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RESEARCH ARTICLE

Multilocus identification and pathogenetic characterization of *Colletotrichum* endophyte and pathogen species isolated from cocoa leaves and pods (*Theobroma cacao*) in Ecuador

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Abstract

Cacao cultivation is one of the main agricultural products of Ecuador, known internationally for its quality and aroma. However, it is affected by fungal diseases including *Moniliophthora roreri, Moniliophthora perniciosa, Phytophthora* spp., and *Colletotrichum* spp. The genus *Colletotrichum* spp. is known for its characteristics that complicate traditional taxonomic identification. In cacao cultivation, it is one of the most frequently found species as an endophyte of leaves and fruits, yet it is also reported to cause the disease known as anthracnose on leaves and fruits. The objective of this work was to identify at the species level 16 *Colletotrichum* isolates, 13 from healthy leaf endophytes and 3 from pods with symptoms, through multilocus analysis of the ITS1, 5.8S, and ITS2 region, and partial sequences of the TUB2 and GAPDH genes. Subsequently, their pathogenicity was evaluated by inoculating healthy cacao pods and measuring the damage caused. The 16 isolates were identified as follows: from the *gloeosporioides* complex, *C. siamense* 6, *C. chrysophilum* 6, *C. theobromicola* 2 and from the boninense complex, *C. karstii* 2. The most frequently found species were those that caused symptoms, especially *C. siamense*, to which the strains were isolated from symptomatic pods belonged. This work provides relevant and accurate information about the diversity of *Colletotrichum* species that colonize cocoa plantations and identifies which species might cause the disease known as anthracnose. Additionally, it allows for a more precise diagnosis and consequently better treatment.

Keywords: Anthracnose; Phylogenetic Analyze; Multi-locus; Endophyte; Pathogen.

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1. Introduction

Cocoa has been a fundamental crop worldwide, not only because of the chocolate industry but also due to its economic and social impact in producing countries. The main global producers are in Africa, with Ivory Coast and Ghana accounting for more than 60% of the world's production. In Latin America, Brazil, Colombia, Peru, and Ecuador stand out, mainly for the qualit y of their product (Soares & Oliveira, 2022)

The cocoa cultivation in Ecuador is considered one of the greatest economic importance for the country, in 2024 was exported 362,296 MT with a total of $2,787.2 million of dollars (Ministerio de Producción, Comercio Exterior, Inversiones y Pesca, 2025) in addition, the country is the world leader in the production and export of fine aroma cocoa with 70% of the world total production and is a livelihood for around one hundred thousand families (ProEcuador, 2019). This production is affected by diseases mainly of fungal origin such as witch´s broom, frosty pod rot (de Novais et al., 2023), in smaller quantity is found the fungi *Colletotrichum* that in pathogenic condition is

found causing the disease known as anthracnose that can affect a number of crops of economic interest (Wijaya et al., 2023).

Many morphological and molecular studies of *Colletotrichum* have been carried out mainly because of the various characteristics it presents (Angeli et al., 2024; Asare et al., 2021), among them its adaptability that makes it easier for it to have a life as an endophyte and has been found in a large number of hosts without causing apparent damage, however, many authors have typecast the endophytes as inactive saprophytes (Ebadi et al., 2024; Whalley, 1996), latent pathogens (Stone et al., 2000) or mutualists (Herre et al., 2007; Mejía et al., 2008).

Endophytic fungi have the ability to stimulate the development of the host plant, enhance the activity of antioxidant defense enzymes, and induce the synthesis and storage of secondary metabolites (Xu et al., 2023). In cocoa cultivation, it has been shown that *Colletotrichum*, as an endophyte, provides protection to the plant by reducing the incidence of diseases caused by fungi, primarily (Tao et al., 2013; Yu et al., 2022).

Previously, identification through taxonomic techniques was very common; however, it presented inconsistencies as it heavily relied on the specific technique used (Cai et al., 2009; Tao et al., 2013), hence the importance of phylogenetic studies in this case the multi-locus analysis by the difficulty of performing a taxonomic identification by the morphological characteristics presented by *Colletotrichum*, In addition, the use of a single gene or part of it is very uninformative as is the case of the ITS region of rDNA. For this reason, it is very important to supplement the use of the ITS region with other genes or parts that are preserved but provide a variability for their use. In order to differentiate the isolated endophytes previously identified as *Colletotrichum* spp, it was considered the objective of inferring their genetic relationship based on a multi-locus phylogenetic analysis of 13 endophytes from *T. cocoa* leaves and 3 isolated strains of cocoa pods with disease symptoms by sequencing three genes (Beta Tubulin 2, Internal Transcribed Spaces, Gliceraldehyde-3-phosphate dehydrogenase) and relate their pathogenic or nonpathogenic capacity by inoculating healthy cobs with their identification, thus providing a guideline for the management of this disease..

2. Methodology

Origin of the strains

For this study, the endophytic strains were isolated from healthy leaves of the National type, cocoa variety, with more than 50 years of age located in the provinces of Guayas and Azuay (Table 1). Small fragments (2x2 cm) were washed with tap water and dried with sterile paper towels. Plant tissues were rinsed with 70% ethanol and 0.5% sodium hypochlorite for 2 minutes and washed with sterile distilled water three times. Eight fragments were placed in a 90 mm diameter Petri dish containing agar with 2% malt extract (Arnold et al., 2003) and incubated at 25 °C in the dark for 10 days. Colonies with different morphology were observed every two days, they were isolated and purified on potato-dextrose-agar. The pure endophytic fungal strains were kept in the Collection of Microorganism Cultures of the Ecuadorian Center for Biotechnological Research (CCM-CIBE).

For the isolation of the pathogenic strains, the fruits with symptoms were collected, brought to the laboratory and followed the protocol of Arnold et al. (2003) for the planting of the plant material (Table 1), later the pure isolates were obtained and deposited in the (CCM-CIBE).

Table 1
Origin of the samples

CCM-CIBE Collection	Straigth	Genus	Origin	S	W
CCMCIBE-H093	C6	*Colletotrichum*	Balao - Guayas	2°30'29,5''	79°46'34,8''
CCMCIBE-H098	C12	*Colletotrichum*	Balao - Guayas	2°30'29,5''	79°46'34,8''
CCMCIBE-H1146	C15	*Colletotrichum*	Molleturo -Azuay	2°30'49,2''	79°26'11,2''
CCMCIBE-H140	C65	*Colletotrichum*	Naranjal - Guayas	2°40'35,2''	79°38'21,2''
CCMCIBE-H148	C75	*Colletotrichum*	Naranjal - Guayas	2°40'35,2''	79°38'21,2''
CCMCIBE-H152	C82	*Colletotrichum*	Balao - Guayas	2°30'29,5''	79°46'34,8''
CCMCIBE-H153	C83	*Colletotrichum*	Balao - Guayas	2°30'29,5''	79°46'34,8''
CCMCIBE-H171	C107	*Colletotrichum*	Balao - Guayas	2°30'29,5''	79°46'34,8''
CCMCIBE-H190	C133	*Colletotrichum*	Molleturo - Azuay	2°30'49,2''	79°26'11,2''
CCMCIBE-H196	C146	*Colletotrichum*	Naranjal - Guayas	2°40'35,2''	79°38'21,2''
CCMCIBE-H206	C160	*Colletotrichum*	Naranjal - Guayas	2°40'35,2''	79°38'21,2''
CCMCIBE-H209	C163	*Colletotrichum*	Naranjal - Guayas	2°40'35,2''	79°38'21,2''
CCMCIBE-H210	C164	*Colletotrichum*	Naranjal - Guayas	2°40'35,2''	79°38'21,2''
CCMCIBE-H1147	PAT1	*Colletotrichum*	Taisha - Morona Santiago	2°30'53"	77°35'51"
CCMCIBE-H1148	PAT2	*Colletotrichum*	Palanda - Zamora Chinchipe	4°38'56,5''	79° 6' 59,7''
CCMCIBE-H1149	PAT6	*Colletotrichum*	Palanda - Zamora Chinchipe	4°40'18,8''	79°2'22,4''

DNA extraction, PCR, sequencing and identifying

The DNA was extracted from the fungal mycelium, obtained from pure cultures in DIFCO Potato Dextrose Agar medium (PDA), following the Cenis protocol (Cenis, 1992). The ITS 1, 5.8S, ITS 2 region was amplified by polymerase chain reaction (PCR), using the universal primers ITS-1F (5'-TCCGTAGGTGAACCTGCGG-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The volume of the final reaction was 25 µl; containing the following mixture at final concentration: 1X buffer solution (Invitrogen), 0.2 mM dNTPs, 1.5 mM Mg2Cl, 0.4 µM of each primer, 0.5 U Taq polymerase per reaction (Invitrogen) and 2 µl of template DNA (10-50 ng). PCR reactions were carried out with an initial denaturation of 94 °C for 1 min followed by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and an extension at 68 °C for 1 min; and a final extension of 68 °C for 3 min for extension. The PCR products were visualized in 2% agarose gel.

The amplified products were sequenced at the Interdisciplinary Center for Biotechnology Research at the University of Florida (ICBR). The quality of the sequences was analyzed with the FinchTV program Version 1.4.0 (http://www.geospiza.com/finchtv). The sequences obtained were compared with the existing information in the database of the gene bank of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), using the BLAST searches and were aligned using the MEGA 6 program (Tamura et al., 2013).

Once the isolates were preliminary identified using the ITS gene, they were further analyzed using partial gene sequences of another two genomic loci: the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β tubulin 2 (TUB2) genes. The primers GDF1 (5'–GCCGTCAACGACCCCTTCATTGA-3') and GDR1 (5'– GGGTGGAGTCGTACTTGAGCATGT-3') were used to amplify and sequence the GAPDH (Guerber et al., 2003), and for TUB2 the primers Btub2Fd (5'-GTBCACCTYCARACCGGYCARTG-3´) and Btub4Rd (5'-CCRGAYTGRTCCRGAYTGRT) were used (Woudenberg et al., 2009).

The PCR conditions for GAPDH were an initial denaturation at 94 °C for 4 min followed by 34 cycles consisting of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min: a final step of 72 °C for 10 min. (Prihastuti et al., 2009). TUB2 PCR consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, and extension at 72 °C for 7 min (Woudenberg et al., 2009).

The purified PCR products were sequenced, in both directions, by Macrogen Inc., Korea. The quality of the nucleotide sequences and the consensus assembly was carried out using Geneious version 2020.1.2. Then, the assembled sequences were compared to the NCBI database using BAST.

Phylogenetic analysis

The phylogenetic analysis included the 16 sequences from the isolates of this study and 62 sequences belonging to 47 species from *gloeosporioides* and *13 boninense* complex, that were downloaded from GenBank at NCBI (https://www.ncbi.nlm.nih.gov/) as model sequences (Table 2) and sequences from *acutatum* complex: *C. acutatum* (CBS112996) and *C. nymphaeae* (CBS_515.78) were used as outgroup. The sequences of ITS, GAPDH, and TUB2 were aligned independently with ClustalW software in MEGA X program (Kumar et al., 2018). Then, a multi-gene analyses were performed using a concatenated dataset of the three loci. The trees were visualized in FigTree v1.4.4 (https://tree.bio.ed.ac.uk/software/figtree/). For the maximum-likelihood method (ML), the Tamura-Nei model + G nucleotide substitution model was implemented with 500 bootstrap repetitions. ML analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA) 10.2 software, and the best substitution model was decided using CIPRES in jModelTest 2.1.6 (Darriba et al., 2012). Bayesian probability (BP) analysis was performed using BEAST v1.10.4 software package. The Hasegawa-Kishino-Yano (HKY) model with a Gamma distribution with an uncorrelated relaxed clock strict clock was selected as the optimal model. the Markov Chain Monte Carlo (MCMC) method was run for 10 million generations and sampled every 5000 steps in two repetitions.

Pathogenicity tests

Inoculation was performed in duplicate at the apical, middle, and terminal parts of approximately 2-month-old national cocoa fruit. For this, a 6 mm diameter portion of the bark was separated using a hole punch, and a disk of the same diameter with the fungal culture grown for seven days was placed. The fruits were individually incubated in polyethylene bags with damp cotton at 28 °C for 7 days. The variables that were evaluated were the external diameter, the internal diameter, and the depth of the damage. The external diameter was measured directly on the surface of the inoculation site, and for the evaluation of the other 2 variables, a longitudinal cut of the pod was made, and the surface of the damage was measured if it existed (Figure 1) (Montri et al., 2009).

Table 2
Strains of *Colletotrichum* used in multilocus analysis in this study. Details are provided about complex, species, strain and GenBank accessions of the reference sequence

Complex	Species	Strain/Type	GenBank Nº Accession		
			ITS	GAPDH	TUB2
gloeosporioides	*C. alienum*	ICMP18621	JX010246	JX009959	JX010386
	C. alienum	ICMP12068	JX010255	JX009925	-
	C. alienum	ICMP 18608	JX010244	JX010044	JX010389
	C. artocarpicola	MFLUCC_18-1167	NR_171192	MN435568	MN435567
	C. asianum	ICMP 18580	FJ972612	JX010053	JX010406
	C. chrysophilum	CMM4363	KX094240	KX094180	KX094283
	C. chrysophilum	CMM4394	KX094239	KX094179	KX094282
	C. chrysophilum	CMM4292	KX094248	KX094182	KX094284
	C. chrysophilum	CMM 4268	KX094252	KX094183	KX094285
	C. chrysophilum	8395	GU994370	KX094176	GU994473
	C. chrysophilum	**CCMCIBE-H098 (C12)**	**PP316988**	**PP502892**	**PP502874**
	C. chrysophilum	**CCMCIBE-H152 (C82)**	**PP316992**	**PP502896**	**PP502878**
	C. chrysophilum	**CCMCIBE-H153 (C83)**	**PP316993**	**PP502897**	**PP502879**
	C. chrysophilum	**CCMCIBE-H171 (C107)**	**PP316994**	**PP502898**	**PP502880**
	C. chrysophilum	**CCMCIBE-H196 (C146)**	**PP316997**	**PP502901**	**PP502883**
	C. chrysophilum	**CCMCIBE-H210 (C164)**	**PP317000**	**PP502904**	**PP502886**
	C. fructicola	CBS:125397	JX010173	JX010032	-
	C. fructicola	LF652	KJ955192	KJ954893	KJ955339
	C. fructicola	LF716	KJ955207	KJ954908	KJ955353
	C. fructicola	3589	-	KX094175	KX094280
	C. fructicola	ICMP18581	JX010165	JX010033	JX010405
	C. fructicola	1087	GU994377	KX094174	KX094279
	C. fructicola	ICMP 18581	JX010165	JX010033	JX010405
	C. gloeosporioides	GA077	KX620305	KX620239	KX620338
	C. gloeosporioides	ICMP 17821	JX010152	JX010056	JX010445
	C. gloeosporioides	CBS 112999	JQ005152	JQ005239	JQ005587
	C. gloeosporioides	ICMP 19121	JX010148	JX010054	-
	C. grevilleae	GgPc22-1-11	LC773714	LC773711	LC773710
	C. grevilleae	WP4	ON849044	ON862125	ON862130
	C. grossum	CAUG7	KP890165	KP890159	KP890171
	C. grossum	CAU31	KP890166	KP890160	KP890172
	C. grossum	CAUG32	KP890167	KP890161	KP890173
	C. grossum	CGMCC3.17614	KP890165	KP890159	KP890171
	C. hystricis	CBS 142411	KY856450	KY856274	KY856532
	C. hystricis	CBS 142411	KY856450	KY856274	KY856532
	C. hystricis	CPC 28154	KY856451	KY856275	KY856533
	C. musae	ICMP 19119	JX010146	JX010050	HQ596280
	C. musae	CMM4423	KX094243	KX094195	KX094294
	C. musae	LPPC389	OR251500	OR295210	OR295213
	C. nupharicola	ICMP 18187	JX010189	JX009936	JX010397
	C. nupharicola	CBS 472.96	JX010188	JX010031	JX010399
	C. pandanicola	MFLUCC 17-0571	MG646967	MG646934	MG646926
	C. pandanicola	MFLU 18-0003	MG646967	MG646934	MG646926
	C. pandanicola	SAUCC200204	MW786641	MW846239	MW888969
	C. perseae	CBS141365	KX620308	KX620242	KX620341
	C. pseudotheobromicola	MFLUCC 18–1602	MH817395	MH853675	MH853684
	C. queenslandicum	ICMP 1778	JX010276	JX009934	JX010414
	C. siamense	LF139	KJ955087	KJ954788	KJ955236
	C. siamense	LF148	KJ955088	KJ954789	KJ955237
	C. siamense	ICMP 18578	JX010171	JX009924	JX010404
	C. siamense	**CCMCIBE-H148 (C75)**	**PP316991**	**PP502895**	**PP502877**
	C. siamense	**CCMCIBE-H190 (C133)**	**PP316996**	**PP502900**	**PP502882**
	C. siamense	**CCMCIBE-H206 (C160)**	**PP316998**	**PP502902**	**PP502884**
	C. siamense	**CCMCIBE-H1147 (PAT1)**	**PP317001**	**PP502905**	**PP502887**
	C. siamense	**CCMCIBE-H1148 (PAT2)**	**PP317002**	**PP502906**	**PP502888**
	C. siamense	**CCMCIBE-H1149 (PAT6)**	**PP317003**	**PP502907**	**PP502889**
	C. tainanense	CBS 143666	MH728818	MH728823	MH846558
	C. theobromicola	ICMP 18649	JX010294	JX010006	JX010447
	C. theobromicola	ICMP 17814	JX010288	JX010003	JX010379
	C. theobromicola	**CCMCIBE-H140 (C65)**	**PP316990**	**PP502894**	**PP502876**
	C. theobromicola	**CCMCIBE-H209 (C163)**	**PP316999**	**PP502903**	**PP502885**
	C. xanthorrhoeae	ICMP 17903	JX010261	JX009927	JX010448
boninense	*C. annellatum*	CBS 129826	JQ005222	JQ005309	JQ005656
	C. beeveri	CBS 128527	JQ005171	JQ005258	JQ005605
	C. boninense	CBS 123755	JQ005153	JQ005240	JQ005588

	C. brassicicola	CBS 101059	JQ005172	JQ005259	JQ005606
	C. chongqingense	CS0612	MG602060	MG602022	MG602044
	C. cymbidiicola	IMI 347923	JQ005166	JQ005253	JQ005600
	C. doitungense	MFLU 14-0128	MF448524	MH049480	MH351277
	C. feijoicola	CBS 144633	MK876413	MK876475	MK876507
	C. karstii	CBS 127597	JQ005204	JQ005291	JQ005638
	C. karstii	CBS 129833	JQ005175	JQ005262	JQ005609
	C. karstii	CBS 132134	HM585409	HM585391	HM585428
	C. karstii	**CCMCIBE-H093 (C6)**	**PP316987**	**PP502891**	**PP502873**
	C. karstii	**CCMCIBE-H1146 (C15)**	**PP316989**	**PP502893**	**PP502875**
	C. phyllanthi	CBS 175.67	JQ005221	JQ005308	JQ005655
	C. watphraense	MFLU 14-0123	MF448523	MH049479	MH351276
acutatum	*C. acutatum*	CBS 979.69	JQ948400	JQ948731	JQ950051
	C. nymphaeae	CBS 515.78	JQ948197	JQ948527	JQ949848

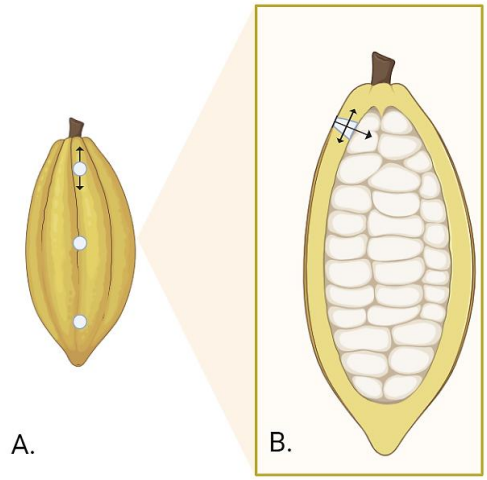

Figure 1. Illustration of a cocoa pod with inoculation points. A. Whole pod showing the evaluation of external diameter. B. Longitudinal cut of the cacao pod showing the evaluation of internal diameter and depth of damage.

The results obtained were analyzed using ANOVA, and the means were compared using Tukey's test at the significance level of $p \leq 0.05$, using INFOSTAT.

3. Results and discussion

Phylogenetic analysis

This study was based on the examination of Colletotrichum, which has been reported as an endophyte, pathogen, and saprobe and is distributed worldwide, colonizing various hosts (Hyde et al., 2014; Jayawardena et al., 2016; Zheng et al., 2022). In cocoa cultivation, it is one of the most commonly found foliar endophytic fungi (Villavicencio-Vásquez et al., 2018), and also causes the disease known as anthracnose in cocoa cultivation (Asare et al., 2021; Rojas et al., 2010).

To elucidate the molecular phylogenetic position of our isolate, a BLAST search was performed in the NCBI database, and phylogenetic analyses were conducted. The isolates were first classified up to the genus level by performing a BLAST of their partial nucleotide sequences of ITS, GAPDH, and TUB2 (Table 3). Their identity was further confirmed at the species level, based on the multi-locus phylogenetic analysis of those three loci using our 16 sequences of Colletotrichum isolates along with reference sequences retrieved from GenBank (Table 2). The final dataset contained 1288 bp, including gaps, comprising 519, 267, and 502 positions from ITS, GAPDH, and TUB2, respectively. The multilocus analysis conducted was primarily based on the difficulty in morphological identification of the genus *Colletotrichum* (Jayawardena et al., 2016), the results obtained from the BLAST analysis with the ITS1, 5.8S, and ITS2 regions were inconclusive, as high-percentage similarity identities were found with several isolates in this study, such as: *C. fructicola, C. siamense, C. theobromicola, C. crysophyllum, C. gloeosporioides, C. pandanicola, C. alienum, C. karstii, and C. phyllanthi*, When analyzing the sequences of the ITS region, ITS1, 5.8S, and ITS2, the results were inconclusive due to a lack of information using only one gene (Yu et al., 2022), which also made differentiation between *C. tropicale* and *C. siamense* or *C. fructicola*, *C. aeschynomene* and *C. chrysophilum* (Weir et al., 2012), nearly impossible, However, it was clearly differentiated that these isolates were entirely related to the *Colletotrichum gloeosporioides* and *boninense* complexes. On the other hand, partial sequences of the TUB2 and GAPDH genes, and their combined use in phylogenetic or Bayesian inference analyses, are frequently employed in the study of these fungi, providing greater accuracy to the results; a study conducted on *C. truncatum*, *C. dematium*, and *C. gloeosporioides* indicated that the GAPDH locus is essential for resolving relationships between closely related Colletotrichum species (Mahmodi et al., 2014; Samarakoon et al., 2018). For this reason, the sequencing of the GAPDH and TUB2 regions was performed, obtaining similar results (Table 3), but also showing similarity with other species.

Therefore, a phylogenetic analysis with Bayesian inference was carried out.

The phylogenetic analysis revealed that the 16 isolates were assigned into two species complexes (Figure 2), 14 allocated within the *C. gloeosporioides* complex, and the remaining two belonged to the *C. boninense* complex. The isolates within the *gloeosporioides* complex are clustered in three clades, six leaf endophytic isolates (C12, C82, C83, C107, C146, and C164) with *C. chrysophilum*, despite the blast indicating mostly *C. fructicola*. This can be explained by the close relationship between *C. fructicola* and *C. chrysophilum*; however, the use of multiple genes for phylogenetic analysis helps to separate them (Vieira et al., 2017), moreover, according to Vieira et al. (2017), studies conducted by Weir et al. (2012) using isolates from *Malus* in the USA and Brazil consider *C. fructicola* as conspecific with *C. chrysophilum*; two isolated were clustered with *C. theobromicola*, and six isolates including those obtained from pods with anthracnose symptoms (PAT1, PAT2, and PAT6) and those from healthy leaves (C75, C133, and C160) clustered with *C. siamense* and *C. pandanicola* This can be explained by their high genetic similarity, as noted by Zhang et al. (2023), who indicate that there are fewer nucleotide differences between *C. pandanicola* and *C. siamense*. However, there are no reports of *C. pandanicola* in cocoa cultivation since it was reported less than six years ago by Tibpromma et al. (2018) in leaves of *Pandanus* sp. For this reason, in this case, we will consider the information obtained in the BLAST that identifies the isolates as *C. siamense*. However, to complement and clarify, an analysis with more genes could be performed as indicated by Chang et al. (2022) and Yu et al. (2022). The 2 isolates within the *boninense* complex clustered with *C. karstii*.

Pathogenicity tests

No significant differences were observed among all treatments; however, a marked difference was observed between the damage caused by *C. siamense* (greater) and *C. crysophilum* (lesser). Of the isolates evaluated, 8 showed external and internal damage on the pods, with 5 of the 6 isolates identified as *C. siamense* (3 from diseased pods and 2 leaf endophytes). The most aggressive was PAT6 (39 mm external diameter, 29.35 mm internal diameter, and 20.22 mm depth).

Table 3
Molecular identification by the three sequenced genes

Straigth	Closest species identification based on GENEBANK					
	TUB2		ITS		GAPDH	
C6	*C. karstii*	MK224865.1	*C. karstii*	MK336581.1	*C. karstii*	MK963100.1
	Percent Identity	99,61	Percent Identity	100	Percent Identity	100
C12	*C. fructicola*	MN982447.1	*Colletotrichum sp.*	OQ793660.1	*C. fructicola*	MN982434.1
	Percent Identity	100	Percent Identity	100	Percent Identity	99,61
C15	*C. karstii*	MN273232.1	*Colletotrichum sp.*	PP316989.1	*C. karstii*	MG602035.1
	Percent Identity	99,42	Percent Identity	100	Percent Identity	100
C65	*C. theobromicola*	MW151284.1	*C. theobromicola*	MK790662.1	*C. theobromicola*	MN939222.1
	Percent Identity	99,62	Percent Identity	100	Percent Identity	99,64
C75	*C. siamense*	OQ079586.1	*C. siamense*	OR807537.1	*C. siamense*	MK693710.1
	Percent Identity	99,22	Percent Identity	99,83	Percent Identity	100
C82	*C. fructicola*	MN982447.1	*C. fructicola*	CP150817.1	*C. fructicola*	MN982433.1
	Percent Identity	99,8	Percent Identity	100	Percent Identity	100
C83	*C. fructicola*	MN982447.1	*C. fructicola*	CP150817.1	*C. fructicola*	MN982434.1
	Percent Identity	99,8	Percent Identity	99,83	Percent Identity	100
C107	*C. fructicola*	MN982447.1	*C. fructicola*	MK874590.1	*C. fructicola*	MN982434.1
	Percent Identity	99,8	Percent Identity	100	Percent Identity	100
C133	*C. siamense*	KC566246.1	*C. siamense*	OR807537.1	*C. siamense*	MK693710.1
	Percent Identity	99,61	Percent Identity	99,31	Percent Identity	100
C146	*C. fructicola*	MN982447.1	*C. fructicola*	CP150817.1	*C. fructicola*	MN982433.1
	Percent Identity	99,6	Percent Identity	100	Percent Identity	97,63
C160	*C. siamense*	KC566246.1	*Colletotrichum sp.*	PP316998.1	*C. siamense*	MK693710.1
	Percent Identity	99,8	Percent Identity	100	Percent Identity	99,16
C163	*C. theobromicola*	MW151284.1	*C. theobromicola*	MK790662.1	*C. theobromicola*	MN939222.1
	Percent Identity	99,62	Percent Identity	100	Percent Identity	99,64
C164	*C. fructicola*	MN982447.1	*C. fructicola*	CP150817.1	*C. siamense*	MH151153.1
	Percent Identity	99,4	Percent Identity	100	Percent Identity	96,43
PAT1	*Colletotrichum sp.*	GU994462.1	*C. siamense*	PP407794.1	*C. siamense*	MK693710.1
	Percent Identity	100	Percent Identity	100	Percent Identity	99,61
PAT2	*Colletotrichum sp.*	GU994462.1	*C. tropicale*	MK874589.1	*C. siamense*	MK693710.1
	Percent Identity	100	Percent Identity	100	Percent Identity	99,45
PAT6	*Colletotrichum sp.*	GU994462.1	*C. siamense*	PP407794.1	*C. siamense*	MK693710.1
	Percent Identity	100	Percent Identity	100	Percent Identity	99,62

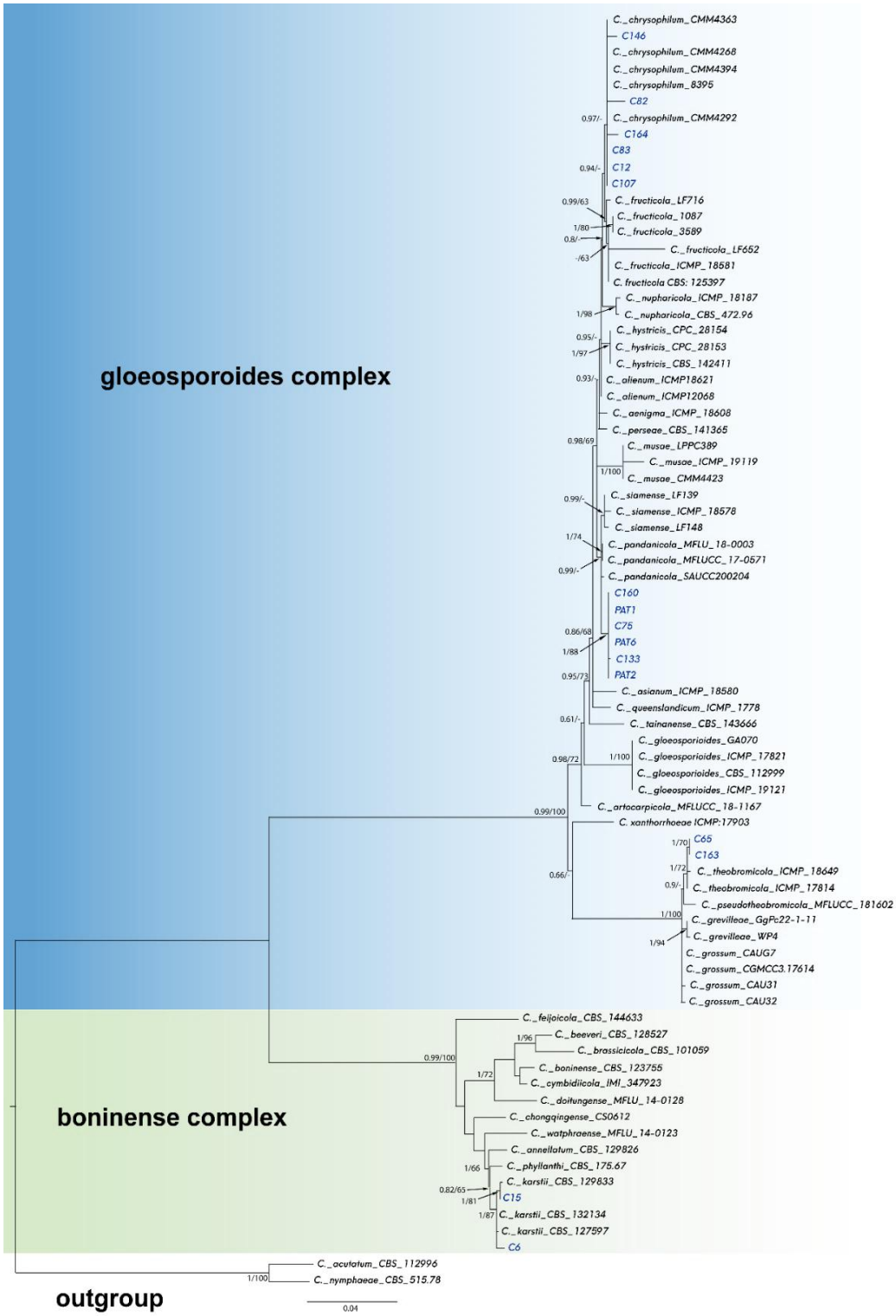

Figure 2. Maximum likelihood (ML) tree of the *gloeosporioides* and *boninense* species complex based on combined data sets of ITS, GAPDH, and TUB2 sequences (1288 bp including gaps). *C. acutatum* (CBS 112996) and *C. nymphaeae* (CBS 515.78) are used as an outgroup. ML bootstrap values and Bayesian posterior probability (BP) analysis are shown at the nodes (BP/ BS). BS > 60% and BP > 0.60 are shown, Branches that are unsupported with BS or BP are denoted by "–". Sequences obtained in the present study are indicated in blue.

Among the isolates identified as *C. chrysophilum* (all endophytes), 3 out of 6 showed symptoms, which were milder than those caused by the *C. siamense* isolates. The most aggressive in this case was C107 (18.98 mm external diameter, 16.25 mm internal diameter, and 10.74 mm depth) (Figure 3). None of

the isolates identified as *C. theobromicola* showed symptoms, and regarding the two isolates from the *boninense* complex, they also did not show any damage in the evaluations.

The species found in this study, mainly those belonging to the *gloeosporioides* complex, have been reported causing damage in different crops worldwide, such as *C. chrysophilum* in blueberry (Brazil) (Soares et al., 2021a) and cassava (Brazil) (Machado et al., 2021), *C. theobromicola* in wild cassava (Brazil) (Oliveira et al., 2018), cocoa (French West Indies) (Rojas et al., 2010), *C. siamense* in cocoa (Puerto Rico) (Serrato-Diaz et al., 2019), wild cassava (Brazil) (Oliveira et al., 2018), mango (China) (Qin et al., 2017), and chili (China) (Liu et al., 2016).

In the case of *C. karstii*, which belongs to the *boninense* complex, it has been reported causing anthracnose in soursop, passion fruit, banana, and tamarillo (Colombia) (Oliveira et al., 2018), strawberry (Brazil) (Soares et al., 2021b), Natal plum (Spain) (Garcia-Lopez et al., 2021), Mango (Brazil) (Zakaria, 2021), Dragon fruit (Brazil) (Nascimento et al., 2019), however, in this study, when pathogenicity tests were carried out, no damage was shown in the inoculated tissues, this is very common since *C. karstii* has been reported as endophytes in other crops such as Citrus (Europe) (Guarnaccia et al., 2017) Coffee (Colombia) (Poma-Angamarca et al., 2024).

On the other hand, it is notable that the four strains identified as *C. siamense* isolated from diseased pods were collected from different localities; however, they present symptoms when inoculated, as did the asymptomatic endophytes belonging to the same species. This could be explained according to (Photita et al., 2004), as endophytes can change their condition to pathogens under certain stress conditions.

Therefore, it is presumed that the endophytic isolates of *C. siamense* and *C. crhysophilum* changed their endophytic condition to pathogenic, even though they originated from leaves. This is well-supported, as *Colletotrichum* is one of the most frequently isolated endophytes from many crops (Baralt et al., 2012; Osorio et al., 2021; Vázquez Cruz et al., 2023).

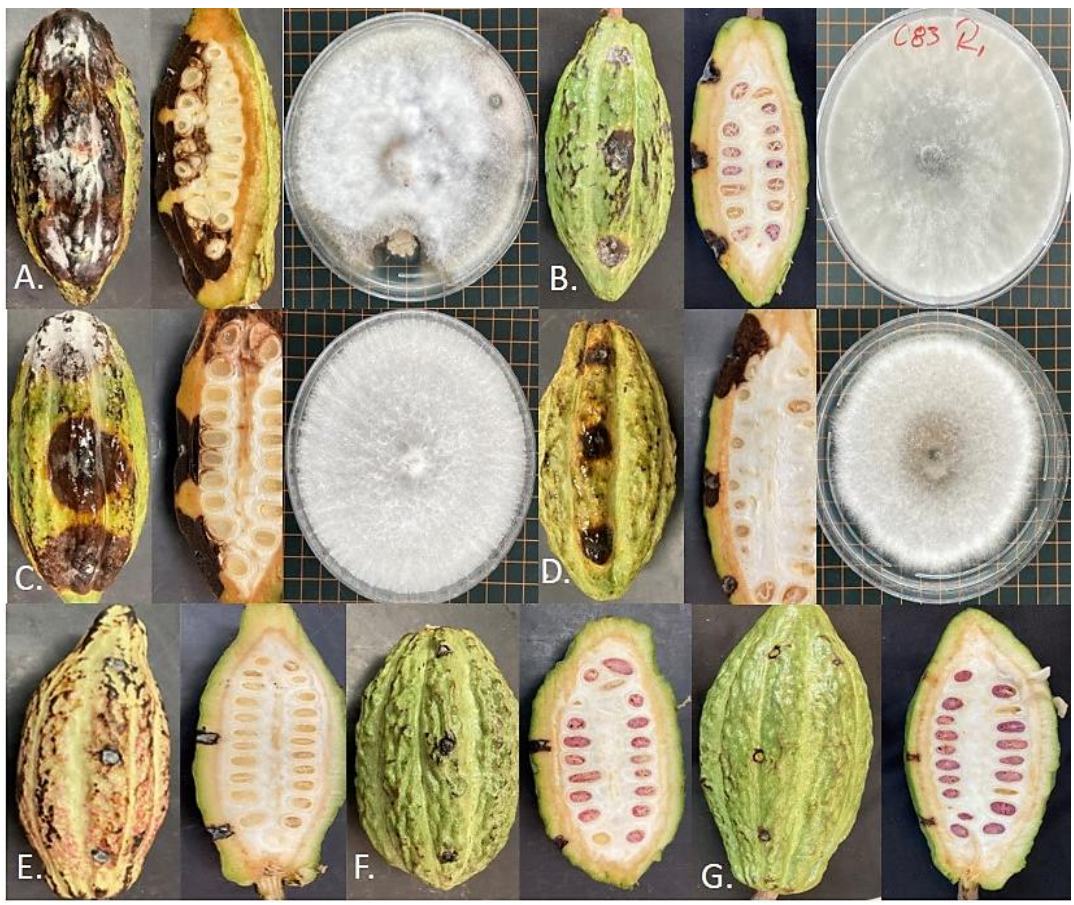

Figure 3. **A – D.** Damage caused by inoculation of *Colletotrichum* isolates, entire pod, longitudinal section, and recovery of isolate in Petri dish with PDA. A. PAT6, **B.** C83, **C.** C133, **D.** C107. **E – G.** Inoculated isolates that did not cause damage to pods. **E.** C65, **F.** C6, **G.** CONTROL.

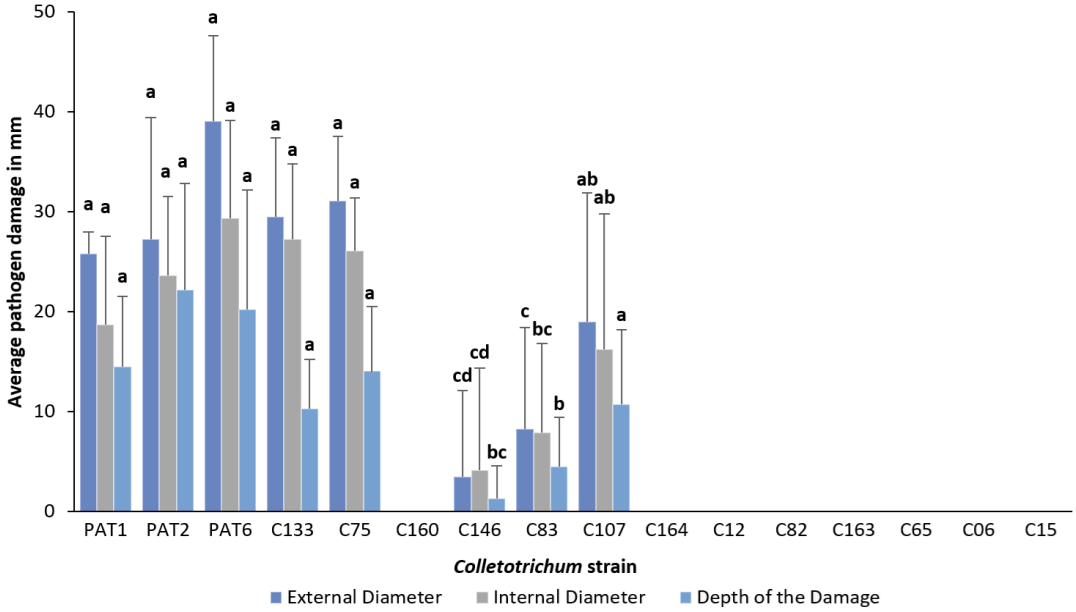

Figure 4. Averages of the three damage variables evaluated in mm (external diameter, internal diameter, and depth of damage) for the *Colletotrichum* isolates.

4. Conclusions

Through phylogenetic analysis, two complexes of the *Colletotrichum* group were identified in cocoa cultivation: the *gloeosporioides* complex (*C. crysophilum*, *C. siamense*, *C. theobromicola*) and the *boninense* complex (*C. karstii*). Among these, *C. crysophilum* has not been previously reported in cocoa cultivation. Pathogenicity tests demonstrate that isolates of *C. siamense* are the main cause of necrosis symptoms in cocoa pods, while isolates of *C. crysophilum* cause much less damage. The isolates *C. theobromicola* and *C. karstii* did not cause any damage in the inoculated pods.

This study reports that the main species causing damage in cocoa cultivation is *C. siamense*. However, the possibility that *C. crysophilum* might change its condition from endophytic to pathogenic cannot be ruled out. Future studies should conduct periodic sampling to identify possible changes in the pathogen population and its geographic distribution in order to develop integrated management strategies that include cultural and biological practices to control the pathogens.

Conflicts of Interest

There are no conflicts of interest.

Authors' Contribution:

F. Espinoza-Lozano: conceptualization, data curation, research, methodology, writing the initial draft, and review. **L. Serrano-Mena**: Research, data curation, software, and review. **M. Villavicencio-Vasquez**: Conceptualization, research, formal analysis, and review. **M. Vera-Morales**: Formal analysis, data curation, and review. **J. Coronel-León**: Formal analysis, review, and supervision. **D. Sosa-Castillo**: Conceptualization, research, formal analysis, review, and supervision.

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