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RESEARCH ARTICLE

Molecular identification of fungi associated with *Lagenaria siceraria* (Molina) Standl necrosis in Côte d'Ivoire

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Abstract

Parasitic pressure, particularly fungal diseases, represents a major constraint to *Lagenaria siceraria* (*L. siceraria*) production. It causes considerable losses of agricultural production. Thus, identification of fungi infecting leaves of *L. siceraria* was necessary. Some infected leaves of *L. siceraria* were collected in 30 fields of 5 localities of Côte d'Ivoire and their examination had shown five types of symptoms. Fungi genera were identified by their morphological and microscopic characteristics. Then, the study of molecular diversity based on rDNA provided a powerful tool for identification of major isolated fungal genera. The nuclear ribosomal DNA region (ITS1, 5,8S and ITS2) of fungal strains was PCR amplified using with the universal primers, ITS1 and ITS4. That amplification generated only one fragment of about 600 bp. The sequence data obtained from the PCR products were aligned and compared with other sequences. Homologous sequences of fungi were obtained from GenBank database.

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Introduction

The bottle gourd *Lagenaria siceraria* (Molina) Standl is a Cucurbitaceae cultivated primarily in the tropical and subtropical localities (Decker-Walters et al., 2001). It is considered as a secondary crop and is cultivated in small areas in association with other crops (Achigan et al., 2006), mainly for its oil-rich seeds and high nutrition value (Zoro Bi et al., 2003). The seeds constitute also a good source of proteins and lipids (Chinyere et al., 2009; Enzonga-Yoca et al., 2011) and energy (Badifu and Ogunsua, 1991). They cover food needs for family unit and represent a potential source of additional income to producers. In spite of its nutritional and socio-economical importance, *L. siceraria* remains a minor crop. It is subjected to a strong parasitic and diseases pressure. Several pathogenic agents cause significant damage in field (Lepoivre and Semal, 1989). The most devastating fungal diseases of *L. siceraria* are downy mildew caused by *Pseudoperonospora cubensis* (Lebeda and Urban, 2004), powerdly mildew caused by *Podosphaera xanthii* (Kousik et al., 2007) and *Fusarium* wilt due to *Fusarium oxysporum* (Trionfetti et al., 2002). Fungi constitute major pathogenic agents on a wide variety of woody and herbaceous plants. They are world-wide distributed, especially in tropical and subtropical regions.

In Côte d'Ivoire, *Lagenaria siceraria* plants are susceptible to infection with pathogens, including fungi which cause disease and enormous loss of the crop. Fungi reduce seeds production, 40 to 70 % (Fondio et al., 2000). They may also cause severe reduction in yield including complete loss of production. Fungal infection reduces seed weight, nutritional value and agronomical qualities (Harman, 1983). No control method of fungi in fields is adopted during plantation because of the lack of precise knowledge of fungi infecting *L. siceraria*. Fungi survey in fields of five localities of Côte d'Ivoire would allow identifying plant fungal agents. However, morphological identification of fungi is not always reliable, thus, for this reason we have used molecular characterization of infected fungi. Molecular tools have been employed to systematically characterize fungi strains, with the application of marker-based methodology and the analysis of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) gene trees (Sreenivasaprasad et al., 1996). More recently, phylogenetic analyses based on sequences of the rDNA regions have been successfully used to understand relationships among strains (Du et al., 2005; Crouch et al., 2006; Farr et al., 2006). Indeed, the ITS1-5.8S-ITS2 rDNA region-based information is a valuable tool of fine, accurate and reliable identification, taxonomy and phylogeny of fungi. This region shows sequence variability for distinguishing individual strains and stability among strains belonging to a given species (Gomes et al., 2002; Horton, 2002; Korabecna, 2007).

The objective of the present study was to identify fungi infecting *L. siceraria* in Côte d’Ivoire, particularly those associated to leaves. Thus, fungal strains were collected in five production localities located in the South, East and North of the country. The nucleotide sequences data of the ITS1+5.8S+ITS2 ribosomal DNA region of these isolated fungi, obtained with universal primers ITS1 and ITS4 were used to compare with others on GenBank database.

Materials and Methods

Localities surveyed

Five localities of *L. siceraria* seeds production were investigated in Côte d’Ivoire. These localities are Alepe (latitude 5°28′ N and longitude 3°46′ W), Bondoukou (latitude 8°1′ N and longitude 3°48′ W), Bongouanou (latitude 6°38′ N and longitude 5°12′ W), Divo (latitude 5°50′ N and longitude 6°22′ W) and Korhogo (latitude 9°26′ N and longitude 6°38′ W. They are situated in southern (Alepe and Divo), in eastern (Bondoukou and Bongouanou) and in northern (Korhogo) Côte d’Ivoire (Fig 1).

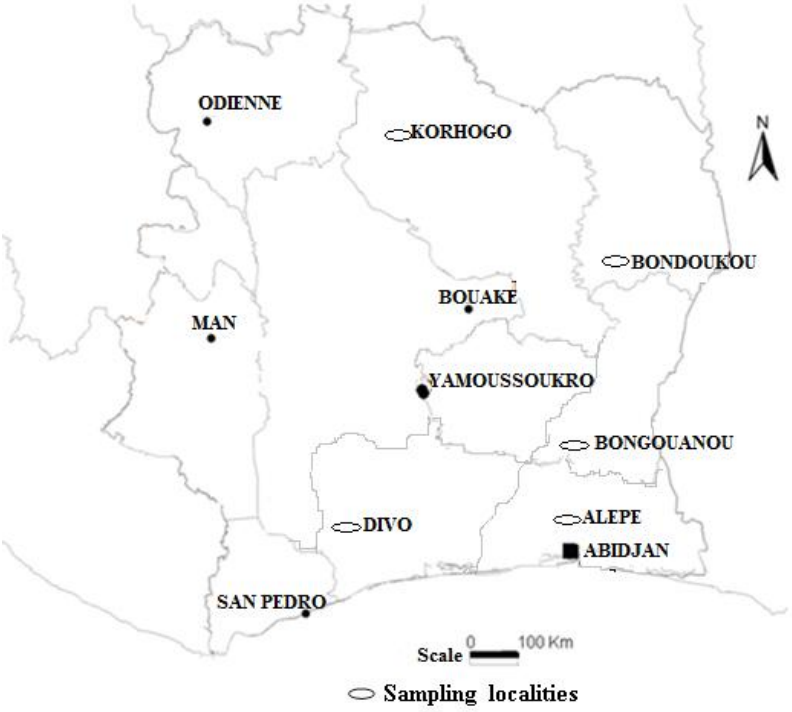

Fig 1. Five localities in Côte d'Ivoire where infected leaves of *Lagenaria siceraria* were collected.

Symptomatology and infected leaves collection

From August 2008 to September 2009, symptoms of fungi infections were observed and described for the form, the size and the color, in thirty randomly selected plantations: ten in Korhogo, the largest surveyed locality, and five in each of the four other localities. In each plantation, five leaves with the same symptoms were collected and placed on blotting paper, then in polyethylene plastic bag and brought to the laboratory. A total of 750 leaves were collected to conduct the fungal identification study.

Fungal genera identification

Three fragments of 2 cm long each cut off the margin of lesions of each collected leaf were disinfected in 10 % sodium hypochlorite for 3 min, twice rinsed with sterile distilled water and air-dried. Dried leaf fragments were placed on Potato Dextrose Agar (PDA) then incubated at room temperature (25 ± 2^{o}C) for three days. Growing mycelial tips of fungal strains were transferred to new PDA plates. A single spore was isolated from the colony, by picking conidia using dilution method on water agar (WA), and cultured on PDA. After seven days growth on PDA, the morphological (structure and color of fungi on PDA plate) and microscopic (structure and form of mycelium and conidia observed by microscopy) characteristics of the colony deriving from the single spore were identified under a phase-contrast photonic microscope (ZEISS) using the identification key of Botton et al. (1990). The description of fungi colonies allowed identifying fungal genera.

Genomic DNA extraction and DNA solution

Genomic DNA was extracted according to the procedure described by Murray and Thompson (1980). Briefly, monoconidial cultures suspensions were subcultivated for at least seven days on PDA plates and incubated at room temperature. In a 2 ml eppendorf tube, 0.2 g of the mycelia was mixed with 500 µl CTAB buffer [50 Mm hexadecyl-trimethyl ammonium bromide, 100 mM NaCl, 100 mM Tris-HCl (pH 8), 20 mM EDTA] and incubated at 65° C for 30 min with gentle intermittent shaking. This mixture was centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a new 2 ml eppendorf tube, then 200 µl chloroform were added and the mixture centrifuged at 14,000 rpm for 10 min. The upper phase was transferred into another 2 ml eppendorf tube, 500 µl of isopropanol were added and the mixture centrifuged at 14,000 rpm for 10 min. The supernatant was discarded. The DNA pellet was washed once with 500 µl of 70% ethanol and dried in a SpeedVac for approximately 10 min. The pellet was dissolved in 100 µl TE and the DNA solution stored at -20°C until use. DNA working solutions, concentrated at 10 ng/µl were obtained by dilution of crude DNA solution with pure water.

PCR amplification and fragment analysis

The universal primer pairs (forward ITS1 and reverse ITS4) described by White et al., (1990) were used to amplify the entire rDNA ITS region (ITS1, 5.8S and ITS2) of the eighty-eight strains collected. Amplification reaction was carried out in a final volume of 25 µl containing 2 µl fungal genomic DNA (10 ng/µl), 2.5 µl Tris-HCl; (50 mM, pH 8.4), 3 µl $MgCl_2$ (25 mM), 1.5 µl of dNTP (10 mM) in a 1:1:1:1 dATP: dTTP: dCTP: dGTP ratio, 2 µl of each primer (10 mM), 0.5 µl of Taq polymerase (Invitrogen, France) and 11.5 µl H_2O Qsp. Negative controls (without DNA template) were included in every experiment to test for the presence of contamination of reagents and reaction mixtures by sample DNA or previously amplified DNA. A DNA thermal cycler (Techne TC-512) was programmed as follows: 1 cycle of denaturation at 94^o C for 5 min, followed by 35 cycles of denaturation at 94^o C for 30 s, annealing at 55^o C for 30 s, and extension at 72^o C for 30 s, and a final extension at 72^o C for 10 min. The amplification products were size-separated in a 1.5 % agarose gel immersed in 0.5X TBE buffer by electrophoresis at 80 V for 45 min, stained with 0.5 ng/ml ethidium bromide and photographed under ultraviolet light. A 100 bp ladder (Invitrogen, France) was used to size the DNA amplified fragments.

DNA amplified fragments sequencing and fungal species identification

After electrophoresis, positive PCR products were subjected to capillary sequencing. Sequences were determined in an Applied Biosystems ABI 3130 DNA sequencer using the foward primer ITS1. Results of sequencing were arranged into two types of files: a file format"ab1" showing the chromatogram and a FASTA file containing the nucleotide sequence. Similarities were checked between nucleotide sequences in FASTA file and their homologous sequences of the National Center for Biotechnology Information (NCBI) GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990). This comparison allowed the identification of fungal species. To this purpose, a given species is assumed to be the closest relative of a strain when they exhibited the highest identity percent. DNA sequences obtained in this paper have been deposited in GenBank.

Results

Symptomatology

Symptoms of fungal infection on *L. siceraria* leaves are spots of various forms, sizes and colorations. Overall, on the seven hundred and fifty leaves collected in the thirty plantations five types of spots were observed (Fig 2). In each of the five localities surveyed, three types of spots out of the five were observed (Table 1). These three types are: small circular brown spots with yellow halation of approximately 1 cm in diameter (Fig 2A) (symptom type A, S_A), brown coalescent spots of approximately 2 cm in diameter of which some are detached (Fig 2B) (symptom type B, S_B) and large brown spots (Fig 2C) (symptom type C, S_C).

In Bondoukou and Bongouanou, located in east Côte d'Ivoire and Korhogo, located in the north, two other types of spots were observed: brown spots extended symmetrically to the main vein to the petiole on the upper surface of leaves (Fig 2D) (symptom type D, S_D) and more or less circular brown spots which are detached thereafter (Fig 2E) (symptom type E, S_E). Thus, all the five types of spots were reported in Bondoukou, Bongouanou and Korhogo, three types of spots out of five were observed in Alepe and Divo located in south Côte d'Ivoire (Table 1).

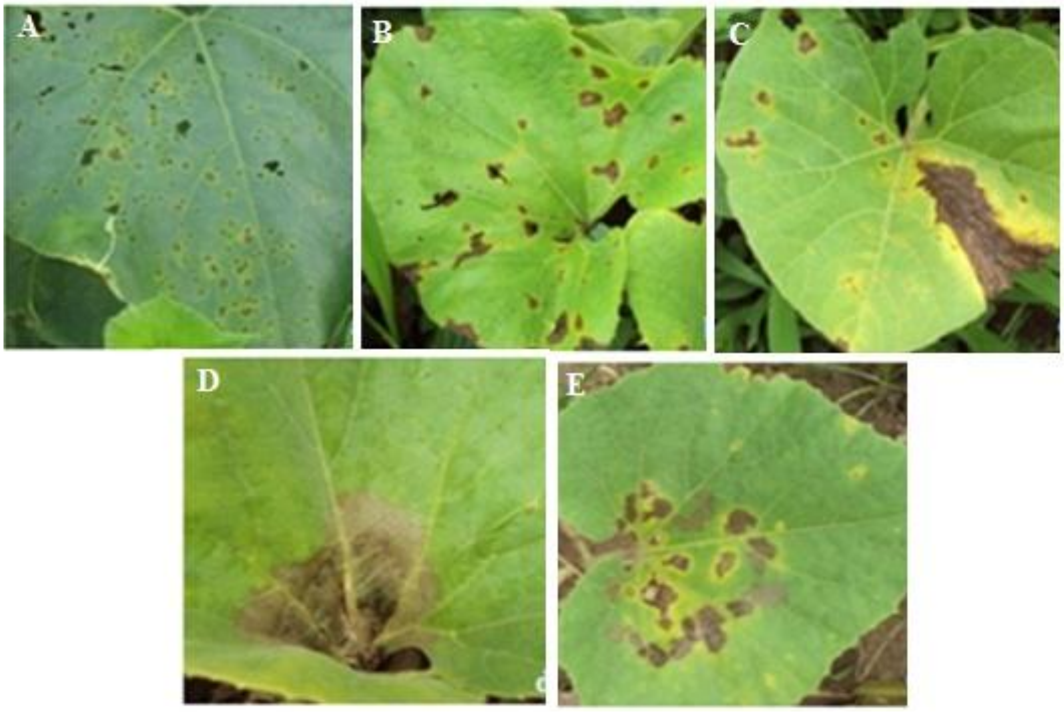

Fig 2. Disease symptoms identified as types of spots of various forms, sizes and colorations on leaves of *Lagenaria siceraria*. (A): symptom type A. Small circular brown spots with yellow halations of approximately 1 mm in diameter; (B): symptom type B. Brown coalescent spots of approximately 2 cm in diameter of which some are detached; (C): symptom type C. large brown spots; (D): symptom type D. Brown spots extended symmetrically to the major vein to the petiole on the upper surface of leaves; (E): symptom type E. More or less circular brown spots which are detached there after.

Table 1. Presence and absence of symptoms of fungi infecting leaves of *Lagenaria siceraria* collected in five localities surveyed in Côte d'Ivoire.

		Surveyed localities				
		Alepe	**Bondoukou**	**Bongouanou**	**Divo**	**Korhogo**
Symptom types	S_A	+	+	+	+	+
	S_B	+	+	+	+	+
	S_C	+	+	+	+	+
	S_D	-	+	+	-	+
	S_E	-	+	+	-	+

S_A Symptom type A; S_B Symptom type B; S_C Symptom type C; S_D Symptom type D; S_E Symptom type E

\+ Presence of a type of symptom in a locality; - absence of a type of symptom in a locality

Fungal genera identification

On the basis of their morphological and microscopic characteristics, different fungal strains were identified. These fungi strains infecting leaves of *L. siceraria* collected in the five surveyed localities were identified to belong to ten genera.

These are *Botryosphaeria* (Figs 3A, 3B), *Cochliobolus* (Figs 3C, 3D), *Colletotrichum* (Figs 3E, 3F), *Diaporthe* (Figs 3G, 3H), *Fusarium* (Figs 3I, 3J), *Lasiodiplodia* (Figs 3K, 3L), *Pestalotiopsis* (Figs 3M, 3N), *Phoma* (Figs 3O, 3P), *Phomopsis* (Figs 3Q, 3R) and *Rhizoctonia* (Figs 3S, 3T). Six genera were identified in Bondoukou, seven were identified in Bongouanou and eight were identified in Alepe, Divo and Korhogo (Table 2).

Six genera, *Botryosphaeria*, *Cochliobolus*, *Colletotrichum*, *Fusarium*, *Lasiodiplodia* and *Phoma*, were observed in each of the five localities. The genus *Diaporthe* was observed in two localities (Bongouanou and Divo). Two other genera, *Phomopsis* and *Rhizoctonia*, were observed in two localities (Alepe and Korhogo), whereas the genus, *Pestalotiopsis* was observed in only one locality (Divo) (Table 2). The number of fungi genera identified by symptom types varied from four, (symptom type E) to nine (symptom type A). A given fungi genus was detected from two symptoms (*Phomopsis*, *Pestalotiopsis*) to five symptoms (*Colletotrichum*, *Fusarium*, *Lasiodiplodia*). Thus, *Botryosphaeria*, *Cochliobolus*, *Colletotrichum*, *Fusarium*, *Lasiodiplodia* and *Phoma* genera isolated in each of the five surveyed localities are from two of the three types of symptom (A, B, C) common to these localities (Table 3).

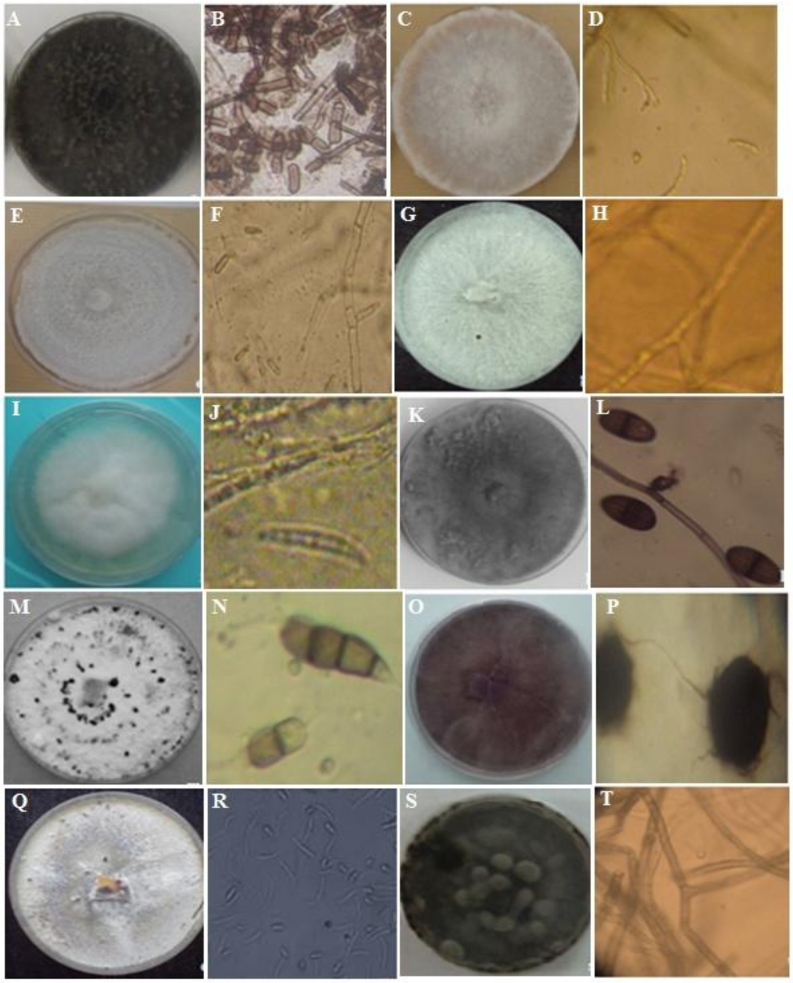

Fig 3. Macroscopic (A, C, E, G I, K, M, O, Q, S,) and microscopic (B, D, F, H, J, L, N, P, R, T) aspects of some fungi isolated on Potato Dextrose Agar medium from different disease symptoms of *Lagenaria siceraria* leaves. (A) Mycelia colonies of *Botryosphaeria* spp.; (B) Conidia of *Botryosphaeria* spp.; (C) Mycelia colonies of *Cochliobolus* spp.; (D) Conidia of *Cochliobolus* spp.; (E) Mycelia colonies of *Colletotrichum* spp.; (F) Conidia of *Colletotrichum* spp.; (G) Mycelia colonies of *Diaporthe* spp.; (H) Conidia of *Diaporthe* spp.; (I) Mycelia colonies of *Fusarium* spp.; (J) Conidia of *Fusarium* spp.; (K) Mycelia colonies of *Lasiodiplodia* spp.; (L) Conidia of *Lasiodiplodia* spp.; (M) Mycelia colonies of *Pestalotiopsis* spp.; (N) partitioned conidia of *Pestalotiopsis* spp.; (O) Mycelia colonies of *Phoma* spp.; (P) Pycnidia of *Phoma* spp.; (Q) Mycelia colonies of *Phomopsis* spp.; (R) Conidia of *Phomopsis* spp.; (S) Mycelia colonies of *Rhizoctonia* spp.; (T) mycelia of *Rhizoctonia* spp.

Table 2. Fungi species identified on infected leaves of *Lagenaria siceraria* based on localities surveyed in which they were identified.

Surveyed localities				
Alepe	**Bondoukou**	**Bongouanou**	**Divo**	**Korhogo**
B. rhodina	*B. rhodina*	*B. rhodina*	*B. rhodina*	*B. rhodina*
Cochliobolus kusanoi	*Cochliobolus kusanoi*	*Cochliobolus kusanoi*	*Cochliobolus kusanoi*	*Cochliobolus kusanoi*
C. gloeosporioides	*C. gloeosporioides*	*C. gloeosporioides*	*C. gloeosporioides*	*C. gloeosporioides*
F. oxysporum	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
Phoma herbarum	*Phoma herbarum*		*Phoma herbarum*	*Phoma herbarum*
	Phoma sorghina	*Phoma sorghina*	*Phoma sorghina*	*Phoma sorghina*
L. theobromae	*L. theobromae*	*L. theobromae*	*L. theobromae*	
	F. equiseti	*F. equiseti*		*F. equiseti*
F. chlamydosporum			*F. chlamydosporum*	*F. chlamydosporum*
	L. pseudotheobromae	*L. pseudotheobromae*		*L. pseudotheobromae*
		D. phaseolorum	*D. phaseolorum*	
Phomopsis glabrae				*Phomopsis glabrae*
Rhizoctonia bataticola				*Rhizoctonia bataticola*
	F. solani	*D. helianthi*	*Pestalotiopsis sp.*	

B. Botryosphaeria *C. Colletotrichum* *D. Diaporthe* *F. Fusarium* *L. Lasiodiplodia*

Table 3. Fungal genera identification from symptoms described on *Lagenaria siceraria* leaves.

Symptom types	**Fungal genera identified**									
	Botryosphaeria	***Cochliobolus***	***Colletotrichum***	***Diaporthe***	***Fusarium***	***Lasiodiplodia***	***Pestalotiopsis***	***Phoma***	***Phomopsis***	***Rhizoctonia***
S_A	+	-	+	+	+	+	+	+	+	+
S_B	+	+	+	+	+	+	-	-	+	-
S_C	+	+	+	-	+	+	+	+	-	+
S_D	+	+	+	+	+	+	-	+	-	-
S_E	-	-	+	-	+	+	-	-	-	+

S_A Symptom type A; **S_B** Symptom type B; **S_C** Symptom type C; **S_D** Symptom type D; **S_E** Symptom type E; + fungal genus identified from the symptom type; - fungal genus non identified from the symptom type

Fungal species identification

The internal transcribed spacer (ITS) of the rDNA region was successfully amplified in the fungal strains analyzed that belong to the ten genera. The PCR amplification with the universal primers pairs (forward ITS1 and reverse ITS4) generated only one and unique fragment of approximately 600 bp (Fig 4). However, the comparison of the sequence data obtained in FASTA file with those in the NCBI GenBank, via BLAST, revealed that fungi collected in the five localities (Alepe, Bondoukou, Bongouanou, Divo and Korhogo) belong to sixteen species (Table 2). The identity percentage varied from 85 to 100 % and they GenBank accessions are presented in Table 4.

The geographical repartition of these species is as follows: nine in Alepe, ten in Bondoukou, Bongouanou and Divo and eleven in Korhogo. A few fungal species is common to the five localities. This common flora is composed of four species *Botryosphaeria rhodina*, *Cochliobolus kusanoi*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. Two other species, out of the sixteen, were reported in two localities. These species were *Phomopsis glabrae* and *Rhizoctonia bataticola* in Alepe and Korhogo. Some species were localized only in one locality: *Diaporthe helianthi* in Bongouanou, *Fusarium solani* in Bondoukou and *Pestalotiopsis* sp. in Divo. Each of the species, *Botryosphaeria rhodina*, *Cochliobolus kusanoi*, *Colletotrichum gloeosporioides*, *Phomopsis glabrae*, *Pestalotiopsis* sp., was the only one identified in their respective genera in this study.

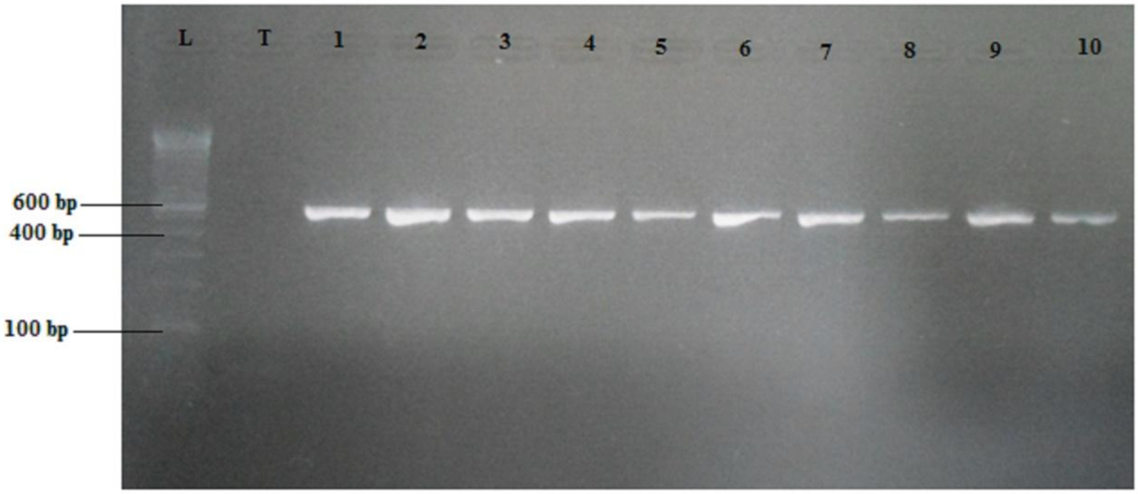

Fig 4. Internal Transcribed Spacer (ITS1+5,8S+ITS2) rDNA PCR product of ribosomal DNA of some fungi strains. Size-resolved in 15% agarose gel for 45 min at 80V in 05 X TBE buffer, L = 100 bp DNA ladder (Invitrogen), T = negative control (without DNA), Lanes 1 to 10 are positive samples generated a product of 600 bp.

Table 4. Homology between the nucleotide sequences of fungal rDNA sequences obtained and species of the GenBank database (NCBI)

Codes	Corresponding species in GenBank database	Accession number	Identity percentages
M12	*Botryosphaeria rhodina*	KF000067	97
M22	*Botryosphaeria rhodina*	KF317650	98
M29	*Botryosphaeria rhodina*	KF317652	95
M47	*Cochliobolus kusanoi*	KF000077	91
M50	*Cochliobolus kusanoi*	KF317657	97
M58	*Colletotrichum gloeosporioides*	KF000080	99
M85	*Colletotrichum gloeosporioides*	KF317659	99
M86	*Colletotrichum gloeosporioides*	KF317660	98
M96	*Colletotrichum gloeosporioides*	KF317661	99
M106	*Colletotrichum gloeosporioides*	KF317662	99
M111	*Colletotrichum gloeosporioides*	KF317663	98

M115	*Colletotrichum gloeosporioides*	KF317664	95
M132	*Colletotrichum gloeosporioides*	KF317665	98
M33	*Diaporthe helianthi*	KF000072	94
M37	*Diaporthe phaseolorum*	KF000073	87
M41	*Diaporthe phaseolorum*	KF317655	99
M112	*Fusarium chlamydosporum*	KF000082	100
M10	*Fusarium equiseti*	KF317644	98
M46	*Fusarium equiseti*	KF000076	98
M48	*Fusarium equiseti*	KF317656	98

Table 4. (continued) Homology between the nucleotide sequences of fungal rDNA sequences obtained and species of the GenBank database (NCBI)

Codes	Corresponding species in GenBank database	Accession number	Identity percentages
M42	*Fusarium oxysporum*	KF000075	98
M66	*Fusarium solani*	KF000081	99
M4	*Lasiodiplodia pseudotheobromae*	KF317642	94
M18	*Lasiodiplodia pseudotheobromae*	KF317649	95
M20	*Lasiodiplodia pseudotheobromae*	KF000069	99
M11	*Lasiodiplodia theobromae*	KF317645	94
M13	*Lasiodiplodia theobromae*	KF317646	95
M15	*Lasiodiplodia theobromae*	KF317647	97
M17	*Lasiodiplodia theobromae*	KF317648	90
M19	*Lasiodiplodia theobromae*	KF000068	99
M35	*Lasiodiplodia theobromae*	KF317653	93
M40	*Pestalotiopsis sp.*	KF000074	90
M8	*Phoma herbarum*	KF317643	97
M53	*Phoma herbarum*	KF000078	89
M57	*Phoma herbarum*	KF317658	98
M56	*Phoma sorghina*	KF000079	97
M31	*Phomopsis glabrae*	KF000071	98
M36	*Phomopsis glabrae*	KF317654	98
M26	*Rhizoctonia bataticola*	KF317651	89
M28	*Rhizoctonia bataticola*	KF000070	85

Discussion

The current work contributes to study fungi infecting *Lagenaria siceraria* (Molina) Standl in Côte d'Ivoire. Leaves of *L. siceraria* carrying various symptoms were collected in thirty plantations of five localities of Côte d'Ivoire. These symptoms reflect the high parasitic infection caused by fungi on the plant of *L. siceraria*. Overall, five types of symptoms were described. These five types were reported in Bondoukou, Bongouanou and Korhogo, whereas three of them were identified in Alepe and Divo. Indeed, the occurrence of a symptom in a locality would be due to the presence of one or more sources of infection, susceptible hosts and favorable conditions for its development. Similar observations were made by Anahosur (1992).

Fungal species were identified to belong to ten genera and sixteen species. Fungal species localized in the five localities would be the common flora and those localized in only one locality (species of *Diaporthe helianthi* in Bongouanou, *Fusarium solani* in Bondoukou and *Pestalotiopsis* sp. in Divo) would be endogenous of these localities. If some fungi were present in all localities, a lot of them were not. There was no specificity fungal genera and type of symptom described. That could be linked to the fact that a given symptom is not caused by fungi of a genus. That corroborates that the same pathogenic agent can cause a multitude of symptoms as several pathogenic agents can cause similar symptoms (Howell, 2003). Some species of these fungi are responsible of diseases. So to prove that, a connection Koch's postulate should be completed for each pathogen species.

The nucleotide sequences obtained in this study were homologous of those existing in the NCBI GenBank database. So, fungi strains in Côte d'Ivoire are also present in several other localities throughout the world. The majorities of fungi have a cosmopolitan distribution and have been found on most continents and on numerous hosts. Many species called “endophytic” fungi live within plant tissues without causing apparent injury to the host plant, growing as symptomless parasitic fungi (Peixoto and al., 2004; Maheshwari, 2006).

ITS sequences, allowed us to identified *L. siceraria* main species. But, according to Crouch et al. (2006), species diversity will be underestimated when the ITS sequence is used alone and confirm the limited usefulness of ITS for evolutionary studies in genera. In one important way this study confirms something already well known about the ITS sequence.

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