kahawae* (blue lines) from *C. cigarro* and close

Table 3. Summary of the features obtained in the LOD test

Marker label	DNA analyzed[a]	LOD95[b]	Tm (°C) (%)[c]	Quality values for LOD tests		
				Efficiency%	Linearity R^2	Ct_SD[d]
Cen-CkM05	Plasmid (PCN)	20	82.0 (10%), 82.2 (90%)*	84.8–85.2	0.997–0.998	0.068–1.173
	Genomic (pg)	50	82.0 (3.5%), 82.2 (96.5%)*	73.8–83.1	0.984–0.994	0.136–1.068
Cen-CkM10	Plasmid (PCN)	20	84.8 (57%)*, 85.0 (42%), 85.2 (1%)	80.9–84.2	0.988–0.998	0.056–2.448
	Genomic (pg)	5	84.8 (49%), 85.0 (51%)	73.0–88.7	0.976–0.994	0.097–0.919

[a] Plasmid containing the target gene or genomic DNA from *Colletotrichum kahawae* reference isolate ICMP17915. PCN = plasmid copy number; pg = picograms.
[b] DNA concentration (PCN or pg) at which fluorescence was detected over the threshold in 95% of the samples, no matter the Ct value. LOD = limit of detection.
[c] Percentage of samples (%) in each melting temperature (Tm) value is presented in parentheses. The most frequent values are those with an asterisk (*).
[d] Ct_SD = standard deviation of the cycle threshold value.

Table 4. Results of the exclusivity test for the markers using 50 ng of genomic DNA per reaction

Taxonomic group	N[a]	Code	Ct group	Marker Cen-CkM05		Marker Cen-CkM10	
				Samples %[b]	Tm values (°C)	Samples %[b]	Tm values (°C)
C. gloeosporioides species complex	17	Coll-Col[c]	<30	0.0	–	35.3	86.0, 86.4, 86.6, 87.0
			>30	58.8	82.0, 82.2, 82.4	17.6	84.8, 86.4
			NA	41.2	–	47.1	–
C. gloeosporioides	1	ICMP12938	<30	0.0	–	100	86.2
C. cigarro	3	R046, S007, ICMP6999	<30	100	82.0, 82.2	66.7	86.8, 87.2
			>30	0.0	–	33.3	87.2, 87.4
C. fructicola	1	ICMP17921	>30	100	82.2	100	86.6, 86.8
C. theobromicola	1	ICMP18649	>30	100	82.0, 82.4	100	86.0, 86.6
C. kahawae	4	ICMP17811, ICMP17816, ICMP17905, ICMP17915	<30	100	81.8, 82.0, 82.2	100	84.8, 85.0, 85.2
Ck-915 (−3)	2	C+ (Plasmid)	<30	100	82.0, 82.2	100	84.8, 85.0, 85.2
C. acutatum species complex	25	Coll-Col[c]	<30	0	–	0.0	–
			>30	60	82.0, 82.2, 82.4, 83.0	60	84.8, 85.0, 85.2
			NA	40	–	40	–
C. acutatum	1	ICMP20568	>30	100	82.2	100	84.8, 85.0
C. boninense species complex	12	Coll-Col[c]	>30	33.3	82.0, 82.2	25	84.8, 85.0
			NA	66.7	–	75	–
Colletotrichum spp.	7	Coll-Col[c]	>30	42.9	82.0, 82.2	28.6	84.8, 85.0
			NA	57.1	–	71.4	
Coffee fungi	14	Fung-Col[c,d]	>30	50	78.2, 82.0, 82.2, 82.4, 82.6, 86.0	0.0	–
			NA	50	–	100	–
Coffea arabica cv. Caturra	2	MEG[e]	>30	100	79.2, 82.4	0.0	–
			NA	0		100	–

[a] Number of samples evaluated in each taxonomic group (Supplementary Table S2).
[b] Percentage of samples in each range of values. Categories according to cycle threshold (Ct) values: (i) less than 30, (ii) higher than 30, and (iii) nonamplified samples (NA). When applicable, the melting temperature (Tm) value was registered for each category.
[c] *Colletotrichum* isolates collected in coffee crops in Colombia as part of this research.
[d] Isolates of coffee-inhabiting fungi collected in this research or donated.
[e] DNA from *C. arabica* leaves donated by Plant Breeding in Cenicafé.

species, identifying *C. kahawae* as a different *Colletotrichum* species. The interpretation of HRM results was made considering the following: (i) the low-resolution melting derivative plot (−d [RFU]/dT against temperature) (Fig. 1A and B), (ii) the normalized data derived from plots of raw data (Fig. 1C and D), and (iii) the difference curve (Fig. 1E and F). The minus difference graphs derived from the normalization data show clear differences between species, as seen by the colored lines (Fig. 1). The HRM analysis did not show discrimination power for the Cen-CkM05 marker. This result was expected, given the slight variation in the Tm value, as a consequence of the similarity of the *GAPDH* gene for the amplified samples.

Inclusivity and repeatability. For inclusivity, the amplifications were as expected according to the three plasmid and genomic DNA dilutions with the four *C. kahawae* reference isolates (Table 5). Results were repeatable in the same trial and DNA concentration. The interassay variation was higher, with significant differences in tests carried out at different times (Supplementary Fig. S6). The SD of the Ct values ranged between 0.08 and 0.96 in the evaluated times (Table 5).

Selectivity. For marker Cen-CkM05, mixtures of *C. kahawae* DNA with DNA from other *Colletotrichum* species generated a variable response, depending on the DNA proportions and the combination of evaluated species. High interference was observed between *C. kahawae* and samples in the *C. gloeosporioides* complex. In the mixtures of *C. kahawae* DNA with nonrelated species, Ct values increased as the concentration of *C. kahawae* DNA decreased, showing that nontarget DNA would not interfere with the detection of *C. kahawae*. The obtained Ct values were under the 30 Ct in mixtures containing DNA of *C. cigarro* (Fig. 2). Although *C. cigarro* is not yet reported to infect coffee plants, its presence as a contaminant in a sample would generate false-positive results. The Tm value

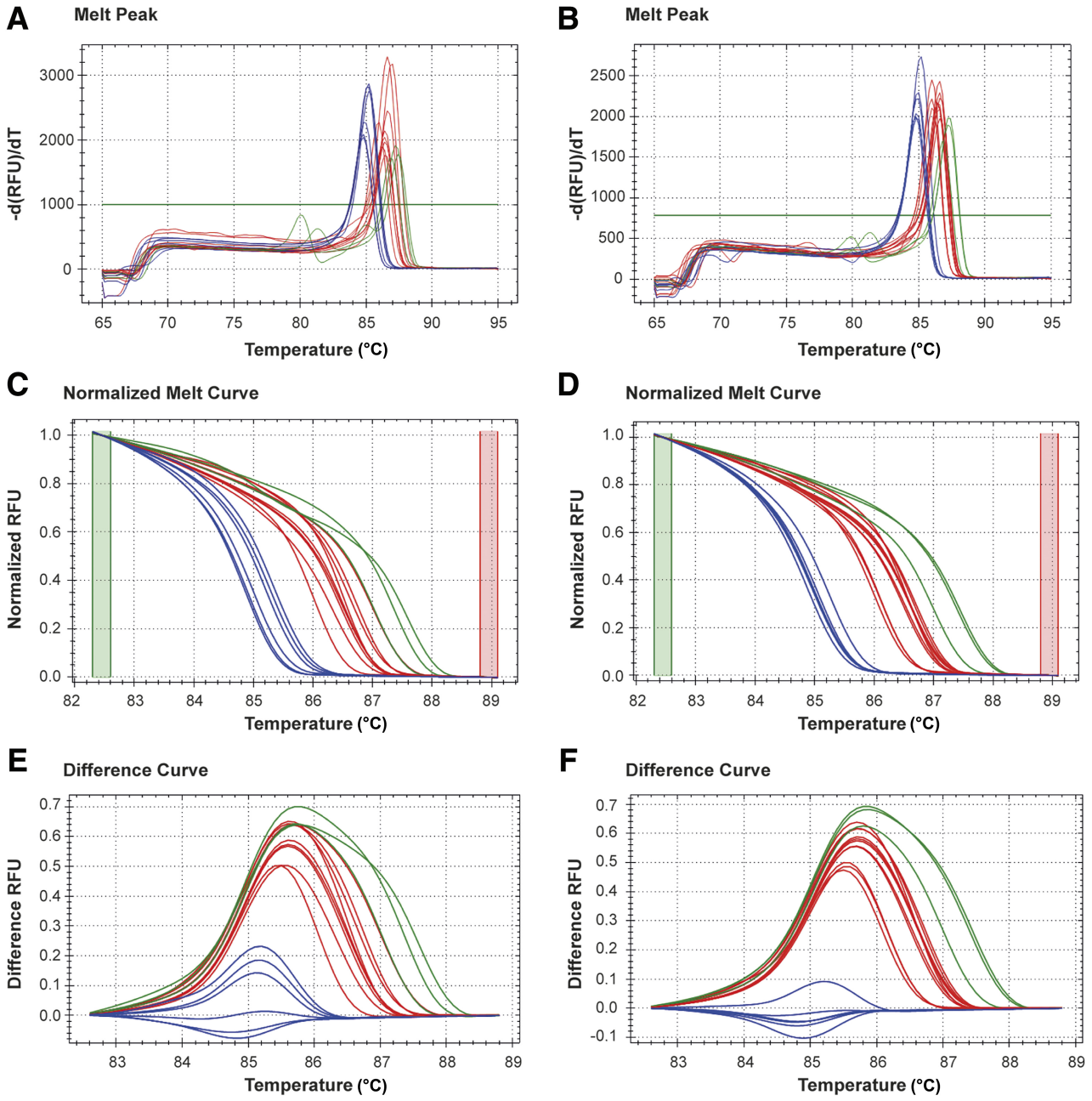

Fig. 1. Discrimination of *Colletotrichum kahawae* isolates for the Cen-CkM10 marker by melting temperature and melting profiles. Results for two independent runs (Run 1: **A, C, and E** and Run 2: **B, D, and F**). Blue lines identify *C. kahawae* samples, green lines *C. cigarro* isolates, and red lines *Colletotrichum* spp. A and B, Melting peak. C and D, Normalized HRM melting curves. E and F, Difference graph using *C. kahawae* as the baseline genotype. RFU = relative fluorescence units.

of the Cen-CkM05 marker was similar for *C. kahawae* and *C. cigarro*. If both are present in the same sample, it is not possible to discriminate the identity as in previous studies (Tao et al. 2013).

The tests with the marker Cen-CkM10 showed that the nontarget *Colletotrichum* DNA interfered with detecting *C. kahawae*. High heterogeneity was observed in the Ct values for most of the assessed DNA combinations, with most Ct values over the threshold (Table 6). In 84% of the cases, the Tm value agreed with the Tm expected for *C. kahawae*, showing that although there was interference in the amplification, the primers preferentially amplified *C. kahawae*. Several peaks were obtained in the Tm curve in some DNA combinations, showing simultaneous amplification of the two species in the DNA mixture (Table 6; Supplementary Fig. S7).

Reproducibility

The obtained Ct values were within those expected for each DNA concentration. Heterogeneity was observed in the results for the same sample when processed by three different operators, and the variation was higher for samples with medium and low concentrations of *C. kahawae* DNA (Fig. 3). These results confirmed the optimal DNA concentration for qPCR as 50 ng of genomic DNA. DNA below the recommended level generated false negatives. With the optimal concentration of *C. kahawae* genomic and plasmid DNA, the three operators usually reached Ct values below the established threshold of 30 cycles.

With the FastGene 2× Optima PCR HotStart kit (Nippon Genetics), the results were according to the expected (Ct values below 30 cycles). The Tm value was preserved, and no differences were associated with the used HRM kit. The results showed that the markers can be used with another brand of HRM kit, at least with the two tested brands. The obtained Ct values were within the expected range for the analyzed DNA levels when processed by different operators with a different brand of HRM kit, highlighting the robustness of the developed method (Groth-Helms et al. 2023) (Supplementary Fig. S8).

Standardization of a marker for end-point PCR

The standardized Cen-CkM22 marker for end-point PCR has an expected amplicon size of 428 bp. For the exclusivity test, amplification was obtained exclusively with *C. kahawae* and *C. cigarro* samples (Supplementary Fig. S9). Although the marker was not specific for *C. kahawae*, the amplification was restricted to these two species. Therefore, this marker is recommended for laboratories without facilities for qPCR. However, samples have to be subject to confirmation with the qPCR Cen-CkM10 marker developed in this research.

Table 5. Ct values obtained in inclusivity and repeatability tests with three concentrations of DNA

			Inclusivity	Repeatability[b]	
Marker	**DNA analyzed**	**DNA level[a]**	**Ct**	**Ct**	**SD**
Cen-CkM05	Plasmid DNA (PCN)	High	20.0–25.6	21.6–22.6	0.08–0.31
		Medium	27.3–34.1	28.9–30.5	0.12–0.96
		Low	35.3–44.3	36.6–38.2	0.20–0.30
	Genomic DNA (ng)	High	26.7–32.1	26.6–27.6	0.13–0.24
		Medium	30.6–33.3	31.1–32.3	0.12–0.48
		Low	34.3–38.3	35.1–36.6	0.19–0.24
Cen-CkM10	Plasmid DNA (PCN)	High	23.4–25.0	21.6–22.6	0.08–0.31
		Medium	31.6–33–3	28.9–30.5	0.12–0.96
		Low	39.5–41.3	36.6–38.2	0.20–0.30
	Genomic DNA (ng)	High	27.6–29.8	26.3–26.7	0.16–0.28
		Medium	31.8–36.4	30.3–31.1	0.08–0.22
		Low	36.2–38.1	34.5–35.3	0.15–0.20

[a] Plasmid DNA concentration (plasmid copy number [PCN]): high: 2×10^6, medium: 2×10^4, and low: 2×10^2. Genomic DNA concentration (ng): high: 50, medium: 5, and low: 0.5.
[b] Range of cycle threshold (Ct) and SD values for three evaluation times.

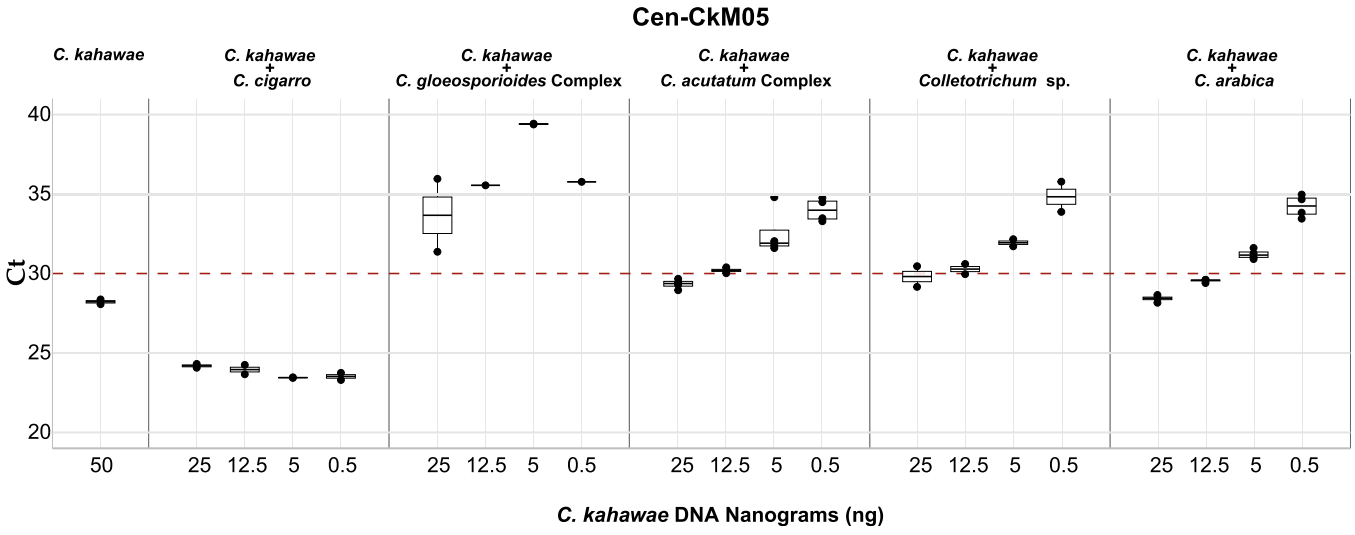

Fig. 2. Cycle threshold (Ct) values of artificial DNA mixtures tested with the Cen-CkM05 marker. The dashed line shows the threshold Ct value of 30 cycles. Each tested sample contained 50 ng of genomic DNA per real-time quantitative PCR reaction. The mixtures were decreasing concentrations of *Colletotrichum kahawae* DNA (50, 25, 12.5, 5, and 0.5 ng) and increasing concentrations of other species' DNA (0, 25, 37.5, 45, and 49.5 ng).

Discussion

C. kahawae is a specific coffee plant pathogenic fungus restricted to Africa. The availability of a method to detect this quarantine fungus is fundamental for the biosecurity of coffee crops worldwide, mainly in the Americas and Asia, which are still free of this fungus and are the largest coffee producers worldwide (International Coffee Organization 2023). Accurate and timely identification is essential to support regulatory control decisions, namely, exclusion, eradication, and containment (Groth-Helms et al. 2023; Martin et al. 2016; Mathys and Baker 1980). Preventing the entry of *C. kahawae* supports the protection of coffee growers and agroecosystems and avoids coffee berry production losses and fungicide use to control the disease, its consequent environmental and economic impacts, and additional investments required for crop management and disease control.

The main restriction of previous diagnosis methods is their inability to separate *C. kahawae* from taxonomically related species associated with coffee plants (Table 1) or from *Colletotrichum* isolates from diverse hosts (unrelated to coffee) and geographic origins that are phylogenetically close and frequently placed as *C. kahawae* (Cabral et al. 2020; Garibaldi et al. 2016; Pardo-De la Hoz et al. 2016; Schena et al. 2014; Wei et al. 2022). The wrong identification of *C. cigarro* as *C. kahawae* has compromised the phytosanitary status

Table 6. Ct and Tm values obtained for marker Cen-CkM10 with individual and artificial mixtures of fungi and coffee plant DNA

			DNA proportions (ng:ng)			
			25:25		12.5:37.5[d]	
Evaluated samples	*N*	Type[a]	Ct[b]	Tm (°C)[c]	Ct	Tm (°C)
C. kahawae	2	Individual	27.1–27.3	**85.0**	NE	NE
C. cigarro	2	Individual	31.8–33.6	87.0, 87.4	NE	NE
C. kahawae + *C. cigarro*	2	Mixture	30.9–34.7	81.6, **85.0**	34.1–36.0	81.6, 85.0
C. gloeosporioides	1	Individual	32.2	86.2	NE	NE
C. kahawae + *C. gloeosporioides*	1	Mixture	29.1	**85.0**	32.8	85.2
C. fructicola	1	Individual	33.8	86.8	NE	NE
C. kahawae + *C. fructicola*	1	Mixture	31.5	**85.0**	34.8	85.2
C. theobromicola	1	Individual	33.9	86.0	NE	NE
C. kahawae + *C. theobromicola*	1	Mixture	30.1	85.0	32.5	85.0
C. gloeosporioides complex	6	Individual	28.2–33.3	86.2, 86.6	NE	NE
C. kahawae + *C. gloeosporioides* complex	6	Mixture	28–31.5	**85.0**, **85.2**, 86.6	–	–
C. acutatum complex[e]	11	Individual	28.6–38.5	**85.0**, 86.6	NE	NE
C. kahawae + *C. acutatum* complex	11	Mixture	29.7–34.6	**85.0**, **85.2**	30.8–36.9	85.0, 85.2, 86.6
Colletotrichum sp.	1	Individual	38.3	86.8	NE	NE
C. kahawae + *Colletotrichum* sp.	1	Mixture	35.1	**85.0**	33.6	85.2
C. boninense	1	Individual	37.9	85.0	NE	NE
C. kahawae + *C. boninense*	1	Mixture	30.4	**85.0**	33.4	**85.0**

[a] Individual samples contained 50 ng of DNA from only one species. They were run once.
[b] Ct = cycle threshold.
[c] Melting temperature (Tm) expected values for *C. kahawae* are highlighted in bold.
[d] NE = not evaluated; – = no amplification.
[e] Eight samples from the *C. acutatum* complex did not amplify.

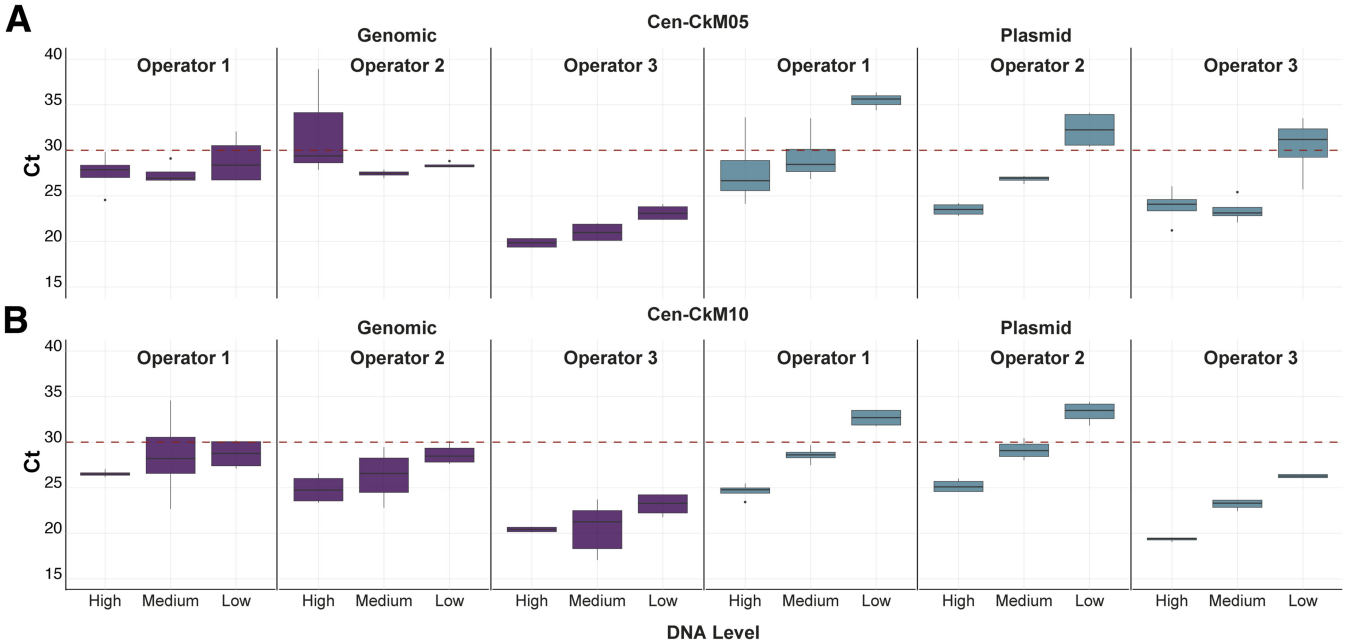

Fig. 3. Cycle threshold (Ct) values obtained by three operators. **A,** Cen-CkM05 marker. **B,** Cen-CkM10 marker. The boxplot shows the results for genomic (left) and plasmid (right) DNA in three concentrations (high: 50 ng of genomic DNA and 2×10^5 copies of the plasmid, medium: 25 ng of genomic DNA and 2×10^4 copies of the plasmid, and low: 5 ng of genomic DNA and 2×10^3 copies of the plasmid per 15 µl real-time quantitative PCR reaction).

of the coffee crop in Colombia (Pardo-De la Hoz et al. 2016; Rojas et al. 2018). This highlights the importance of developing and updating the methods according to changes in the pathogen's taxonomy.

Previous methods for *C. kahawae* diagnosis (qPCR and LAMP) used the *GAPDH* gene and Apn2/MAT locus as target regions (Tao and Cai 2013; Tao et al. 2013). Those genes are helpful for *Colletotrichum* taxonomic studies (Silva et al. 2012; Vieira et al. 2020), but not to distinguish *C. kahawae* from their relatives (Cabral et al. 2020; Liu et al. 2015). We conducted a bioinformatics analysis with primers and probes reported by Tao et al. (2013) and found that they have 96 to 100% identity with other *Colletotrichum* species, including *C. cigarro*. Primers for the LAMP test (Tao and Cai 2013) had homology with *C. cigarro* and *C. jiangxiense*; the number of sequences for the Apn2/MAT locus was low, limiting the analysis and conclusions about their convenience (Supplementary Figs. S10 and S11).

The present research study provides a more precise method for distinguishing *C. kahawae* from many related *Colletotrichum* species found naturally in coffee crops. We designed and tested two qPCR markers (Cen-CkM05 for the *GAPDH* gene and Cen-CkM10 for the *GS* gene) and one marker for end-point PCR (Cen-CkM22 on the *MAT1-2-1* gene). Primers designed for genes *GAPDH* and *MAT1-2-1* still have homology with *C. cigarro* and *C. jiangxiense*.

Previous studies have shown that *C. kahawae* is distinguished from its relatives based on the *GS* gene (Cabral et al. 2020; Liu et al. 2015; Weir et al. 2012). The marker designed in this study on the *GS* gene differentiates *C. kahawae* by the Tm value or HRM analysis. The Tm value was specific, and the normalized HRM curves generated particular melting profiles for *C. kahawae* (Fig. 2). HRM analysis complements *C. kahawae* differentiation. However, it is optional given that the differences in the amplicon Tm value were sufficient to separate *C. kahawae* from nearby species.

The initial discrimination of species was based on a 30 cycles (Ct) threshold for both qPCR markers. Ct values were consistently less than 30 cycles for *C. kahawae* DNA and higher for most analyzed taxonomic groups when we used 40 to 50 ng of DNA per qPCR reaction. To define a Ct threshold is essential because it minimizes the risk of false positives caused by sample contamination or the late amplification of related species (Bustin et al. 2009; Caraguel et al. 2011; Grosdidier et al. 2017; Groth-Helms et al. 2023).

The parameters and the strategy used for validation were based on the recommendations of international standards (International Organization for Standardization 2019, 2021, 2022; OEPP/EPPO 2021; U.S. Food and Drug Administration 2020). Because *C. kahawae* is a quarantine pathogen still not present in Colombia, we faced several restrictions that limited the resources available for validation. For example, DNA extraction from *C. kahawae* mycelium could not be standardized, and the defined optimal concentration of genomic DNA could change with an optimized DNA extraction protocol from the mycelium of the fungus. Additionally, we could not assess a wider diversity of *C. kahawae* isolates during the inclusivity test as desirable (AOAC International 2019; Cardwell et al. 2018; Groth-Helms et al. 2023; OEPP/EPPO 2021; U.S. Food and Drug Administration 2020). However, the number of *Colletotrichum* species tested in the exclusivity analysis may represent the natural diversity of species in coffee crops in the central production zone of Colombia because they included *C. gloeosporioides*, *C. acutatum*, and *C. boninense* complexes, currently reported as the most frequent in coffee plants worldwide (Damm et al. 2012a, b; Nguyen et al. 2009; Weir et al. 2012) (Table 1).

In the artificial mixtures with *C. kahawae* DNA, the greatest interferers were other *Colletotrichum* species, particularly those from the *C. gloeosporioides* species complex. Interference from nontarget *Colletotrichum* species was evident by cross-amplification or heterogeneity and higher Ct values. Currently, 17 species of *Colletotrichum* are reported to be associated with coffee plants (Table 1) (Damm et al. 2012b, 2019; Liu et al. 2014; Nguyen et al. 2009; Weir et al. 2012). All of them can coexist with *C. kahawae*, and some can prevail over *C. kahawae* in advanced infections (Chen et al. 2005). Given that, in a sample, it is not possible to know a priori whether there is more than one *Colletotrichum* species mixed with *C. kahawae* or which of them is prevalent, it is necessary to analyze a single species obtained from monoconidial cultures. This avoids erroneous results in the diagnostic test by cross-amplification and prevents the risk of detecting DNA from dead *C. kahawae* cells in the tested sample (Henson and French 1993).

The results for the molecular markers developed in this research were replicable and reproducible under independent conditions. Ct values were those expected when using the optimal DNA concentrations. Low intra-assay variation was observed in tests carried out over short time intervals, with the same DNA concentrations, in the same laboratory, executed by the same operator, and with the same equipment.

We reported an improved method for detecting and discriminating *C. kahawae* from closely related species, particularly *C. cigarro*, which is widely distributed and frequently confused with *C. kahawae* even in crops different than coffee. The method is recommended for laboratories performing qPCR and Tm analysis for the Cen-CkM10 marker. The end-point PCR marker (Cen-CkM22) is not specific for *C. kahawae*, but its amplification was limited to *C. kahawae* and *C. cigarro*, and it can be used as a preliminary test in laboratories without qPCR facilities; the results must be confirmed with the Cen-CkM10 marker. As a summary, a sample test is positive for *C. kahawae* if the following parameters are obtained: (i) a band of approximately 428 bp with the end-point PCR marker (Cen-CkM22), (ii) Ct values less than 30 cycles for the Cen-CkM10 qPCR marker, and (iii) a Tm value of 84.8 or 85°C or differential curves using HRM analysis for the Cen-CkM10 marker.

In the future, it will be possible to have additional markers to complement the method presented here. Whole-genome sequencing and comparative genomics open new possibilities for developing specific markers for diagnosing *C. kahawae* and related species based on variations associated with the *Colletotrichum* species.

Acknowledgments

The authors gratefully acknowledge Dr. Katharina Hofer and Dr. Brett Alexander from the Plant Health and Environment Laboratory (New Zealand Ministry for Primary Industries) who provided the DNA of *C. kahawae* used as controls. The authors thank the researchers at Cenicafé who provided controls (Luisa López, Nancy Arciniegas, and Dr. Carmenza Góngora). The authors also thank Professor Lucia Afanador (National University of Colombia, Medellin campus) for sourcing two isolates of *C. cigarro*. The authors also acknowledge the researchers from Cenicafé's Entomology and Phytopathology who carried out the reproducibility test and the scientist Carlos Ernesto Maldonado from Cenicafé for the technical advice and the helpful recommendations on the manuscript. Finally, the authors thank Senior Editor Kenneth Frost and the anonymous reviewer for the valuable suggestions that helped us improve the manuscript.

Literature Cited

Afanador-Kafuri, L., González, A., Gañán, L., Mejía, J. F., Cardona, N., and Alvarez, E. 2014. Characterization of the *Colletotrichum* species causing anthracnose in Andean blackberry in Colombia. Plant Dis. 98:1503-1513.

AOAC International. 2019. Appendix F: Guidelines for standard method performance requirements. https://www.aoac.org/wp-content/uploads/2019/08/app_f.pdf

Augustin, S., Boonham, N., De Kogel, W. J., Donner, P., Faccoli, M., Lees, D. C., Marini, L., Mori, N., Petrucco Toffolo, E., Quilici, S., Roques, A., Yart, A., and Battisti, A. 2012. A review of pest surveillance techniques for detecting quarantine pests in Europe. EPPO Bull. 42:515-551.

Bridge, P. D., Waller, J. M., Davies, D., and Buddie, A. G. 2008. Variability of *Colletotrichum kahawae* in relation to other *Colletotrichum* species from tropical perennial crops and the development of diagnostic techniques. J. Phytopathol. 156:274-280.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55: 611-622.

CABI. 2024. *Colletotrichum kahawae* (coffee berry disease). In: PlantwisePlus Knowledge Bank. https://doi.org/10.1079/pwkb.species.14916

Cabral, A., Azinheira, H. G., Talhinhas, P., Batista, D., Ramos, A. P., Silva, M. d. C., Oliveira, H., and Várzea, V. 2020. Pathological, morphological, cytogenomic, biochemical and molecular data support the distinction between *Colletotrichum cigarro comb. et stat. nov.* and *Colletotrichum kahawae*. Plants (Basel) 9:502.

Cannon, P. F., Damm, U., Johnston, P. R., and Weir, B. S. 2012. *Colletotrichum*: Current status and future directions. Stud. Mycol. 73:181-213.

Caraguel, C. G. B., Stryhn, H., Gagné, N., Dohoo, I. R., and Hammell, K. L. 2011. Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: Analytical and epidemiologic approaches. J. Vet. Diagn. Invest. 23:2-15.

Cardwell, K., Dennis, G., Flannery, A. R., Fletcher, J., Luster, D., Nakhla, M., Rice, A., Shiel, P., Stack, J., Walsh, C., and Levy, L. 2018. Principles of diagnostic assay validation for plant pathogens: A basic review of concepts. Plant Health Prog. 19:272-278.

Chen, Z., Liang, J., and Rodrigues, C. J., Jr. 2005. *Colletotrichum gloeosporioides* can overgrow *Colletotrichum kahawae* on green coffee berries first inoculated with *C. kahawae*. Biotechnol. Lett. 27:679-682.

Cruz, L. P., Gaitan, A. L., and Gongora, C. E. 2006. Exploiting the genetic diversity of *Beauveria bassiana* for improving the biological control of the coffee berry borer through the use of strain mixtures. Appl. Microbiol. Biotechnol. 71:918-926.

Damm, U., Cannon, P. F., Woudenberg, J. H. C., and Crous, P. W. 2012a. The *Colletotrichum acutatum* species complex. Stud. Mycol. 73:37-113.

Damm, U., Cannon, P. F., Woudenberg, J. H. C., Johnston, P. R., Weir, B. S., Tan, Y. P., Shivas, R. G., and Crous, P. W. 2012b. The *Colletotrichum boninense* species complex. Stud. Mycol. 73:1-36.

Damm, U., Sato, T., Alizadeh, A., Groenewald, J. Z., and Crous, P. W. 2019. The *Colletotrichum dracaenophilum*, *C. magnum* and *C. orchidearum* species complexes. Stud. Mycol. 92:1-46.

Ebbels, D. L. 2003. Principles of Plant Health and Quarantine. CABI, Wallingford, U.K.

Etana, M. B. 2018. Review on the management of coffee berry disease (*Colletotrichum kahawae*) in Ethiopia. Food Sci. Qual. Manage. 76:73-76.

Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., and Kubista, M. 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol. Detect. Quantif. 12:1-6.

Garibaldi, A., Gilardi, G., Puglisi, I., Cacciola, S. O., and Gullino, M. L. 2016. First report of leaf spot caused by *Colletotrichum kahawae* on cultivated rocket (*Eruca sativa*) in Italy. Plant Dis. 100:1240.

Ghelardini, L., Luchi, N., Pecori, F., Pepori, A. L., Danti, R., Della Rocca, G., Capretti, P., Tsopelas, P., and Santini, A. 2017. Ecology of invasive forest pathogens. Biol. Invasions 19:3183-3200.

Giddisa, G. 2016. A review on the status of coffee berry disease (*Colletotrichum kahawae*) in Ethiopia. J. Biol. Agric. Healthc. 6:140-151.

Grosdidier, M., Aguayo, J., Marçais, B., and Ioos, R. 2017. Detection of plant pathogens using real-time PCR: How reliable are late C_t values? Plant Pathol. 66:359-367.

Groth-Helms, D., Rivera, Y., Martin, F. N., Arif, M., Sharma, P., and Castlebury, L. A. 2023. Terminology and guidelines for diagnostic assay development and validation: Best practices for molecular tests. PhytoFrontiers 3:23-35.

Henson, J. M., and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol. 31:81-109.

International Coffee Organization. 2023. Coffee Report and Outlook. https://icocoffee.org/documents/cy2023-24/Coffee_Report_and_Outlook_December_2023_ICO.pdf

Instituto Colombiano Agropecuario. 2015. Resolución 3593 de 2015. Por medio del cual se crea el mecanismo para establecer, mantener, actualizar y divulgar el listado de plagas reglamentadas de Colombia. https://www.ica.gov.co/getattachment/a6a72675-e009-42f7-8c25-89b406e494d9/2015R3593.aspx

International Organization for Standardization. 2019. ISO 20395:2019: Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR. https://www.iso.org/standard/67893.html (accessed 5 May 2023).

International Organization for Standardization. 2021. ISO 16140-3:2021. Microbiology of the food chain — Method validation — Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory. https://www.iso.org/standard/66324.html (accessed 5 May 2023).

International Organization for Standardization. 2022. ISO 16577:2022. Molecular biomarker analysis — Vocabulary for molecular biomarker analytical methods in agriculture and food production. https://www.iso.org/obp/ui/en/#iso:std:iso:16577:ed-2:v1:en (accessed 4 May 2023).

International Plant Protection Convention. 2018. Surveillance. https://openknowledge.fao.org/server/api/core/bitstreams/ebc777e5-cf0c-4064-9bbf-2198790e2fff/content

Jayawardena, R. S., Bhunjun, C. S., Hyde, K. D., Gentekaki, E., and Itthayakorn, P. 2021. *Colletotrichum*: Lifestyles, biology, morpho-species, species complexes and accepted species. Mycosphere 12:519-669.

Kahn, R. P. 1991. Exclusion as a plant disease control strategy. Annu. Rev. Phytopathol. 29:219-246.

Kanderian, S., Jiang, L., and Knight, I. 2015. Automated classification and cluster visualization of genotypes derived from high resolution melt curves. PLoS One 10:e0143295.

Kassambara, A. 2022. rstatix: Pipe-friendly framework for basic statistical tests. https://CRAN.R-project.org/package=rstatix (accessed 3 January 2023).

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., and Richter, C. A. 2020. Reporting the limits of detection and quantification for environmental DNA assays. Environ. DNA 2:271-282.

Lipsky, R. H., Mazzanti, C. M., Rudolph, J. G., Xu, K., Vyas, G., Bozak, D., Radel, M. Q., and Goldman, D. 2001. DNA melting analysis for detection of single nucleotide polymorphisms. Clin. Chem. 47:635-644.

Liu, F., Cai, L., Crous, P. W., and Damm, U. 2014. The *Colletotrichum gigasporum* species complex. Persoonia 33:83-97.

Liu, F., Weir, B. S., Damm, U., Crous, P. W., Wang, Y., Liu, B., Wang, M., Zhang, M., and Cai, L. 2015. Unravelling *Colletotrichum* species associated with *Camellia*: Employing ApMat and GS loci to resolve species in the *C. gloeosporioides* complex. Persoonia 35:63-86.

Martin, R. R., Constable, F., and Tzanetakis, I. E. 2016. Quarantine regulations and the impact of modern detection methods. Annu. Rev. Phytopathol. 54:189-205.

Mathys, G., and Baker, E. A. 1980. An appraisal of the effectiveness of quarantines. Annu. Rev. Phytopathol. 18:85-101.

Nguyen, T. H. P., Säll, T., Bryngelsson, T., and Liljeroth, E. 2009. Variation among *Colletotrichum gloeosporioides* isolates from infected coffee berries at different locations in Vietnam. Plant Pathol. 58:898-909.

OEPP/EPPO. 2021. PM 7/98 (5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bull. 51:468-498.

Paini, D. R., Sheppard, A. W., Cook, D. C., De Barro, P. J., Worner, S. P., and Thomas, M. B. 2016. Global threat to agriculture from invasive species. Proc. Natl. Acad. Sci. U.S.A. 113:7575-7579.

Papavasileiou, A., Madesis, P. B., and Karaoglanidis, G. S. 2016. Identification and differentiation of *Monilinia* species causing brown rot of pome and stone fruit using high-resolution melting (HRM) analysis. Phytopathology 106: 1055-1064.

Pardo-De la Hoz, C. J., Calderón, C., Rincón, A. M., Cárdenas, M., Danies, G., López-Kleine, L., Restrepo, S., and Jiménez, P. 2016. Species from the *Colletotrichum acutatum*, *Colletotrichum boninense* and *Colletotrichum gloeosporioides* species complexes associated with tree tomato and mango crops in Colombia. Plant Pathol. 65:227-237.

Prihastuti, H., Cai, L., Chen, H., McKenzie, E. H. C., and Hyde, K. D. 2009. Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. Fungal Divers. 39:89-109.

Ristaino, J. B., Anderson, P. K., Bebber, D. P., Brauman, K. A., Cunniffe, N. J., Fedoroff, N. V., Finegold, C., Garrett, K. A., Gilligan, C. A., Jones, C. M., Martin, M. D., MacDonald, G. K., Neenan, P., Records, A., Schmale, D. G., Tateosian, L., and Wei, Q. 2021. The persistent threat of emerging plant disease pandemics to global food security. Proc. Natl. Acad. Sci. U.S.A. 118: e2022239118.

Rojas, P., Pardo-De la Hoz, C. J., Calderón, C., Vargas, N., Cabrera, L. A., Restrepo, S., and Jiménez, P. 2018. First report of *Colletotrichum kahawae* subsp. *ciggaro* causing anthracnose disease on tree tomato in Cundinamarca, Colombia. Plant Dis. 102:2031.

Schena, L., Mosca, S., Cacciola, S. O., Faedda, R., Sanzani, S. M., Agosteo, G. E., Sergeeva, V., and Magnano di San Lio, G. 2014. Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated with olive anthracnose. Plant Pathol. 63:437-446.

Schiwek, S., Beule, L., Vinas, M., Pfordt, A., von Tiedemann, A., and Karlovsky, P. 2020. High-resolution melting (HRM) curve assay for the identification of eight *Fusarium* species causing ear rot in maize. Pathogens 9:270.

Sharma, G., Kumar, N., Weir, B. S., Hyde, K. D., and Shenoy, B. D. 2013. The *ApMat* marker can resolve *Colletotrichum* species: A case study with *Mangifera indica*. Fungal Divers. 61:117-138.

Silva, D. N., Talhinhas, P., Várzea, V., Cai, L., Paulo, O. S., and Batista, D. 2012. Application of the *Apn2/MAT* locus to improve the systematics of the *Colletotrichum gloeosporioides* complex: An example from coffee (*Coffea* spp.) hosts. Mycologia 104:396-409.

Słomka, M., Sobalska-Kwapis, M., Wachulec, M., Bartosz, G., and Strapagiel, D. 2017. High resolution melting (HRM) for high-throughput genotyping—limitations and caveats in practical case studies. Int. J. Mol. Sci. 18:2316.

Tao, G., and Cai, L. 2013. Molecular diagnosis of *Colletotrichum kahawae* by loop-mediated isothermal amplification (LAMP). J. Plant Pathol. 95: 519-524.

Tao, G., Hyde, K. D., and Cai, L. 2013. Species-specific real-time PCR detection of *Colletotrichum kahawae*. J. Appl. Microbiol. 114:828-835.

U.S. Food and Drug Administration. 2020. Guidelines for the validation of analytical methods for nucleic acid sequence-based analysis of food, feed, cosmetics and veterinary products. https://www.fda.gov/media/121751/download

Varzea, V. M. P., Rodrigues, C. J., Jr., and Lewis, B. G. 2002. Distinguishing characteristics and vegetative compatibility of *Colletotrichum kahawe* in comparison with other related species from coffee. Plant Pathol. 51:202-207.

Vieira, W. A. D. S., Bezerra, P. A., Silva, A. C. D., Veloso, J. S., Câmara, M. P. S., and Doyle, V. P. 2020. Optimal markers for the identification of *Colletotrichum* species. Mol. Phylogenet. Evol. 143:106694.

Vincelli, P., and Tisserat, N. 2008. Nucleic acid-based pathogen detection in applied plant pathology. Plant Dis. 92:660-669.

Waller, J. M., Bridge, P. D., Black, R., and Hakiza, G. 1993. Characterization of the coffee berry disease pathogen, *Colletotrichum kahawae* sp. nov. Mycol. Res. 97:989-994.

Wei, X., Yang, S. Q., Jiang, Z. G., Cui, M. J., Deng, J. X., and Zhang, Y. 2022. *Colletotrichum juglandis* sp. nov. (Ascomycota: Glomerellaceae) associated with walnut leaf spot in China. Phytotaxa 556:256-268.

Weir, B. S., Johnston, P. R., and Damm, U. 2012. The *Colletotrichum gloeosporioides* species complex. Stud. Mycol. 73:115-180.

Whelan, J. A., Russell, N. B., and Whelan, M. A. 2003. A method for the absolute quantification of cDNA using real-time PCR. J. Immunol. Methods 278:261-269.

White, T. J., Bruns, T. D., Lee, S. B., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.

Wickham, H. 2009. ggplot2: Elegant graphics for data analysis. Springer, New York, NY. https://link.springer.com/book/10.1007/978-0-387-98141-3 (accessed 29 December 2022).

Winder, L., Phillips, C., Richards, N., Ochoa-Corona, F., Hardwick, S., Vink, C. J., and Goldson, S. 2011. Evaluation of DNA melting analysis as a tool for species identification. Methods Ecol. Evol. 2:312-320.

Wubshet, Z., and Merga, D. 2020. Biology, dispersal and management of coffee berry disease: A review. J. Biol. Agric. Healthc. 10:14-25.