

First report of a styler end rot disease of guava fruits caused by *Lasiodiplodia pseudotheobromae* in Ghana

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ABSTRACT

An investigation was conducted to identify the causal agent of a new styler end rot disease affecting guava (*Psidium guajava* L.) fruits in Ghana. Symptomatic fruits were collected at random from fields in the Eastern region of Ghana, and fungal isolates were identified based on cultural and morphological characteristics, supported by sequence analysis of the internal transcribed spacer (ITS) and translation elongation factor 1-alpha (*tef1-α*) regions. *Lasiodiplodia pseudotheobromae* was consistently isolated and confirmed as pathogenic through Koch's postulates, producing similar symptoms on inoculated fruits. Phylogenetic analysis clustered the isolates with reference *L. pseudotheobromae* strains with high bootstrap support (99%). This represents the first report of *L. pseudotheobromae* associated with guava styler end rot in Ghana, warranting inclusion in the country's plant disease checklist.

1. Introduction

Guava (*Psidium guajava* L.), is a significant fruit crop grown in many subtropical and tropical climates worldwide [1]. It is a perennial plant, that is the toughest among tropical fruiting trees, with a high production rate, and adaptability to many environments [2]. Guava originated from Tropical America, but currently, may be assuming economic importance in more than 50 countries in the world [3,4]. Apart from the nutritional importance of the crop [5,6], it also serves as an important source of foreign exchange for producer countries. As of 2019, major guava exporting countries include Thailand, Mexico, Netherlands and Vietnam [7]. In Africa, with the exception of Sudan, Egypt and South Africa, guava is often considered a minor crop and much attention has not been provided for its improvement [8]. Ghana was reported as the 14th highest exporter of the crop in 2022, raking in more than USD 55,000 in foreign exchange [7]. Guava therefore, has the potential to contribute substantially to the food security and economic development of Ghana, if proper attention is given to it as one of the non-traditional export crops.

Despite the global significance of guava, information on its diseases in Ghana remains scarce Worldwide, diseases reported on guava include

anthracnose, attributed to *Colletotrichum* spp. [9], the styler end rot disease attributed to *Phomopsis psidii* in India [1] and other rots, attributed to *Botryosphaeria* sp. in Malasia [10]. These diseases have been described as causing destruction of guava fruits, both in the field and after harvest [9,1]. Yield losses ascribed to such diseases, ranged from 10 to 60% in India [10,11]. Until recently, there had been no record of diseases affecting guava fruits in Ghana. This could have been either due to a lack of interest in studying such diseases or a complete absence of the diseases due to hardness of local varieties, which were considered to be of no commercial value.

In recent times, interest in cultivating the exotic varieties of guava in Ghana has increased, driven mainly by the high prices being offered for such fruits on the international markets [7]. This has led to the establishment of guava orchards in several parts of the country, with the main aim of supplying exotic guava fruits to the international markets. In a recent visit, to some of these orchards, located in the coastal savannah zone of Ghana, it was observed that some of the fruits on the trees were infected with an unknown disease, characterised by dark-brown spots, on the fruits surface. Infected fruits were unsightly and were widespread in the field, raising concerns about the overall effect of the disease on the crop's productivity. Since the disease was being observed for the first

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time in the country, there had been no information regarding the causal agent and consequently no guidelines for its control. In such a situation, the nation risks the spread of this debilitating diseases among the guava growing areas of the country, which could hamper any future plans for expansion of the guava industry in the country. It is therefore important, that work be done to identify the causal agent of the disease. This information will be essential in formulating a control measure against the disease, since, this will be primarily targeted at the causal agent [12]. This study reports, for the first time in Ghana, the occurrence of stylar end rot disease of guava caused by *L. pseudotheobromae*, supported by morphological, molecular, and pathogenicity evidence.

2. Materials and methods

2.1. The study area

The study was carried out on a commercial exotic guava farm, located in the Lower Manya Krobo District of the Eastern Region of Ghana (Fig. 1). The soil type in the area is dominated by the vertisols, which are difficult for vegetable cultivation. Due to this most of the lands are cultivated to tree crops such as mango, citrus and pawpaw. Exotic guava has been introduced recently, and hence very few guava orchards are currently producing fruits, while several others have trees that are at the seedling stage. The climate in the area is conducive for flowering of trees, all year round and is one of the fewer areas in Africa, where the climate permits flowering of mango, twice in a calendar year.

2.2. Collection of diseased fruits

Fruit bearing trees in the field were selected at random and fruits hanging on trees were observed carefully noting disease symptoms. Some symptomatic fruits were sliced opened to determine the penetration of the disease lesions into the fruit pulp. A total of 45 diseased fruits harvested, were sent to the Plant Pathology Laboratory of the Soil and Irrigation Research Centre of the University of Ghana, located at Kpong, for the study of the disease etiological agent.

2.3. Isolation and cultural and morphological identification of fungi

Fungi associated with the diseased fruits, were isolated by excising pieces of the diseased fruit tissues, which were subsequently plated on potato dextrose agar (39 gL^{-1}), after surface sterilizing with 1.5 % potassium hypochlorite for 15 s. The plates were incubated at $25 \pm 2^\circ\text{C}$ and 65 % RH for 5 days under alternating 12 h daylight and 12 h darkness. Single spore cultures of isolates were produced on Water Agar (WA), after which they were sub-cultured on Potato Dextrose Agar (PDA) and incubated for a further seven days as described above. As the isolates grew, the colour of their mycelia and diameter of the mycelium were recorded. Also, the colour, shape and dimensions of the conidia produced by isolates, were recorded under the $\times 20$ magnification of the light microscope.

2.4. Molecular characterisation of isolates

Five isolates, selected at random and designated as DOREEN 1–5, were subjected to molecular characterisation. Total DNA was extracted from the five isolates, with the aid of the GenFlute Plant Genomic DNA Miniprep Kit, obtained from Sigma (St. Louis, MO, USA) and used as templates for polymerase chain reaction (PCR) with the primer pair ITS1/ITS4 to amplify the ITS region [13] and EF1-728F/EF1-986R, to amplify the translation elongation factor 1 alpha (*tef1- α*) gene [14]. The reaction mixture was made up of 5 μL of the template DNA, 25 μL of mastermix (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 5 % Glycerol, 0.08 % IGEPAL® CA-630, 0.05 % Tween® 20, 25 units/ml Taq DNA Polymerase, pH 8.6@25°C) (New England Biolabs, UK), 2.5 μL of each of the forward and reverse primers and 13.75 μL of deionized autoclaved water. The PCR conditions were 30 s of initial denaturing at 95°C , followed by 35 cycles of denaturing at 95°C for 10 s, annealing at 59°C for 15 s and extension at 72°C for 30 s, followed by final extension at 72°C for 5 min. The amplified products were separated by 1.5 % w/v agarose gel (Invitrogen, Carlsbad, CA), stained with Ethidium bromide alongside 1.0 kb marker at 100 V for about 1.5 h. Bands were observed under UV light. The PCR products were purified and sequenced directly at Inqaba Biotech in South Africa (Inqaba Biotechnical Industries Ltd.). Consensus sequences were generated with the help of BIOEDIT 7.2

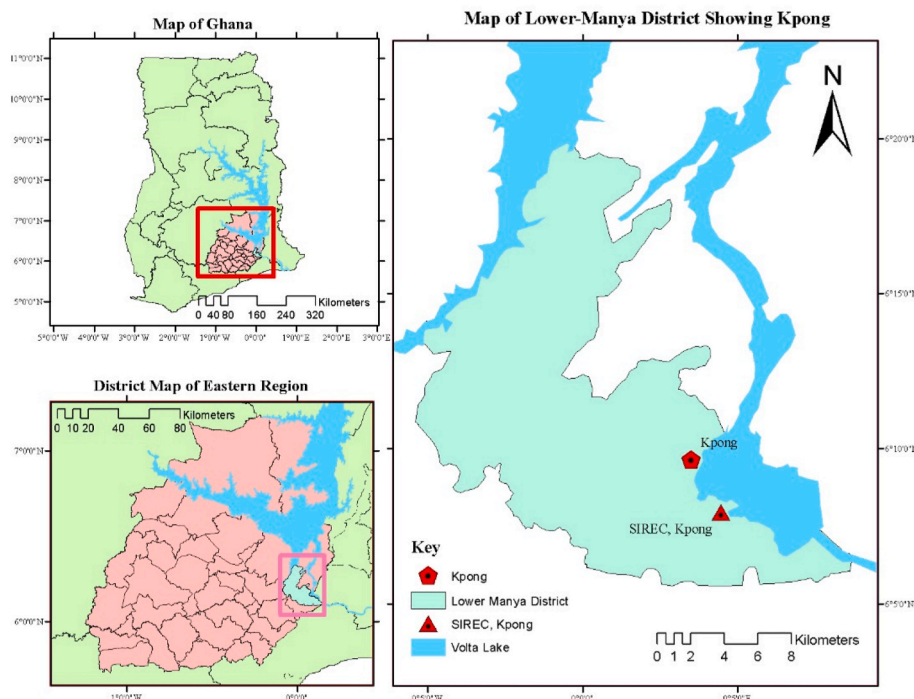


Fig. 1. Map of Ghana showing the Lower Manya District where the study was carried out.

software. Sequences were deposited at the EMBL GeneBank under accession numbers OR211544-OR211548 (ITS) and PP583663-PP583667 (tef1-α) and also used for phylogenetic studies.

2.5. Phylogenetic analysis

The nucleotide sequences of the two gene regions of the five isolates in this study, were concatenated and compared to concatenated sequences of the same gene regions of 30 other *Lasiodiplodia* species obtained from the Genebank. Sequences of *Botryosphaeria viticola* and *Spenceriartinsia* sp. were also included and used as out-groups, giving a total of 35 isolates (Table 1). The sequences were entered into the MEGA11 software [15] and the maximum parsimony (MP) option was selected for the inference of evolutionary history. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [16] with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Bootstrapping, with 1000 replicates [17], was used to evaluate the strength of the clustering of isolates. The manner of clustering of isolates in the generated phylogram, was used to confirm the species status of isolates.

2.6. Pathogenicity test of isolates

Clean and physiologically mature fruits of guava were harvested, surface sterilised with household bleach (5 %) and rinsed under running tap water for 5 min after which they were dried, under shade. Two types of wounding of fruits were investigated; wounding at the styler end and at the side of the fruit. All wounds were created using a sterile 5-mm cock-borer. Wounded fruits were inoculated with mycelia of the five isolates of the fungus, selected at random. Each isolate was used to

inoculate six fruits, to serve as the replicate of each treatment. Each treatment was replicated three times. Fruits inoculated with plugs of PDA only served as control. Wounds were sealed with parafilm tape. Inoculated fruits were placed in cardboard boxes in the laboratory, and incubated for 7 days, using the completely randomised design with 3 replicates. Re-isolation of the isolates from symptoms on the artificially inoculated fruits, was done to complete Koch's postulates [12]. An isolate that caused the disease on the host, was confirmed as the causal agent of the disease. After incubation of inoculated fruits, the area of the inoculated fruit covered by the disease lesion was rated visually on a scale of 0–4, where 0 = no visible symptoms, 1 = 1–25 % of fruit surface area covered by the disease lesion, 2 = 26–50 % of the fruit surface area covered by the disease lesion, 3 = 51–75 % of fruit surface area covered by the disease lesion and 4 =>76 % of fruit surface covered by the disease lesion.

The score obtained for each isolate was then used to calculate the disease severity index (DSI) by the following formula;

$$DSI = \frac{\text{sum of individual ratings}}{\text{number of fruits assessed} \times \text{maximum rating}} \times 100$$

2.7. Data analysis

The experiment was repeated once and the two sets of data were combined and analysed once. The data obtained was arcsine transformed and subjected to a two-way ANOVA using Genstat software. Means were separated using LSD at 5 %.

Table 1
Isolates of *Lasiodiplodia* species used in the phylogenetic study and their GenBank accession numbers.

Species	Designation	Host	Country	Accession numbers	
				ITS	TEF-1α
<i>Botryosphaeria viticola</i>	CBS117006	<i>Vitis vinifera</i>	Spain	AY905555	AY905562
<i>Lasiodiplodia citricola</i>	IRAN1521C	<i>Citrus</i> sp	Iran	GU945354	DQ103559
<i>L. citricola</i>	IRAN1522C	<i>Citrus</i> sp	Iran	DQ103552	DQ103552
<i>L. crassipora</i>	CMW 13488	<i>Eucalyptus urophylla</i>	Venezuela	DQ103552	DQ103559
<i>L. crassipora</i>	WAC 12533	<i>Santalum album</i>	Australia	DQ103550	DQ103557
<i>L. gilansensis</i>	IRAN 1523C	Unknown	Iran	GU945351	GU945342
<i>L. gilansensis</i>	IRAN 1501C	Unknown	Iran	GU945352	GU945341
<i>L. gonubiensis</i>	CBS 115812	<i>Syzygium cordatum</i>	South Africa	DQ458892	DQ458877
<i>L. gonubiensis</i>	CMW 14078	<i>Syzygium cordatum</i>	South Africa	AY639594	DQ103567
<i>L. homozganensis</i>	IRAN1500C	<i>Olea</i> sp.	Iran	GU945355	GU945343
<i>L. homozganensis</i>	CJA57	<i>Mangifera indica</i>	Iran	GU945357	GU945345
<i>L. homozganensis</i>	IRAN1498C	<i>Mangifera indica</i>	Iran	GU945356	GU945344
<i>L. margaritacea</i>	CBS122159	<i>Adansonia Gibbosa</i>	Western Australia	EU144050	EU144065
<i>L. margaritacea</i>	CBS122065	<i>Adansonia Gibbosa</i>	Western Australia	EU144051	EU144066
<i>L. parva</i>	CBS494.78	<i>Cassava field soil</i>	Columbia	EF622084	EF622064
<i>L. parva</i>	CBS456.78	<i>Cassava field soil</i>	Columbia	EF622083	EF622063
<i>L. pseudotheobromae</i>	CBS116459	<i>Gmelina arborea</i>	Portugal	EF622077	EF622057
<i>L. pseudotheobromae</i>	CBS374.54	<i>Coffea</i> sp.	Zaire	EF622080	EF622059
<i>L. pseudotheobromae</i>	IRAN1518C	<i>Citrus</i> spp.	Iran	GU973874	GU973868
<i>L. pseudotheobromae</i>	CJA36	Unknown	Iran	GU973875	GU973867
<i>L. pseudotheobromae</i>	DOREEN 16	<i>Psidium guajava</i>	Ghana	OR211544	PP583663
<i>L. pseudotheobromae</i>	DOREEN 17	<i>Psidium guajava</i>	Ghana	OR211545	PP583664
<i>L. pseudotheobromae</i>	DOREEN 18	<i>Psidium guajava</i>	Ghana	OR211546	PP583665
<i>L. pseudotheobromae</i>	DOREEN 19	<i>Psidium guajava</i>	Ghana	OR211547	PP583666
<i>L. pseudotheobromae</i>	DOREEN 20	<i>Psidium guajava</i>	Ghana	OR211548	PP583667
<i>Spenceriartinsia</i> sp.	CMW35505	<i>Podocarpaceae</i> spp.	South Africa	KM103241	KM103175
<i>L. theobromae</i>	IRAN1496C	<i>Mangifera indica</i>	Iran	GU973869	GU973861
<i>L. theobromae</i>	IRAN1499C	<i>M. indica</i>	Iran	GU973870	GU973862
<i>L. theobromae</i>	IRAN1233C	Unknown	Iran	GU973868	GU973860
<i>L. theobromae</i>	CJA198	Unknown	Iran	GU973871	GU973863
<i>L. theobromae</i>	CJA199	<i>M. indica</i>	Iran	GU973873	GU973865
<i>L. theobromae</i>	CJA279	<i>Cocos</i> sp.	Unknown	GU973872	GU973864
<i>L. theobromae</i>	CBS 164.96	Unknown	France	OR077890	AY640258
<i>L. venezuelensis</i>	WAC12539	<i>Acacia magnum</i>	Australia	DQ103547	DQ103568
<i>L. venezuelensis</i>	CMW13513	<i>Acacia magnum</i>	Australia	DQ103552	DQ103557

Isolates in bold were obtained from this study.

3. Results

3.1. Description of disease symptoms observed on guava fruits in the field

The healthy fruits were characterised by uniform green colouration of the epicarp when unripe, but becomes yellow upon ripening. The disease symptoms were found on both ripe and unripe fruits hanging on the trees. It started as discoloured portion of the fruit epicarp near the persistent calyx, which later turned dark (Fig. 2A and B). With time, the lesion expanded to cover extensive area of the epicarp, which then shrunk and became wrinkled (Fig. 2C). In severe cases, the entire fruit surface was covered with the disease lesions. The symptoms penetrated the fruit pulp, irrespective of the size of the lesion covering the skin, thereby rendering the affected fruit, unfit for consumption.

3.2. Cultural and morphological identification of the fungi isolated from diseased fruits

The isolates produced mycelium which was initially white but turned dark as the culture aged (Fig. 2D). Mycelia grew and filled the entire 9 mm plate in four days. The septated hyphae, were hyaline but turned dark as the culture aged. The isolates produced immature conidia that were hyaline, non-septate, ovoid in shape and thick-walled and mature

conidia that had one septation, brown-walled and had longitudinal striations (Fig. 2E and F). Dimensions of the mature conidia were 25.4–29.5 μm in length and 15.0–16.8 μm in width ($\pi = 27.5 \mu\text{m} \times 15.9 \mu\text{m}$).

3.3. Pathogenicity of the *L. pseudotheobromae* isolates

All the isolates obtained from guava were able to cause similar rot symptoms on the artificially inoculated guava fruits. The symptoms developed on fruits when inoculated both at the styler end and at the side of the fruit (Fig. 2G and H). In all cases, the symptoms were first observed around the point of inoculation as a colourless, water-soaked necrotic area. With time, the affected area became dark and spread to the other areas of the fruit surface, same as what was observed in the field. The disease severity among the five isolates ranged from 34.2 % to 40.9 % (Table 2). However, the difference among the isolates was not significant. Similarly, there was no significant difference in the disease severity between the two wounding locations of the fruit. (Table 2). Symptoms were not observed on the fruits inoculated with PDA only. The fungus was re-isolated from the disease lesions to confirm pathogenicity.

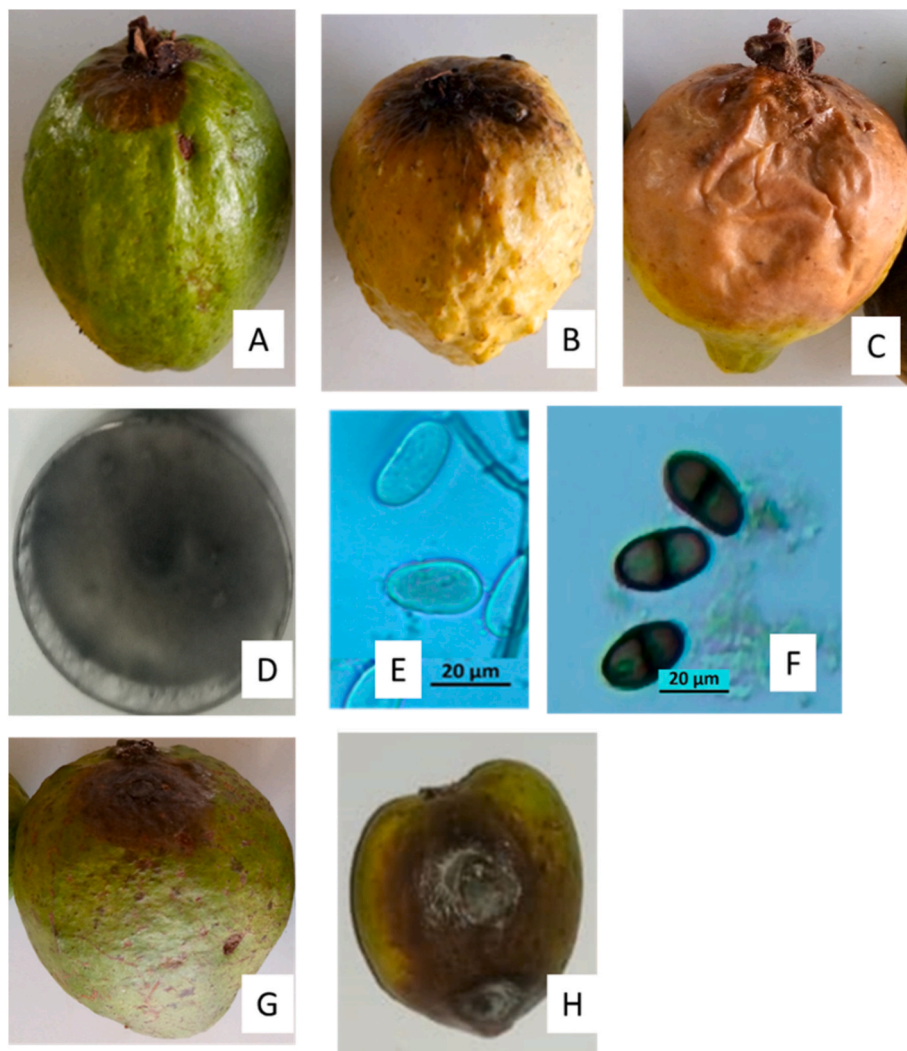


Fig. 2. Disease symptoms of guava styler end rot, cultural and morphological features of *L. pseudotheobromae* isolated from guava and symptoms induced on artificially inoculated guava fruits. A and B, symptoms on unripe and ripe fruits, C = symptoms at advanced stage, D = mycelial growth on PDA, E = immature hyaline conidia, F = mature septated dark conidia, G = guava fruit inoculated at the styler end, H = guava fruit inoculated on the side.

Table 2
Conidial dimensions and disease severity induced on artificially inoculated guava fruits.

Isolate designation	Location of wound for inoculation/Disease severity index (%) $\pi \pm se$		Mean
	Stylar end	Side	
DOREEN1	33.3 \pm 3.4	35.2 \pm 6.9	34.2
DOREEN2	34.3 \pm 1.4	43.0 \pm 5.0	38.9
DOREEN3	37.5 \pm 4.8	36.1 \pm 5.0	36.8
DOREEN4	33.8 \pm 3.6	38.9 \pm 4.1	36.3
DOREEN5	37.5 \pm 4.1	44.4 \pm 3.3	40.9
Mean	35.3	39.8	

p values: Isolate = 0.69; Location of wound = 0.19; isolate*location of wound = 0.85.

3.4. Phylogenetic study

The individual gene trees drawn with the sequences of the ITS region and the *tef1- α* gene, were similar, in terms of topology. The most parsimonious tree obtained with a concatenation of the sequences of the two gene regions (Fig. 3), had a length of 230, a consistency, retention and composite indices of 0.781915, 0.897243 and 0.737300 (0.701568), respectively, for all sites and parsimony-informative sites (in parentheses) respectively. There was a total of 661 positions in the final dataset. In the phylogram, all the five isolates of the pathogen obtained from guava, clustered in the *L. pseudotheobromae* clade, supported by a high bootstrap value of 99% (Fig. 3), indicating these isolates belong the *L. pseudotheobromae* species.

4. Discussion

Unlike mango fruit cultivation in Ghana, in which diseases have been well documented [18,19,20], diseases affecting guava in the country have rarely been documented. In this study, symptoms of the new guava

disease, started from the stylar end of the fruit, in form of colourless spots, darkened with time and spread to the distal parts of the infected fruit. The infected fruit eventually shrunk and became mummified. These symptoms were similar to what have been reported for the stylar end rot disease of guava, reported in India and Malasia [9,21].

Stylar end rot disease of guava has been associated with *Phomopsis psidii* [21]. In this present study, the fungus, consistently isolated from all the diseased conditions had characteristic features of members of the *Lasiodiplodia* genus. The type of immature and mature conidia, produced by the isolates, and presence of conidioamata, are diagnostic features of members of the genus [22,23]. Within this genus, *L. theobromae* is commonly associated with rots of fruits in the tropics and has been reported on mango in Ghana [18]. However, the isolates obtained in this study produced larger conidia (25.4–29.5 $\mu\text{m} \times$ 15.0–16.8 μm) compared to conidia, reportedly, produced by *L. theobromae* (26.2–27 $\mu\text{m} \times$ 14–14.4 μm), indicating they were not *L. theobromae*. On the other hand, the dimensions of their conidia fall within the range prescribed for *L. pseudotheobromae* (23.5–32 $\mu\text{m} \times$ 14–18 μm) [24], an indication that they may belong to the *L. pseudotheobromae* species. In the phylogram drawn with concatenated sequences of the ITS region and the *tef1- α* gene, all the isolates of the fungus clustered with the type strain of *L. pseudotheobromae*, a confirmation that they belong to the species. The use of the sequences of the ITS region and the *tef1- α* gene, for species delineation in *Lasiodiplodia* genus has been reported to be very robust [24,22,9]. This confirms, unequivocally that the isolates were *L. pseudotheobromae*. The ability of the fungus to cause similar disease symptoms, was an indication that it was responsible for the disease. Though *L. theobromae* has been confirmed as the causal agent of post-harvest fruit rot of mango [18] and soursop fruits [25], while *L. parva* has been associated with black rot of yam tubers [26], this is the first report of *L. pseudotheobromae* causing disease of any kind in Ghana.

Lasiodiplodia pseudotheobromae is one of the cryptic species, previously lumped together as *L. theobromae* [24]. Findings from this work support the reports that the two species are distinct. It also supports the

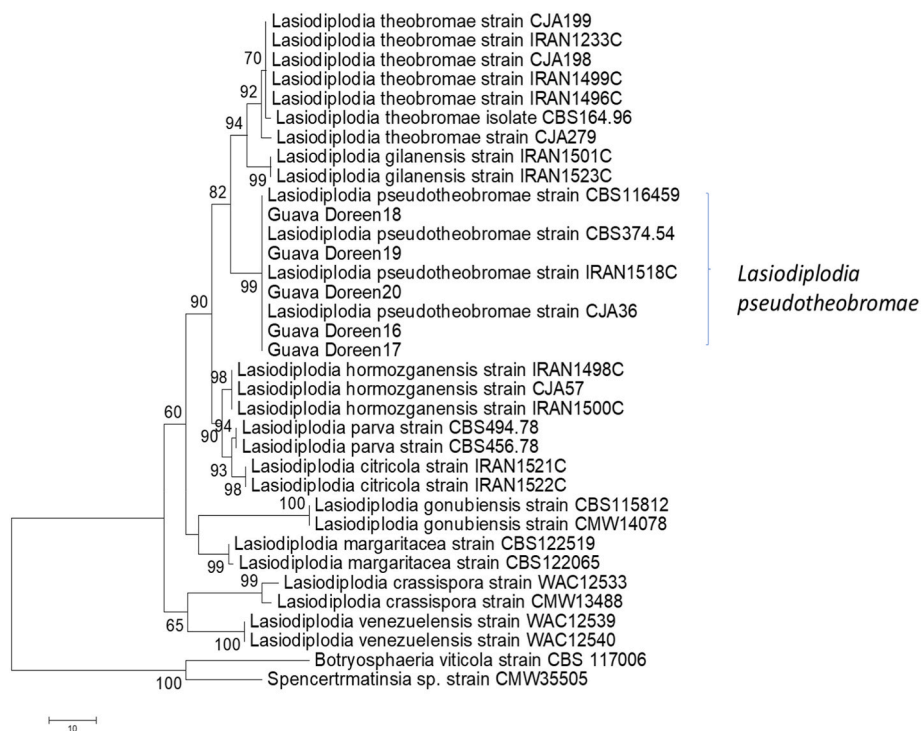


Fig. 3. A maximum parsimony tree drawn with the concatenated sequences of the ITS region and translation elongation factor gene of 35 *Lasiodiplodia* isolates. Isolates whose designation begin with DOREEN were obtained from this study while the rest were downloaded from the EMBL database. *Botryosphaeria viticola* and *Spencertmatinsia* sp. were used as outgroups.

report that members of the *Lasiodiplodia* genus are widely distributed in the tropics and subtropics [24], with the discovery of the pathogen in Ghana. Its association with guava fruits, has demonstrated the expansiveness of the host range of members of the genus, being reportedly associated with more than 500 plant hosts, worldwide [27]. Though the path of introduction of the pathogen into Ghana was not investigated, it is more likely, that it was through planting materials, same as what led to the introduction of the citrus canker and the mango bacterial black spot disease into the country [19,28]. This is because, the disease was found only on the newly introduced exotic varieties of guava, but not the indigenous local guava trees. According to Ref. [29], the proliferation of such pathogens is aided by the warmer climate, currently being experienced in the country.

While we report the stylar end rot disease of guava, for the first time in Ghana, its destructive nature in the field, means it can be detrimental to the guava industry in the country. To avert this, more studies can be conducted in the future to obtain more information for its management. For example, we found the disease symptoms only on the exotic cultivars of guava fruit, but not on the few local trees present in the study area. A future study, to determine whether these cultivars were resistant to the disease could yield important information, for its control. Also, while we suspected that warming temperatures in Ghana, could be influencing the severity of the disease, this was not investigated and it could be a subject matter for a future study of the disease.

It can be concluded that the new disease observed in this study is stylar end rot caused by *L. pseudotheobromae*. It is therefore recommended that the information be used to update the checklist of plant diseases in Ghana and also to be considered in formulating a control measure against the disease in the near future.

CRediT authorship contribution statement

Joseph Okani Honger: Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Christiana Adukwei Amoatey:** Resources, Project administration, Funding acquisition. **Isaac Bedu:** Methodology, Investigation. **Doreen Naa Sackey:** Methodology, Investigation, Formal analysis. **Karen Saahene Agyekum:** Investigation, Formal analysis, Data curation. **Shadrach Coffie:** Formal analysis, Data curation. **Benjamin Otu:** Formal analysis, Data curation.

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Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Data availability

Data will be made available on request.

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