Differentiation of two Botryosphaeriaceae species isolated from declining mango trees in Ghana

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Abstract

Lasiodiplodia theobromae is the only pathogen reported to cause mango tree decline disease in Ghana. In this study, several Botryosphaeriaceae isolates were obtained from mango tree decline disease symptoms and were identified using both phenotypic and genotypic characteristics and inoculation studies. The methods employed differentiated the isolates into two species, *Lasiodiplodia theobromae* and *Neofussicoccum parvum. L. theobromae* sporulated freely on media while N. parvum did not. Also, the species specific primer, Lt347-F/Lt347-R identified only *L. theobromae* while in the phylogenetic studies, *L. theobromae* and *N. parvum* clustered in different clades. *L. theobromae* caused dieback symptoms on inoculated mango seedlings while *N. parvum* did not. However, both species caused massive rot symptoms on inoculated fruits. *L. theobromae* was therefore confirmed as the causal agent of the tree decline disease in Ghana while *N. parvum* was reported for the first time as a potential pathogen of mango fruits in the country.

Introduction

Mango (Mangifera indica L.) is one of the most important non-traditional export crops from Ghana, bringing in much needed foreign exchange to the country. The mango industry employs many people including farmers, labourers and traders. Currently, mango farms have been set up in almost all the agro-ecological zones of the country which has the potential of being an important exporter of the crop due to the availability of two production seasons per calendar year. Consequently, the crop has been projected to make significant contributions to the export portfolio of Ghana (Zakari, 2012).

Mango cultivation in Ghana is hampered by the incidence of pests and diseases, which affects the crop both in the field and after harvest (Offei et al., 2008). One of the most damaging diseases recently identified in the country is mango tree decline. Mango decline disorders have been reported in nearly all mango growing areas of the world (Alveraze and Lopez, 1971; Rawal, 1998; Schaffer et al., 1988). In India it has been reported that the disease had been a very significant one since 1940 (Khanzanda et al., 2004) with the disease incidence ranging between 30-40% of plantations in one region alone (Prakash and Srivatava, 1987).

The disease has also been reported in Florida (Ploetz et al., 1996) and in Omar, where about 60% of the trees grown in one of the regions were affected (Al Adawi et al., 2003). One of the major symptoms of mango tree decline is the death and shedding-off of the tree bark. This means transfer of food and nutrients from the sources to sinks are disrupted (Khanzanda et al., 2004). In young seedlings this is likely to prevent growth and development. Tree growth therefore stagnates and the tree will eventually die.

Several fungi pathogens of different taxonomic groupings have been associated with the mango tree decline disease worldwide. These include *Alternaria alternata, Cladosporium sp.,*

Colletotrichum gloeosporioides, Dothiorella dominicana, Fusarium spp., *Lasiodiplodia theobromae, Penicillium* sp., *Pestalotiopsis* sp. and *Phomopsis* spp. (Khanzada *et al*., 2004; Ploetz *et al.*, 1996). Among these diverse number of fungi species, only *L. theobromae* has been confirmed as the causal agent of the disease in Pakistan (Khanzanda *et al*., 2004) and in Ghana (Honger *et al*., 2018). However, in Florida, *Phomopsis* species have also been found to produce almost all the different symptoms associated with the decline disease on mango (Ploetz *et al.*, 1996).

Though *L. theobromae* has been reported as the causal agent of the disease in Ghana, it's not uncommon to find more than one type of fungi species causing the same disease symptoms on susceptible hosts. For example, *Colletotrichum gloeosporioides, C. siamense* and *C. asianum* have been reported as the causal agent of mango anthracnose in Ghana (Honger *et al.*, 2014). Also, *L. theobromae* and *Phomopsis* species have been found to cause the decline disease in Florida (Ploetz *et al*., 1996). Recent observations have shown that several isolates suspected to be *L. theobromae,* obtained from declining mango trees in Ghana, may be of different taxonomic groupings. In fact, some of these isolates failed to sporulate under the same conditions in which *L. theobromae* isolates sporulated freely (Ablormeti, 2017). These observations have raised questions as to whether other fungi species, apart from *L. theobromae*, were involved in the disease epidemiology of the mango tree decline disease. While sporulation alone may not be enough to discriminate among closely related species, it nevertheless gives an indication of a possible variation among unknown species.

The identification of *L. theobromae* as the causal agent of the mango tree decline disease in Ghana was achieved using both phenotypic and genotypic methods coupled with inoculation studies (Honger *et al.*, 2018). However, it is also important that any other isolates obtained from the decline disease symptoms are identified and their possible role in the disease epidemiology ascertained. This is necessary to clear any doubt surrounding the aetiology of the mango tree decline disease in Ghana. In this study, therefore, different Botryosphaeriaceae species isolated from mango tree showing the declining symptoms were differentiated using phenotypic, pathological and molecular methods.

Materials and methods

Isolation of the causal agent

Farm visits were made to the coastal savannah zone of Ghana, where the bulk of Ghana's mangoes for the export market are produced. Commercial mango farms in the area were selected at random and both the local and keitt varieties of the crop were inspected for the symptoms of mango tree decline disease. Leaves, twigs, and bark showing mango decline symptoms were collected and sent to the Plant Pathology Laboratory of The Department of Crop Science, of the University of Ghana for isolation of the causal agents.

The isolation of causal agent was first done on water agar (WA) and then on Potato Dextrose Agar (PDA). WA (15 g/L) and PDA (39 g/L) were prepared and each mixture was autoclaved, cooled and poured into clean sterilized petri-dishes and allowed to set. Pieces (1-2 cm) of the sampled plant parts (leaves, twigs, bark and vascular tissues) were taken from the advancing edge of infection with a sterile scalpel. These excised tissues were then surface sterilized in a 1% Sodium

hypochlorite for 5 minutes, rinsed twice in sterile distilled water and blotted dry using a sterile tissue paper. These were plated singly on water agar plates and incubated for 6-8 days. The growth were then sub-cultured on PDA and incubated for the same period (6-8 days) to obtain pure cultures.

Identification of the isolated fungal pathogens. Cultural and morphological identification of isolates

Mycelial discs (4 mm diameter), taken from actively growing edge of seven-day-old cultures of each isolate were transferred to PDA and incubated at 24±1ºC and RH=60-65%. Colony diameter was measured daily for 4 days when the mycelia had covered the entire plate and growth rate was calculated as the four-day average of mean daily growth (millimetres per day). Mycelial bits and conidia of isolates that sporulated were fixed on slides and the septation, length and breadth of 50 conidia harvested from the cultures were recorded and means calculated (Alves *et al*., 2008).

Molecular characterisation

Twenty isolates of the fungi, made up of 10 that sporulated readily in culture and other 10 that did not sporulate, were selected, for the molecular characterisation. DNA was extracted from the isolates using the Sigma's GenFlute Plant Genomic DNA Miniprep Kit, following the manufacturer's instructions.

The extracted DNA was used as templates in polymerase chain reaction with the *L. theobromae* species specific primer Lt347-F (AACGTACCTCTGTTGCTTTGGC) and Lt 347-R (TACTACGCTTGAGGGCTGAACA) (Xu *et al*., 2015). A second set of PCR was performed using the universal primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al*., 1990) to amplify the internal transcribed spacer (ITS) region of the isolates. PCR was carried out in a total reaction volume of 50µl. The reaction mixture was made up of 34.25 µl of double distilled water, 5 µl of 10X PCR buffer (Invitrogen, Carlsbad, CA), 2.5 µl of deoxynucleoside-triphosphate (DNTP) mix (2.5 mM each), 0.25 µl bovine serum albumin (20 mg/ml), 2 µl each of the forward and reverse primer, and 0.2 µl of taq polymerase, 1.8 µl of magnesium chloride (50 mM) and 2 µl target DNA. The reaction was carried out in a Thermo Hybaid PXE Thermal Cycler. The reaction cycles were denaturing for 2 min at 94°C followed by 35 cycles of 1min at 94°C, 1 min at 55°C, 2 min at 72°C and a final of 10 min at 72°C. Amplification products were separated by 1.5% w/v agarose gel stained with Ethidium bromide alongside 1.0 kb marker at 80 V for about 1 hour. Bands were observed under UV light and Polaroid photographs taken or viewed using the Gene Flash Documentation System (Snygene Bio Imaging).

Purification and sequencing of amplified product of the ITS region

The PCR amplified product of the ITS region were sent to ATGC in Germany for purification and sequencing. Ten (10) picomole of each primer pair (ITS1/ITS4) was used to sequence the products directly from both directions.

Sequence analysis and phylogenetic studies of the ITS region

The sequences were entered into BioEdit and consensus strands were generated. The sequences of the internal transcribed spacer region (ITS) of a total of 38 isolates were used

in the phylogenetic studies. These were made up of 20 isolates obtained from the tree decline symptoms in this study whose sequences were deposited in the gene bank (Table 1) and 18 sequences of ex-types and isolates of confirmed identities (downloaded from EMBL database) (Table 2). Included in the downloaded sequences was that of *Guinardia citricarpa* which was used as the out-group. The sequences of the rDNA-ITS region of the isolates were aligned using Clustal W. The multiple sequence alignments obtained were used in a phylogenetic analysis using MEGA5 (Tamura *et al.*, 2011).

The Maximum Parsimony (MP) analysis was performed. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree was drawn to scale with branch length calculated using the average pathway method (Nei and Kumar, 2000) and were in units of number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). Clade stability of the tree resulting from maximum parsimony analysis was assessed by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

Pathogenicity test

Inoculation of seedlings

The pathogenicity test was carried out on ~10 months old, local mango variety seedlings. The experiment was set up in randomized

TABLE 1
Isolates of Botryosphariaceae species isolated from the diseased mango tree parts used in the study and their GenBank accession numbers

Strain Identification	Host	Sporulation on media	Location	ITS
LMTD-GH1	*Mangifera indica*	+	Ghana	KY657456
LMTD-GH2	*Mangifera indica*	+	Ghana	KY657457
LMTD-GH3	*Mangifera indica*	+	Ghana	KY657458
LMTD-GH4	*Mangifera indica*	+	Ghana	KY657459
LMTD-GH5	*Mangifera indica*	+	Ghana	KY657460
LMTD-GH6	*Mangifera indica*	+	Ghana	KY657461
LMTD-GH7	*Mangifera indica*	+	Ghana	KY657462
LMTD-GH8	*Mangifera indica*	+	Ghana	KY657463
LMTD-GH9	*Mangifera indica*	+	Ghana	KY657464
LMTD-GH10	*Mangifera indica*	+	Ghana	KY657465
ORID-NP1	*Mangifera indica*	-	Ghana	KY657466
ORID-NP2	*Mangifera indica*	-	Ghana	KY657467
ORID-NP3	*Mangifera indica*	-	Ghana	KY657468
ORID-NP4	*Mangifera indica*	-	Ghana	KY657469
ORID-NP5	*Mangifera indica*	-	Ghana	KY657470
ORID-NP6	*Mangifera indica*	-	Ghana	KY657471
ORID-NP7	*Mangifera indica*	-	Ghana	KY657472
ORID-NP8	*Mangifera indica*	-	Ghana	KY657473
ORID-NP9	*Mangifera indica*	-	Ghana	KY657474
ORID-NP10	*Mangifera indica*	-	Ghana	KY657475

+=sporulates in culture, -=does not sporulate in culture (culture was PDA incubated at 24±1ºC and RH=60-65% for 14 days)

TABLE 2
A list of isolates downloaded from EMBL Database and used in the study and their GenBank accession numbers

Species	Strain identification	Host	Country	GenBank accession numbers (ITS)
Botryosphaeria eucalypticola	CMW6539	*Eucalyptus sp.*	Australia	AY615141
Botryosphaeria australis	CMW6837	Acacia Sp.	Australia	AY339262
Diplodia cupressi.	CBS 261.85	*Cupressus sp.*	Israel	DQ458894
Diplodia mutila.	CBS112553	*Vitis vinifera*	Portugal	AY259093
Diplodia seriata	CBS112555	*Vitis vinifera*	Portugal	AY259094
Dothiorella iberica	CBS 115041	*Quercus ilex*	Spain	AY573202
Dothiorella sp.	CAA005	*Pistachia vera*	USA	EU673312
Dothiorella sp.	CAP187	*Brunus dulchis*	Portugal	EU673313
Dothiorella sp	JL599	*Corylus aveliana*	Spain	EU673281
Guinardia citricarpa	CBS102374	*Citrus aurantifolia*	Brazil	FJ824767
L. parva	CBS 494.78	*Cassava field soil*	Colombia	EF622084
L. psuedotheobromae	CBS116459*	*Gmelina arborea*	Costa Rica	EF622077
L. theobromae	CAA 006	*Vitis vinifera*	USA	DQ458891
L. theobromae	CBS124.13	Fruit along coral reef coast	USA	DQ458890
L. theobromae	CBS 164.96*	Fruit along coral reef coast	New Guinea	AY640255
Neofussicoccum parvum	CMW27135	*Eucalyptus sp.*	China	HQ332205
Neofusicoccum parvum	CMW27110	*Eucalyptus sp.*	China	HQ332218
Neofusicoccum mangiferum	CMW7797	*Mangifera indica*	Australia	AY615186

*=Type strain

complete block design (RCBD) in a screened house (25-28^{o}C and RH 65-70%) at the Soil and Irrigation Research Centre, University of Ghana. Test plants (3 each per test fungus) were inoculated artificially by cutting a flap on the basal portion of the stem using a sterilized knife and inserting a 3 mm agar disc obtained from the advancing zone of the test fungus in Petri plates. In the control trials (1 each per test fungus), plants were inoculated with plain sterile agar disc without the test fungus. The inoculated portions were wrapped with a plastic paraffin film. Plants were irrigated after inoculation and the wrapping material was removed from the stems after 2 weeks of inoculation. Plants were then monitored for possible developments of the decline syndrome symptoms for 2 months. The pathogen was re-isolated from the test plants to confirm the pathogenicity.

Inoculation of fruits

The pathogenicity test of isolates was repeated using palmer mango fruits. Freshly harvested untreated, unwaxed and physiologically mature fruits were washed, surface sterilized with 1% sodium hypochlorite and air dried. Plugs (4mm diameter) of mycelia of isolated causative agent were cut from actively-growing areas near the edge of an eight-day old culture of each isolate. One 7mm deep hole was aseptically punched with cockborer (4mm diameter) around the stem end of each fruit and a plug of the isolate was placed into each of the holes. Each isolate was used to inoculate 3 fruits while one fruit, serving as control was inoculated with PDA only. Fruit plugs removed from punched holes were replaced and covered with parafilm. The

inoculated fruits were incubated upright at the prevailing relative humidity and temperature conditions in the laboratory (60-65% RH and 23-25°C). Inoculated fruits were observed daily till symptoms appeared. An isolate was considered pathogenic to the fruit when it was able to cause rot symptoms on inoculated fruits. Subsequently, it was re-isolated from the disease lesions to satisfy Koch's postulates.

Results

Cultural, growth rate and morphological characteristics of isolates

Isolates obtained could be placed into two groups based on cultural and morphological characteristics. The first group initially produced a thin mycelium which covers the entire 9 mm plate in three days. The mycelium grew fluffy in 5 days (Fig. 1A). The colour of the mycelium was initially white but changed to grey and finally black within 7 days. The hyphae were initially hyaline and non-septate with a diameter of ≤2.0±0.5µm. The diameter increased with age from about ≤2.0±0.5 µm in 2 days to about ≤8.0±1.2 µm in 21 days. It took 12 days for pycnidia to form on PDA kept under continuous darkness and 25 days to form conidia. The conidia were initially unicellular subovoid to ellipsoidal in shape. At maturity, conidia became bi-celled, thick walled and ellipsoidal in shape (Fig. 1B). Dimensions of the conidia on PDA were 10.0-12.0±1.5 µm wide and 20.0-30.0±2.0 µm long.

The second group of isolates also produced thin mycelium which covered the 9 mm plate within 3 days and eventually produced a thick mass of mycelial mat in 5 days (Fig. 1C). The colour of mycelium was grey and changed to black as the culture aged. The hyphae were also non septated with same dimensions as what was recorded for the first group. These isolates failed to sporulate no matter how long the cultures were kept and the mycelium eventually dried up after more than 30 days of incubation.

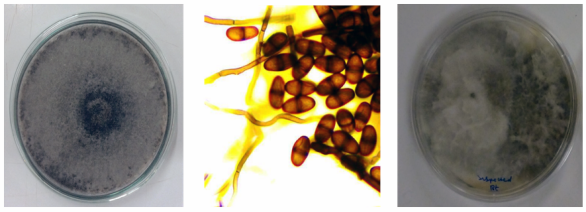

Fig.1 Cultural and morphological characteristics of isolates obtained in the study. Culture on PDA (left) and bi-celled mature conidia (middle) of isolates that sporulated on media and culture of non-sporulating isolate on media (right). Mg. X400

Polymerase chain reaction using specific primers

A 347 bp PCR product was amplified from DNA of the isolates that produced the characteristic spores of *Lasiodiplodia theobromae*, with species specific primers Lt347-F/ Lt347-R (Fig. 2). No amplification product was observed in the DNA of the isolates that did not sporulate.

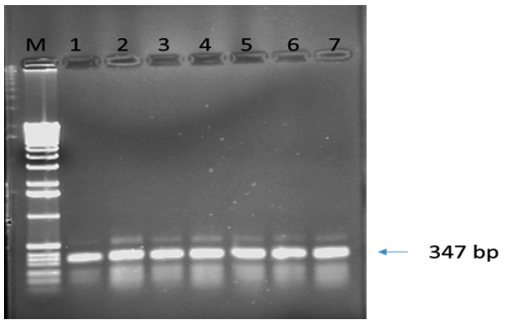

Fig. 2. A gel showing the amplification of a PCR product of 347 bp. M=1 Kb marker, Lane1-7=Isolates of *L. theobromae* from mango tree

Sequences and phylogenetic studies of the ITS region

An approximately 600 bp product of the ITS region was amplified using the primer pair ITS1/ITS4 from the isolates. The assembled sequences were 535 bp long which have been

deposited in the GenBank and their accession numbers indicated (Table 2). The evolutionary history was inferred using the Maximum Parsimony method. Tree number 1 out of 9 most parsimonious trees with length of 172 is shown as fig. 3. The consistency index is 0.693069, the retention index is 0.950872) and the composite index is 0.779494 (0.659020) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985).The analysis involved 38 nucleotide

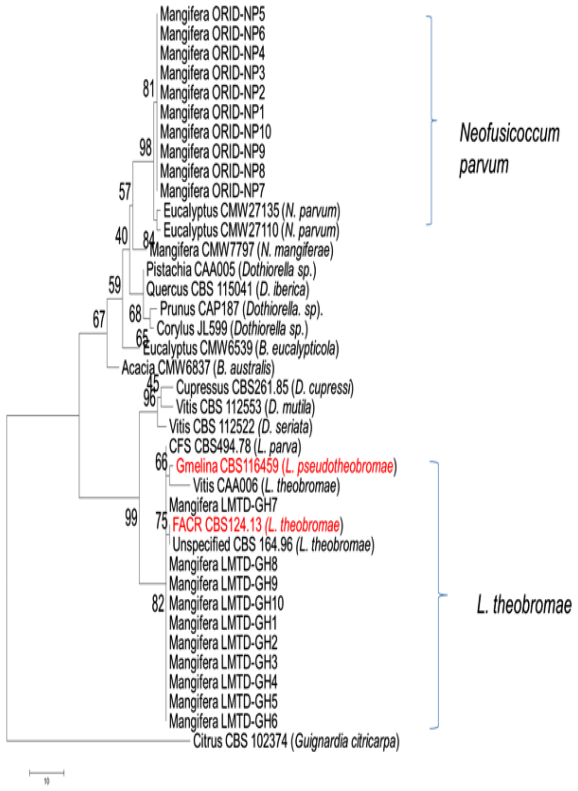

Fig. 3. Maximum parsimony phylogram constructed from the multiple sequence alignment of the nucleotide sequences of the rDNA-ITS region. *G. citricarpa* was used as the outgroup. With the exception of strains whose names were preceded by either LMTD or ORID, all strains are either isolates of confirmed identities or are ex-type strains, with names of ex-type strains in red. Sequences of ex-type strains or strains with confirmed identities were downloaded from the EMBL database. The genus name of host, strain identification and species name of isolates have been provided (CFS=Cassava field soil; FACRC=Fruits along coral reef coast)

sequences. There were a total of 467 positions in the final dataset. The 20 isolates obtained from this study clustered into 2 distinct clades. All 10 isolates of the fungi that did not sporulate during the period of the study clustered in the *Neofusicoccum parvum* clade with a high bootstrap support of 98% (Fig. 3). On the other hand, all the isolates identified as *L. theobromae* clustered with several *Lasiodiplodia* species in the *L. theobromae* clade. The clade was supported with a high bootstrap support of more than 80% (Fig. 3). Clades of other species belonging to *Dothiorella* and *Diplodia* were clearly formed but none contained any of the *L.* isolates obtained in this study.

Pathogenicity test

All isolates of *L. theobromae* used to inoculate the mango seedlings were able to induce the mango tree decline disease symptoms (Fig. 4). Dieback coupled with gum exudation symptoms were first observed 42 days after inoculation. After 70 days the gum exudation disappeared. The inoculated seedlings equally exhibited leaf necrosis which remained attached to the dying stem though some of the leaves defoliated. The entire seedlings died after four months. A vertical section through the affected stems showed vascular discolouration. The roots developed showed root rot but the control plants had no symptoms of the disease. The pathogen was re-isolated from the diseased lesions to confirm the fungus as pathogenic to mango plants. The symptoms were absent on seedlings inoculated with the *Neofusicoccum parvum* isolates and the PDA only.

On the other hand, all isolates were able to induce rotten symptoms on the inoculated mango fruits. Symptoms began to show at

three days after inoculation. This was made up of dark brown and watery portions of the fruit surface. Seven days later, extensive areas of the fruits were diseased. This was accompanied by ruptured points and oozing of foul smelling liquid. However, the rotten symptoms were absent in fruits inoculated with PDA only.

Fig. 4. Inoculated mango seedlings showing symptoms of the disease. (C=control seedlings inoculate with PDA only and D=seedlings inoculated with plugs of mycelium of *L. theobromae*)

Fig. 5. Rotten symptoms induced on inoculated mango fruits by isolates of *L. theobromae* (left) and *N. parvum* (right)

Discussion

Several disease symptoms have been attributed to *Botryosphaeriaceae* species worldwide. These include fruit rot, leaf spots, seedling damping-off, cankers, shoot blight, dieback and tree death (Slippers and Wingfield 2007). They have also been specifically implicated in the mango tree decline disease world (Ploetz *et al.*, 1996). In Ghana, *L. theobromae* has been reported as the causal agent of the disease in the country (Adiku, 2014; Honger *et al*., 2018). Symptoms of the disease observed in the field and the identity of the causal agent recorded in this study confirms the earlier reports that *L. theobromae* was responsible for the mango decline disease in Ghana.

In this current study, several isolates belonging to the *Botryosphaeriaceae* were obtained from the diseased samples collected from the various farms and mango genotypes in Ghana. Culturally, all isolates were identical, producing the characteristic grey to dark coloured thick mass of mycelium within a very short period of time (Phillips *et al*., 2008). Within the period of study, some of the isolates produced the diagnostic spores of *L. theobromae,* making their identification based on cultural and morphological features, possible. However, within the same period, some isolates did not sporulate and hence without the characteristic dark coloured and septated conidia with longitudinal striations, associated with *L. theobromae* (Phillips *et al.*, 2008), it was impossible to confirm whether these belongs to the species or not. Questions that arose included whether these were strains of *L. theobromae* with deviant characteristics or were different species altogether. There was therefore the need to carry out further studies to confirm the identity of these isolates, since this would determine whether other fungi species (and not *L. theobromae* alone), are responsible for the tree decline disease in Ghana.

One of the most reliable methods of distinguishing between *L. theobromae* and other *Botryosphaeriaceae* species is polymerase chain reaction with the species specific primer Lt347-F/ Lt347-R (Xu *et al.*, 2015). This primer set is known to amplify a PCR product of 347 bp only in *L. theobromae* species. In this study, the expected product was amplified in the isolates that produced the dark septated conidia of *L. theobromae*, confirming

the identity of these isolates. On the other hand, the product was not amplified in the other isolates that were not sporulating. This was an indication that at least two types of fungi of different taxonomic groupings were present in the disease samples collected. In many parts of the world, it is not uncommon to isolate different *Botryosphaeriaceae* species on the same host. For example, it has been reported that three *Botryosphaeriaceae* spp., namely *L. theobromae, Botryosphaeria dothidea* and *Neofusicoccum parvum* could be isolated from symptomatic blueberry plants in China (Xu *et al*., 2015), while different *Botryosphaeriaceae* species including *L. theobromae*, have been reported to be the cause of blue berry stem decline disease in several growing areas of the crop (Espinoza *et al,*. 2009). It was therefore possible that *L. theobromae* alone was not responsible for the disease.

Though PCR with species specific primer was able to distinguish between two species among the isolates obtained in this study, the method was nonetheless limited in delineating the identity of the non-sporulating isolates. To achieve this, the phylogenetic study method (Taylor *et al*., 2000; Phillips *et al*., 2008), was employed. The phylogenetic species recognition (PSR) has been found to be consistent with the evolutionary species concept, as compared to other concepts such as morphological species recognition (MSR) and biological species recognition (BSR) in species recognition (Taylor *et al.* 2000). The method is therefore very reliable and has been employed to delineate the species status of *L. parva* isolates causing yam rot in Ghana (Honger *et al*., 2017).

In this current study, the phylogenetic study involved the use of nucleotide sequences of the rDNA-ITS region. The rDNA-ITS region has been used as bar code for the identification of fungi from several taxonomic groupings and is usually sufficient for placing isolates into their genus (Weir *et al*., 2000). The region has been used either solely or in combination with the beta-tubulin or the alpha elongation factor gene, to resolve species within the genus *Botryosphariaceae* (Phillips *et al.*, 2005; Alves *et al*., 2008). The phylogram drawn with the rDNA-ITS sequences correctly placed the *L. theobromae* isolates obtained in this study in the *L. theobromae* clade, a confirmation of their identification based on morphology and PCR with species specific primer. The clade contained other isolates of *L. theobromae* of confirmed identities and the type strain of *L. theobromae* and was supported by a high bootstrap support (Fig. 3). In the same phylogram, the isolates that were not sporulating were placed in the *N. parvum* clade. The clade was also supported by a high bootstrap value. These results showed without doubt that the isolates that were isolated from the mango tree and which failed to sporulate were *N. parvum*. Therefore, the phylogenetic study showed without doubt that two distinct species were isolated in this study. This is similar to the identification of three distinct *Botryosphaeriaceae* species, namely, *Botryosphaeria sp*., *Pseudofusicoccum sp*., and *L. theobromae* on mango fruits in Ghana (Saeed, 2012).

Neofusiccoccum parvum is a known pathogen of mango worldwide and has been associated with mango tree decline in Italy (Ismail *et al*., 2013). The pathogen appeared not to sporulate easily in PDA and in most cases elsewhere, a specialised medium made up of water agar and pine needles was needed to induce sporulation (Slippers *et al*., 2005). The isolates of the fungus (*N. parvum*) obtained in this study also

failed to sporulate on PDA, another feature confirming its identity in Ghana.

Pathogenicity tests showed that, while *L. theobromae* was able to induce the die-back symptoms on inoculated seedlings, *N. parvum* did not. This was a confirmation that *L. theobromae* was solely responsible for the disease in Ghana, while *N. parvum* may be existing on the diseased symptoms as a saprophyte. However, its ability to cause the rot on the inoculated mango fruits was an indication that it was of post-harvest importance in Ghana. The pathogen has been associated with stylar-end rot of guavas in Brazil (Junior *et al*., 2016), confirming its status as a pathogen that could be very destructive. There is therefore the need for control measures to be put in place to control the pathogen on mango as well.

Conclusion

In this study, two major fungi species of the *Botryosphaeriaceae*, namely, *L. theobromae* and *N. parvum* were isolated and identified from mango trees showing symptoms of mango tree decline disease. Between the two species, only *L. theobromae* was able to cause the symptoms on inoculated mango seedlings, confirming that it was the causal agent of the disease in Ghana. On the other hand, *N. parvum* could only cause disease on inoculated mango fruits, making it one of the important fungi species infecting mangoes in Ghana and hence requires urgent attention. This study further showed that molecular methods are very essential in distinguishing among species within the *Botryosphaeriaceae* family. *Neofussicoccum parvum* was being reported for the first time as a pathogen of mango fruits in Ghana and therefore the update of the pathogen checklist in Ghana to reflect this finding is recommended.

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