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Full Length Article

Morphological and Molecular Diversity of Fungal Microflora Associated with Kola Tree Suffering from Witches' Broom Disease (*Cola* sp.) in Côte d'Ivoire

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Abstract

In Côte d'Ivoire, kola tree cultivation is a real source of income for thousands of people. However, it is threatened by witches' broom disease, which causes significant yield losses. The aim of this study was to characterize the fungal microflora associated with witches' broom disease of the kola tree. To this end, symptomatic branches from six major agro-ecological zones were sampled. Associated fungi were isolated on agar medium and analyzed on molecular basis. Fungi belonging to four genera namely *Lasiodiplodia*, *Pestalotiopsis*, *Fusarium* and *Trichoderma* were isolated. The *Lasiodiplodia* genus was the most frequently associated with the diseased plants, with 93% occurrence. Four species of *Lasiodiplodia* identified in this study included *L. theobromae* with 179 isolates, *L. brasiliensis, L. iranensis* and *L. jatrophicola*. For the genus *Pestalotiopsis,* 5 fungal species namely *P. microspora, P. saprophytica, P. theae, P. clavispora* and *P. formicarum* were identified. Two species of *Fusarium* (*F. fujikuroi* and *F. oxysporum*) and one species of *Trichoderma* namely *T. harzianum* were also identified. Sequences of the fungal species were deposited in GenBank with accession numbers ranging from PP824668 to PP824719. DNAs from species of all the 5 fungal genera had 98–100% identity compared with those existing in GenBank. These results open up new avenues of research into the involvement of these fungal genera in symptom expression.

Keywords: Côte d'Ivoire; Fungi; Kola tree; Molecular characterization; Witches' broom

Introduction

Witches' broom is a stem branching anomaly that results in an abundant proliferation of twigs with shortened internodes and small, often deformed leaves. These symptoms develop as a result of abnormal bud excitation, caused by trauma or parasites (Lepoivre 2007). This exogenous disease is currently rampant in South America (Ecuador, Brazil and Colombia), causing enormous yield losses in cocoa orchards (Griffith *et al*. 2003). Its possible introduction into a cocoa-growing region can be devastating, causing losses ranging from 30–100% in endemic areas. The causal agent of this disease on cocoa tree is *Moniliophthora perniciosa.* Although *M. perniciosa* induces numerous symptoms on vegetative shoots, flowers, flower pads and pods, hypertrophy of the growth of infected vegetative meristems (brooms) remains the most characteristic symptom of witches' broom disease. The pathogen has two phases in its disease cycle; the biotrophic phase (Scarpari *et al*. 2002) and the necrotrophic phase (Silva *et al*. 2002).

In Côte d'Ivoire, witches' broom has been observed on kola tree. Recent work on this tree by the National Center for Agronomic Research (CNRA) has shown *Moniliophthora* sp. strains associated with diseased plants. However, no

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pathogenicity tests have been carried out to show the involvement of this fungus in witches' broom's expression. Furthermore, no recent research has suggested that this is the same species as that found on cocoa trees and endemic to South America. Other fungal pathogens could be associated with witches' broom disease. It is therefore important to identify and characterize the fungal agents associated with this disease in order to better guide control. The aim of this study was to characterize the fungal microflora associated with witches' broom disease of the kola tree. Sample collection and DNA sequencing of isolated fungi were used to determine the molecular diversity of the fungi associated with kola tree witches' broom disease.

Materials and Methods

Study area

This study was carried out in five of the seven major agroecological zones of Côte d'Ivoire (Fig. 1). These included the southern humid dense zone (I), the western humid dense zone (II), the semi-mountainous zone (III), the semi-deciduous humid dense zone (IV) and the transitional forest zone (V) Twenty localities were visited. In each locality, 5 kola orchards of sole or mixed cropping were surveyed. Samples were taken from ten kola trees per orchard, at a rate of one sample per kola tree showing symptoms of witches' broom. The samples were stored in plastic bags, labelled and transferred to the laboratory for analysis.

Surveys and sample collection

Twenty localities were visited. In each locality, 5 kola orchards of sole or mixed cropping were surveyed. One sample was taken per kola tree, at a rate of 10 kola trees per orchard exhibiting witches' broom symptoms. The samples were stored in plastic bags, labelled and transferred to the laboratory for analysis.

Isolation and purification of fungal strains

Fresh and dry branches of kola tree exhibiting witches' broom symptoms were collected, cut into explants and disinfected with 1% sodium hypochlorite for five minutes. They were then rinsed three times with distilled water and dried in a laminar flow hood. The disinfected explants were seeded on potato dextrose agar (PDA) medium in 8.5 cm Petri dishes. The Petri dishes were then stored at laboratory room temperature (25°C) for three days, in order to allow mycelial colonies to develop. The developed mycelial colonies were sub-cultured so as to obtain pure colonies using the method of Davet and Rouxel (1997). Mycelial colony isolation frequencies were calculated using the formula of Iqbal and Saeed (2012).

$$Ti\ (\%) = \frac{Ni}{Nt} \times 100$$

Where, Ti: Isolation rate in percentage; Ni: Number of isolates for a given genus and Nt: Total number of isolates of all genera obtained.

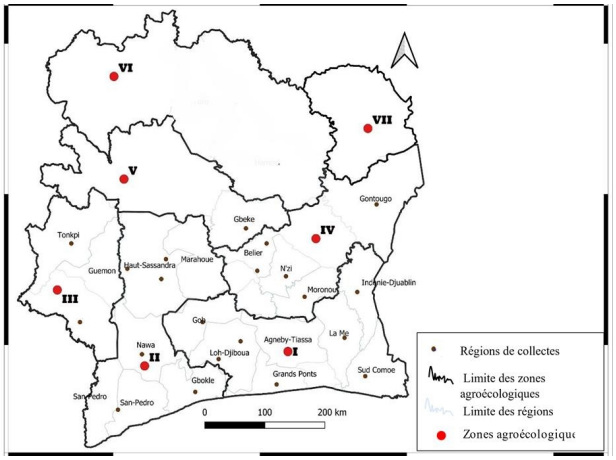

Fig. 1: Map of Côte d'Ivoire showing agro-ecological zones and collection regions

Identification of fungi associated with witches' broom diseases

Fungal strains were grouped on the basis of cultural characteristics such as colony color, mycelial appearance, relief, pigment production and average mycelial growth diameter. Microscopic observations were made at 40x magnification using an Optika light microscope. Structures such as spore shape, number of partitions, branching and mycelial color were used for identification. Fungal strains were identified using the identification key of Botton *et al*. (1990).

DNA extraction

DNA extraction from isolates was performed according to the protocol of Doyle and Doyle (1990). The 7-day-old fungal colonies were scraped separately with a sterile spatula using the method of Than *et al.* (2008). Thus, a quantity of 0.2 g of mycelium was weighed and ground in 1 mL of CTAB buffer preheated to 65°C for 10 min. One milliliter of crushed material from each fungal isolate obtained was transferred to sterile 2 mL Eppendorf tubes and gently vortexed. Tubes were then incubated in a water bath at 65°C for 10 min, homogenized by inversion every 10 min. On exit, tubes were left at room temperature for 2 min and then 500 μL of Chloroform Isoamyl alcohol was added to each tube. The tubes were vortexed and centrifuged at 13500 rpm for 10 min. The supernatant (750 μL) was transferred back to 2 mL Eppendorf tubes, where a further 500 μL of Chloroform was shaken, vortexed and centrifuged at 13500 rpm for 10 min. Six hundred and fifty (650) microliters of the supernatant was removed and transferred to 1.5 mL Eppendorf tubes, then precipitated with 650 μL cold Isopropanol (4°C). The solution was mixed by inversion and then incubated in the freezer at -20°C for over 2 h. After incubation, the solution was centrifuged at 13500 rpm for 10 min. The pellet of each solution in each tube was then recovered and dried on

blotting paper at room temperature. Next, 500 µL of 70% ethanol was added to each Eppendorf tube and centrifuged for 5 min at 13500 rpm. After centrifugation, the supernatant was removed, retaining the DNA deposit, and the tubes were drained and left to dry at room temperature. Finally, the DNA pellet was recovered in 50 μL TE and stored in a freezer at -20°C.

Amplification of DNA fragments

The universal primer pairs ITS1 (TCCGTAGGTGAACCTGCGC) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al*. 1990) was used. Both primers were indicated in the 5' - > 3' direction and were located at the 18 S (ITS1) and 28 S (ITS4) subunits of the ribosomal DNA genes. Each amplification reaction was performed in a total reaction volume of 25 μL. Two microliters of purified fungal DNA (10 ng μL^{-1}), 2.5 μL of buffer (10 X, Invitrogen-France), 3 μL of $MgCl_2$ (25 m*M*, Invitrogen-France), 1.5 μL of dNTP (10 m*M*, Invitrogen-France), 0.5 μL of Taq polymerase and 2 μL of primer pairs ITS1 and ITS4 at 10 m*M*. This reaction volume was adjusted to 25 μL by 11.5 μL of ultrapure water (QSP). A negative control containing all PCR components except DNA was added to the samples. PCR reactions were carried out in the Techne TC 512 thermal cycler according to the following program: 1 cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles each comprising denaturation for 30 s, hydration at 55°C for 30 s and elongation at 72°C for 30 s and final elongation at 72 °C for 10 min.

Electrophoresis and purification of PCR product

Amplification products were size-separated by electrophoresis on a 1% agarose gel. The gel was stained with 0.5 ng mL^{-1} ethidium bromide and immersed in 0.5X TBE buffer. For each amplification reaction, 10 μL of PCR product was mixed with bromothymol blue. The resulting mixture was deposited in agarose gel wells. Migration was performed at 110 V for 30 min. A size marker (Ladder 100 bp, Invitrogen, France) was used to estimate the size of the different amplified DNA fragments. The gel was then visualized under UV light at 360 nm. PCR amplicons migrated at a molecular weight of 800 base pairs. They were sequenced using the Sanger sequencing method by Macrogen Europe (Netherlands). The 5.8 S-ITS region of each sequence was amplified.

Phylogenetic analyses

Sequences of fungal strains were edited and assembled using Mega 5 software. The nucleotide sequences were subjected to a BLAST search for preliminary species assignment. Alignments of query sequences with representative fungal sequences available from GenBank were performed using the ClustalW alignment tool and edited by eye. In order to assign sequences to known fungal species, pairwise identity comparisons of nucleotide sequences were performed with pairwise gap deletion (Muhire *et al.* 2013). Maximum likelihood phylogenetic trees were constructed using FigTree software.

Fig. 2: Witches' broom on a kola tree

Results

Symptom of witches' broom observed on kola tree

Organ deformation followed by internode shortening is the only symptom observed on kola trees in the different localities. This was followed by abundant twig proliferation and hypertrophic growth of flower buds (Fig. 2).

Fungi associated with kola tree witches' broom

Four fungal genera were isolated from samples of symptomatic kola tree branches. These included *Lasiodiplodia* spp., *Pestalotiopsis* spp., *Fusarium* spp. and *Trichoderma* spp. (Fig. 3). The macroscopic and microscopic characteristics of the identified fungal isolates are listed in Table 1.

Molecular characteristics of associated fungi's DNA

One hundred and ninety-nine (199) samples of 210 isolates analyzed, were successfully amplified by the ITS1/ITS4 universal primer pairs. The DNAs migrated at 800 base pairs which is characteristic of fungi (Fig. 4). The sequencing results revealed the presence of several fungi. The genus *Lasiodiplodia* was the most detected, with 4 fungal species and 183 isolates. These included *L. Theobromae* with 179 isolates and *L. brasiliensis, L. iranensis* and *L. jatrophicola* with 2 and 1 isolate each,

Table 1: Macroscopic and microscopic characteristics of the fungi associated with the witches' broom of the colatier

Isolated fungi	Macroscopic characteristics	Microscopic characteristics
Lasiodiplodia spp.	Color: blackish grey on the front and black on the back Appearance: flaky Size: invasive Relief: high	Mycelium: hyaline, branched, septate with pycnidia on surface Conidia: oval, mature, plain, septate; immature, not
Pestalotiopsis spp.	Color: white on the front and yellow on the back Appearance: cottony Size: extended Relief: cerebriform	Conidia: fusiform, very slightly curved, penta-septate, hyaline end segments bearing antennae
Fusarium spp.	Color: white on the front and back Appearance: powdery Size: extended Relief: flat	Spindle-shaped, curved, multi-partitioned macroconidia and microconidia
Trichoderma spp.	Color: green on the front, light yellow on the back Appearance: granular Size: extended Relief: flat	Mycelia: hyaline, non-partitioned and perpendicularly branched

respectively. For the genus *Pestalotiopsis* sp., 5 fungal species were identified, namely *P. microspora, P. saprophytica, P. theae, P. clavispora, P. formicarum*. Two species of *Fusarium* (*F. fujikuroi, F. oxysporum)* and *Trichoderma harzianum* were also identified (Table 2). Similarity searches of our sequences with those of the NCBI (National Center for Biotechnology Information) database through BLAST enabled us to identify these different fungal species with different accession numbers from PP824668 to PP824719. The percentages of identity, which express the relationship between the sequences detected and those present in GenBank, are given in Table 3. The *Lasiodiplodia* spp. species identified had DNAs identical to those in GenBank, with percentages of identity ranging from 88.68% to 100%. As for DNAs of *Pestalotiopsis* spp., *Fusarium* spp. and *Trichoderma* sp. were concerned, they were 98% to 100% homologous with those on Genbank (Table 3). The sequences obtained from our positive samples are positioned on a phylogenetic tree rooted on the *Diplodia* sp. OR205236 sequence (Fig. 5). Four major groupings were formed, representing the different fungal genera. *Lasiodiplodia*, was the most represented with 179 isolates. In addition to the sequences of isolates EB 66-CI and EB 68-CI, all *Lasiodiplodia* sequences were closely related. *Pestalotiopsis,* was the second most represented genus with isolates whose DNA sequences were close to each other, followed by the genera *Fusarium* and *Trichoderma*.

Discussion

During this study, the morphological characteristics of the fungi isolated from kola tree witches' broom allowed us to identify four genera, including *Lasiodiplodia, Pestalotiopsis, Fusarium* and *Trichoderma*. This fungal diversity on the kola tree may be due to the fact that fungi are dependent on certain substrates or supports for their development, either for their adhesion or for their nutrition. Their presence can lead to morphological or physiological changes.

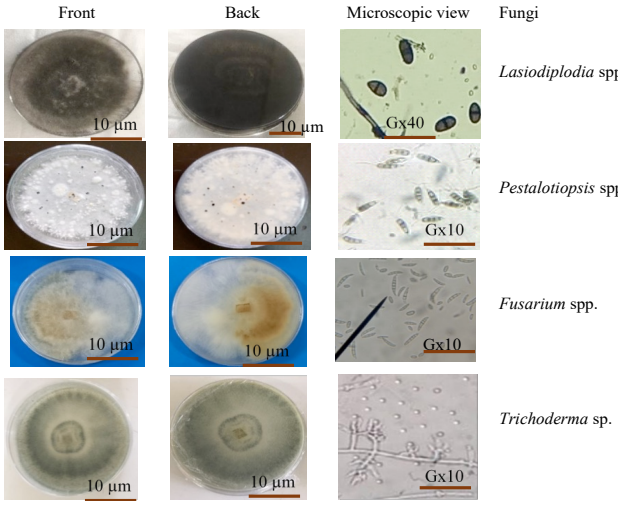

Fig. 3: Morphological characteristics of the fungi associated with kola tree witches' broom

In all the areas surveyed, environmental factors such as temperature, precipitation and relative humidity are conducive to fungi development. In addition, the development of fungi may be favored by the shade, the high relative humidity and the high heat prevailing in kola tree cultivation. These results are similar to those of Pohe *et al*. (2013), who showed that in cocoa cultivation, fungal development is favored by shade, heat and high humidity. The genus *Lasiodiplodia* sp. showed the highest isolation rate with 90.90% in these localities, which could be explained by the fact that this genus is very much present in the environment, either associated with a pathogen, a saprophyte or even the pathogen of the plant. These results are in line with those of Odamtten *et al*. (2018) and Zinsou *et al*. (2021). These authors, working on fruit plants, have isolated and identified *Lasiodiplodia* at a frequency of 90%. The nucleotide sequences obtained in this study had a homology rate of over 90% compared with the one existing in the NCBI GenBank databases. The fungal strains

Table 2: Distribution of fungal isolates associated with kola tree witches' broom per genus and species

Genus	Species	Number of isolates	Isolate (%)
	L. theobromae	179	89.9
	L. brasiliensis	2	1
Lasiodiplodia	*L. iranensis*	1	0.5
	L. jatrophicola	1	0.5
	P. microspora	5	2.50
	P. saprophytica	2	1
Pestalotiopsis	*P. theae*	1	0.50
	P. clavispora	1	0.50
	P. formicarum	1	0.50
Fusarium	*F. oxysporum*	2	0.50
	F. fujikuroi	1	1
Trichoderma	*T. harzianum*	2	0.50

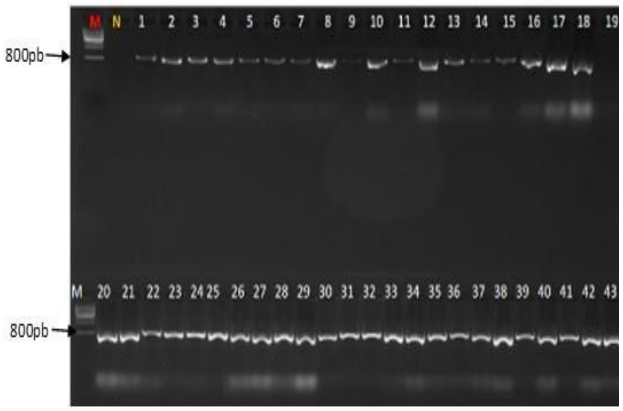

Fig. 4: Electrophoresis gel (1% agarose) of PCR amplicons from DNAs of migrated witches' broom-associated fungi of kola tree. Fragment size is indicated (in base pairs) by the BenchTop 100 bp size marker DNA Ladder (Promega)

identified in Côte d'Ivoire are close to those presented in the sub-regions (in Ghana) and in other countries. According to Burgess *et al.* (2006), the majority of fungal species has a cosmopolitan distribution and are found in several continents and on several hosts. Indeed, many fungal species grow in plant tissues without causing any apparent damage to host plants (Peixoto *et al*. 2004). ITS sequences enabled us to identify the main fungal genus and species infecting kola tree. Indeed, according to Crouch *et al.* (2006), It could be a good marker of inter-generic differentiation. In many recent studies ITS primer was used to identify various post-harvest fungal pathogens such as *Mucor fragilis* in seychelles pole bean, *Penicillium citrinum* in garlic cloves and *Alternaria radicina* in carrot (Javed and Javaid 2021; Khan and Javaid 2022, 2023).

The different fungal genera are positioned on the phylogenetic tree, forming 4 major clades. Species of the *Pestalotiopsis* genus, mostly detected in the Agneby-Tiassa and Moronou regions, are close to sequences from Brazil, India and Malaysia already found on GenBank. The two intermediate genera *Trichoderma* and *Fusarium*, representing clades 2 and 3, have species whose sequences are close to those of India and China on GenBank. Finally, in the largest clade grouping species of the *Lasiodiplodia* genus, four sub-clades exist. In the first 3 sub-clades, as for the other species, *Lasiodiplodia* sequences are close to those of China. In the 4th and last sub-clade, the sequences of *Lasiodiplodia* species detected in Sud-Comoé are close to those of China, India and

Table 3: Sequence names and percentage identity with respect to those existing on GenBank

Sample no.	Insolates	Identity percentage (%)	Accession number of closest sequences on GenBank
EB1	*pestalotiopsis theae*	100	MF495464.1
EB2	*pestalotiopsis saprophytica*	100	MT576586.1
EB3	*Pestalotiopsis microspora*	100	MN856235.1
EB4	*Lasiodiplodia theobromae*	100	MN818616.1
EB5	*Lasiodiplodia theobromae*	100	MK529970.1
EB6	*Lasiodiplodia theobromae*	100	OR963970.2
EB8	*pestalotiopsis saprophytica*	100	LC685759.1
EB9	*Fusarium fujikuroi*	99.82	MH084746.1
EB10	*Pestalotiopsis microspora*	100	KU365133.
EB12	*Lasiodiplodia theobromae*	99.79	MK530049.1
EB13	*Lasiodiplodia theobromae*	98.01	OR361681.1
EB14	*Lasiodiplodia theobromae*	99.36	MT644474.1
EB15	*Lasiodiplodia theobromae*	99.31	OR361681.1
EB16	*Lasiodiplodia theobromae*	100	MN818616.1
EB17	*Lasiodiplodia theobromae*	99.81	MT043789.1
EB19	*Lasiodiplodia theobromae*	99.49	EU860391.1
EB20	*Pestalotiopsis microspora*	98.92	MT568595.1
EB21	*Lasiodiplodia theobromae*	100	MN341226.1
EB23	*pestalotiopsis clavispora*	100	OQ572345.1
EB24	*Lasiodiplodia theobromae*	100	PP209594.1
EB25	*Lasiodiplodia theobromae*	99.80	MT043795.1
EB26	*Lasiodiplodia theobromae*	99.42	ON908473.1
EB28	*Lasiodiplodia brasiliensis*	99.65	KY655212.1
EB29	*Lasiodiplodia theobromae*	100	MN341226.1
EB30	*Lasiodiplodia theobromae*	100	KY052902.1
EB31	*Lasiodiplodia theobromae*	99.81	MK530006.1
EB33	*Fusarium oxysporum*	100	MT420651.1
EB34	*Lasiodiplodia theobromae*	99.77	MT507809.1
EB35	*Fungal endophyte*	98.18	KF435451.1
EB36	*Lasiodiplodia theobromae*	100	OR963970.2
EB37	*Lasiodiplodia theobromae*	100	MT332314.1
EB38	*Lasiodiplodia theobromae*	97.74	MW132905.1
EB40	*Lasiodiplodia theobromae*	100	OR963947.2
EB41	*Lasiodiplodia theobromae*	100	OR963970.2
EB42	*pestalotiopsis formicarum*	100	MN635622.1
EB43	*Lasiodiplodia theobromae*	100	OR963970.2
EB44	*Trichoderma harzianum*	100	MK015045.1
EB45	*Lasiodiplodia theobromae*	100	OR963970.2
EB46	*Lasiodiplodia theobromae*	99.75	OR963947.2
EB47	*Lasiodiplodia theobromae*	100	MT507809.1
EB48	*Trichoderma harzianum*	100	MK015045.1
EB49	*Lasiodiplodia theobromae*	100	MT332314.1
EB50	*Lasiodiplodia theobromae*	100	MK530050.1
EB51	*Pestalotiopsis microspora*	100	MT568595.1
EB61	*Lasiodiplodia theobromae*	100	OR963970.2
EB65	*Lasiodiplodia iranensis*	98.74	KU997381.1
EB66	*Lasiodiplodia theobromae*	95.58	MZ496574.1
EB67	*Lasiodiplodia theobromae*	100	OR963970.2
EB68	*Lasiodiplodia theobromae*	88.68	OR880552.1
EB70	*Lasiodiplodia theobromae*	98.84	OR963970.2
EB71	*Lasiodiplodia theobromae*	98.89	PP026055.1
EB73	*Lasiodiplodia theobromae*	99.82	MT913570.1
EB76	*Lasiodiplodia theobromae*	100	MN294610.1
EB78	*Lasiodiplodia theobromae*	99.82	MT913570.1
EB79	*Lasiodiplodia theobromae*	100	OR963970.2
EB80	*Lasiodiplodia theobromae*	99.57	MN341226.1

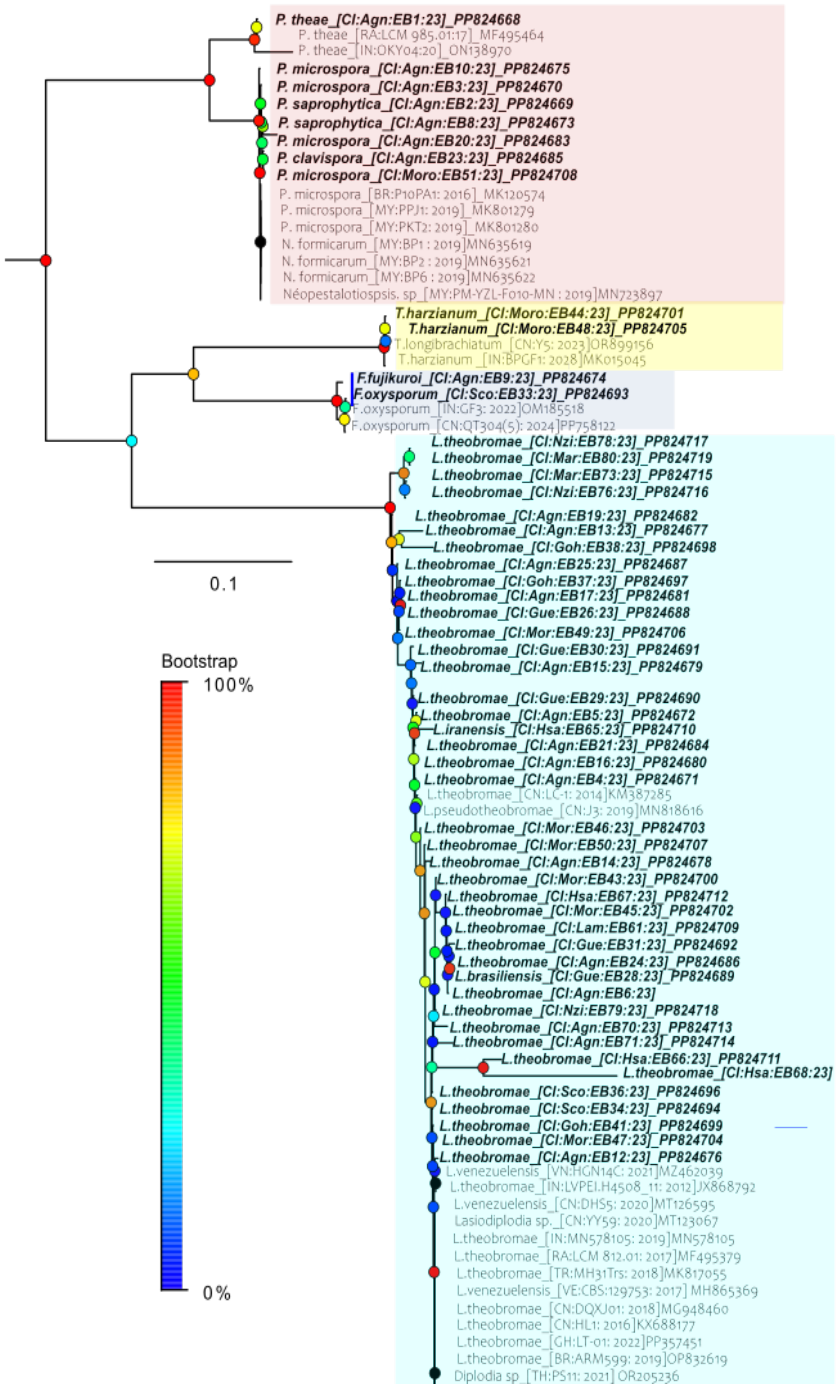

Fig. 5: Maximum likelihood phylogenetic tree deduced from partial sequence alignments of fungi isolated from kola tree witches' broom. The tree was rooted on *Diplodia* sp. (OR205236). New sequences characterized in this study are shown in bold. The scale indicates the genetic distance

Ghana. The lack of comparison with *Lasiodiplodia* sequences from the sub-region is explained by the fact that fewer sequences of these fungi detected on these specimens in the sub-regions exist on GenBank.

Conclusion

A diversity of fungal genera has been associated with kola tree witches' broom. These include *Lasiodiplodia, Pestalotiopsis, Fusarium* and *Trichoderma.* The genus *Lasiodiplodia* followed by the genus *Pestalotiopsis* were the most isolated ones. Molecular analysis revealed 183 isolates of *Lasiodiplodia* sp. versus 10 isolates of *Pestalotiopsis* out of 199 sequenced isolates. This study is the first to identify

and characterize the fungal agents associated with kola tree witches' broom disease in Côte d'Ivoire. Further studies will help to clarify the involvement of these fungal genera in symptom expression.

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Author Contributions

EM, KKT, SK and AK; methodology design. EM; data collection with support from YWJ, DGM and NADC. EM, KKT, SK, AK and NAJ; data analysis and drafting of the manuscript. All authors have read and approved the final manuscript.

Conflict of Interest

The authors have declared no conflicts of interest.

Data Availability

The data presented in this study will be available on request from the corresponding author.

Ethical Approval

Not applicable to this paper

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