



Research article

Identification of *Lasiodiplodia* species causing brown leaf spot disease of cassava in Ghana

Muntala Abdulai^{a,*}, Frank Ankomah-Boamah^a, Joseph Adomako^b, Yükselbaba Utku^c, Kwadwo Gyasi Santo^a, Shadrack Asomah^a, Bernard Armooh^d

^a Department of Horticulture and Crop Production, School of Agriculture and Technology, University of Energy and Natural Resources, Sunyani, BR-001-5677, Ghana

^b CSIR-Crop Research Institute, Plant Pathology Unit, Fumesua, AE-0680-9724, Ghana

^c Department of Plant Protection, Faculty of Agriculture, Akdeniz University, Antalya 07070, Turkey

^d Cocoa Research Institute of Ghana, Tafo, Akim, EE-1230-5080, Ghana

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Abstract

Importance of the work: This research connected *Lasiodiplodia* species to brown leaf spot (BLS) disease of cassava in Ghana, since there is a lack of published data regarding *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Lasiodiplodia mediterranea* Linald., Deidda & Berraf-Tebbal and *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, as causative agents of BLS disease in cassava in Ghana.

Objectives: To isolate, identify and describe the fungal pathogens that cause cassava BLS in Ghana.

Materials and Methods: Diseased cassava leaves with BLS symptoms were collected from farms in five communities, selected from Sunyani Municipality, Sunyani West Municipality and Dormaa East District, Ghana. Morphological (conidial size and shape, colony diameter, colony growth rate) characterization was applied in identifying *Lasiodiplodia* spp. to the genus level, while a multi-locus study of the internal transcribed spacer (ITS) and β -tubulin (TUB2) regions was used to identify these pathogens to the species level.

Results: The fungal pathogens *L. theobromae*, *L. mediterranea* and *L. pseudotheobromae* were identified morphologically and molecularly using their ITS and TUB2 sequences. Together, these three isolates produced the characteristic BLS symptoms that were seen in farmers' fields. Conidia widths were not significantly different, except for the Nsoa community (5.477 μ m). The conidia lengths of the Asua (35.05 μ m), Asuo (73.90 μ m), Kyere (46.20 μ m), Nsoa (37.43 μ m) and Wam (48.78 μ m) communities were not significantly different, while the D.A (38.22 μ m), K.B (36.45 μ m), Yaw (27.67 μ m) and Akon (39.38 μ m) communities were significantly different from Asuo (73.90 μ m). For both the colony diameter and average mycelial growth rate, all communities were significantly ($p < 0.001$) different, except for Nsoa (21.1mm, 2.7 mm/day). Pathogenicity testing on healthy plants produced similar symptoms, validating Koch's postulates.

Main finding: The present study was the first of its kind implicating *L. theobromae*, *L. mediterranea* and *L. pseudotheobromae* as causal agents of BLS on cassava in Ghana.

* Corresponding author.

E-mail address: muntala.abdulai@uenr.edu.gh (M. Abdulai)

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Introduction

Cassava (*Manihot esculenta* Crantz) is in the family Euphorbiaceae and has been a vital food source in Ghana for the past 4,000 years, as well as being a predominant carbohydrate source eaten by animals and humans in tropical regions (Adeoti, 2010; Liu et al., 2014). Characteristically, cassava is able to withstand harsh edaphic and abiotic conditions and is therefore useful in third world countries for poverty reduction and as a rural migration strategy tool due to its numerous uses (Machado et al., 2014b; Vilas Boas et al., 2017). It is estimated that actual cassava losses as a percentage of projected yield exceed 60% in Central and Western Europe and North America, but are lower than 50% for North Africa and Latin America while, south of the Sahara, East-Central Europe, the Caribbean and Central America account for 30% (FAO, 2012; Valipour et al., 2015).

In Ghana, cassava productivity is affected by different diseases, including brown leaf spot (BLS), cassava mosaic disease, cassava brown streak virus disease, cassava bacterial blight (*Xanthomonas axonopodis* pv. *manihotis*), cassava root rot and cassava anthracnose (*Colletotrichum gloeosporioides*) (Abaca et al., 2014).

The genus *Lasiodiplodia*, especially *L. theobromae*, is prevalent and has more than 500 hosts connected to it, primarily in tropical and subtropical regions (Punithalingam, 1980). *L. theobromae* has been reported to affect host plants such as cassava [CABI CPC, 2022], mango (Tan et al., 2019; Burgess et al., 2019), coconut (Dissanayake et al., 2016), papaya (Dissanayake et al., 2016), tea (Pandey et al., 2024), maize (Diab et al., 1984), cocoa (CABI CPC, 2022) and apple (CABI CPC, 2022). The pathogen causes severe damage and leads to major losses, especially when stress is present in the host flora (Slippers and Wingfield, 2007).

Various cultural, morphological and physiological characteristics have led to the incorrect identification of the *L. theobromae* species complex due to aphanic speciation (Slippers et al., 2004; Alves et al., 2008; Abdollahzadeh et al., 2010; Hyde et al., 2014). Currently, the combination of morphological and molecular traits has been acknowledged widely as a common procedure for pathogen taxonomy and species identification (Machado et al., 2014a, 2014b). The general internal transcribed spacer (ITS) of genomic rDNA, in addition to protein-coding genes like β -tubulin and translation elongation factor 1- α has been utilized to accurately identify *Lasiodiplodia* spp., including *L. theobromae* and *L. pseudotheobromae* (Brito et al., 2020).

The evolutionary development of organisms in the Botryosphaeriaceae shows that a unitary gene region alone is insufficient to differentiate aphanic species (Slippers and Wingfield, 2007; Phillips et al., 2013); therefore, concatenated alignment of a minimum of two gene segments is needed (Chen et al., 2014; Slippers et al., 2014; Machado et al., 2014a; Zhou et al., 2015; Coutinho et al., 2017). *L. theobromae* has been reported as the cause of BLS on pepper, *Kadsura longipedunculata* (Fan et al., 2020), mulberry (Luo et al., 2020), tea (Zhu et al., 2014) and *Bruguiera gymnorrhiza*, a mangrove forest tree species (Wang et al., 2023). Ngobisa et al. (2015) isolated and identified *Botryodiplodia theobromae* (synom *L. theobromae*) as a disease-causing fungi connected to Cameroonian cassava (*Manihot esculenta* Crantz). In addition, Machado et al. (2014a) documented the presence of *L. pseudotheobromae*, *Neoscytalidium hyalinum* and *L. euphorbicola* on cassava in Brazil. However, there is a paucity of published information on *L. theobromae* causing BLS disease on cassava in Ghana. Consequently, the aims of the current research were: 1) to conduct a comprehensive identification of leaf spot-inducing pathogens using micro-morphological and molecular analysis; and 2) to link *Lasiodiplodia* species associated with Ghanaian BLS disease to cassava.

Materials and Methods

Study area and fungal isolation

From 2021 to 2023, 135 leaf samples of cassava with symptoms of BLS were randomly collected in five communities in the Dormaa East District, Sunyani Municipality and Sunyani West Municipality in the Bono Region of Ghana. In total, 27 cassava farms were surveyed in the studied Municipalities/District throughout both the rainy and dry seasons. Samples were collected to represent 45 farmers' fields within prominent cassava cultivation areas. Each sample was labelled carefully. Fungal isolation from the samples followed the technique outlined by Thilagam et al. (2018), utilizing potato dextrose agar (PDA) amended with 500 mg of ampicillin as the cultural medium. The excised lesions were subjected to 30 s of disinfection in 70% ethanol, followed by 1.3% (volume per volume, v/v) NaOCl disinfection for 1 min and then rinsing with three exchanges of deionized water. Then, each lesion was dried on chromatography paper, plated on a Petri dish and allowed to incubate at $28 \pm 2^\circ\text{C}$ for 3–7 d. Three replications were used for each isolate.

Isolates were purified based on mono-conidial culture (Choi et al., 1999), based on studying differences in the colony growth and conidia of the BLS pathogens. Sterile distilled water (100 mL) was used to form an inoculum suspension and adjusted to 1×10^5 conidia/mL concentration. The spore suspension was disseminated on a parafilm medium and incubated at $28 \pm 2^\circ\text{C}$ for 24 hr with triplication.

Morphological characterization

Macroscopic observations of the cultural characteristics, (colony color, texture, colony margin and form, colony elevation, aerial hyphae), were recorded on PDA. A Rayner (1970) chart was used to determine the colony colors. Common laboratory methods for detecting fungi were used to describe the colony morphology (Mathur and Kongsdal, 2004). All the isolated fungi were cultivated for 7 d on PDA to perform micro-morphological characterisation. The hyphae were examined for the presence or lack of septa to identify their taxonomic classes. In addition, other characteristics were considered such as spore shape and size, colony growth and growth rate, (Jahan et al., 2013).

A sterile pin was used to make a number of pricks on the detached cassava leaf surfaces in accordance with the method of Ager (2023) to confirm Koch's postulates.

Molecular characterization

Genomic DNA extraction, polymerase chain reaction and gel electrophoresis

Polymerase chain reaction (PCR) was used for the molecular diagnosis of the BLS pathogens and for the sequencing of a region of the DNA from the isolated fungal samples. The DNA was recovered using the technique by Doyle and Doyle (1987). Isolate identification used two genomic loci—general purpose ribosomal ITS (White et al., 1990) and species-specific Primer Glass and Donaldson (1995)

Beta tubulin (TUB2) genes. Table 1 lists the primers that were utilized along with their sequences. Then, 2.0 μL of 100 ng gDNA, 6.8 μL of distilled water, 0.6 μL each of 10.0 μM sense and antisense primers and 10.0 μL of master mix (Taq polymerase, Mg^{2+} , buffer and deoxynucleotide triphosphates) were used in a reaction volume of 20 μL . A standard PCR apparatus with variable denaturation, annealing and extension temperature profiles was utilized. The amplicons were maintained at 4°C and analyzed on 2% agarose gel.

The phylogenetic tree was constructed using the MEGA 11 software (Tamura et al., 2021). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980).

Pathogenicity tests

To satisfy Koch's postulates, fresh, healthy cassava leaves aged 3–8 mth were rinsed with running tap water to eliminate surface debris and subsequently sterilized in 70% ethanol for 30 s, followed by disinfection with 1.3% (v/v) of NaOCl for 1 min. Pin prick holes on a disk cut from cassava leaves at day 7 on PDA culture were inoculated with isolates. The control was inoculated with fresh PDA without any pathogen.

Statistical analysis

Morpho-cultural parameters were analyzed based on two-way analysis of variance using the Genstat software (version 12.1; Paynes et al., 2009) followed by Tukey's post hoc test, to identify significant differences. All tests were considered significant at $p < 0.05$.

Table 1 Detailed information on polymerase chain reaction primers used.

Gene	Product	Primer	Direction	$^\circ\text{C}$	Sequence (5'-3')	Reference
ITS	Internal transcribed spacer	ITS 1	Forward	56	GAAGTAAAAGTCGTAACAAGG	White et al. (1990)
ITS		ITS 4	Reverse	56	TCCTCCGCTTATTGATATGC	White et al. (1990)
BT2	β -tubulin	Bt1a	Forward	50	TTCCCCCGTCTCCACTTCTTCATG	Glass and Donaldson (1995)
		Bt1b	Reverse	50	GACGAGATCGTTTCATGTTGAACTC	Glass and Donaldson (1995)

$^\circ\text{C}$ = annealing temperature; ITS = internal transcribed spacer; BT2 = β -tubulin.

Results

Studies on morphological variability of Lasiodiplodia species

The symptoms observed on the infected cassava hosts in the field were characterized as brown necrotic spots on the leaf abaxial surface, followed by their appearance on the adaxial surface. In total, 21 isolates (Table 3) of *Lasiodiplodia* spp. were used for morphological characterization and were differentiated on the basis of conidial morphology, colony growth rate, colony diameter and cultural variabilities. Initially, all the colonies were white to pale gray, later becoming dark grayish with time, with black-to-tan aerial, fluffy, woolly mycelia on the reverse plate (Fig. 1). In total, 81 conidia were measured from nine communities (three infected leaves from three farmers' fields in each community). Each community was represented by average values for conidia size, colony diameter and growth rate (Burgess et al., 2006).

There were no significant differences ($p > 0.05$) in the conidia width (W) for all the *Lasiodiplodia* spp., except for the Nsoa community, ($p < 0.001$). The conidia length (L) of the Asua, Asuo, Kyere, Nsoa and Wam communities were not significantly different, while the D.A, K.B, Yaw and Akon communities varied from Asuo. Except for the Nsoa community, there were significant ($p < 0.001$) differences in both the colony diameter and average mycelial growth rate among communities (Table 2).

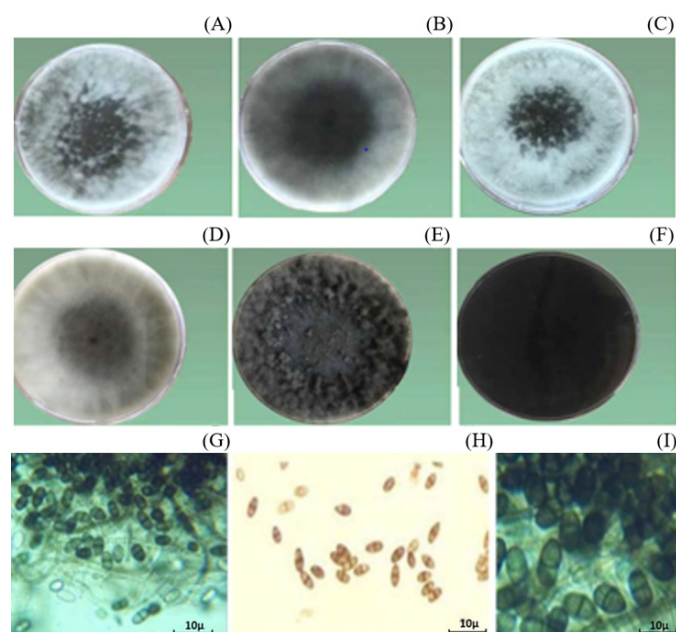


Fig. 1 Morphological characteristics of *Lasiodiplodia* spp. on potato dextrose agar (PDA) at 10 d after inoculation at 28°C for obverse and reverse colonies: (A, B) *L. pseudotheobromae*; (C, D) *L. mediterranea*, conidia hyaline and septate; (E, F) *L. theobromae*, with very fast growth, many cotton-like aerial mycelia on PDA, colorless initially and becoming deep grayish or darkish with age and black pigmentations on reverse plate on PDA; (G) *L. pseudotheobromae* with conidia hyaline and septate; (H) *L. theobromae* with conidia hyaline and single septate; (I) *L. mediterranea* with white cotton-like mycelia after 48 hrs., initially light fluffy grayish and then later grayish in appearance, crateriform with a filiform margin and a fuzzy surface, translucent, dull tan on reverse; where A-F= 40x.

Table 2 Mean $\pm$ SD for conidia size, colony diameter and growth rate of *Lasiodiplodia* spp.

Community	Colony diameter (mm)	Growth rate (unit)	Conidia length (μ m)	Conidia width (μ m)
Akon	79.2 $\pm$ 4.4 ^a	11.3 $\pm$ 1.5 ^a	39.4 $\pm$ 20.2 ^b	1.9 $\pm$ 0.4 ^b
Asua	78.6 $\pm$ 6.9 ^a	11.2 $\pm$ 1.7 ^a	35.1 $\pm$ 5.5 ^{ab}	2.3 $\pm$ 0.4 ^b
Asuo	78.7 $\pm$ 4.7 ^a	11.2 $\pm$ 1.1 ^a	73.9 $\pm$ 26.0 ^a	2.3 $\pm$ 0.4 ^b
D.A	75.9 $\pm$ 7.2 ^a	10.8 $\pm$ 1.9 ^a	38.2 $\pm$ 16.7 ^b	2.4 $\pm$ 0.7 ^b
K.B	69.6 $\pm$ 12.0 ^a	10.0 $\pm$ 2.1 ^a	36.4 $\pm$ 15.6 ^b	2.5 $\pm$ 0.8 ^b
Kyere	81.3 $\pm$ 3.2 ^a	11.6 $\pm$ 1.1 ^a	46.2 $\pm$ 22.5 ^{ab}	2.3 $\pm$ 0.4 ^b
Nsoa	21.1 $\pm$ 1.0 ^b	2.7 $\pm$ 0.5 ^b	37.4 $\pm$ 1.2 ^{ab}	5.5 $\pm$ 0.6 ^a
Wam	77.4 $\pm$ 5.1 ^a	11.1 $\pm$ 1.8 ^a	48.8 $\pm$ 22.8 ^{ab}	2.4 $\pm$ 0.5 ^b
Yaw	68.9 $\pm$ 7.9 ^a	9.9 $\pm$ 1.8 ^a	27.7 $\pm$ 13.9 ^b	2.9 $\pm$ 0.5 ^b
<i>p</i> value	< 0.001	0.000	0.025	< 0.001
LSD	8.361	2.015	25.190	0.633
CV (%)	8.9	15.1	45.0	19.8

CV = coefficient of variation; LSD = least significant difference of means.

Mean $\pm$ SD within the same row with different lowercase superscripts are significantly ($p < 0.001$) different.

Table 3 Isolates of *Lasiodiplodia* spp. selected for morphological and molecular identification

Code	Community	Isolate (ITS and β -tubulin)	Host	Location	
1	Akon F1C1	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
2	Akon F2A3	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
3	Akon F3A3	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
4	K.B F1B1	<i>L. theobromae</i>	Cassava	Sunyani	Municipality
5	K.B F3C1	<i>L. theobromae</i>	Cassava	Sunyani	Municipality
6	Yaw F3C1	<i>L. pseudotheobromae</i>	Cassava	Sunyani	Municipality
7	Nsoa F1	<i>L. theobromae</i>	Cassava	Sunyani	West Municipality
8	Nsoa F3	<i>L. theobromae</i>	Cassava	Sunyani	West Municipality
9	Asuo F1C3	<i>L. mediterranea</i>	Cassava	Dormaa	East District
10	Asuo F2C1	<i>L. mediterranea</i>	Cassava	Dormaa	East District
11	Asuo F2C3	<i>L. mediterranea</i>	Cassava	Dormaa	East District
12	Asuo F3A3	<i>L. mediterranea</i>	Cassava	Dormaa	East District
13	Kyere F1C1	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
14	Kyere F3C1	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
15	Kyere F3A3	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
16	Wam F1C3	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
17	Wam F2B1	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
18	Wam F3A3	<i>L. pseudotheobromae</i>	Cassava	Dormaa	East District
19	D.A F1C3	<i>L. theobromae</i>	Cassava	Dormaa	East District
20	D.A F2A2	<i>L. theobromae</i>	Cassava	Dormaa	East District
21	Asua F1B2	<i>L. theobromae</i>	Cassava	Sunyani	West Municipality

Akon = Akontanin; DA = Dormaa Akwamu; Asuo = Asuotiano; Wam = Wamfie; Kyere = Kyeremasu; Nsoa = Nsoatre; Asua = Asuakwa; K.B = Kyiribogya; Yaw = Yawsae; ITS = internal transcribed spacer.

Pathogenicity

The separate cassava leaves were inoculated using the wounding technique, with tested *Lasiodiplodia* species producing BLS lesions (Fig. 2). In contrast, the leaves treated with PDA plugs (without mycelia) showed no lesions. Koch's postulate was confirmed by successfully reculturing the infected tissues and isolating *Lasiodiplodia* spp. again.

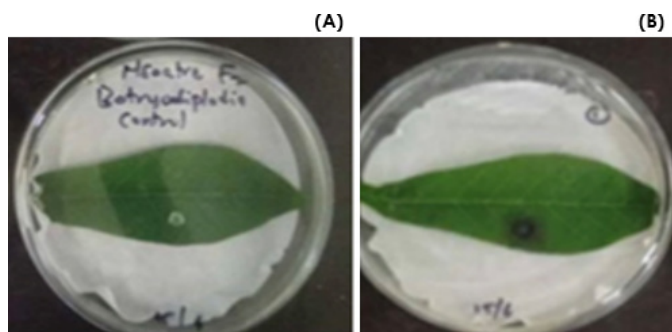


Fig. 2 Detached leaf bioassay: (A) Control using only agar (B) Infected cassava sample showing BLS symptoms

Molecular identification

Each isolate was identified based on the acquired ITS and TUB2 fragments. The *Lasiodiplodia* species were tested using the ITS1/ITS4 primer pairs. All species had 550 bp amplicons using the ITS1/ITS4 primer (Fig. 3). A beta-tubulin (Bt1a/ Bt1b) primer pair was used to target the β -tubulin genes of the isolates (*Lasiodiplodia* spp.). The amplified product with this primer produced an amplification fragment of 358 bp (Fig. 4). Sample 6 on both gels failed to amplify, maybe partly because it does not have genes for ITS/TUB2 established for BLS symptoms on cassava leaves.

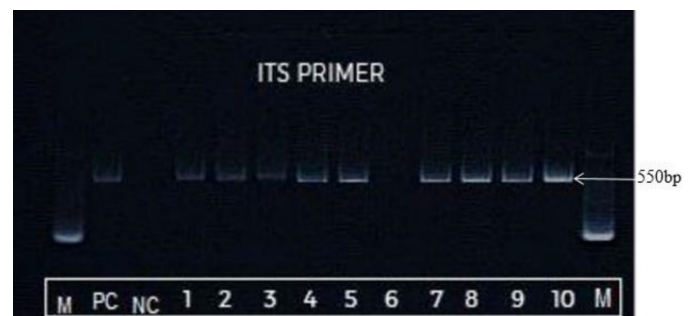


Fig. 3 Gel showing polymerase chain reaction amplified products for *Lasiodiplodia pseudotheobromae* (lanes 1–10) using ITS1/ITS4 primer, where ITS = internal transcribed spacer, M = DNA marker, PC= positive control and NC= negative control.



Fig. 4 Gel showing polymerase chain reaction amplified products for *Lasiodiplodia mediterranea* (lanes 1–4) and *L. theobromae* (lanes 5–10) using Bt1a and Bt1b primers, where M = DNA marker, PC= positive control and NC= negative control.

Following the sequencing of the generated PCR products, the resulting accession numbers were deposited in the NCBI GenBank (Table 4). These accession numbers were compared with the related *Lasiodiplodia* species in the GenBank, some of

which were used for evolutionary development of the tree for the putative organisms.

Evolutionary tree analysis of internal transcriber spacer and β -tubulin genes

The ITS and TUB2 loci were sequenced to verify the genetic identity of the representative isolates of fungi. The PCR products were purified, sequenced and BLASTn searched. *Lasiodiplodia* isolates references, such as *Lasiodiplodia egyptiaca* and *Pseudofusicoccum stromaticum*, that were used as outgroups, were recovered from the NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/). The maximum composite likelihood method was used to compute the evolutionary distances (Tamura et al., 2021), whereas the neighbor-joining approach was used to deduce the evolutionary history (Saitou and Nei, 1987).

Table 4 GenBank accession numbers of *Lasiodiplodia* species used for phylogenetic analysis

Species	Strain	Source	GenBank Accession Number	
			ITS	TUB2
<i>Lasiodiplodia mediterranea</i>	LDRM28	This study		PP847205
<i>Lasiodiplodia theobromae</i>	LSNY18	This study		PP847204
<i>Lasiodiplodia theobromae</i>	LSNW44	This study		PP847206
<i>Lasiodiplodia mediterranea</i>	13-1214	GenBank		KU720483
<i>Lasiodiplodia mediterranea</i>	13-1215	GenBank		KU720484
<i>Lasiodiplodia mediterranea</i>	13-1213	GenBank		KU720482
<i>Lasiodiplodia theobromae</i>	M2RA2PA	GenBank		OP172845
<i>Lasiodiplodia theobromae</i>	Ltheo055	GenBank		PP495255
<i>Lasiodiplodia theobromae</i>	M7RA1PA	GenBank		OP172844
<i>Lasiodiplodia theobromae</i>	Ltheo031	GenBank		PP495251
<i>Lasiodiplodia theobromae</i>	Ltheo026	GenBank		PP495249
<i>Lasiodiplodia theobromae</i>	Ltheo033	GenBank		PP495252
<i>Lasiodiplodia theobromae</i>	Ltheo042	GenBank		PP495253
<i>Lasiodiplodia theobromae</i>	Ltheo052	GenBank		PP495254
<i>Lasiodiplodia theobromae</i>	Ltheo030	GenBank		PP495250
<i>Lasiodiplodia pseudotheobromae</i>	MZA038	GenBank		MH453390
<i>Lasiodiplodia pseudotheobromae</i>	MZA054	GenBank		MH453417
<i>Lasiodiplodia egyptiaca</i>	MT	GenBank		MT592618
<i>Lasiodiplodia pseudotheobromae</i>	CMW40982	GenBank	KP872339	
<i>Lasiodiplodia pseudotheobromae</i>	BOT-25	GenBank	JN814393	
<i>Lasiodiplodia pseudotheobromae</i>	IRAN1518C	GenBank	GU973874	
<i>Lasiodiplodia pseudotheobromae</i>	CBS121773	GenBank	EU101311	
<i>Lasiodiplodia pseudotheobromae</i>	Wamfile	This study	PQ305364	
<i>Lasiodiplodia pseudotheobromae</i>	Kyremasu	This study	PQ305365	
<i>Lasiodiplodia pseudotheobromae</i>	MW	GenBank	MW862155	
<i>Lasiodiplodia pseudotheobromae</i>	KP	GenBank	KP872326	
<i>Lasiodiplodia pseudotheobromae</i>	KT	GenBank	KT075144	
<i>Lasiodiplodia pseudotheobromae</i>	LpLp7	GenBank	OM912717	
<i>Lasiodiplodia pseudotheobromae</i>	KX	GenBank	KX244814	
<i>Lasiodiplodia pseudotheobromae</i>	MN	GenBank	MN718909	
<i>Lasiodiplodia pseudotheobromae</i>	DOREEN16	GenBank	OR211544	

Table 4 Continued

Species	Strain	Source	GenBank Accession Number	
			ITS	TUB2
<i>Lasiodiplodia pseudotheobromae</i>	EU	GenBank	EU860391	
<i>Lasiodiplodia pseudotheobromae</i>	MK	GenBank	MK693210	
<i>Lasiodiplodia pseudotheobromae</i>	CBS447.62	GenBank	EF622081	
<i>Lasiodiplodia pseudotheobromae</i>	CBS304.79	GenBank	EF622079	
<i>Lasiodiplodia pseudotheobromae</i>	CBS116459	GenBank	EF622078	
<i>Lasiodiplodia pseudotheobromae</i>	CMW22667	GenBank	FJ888471	
<i>Lasiodiplodia pseudotheobromae</i>	FJ	GenBank	HQ332191	
<i>Lasiodiplodia pseudotheobromae</i>	KP	GenBank	KP872327	
<i>Lasiodiplodia viticola</i>	CBS128313	GenBank	KP872341	
<i>Lasiodiplodia viticola</i>	CBS	GenBank	MH864856	
<i>Lasiodiplodia citricola</i>	MH	GenBank	MH183370	
<i>Lasiodiplodia parva</i>	CBS495.78	GenBank	EF622085	
<i>Lasiodiplodia parva</i>	CBS456.78	GenBank	EF622083	
<i>Lasiodiplodia theobromae</i>	MG	GenBank	MG870601	
<i>Lasiodiplodia theobromae</i>	EF	GenBank	EF622067	
<i>Lasiodiplodia egyptiaca</i>	BOT-29	GenBank	JN814401	
<i>Lasiodiplodia margaritacea</i>	KP	GenBank	KP872320	
<i>Lasiodiplodia margaritacea</i>	KP	GenBank	KP872321	
<i>Lasiodiplodia rubropurpurea</i>	WAC12538	GenBank	DQ103556	
<i>Lasiodiplodia rubropurpurea</i>	WAC12537	GenBank	DQ103555	
<i>Lasiodiplodia crassisporea</i>	CBS110492	GenBank	EF622086	
<i>Lasiodiplodia crassisporea</i>	CMW13488	GenBank	DQ103552	
<i>Lasiodiplodia venezuelensis</i>	WAC12539	GenBank	DQ103547	
<i>Lasiodiplodia venezuelensis</i>	CMW13513	GenBank	DQ103549	
<i>Pseudofusicoccum stromaticum</i>	CMW13366	GenBank	KP872348	

The ITS and TUB2 loci sequences were distinguished using the basic local alignment tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) program, which includes the NCBI GenBank database's records of other *Lasiodiplodia* spp. and isolates from different host plants. The BLAST analysis indicated that the ITS sequences (GenBank accession numbers PQ305364, PQ305365) and TUB2 (GenBank accession numbers PP847204, PP847205, PP847206) locus shared by all representative isolates had the following percentage identities with *L. pseudotheobromae*, *L. mediterranea* and *L. theobromae*: for ITS, 100% PQ305364, PQ305365; for TUB2, *L. mediterranea* 99.49% (KU720482, KU720483, KU720484) and 99.16% with *L. theobromae* (OP172844, OP172845, PP495249, PP495250, PP495251, PP495252, PP495253, PP495254, PP495255). *L. theobromae* LSNY18

strain had 100% homology with OP172844, OP172845, PP495249, PP495250, PP495251, PP495252, PP495253, PP495254 and PP495255 and 99.24% with *L. mediterranea* (KU720482, KU720483, KU720484). *L. theobromae* LSNW44 strain shared 99.70% similarity with OP172844, OP172845, PP495249, PP495250, PP495251, PP495252, PP495253, PP495254 and PP495255, 99.90% with *L. mediterranea* (KU720482, KU720483, KU720484) and 99.40% with *L. pseudotheobromae* (MH453390, MH453417) derived from several host plant species, including orange, mango, cashew, cacao and peanut. Sequences of the *Lasiodiplodia* isolates from the current study were submitted to the NCBI GenBank. Tables S1 and S2 provide all the accession numbers used in current study.

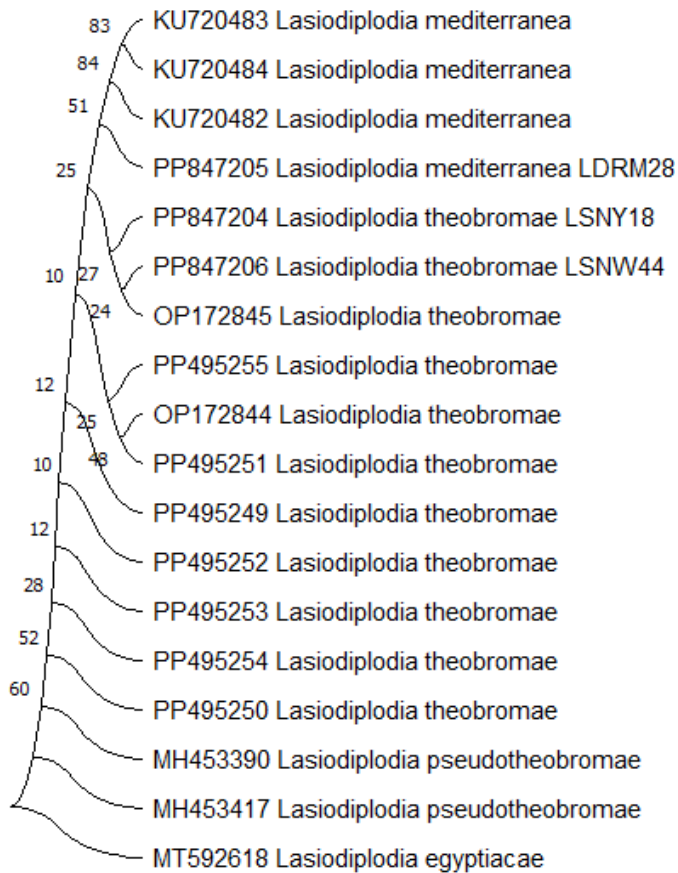


Fig. 5 *Lasiodiplodia* isolates from diseased cassava plants in Ghana's Bono Region, where TUB2 sequences were obtained using neighbor-joining method (Saitou and Nei, 1987), bootstrap values are displayed as percentages of 1,000 replications at nodes, *Lasiodiplodia egyptiaca* was used as outgroup, while *Lasiodiplodia mediterranea* and *Lasiodiplodia theobromae* were identified organisms in the tree and other group members are shown with strain names

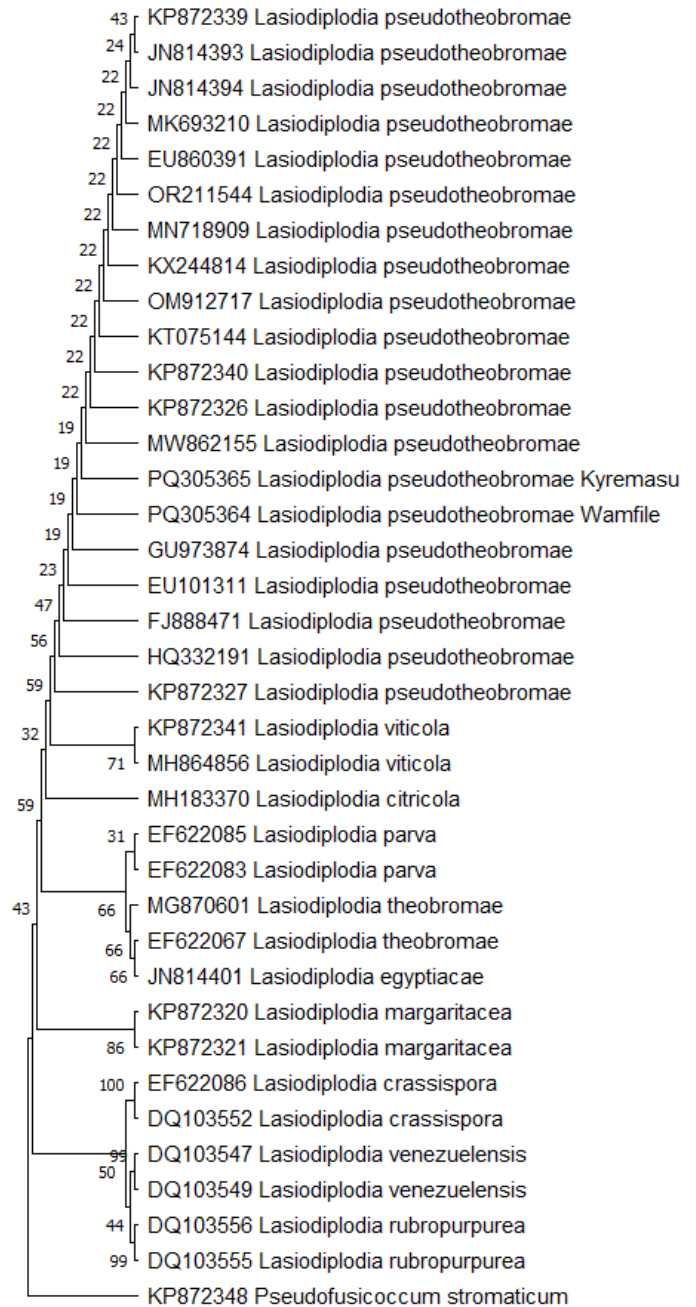


Fig. 6 *Lasiodiplodia* isolates from infected cassava plants in Ghana's Bono Region subjected to the maximum composite likelihood technique using internal transcribed spacer sequences (Tamura et al., 2021), where bootstrap values are displayed as percentages of 1,000 replications at nodes, *Pseudofusicoccum stromaticum* was used as outgroup with *L. pseudotheobromae* as the identified organism in the tree and other group members are shown with strain names

Discussion

Lasiodiplodia theobromae, *L. mediterranea* and *L. pseudotheobromae* were implicated as the pathogens associated with BLS on cassava in the Bono Region of Ghana. The morphological and evolutionary in-depth investigations of the ITS and TUB2 regions suggested that *L. theobromae*, *L. mediterranea* and *L. pseudotheobromae* were all in the genus *Lasiodiplodia* and responsible for the suspected BLS disease of cassava in the surveyed area. *Lasiodiplodia* species are connected with numerous hosts, exhibiting as disease causing organisms (Abdollahzadeh et al., 2010), endophytes (Slippers and Wingfield, 2007; Chen et al., 2015) and saprobes (Abdollahzadeh et al., 2010; Liu et al., 2012; Dissanayake et al., 2016; Hyde et al., 2019). To the best of the current authors' knowledge, the current report was the first exposing *L. theobromae*, *L. mediterranea* and *L. pseudotheobromae* as referenced pathogens of BLS of cassava in Ghana. These pathogens have been reported to infect a diverse array of unrelated hosts, although certain instances seemed to be unique to each host (De Wet et al., 2008).

The fungal infection that causes cassava BLS has seen major changes in taxonomy over time. For example, Pei et al. (2014) referred to it as *Passalora henningsii*, while Babu et al. (2009) and Ayesu-Offei and Antwi-Boasiako (1996) referenced it as *Cercospora henningsii* (Allesch). Videira et al. (2017) recently renamed *Clarohilum henningsii* into the genus *Clarohilum*. In the past, cassava BLS disease was thought to be less economically important because it causes less damage than other cassava infections (Powbunthorn et al., 2012). However, Pei et al. (2014) and Julião et al. (2020) reported on the substantial effects of BLS on cassava production in Asia and South America, respectively. Other research has confirmed the global presence of cassava BLS in Kenya (Ng'ang'a et al., 2019; Ager, 2023), China (Chaoping et al., 2015), Togo (Verdier et al., 2007) and Ghana (Leather, 1959; Ayesu-Offei and Antwi-Boasiako, 1996).

Even though morphological characteristics can cross over (Kim et al., 2005; Slippers et al., 2004; Abdollahzadeh et al., 2010), they continue to be useful when used in conjunction with molecular methods to differentiate novel species within the Botryosphaeriaceae. Hence, using physiological characters as a discriminatory standard alone ought to be approached with caution. The current study shows the color of most of the studied isolates was whitish-gray mycelia that were light, fluffy and grayish in appearance for all the isolates in

the various communities. The morphological characteristics of this genus include conidia that mature slowly, have strong walls and have longitudinal striations on the inner wall surface due to melanin deposition in the species (Punithalingam, 1976,1980); these were confirmed based on the results of the current study. As stated by Alves et al. (2008), despite related similarities, the size and form of their conidia can be used to differentiate *L. pseudotheobromae* and *L. theobromae*, with those of *L. pseudotheobromae* being bigger, not tapering as much toward the base and being more ellipsoid. This assertion was not different from the current study. *L. pseudotheobromae* and *L. theobromae* might have been separated due to cryptic speciation.

Septate paraphyses were identified in all isolates in the current study of *L. pseudotheobromae*, *L. theobromae* and *L. mediterranea*. Burgess et al. (2006) used the paraphyte septation to distinguish between *Lasiodiplodia* species. They found that although other species had septate paraphyses, *L. crassispora*, *L. gonubiensis* and *L. venezuelensis* (among others) had aseptate paraphyses. Damm et al. (2007) and Abdollahzadeh et al. (2010) utilized paraphyse morphology to differentiate *L. plurivora* from *L. crassispora* and *L. venezuelensis*, as well as *L. gilanensis* from *L. plurivora* and also to differentiate *L. hormozganensis* from *L. parva* and *L. citricola*. Additionally, Ismail et al. (2012) used the paraphyse morphology to differentiate *L. laeliocattleya* from the evolutionary related *L. hormozganensis*.

The morpho-cultural traits of *Lasiodiplodia* spp. in the current investigation were consistent with Ismail et al. (2012) and Machado et al. (2014b). Cultural traits were important in the current study to differentiate *Lasiodiplodia* species, where most isolates had pink coloration. Alves et al. (2008) distinguished *L. theobromae* from *L. parva* and *L. pseudotheobromae* by looking at how the latter produced a pink pigment in culture. *L. pseudotheobromae* has been known to be one of the prime species within the genus *Lasiodiplodia*, which shares a close relationship with *L. theobromae* (Alves et al., 2008), as was also confirmed by the current study since they shared the most similar morpho-cultural characteristics.

In the current investigation, *L. theobromae*, *L. mediterranea* and *L. pseudotheobromae* were identified molecularly, based on the multi-locus examination of the TUB2 and ITS regions. Using two-locus phylogeny and morphology, Gomdola et al. (2020) presented a fresh record of *L. pseudotheobromae* generating leaf spots on *Cynometra malaccensis* from northern Thailand. According to Rathnayaka et al. (2023), the most accurate method for identifying *Lasiodiplodia* species is

to use ITS + *tefl-α* + *tub2* + *rpb2* markers. Various researchers have affirmed that utilizing a gene region was inadequate to define aphanic species in the Botryosphaeriaceae family (de Wet et al., 2003; Slippers et al., 2004); hence, more than one gene region is needed to establish species boundaries in the *Lasiodiplodia* genus (Alves et al., 2008; Abdollahzadeh et al., 2010). The ITS1/ITS4 primer identified putative isolates as *L. pseudotheobromae* with 550 bp, while the monomeric globular protein (β -tubulin) targeting primer was applied for species delineation of *L. theobromae* and *L. mediterranea*. The primer used in the current study produced an amplification product of 358 bp. Munirah et al. (2017) studied the characteristics of *L. theobromae* and *L. pseudotheobromae* that cause fruit rot on Malaysian mangos before harvest, with the amplicon sizes for ITS and TUB2 in the ranges 500–600 bp and 300–400 bp, respectively, which were corroborated by Gnanesh et al. (2022) for *L. theobromae* causing black root rot, who also identified prime sources of resistance in mulberry collections.

The pathogenicity test results using the pin-prick method validated Koch's postulates, as *L. theobromae*, *L. mediterranea* and *L. pseudotheobromae* were reisolated from inoculated healthy cassava leaves. Although wounding is not necessarily the only requirement for infection, the current study tested certain speculated hypotheses, which this result confirmed.

In view of the sparse information on BLS disease in Ghana, wider surveys are required, covering the entire cassava-growing regions of the country to identify different pathogens that are limiting cassava production, based on using both morphological and molecular tools for more in-depth and accurate identification of the pathogens to the species level. Furthermore, future studies on BLS disease should examine the impact of it has on the productivity of cassava and on the income levels of farmers in Ghana. Based on the current findings, priority should be given to the search for resistant genotypes, as well as intensifying farmer education regarding disease detection and control.

BLS and other cassava diseases could be controlled by rotating grains and cereal crops with cassava at least once every three seasons of each crop. Rotating crops from various families interferes with the life cycles of important pathogens, contributing to effective disease control (Tilman et al., 2002). Good planting stock and hygiene should reduce BLS. To prevent BLS from infecting the subsequent planting, all crop husks carrying the pathogen should be destroyed. If good planting material is not available, the fungus can be killed before planting by dipping the cassava stem in a diluted

fungicide solution. Fungicides of various kinds will stop the disease from spreading, including chlorthalonil, carbendazim or any products ending in “-azole” (www.plantwise.org). Metalaxyl is commonly used and will control a variety of diseases caused by water molds (www.plantwise.org). The typical method of producing cassava in the tropics is reported to be its intercropping with other main crops (Olasantan et al., 1994), with more than 60% of farmers intercropping cassava with maize (Biaou and Issaka, 1997). Intercropping can affect disease and pest incidence and their severity (Trenbath, 1993). For example, intercropping cassava with cowpea reduced egg populations of *Aleurotrachelus socialis* and *Trialeurodes variabilis*, compared to those in monoculture (Gold et al., 1990), while intercropping cassava with maize did not reduce egg populations (Gold, 1993), indicating that the success of this technique can depend on the intercropped species.

The current study established *Lasiodiplodia* species as the causative organism of BLS on cassava and thereby implicated *L. theobromae*, *L. mediterranea* and *L. pseudotheobromae* as the main agents of BLS on cassava in Ghana. Every studied species of *Lasiodiplodia* had the capacity to proliferate within the internal cells at both the upper and lower sites of infection, resulting in necrotic lesions ranging from brown to black.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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