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NEW OR UNUSUAL DISEASE REPORTS

First report of leaf spot caused by *Alternaria argyroxiphii* on African mahogany trees (*Khaya senegalensis*)

Lívia PIMENTA TEIXEIRA[1], Thaissa DE PAULA FARIAS SOARES[1], Leonardo SARNO SOARES OLIVEIRA[2], Sandra Marisa MATHIONI[3] and Maria ALVES FERREIRA[1]

[1] Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, MG, Brazil, 37200-000
[2] Plant Protection, Research and Development, Sinarmas Forestry, Perawang, Riau, Indonesia, 28772
[3] Donald Danforth Plant Science Center, Saint Louis, Missouri, USA, 63132

Summary. *Alternaria argyroxiphii* is reported for the first time as the cause of leaf spot on African mahogany trees (*Khaya senegalensis*). The disease was first observed in African mahogany field plantations in Perdões, Minas Gerais, Brazil, in October, 2012. Morphological characteristics and phylogeny of the pathogen were determined, and pathogenicity assays assessed the species specificity. Leaf spot was the major symptom observed. Phylogenetic analyses of the combined dataset of the *Alternaria* major allergen gene (*Alt a 1*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) showed that the recovered isolates clustered into *Alternaria* sect. *Porri* and much resembled *A. argyroxiphii*, with 99% booststrap support and Bayesian posterior probability of 1.0. This was largely concordant with morphological characteristics (on potato carrot agar) of *A. argyroxiphii*, including large conidia and long beaks. A pathogenicity test, carried out to fulfil Koch's postulates, confirmed *A. argyroxiphii* as a causal agent of leaf spot of *K. senegalensis*.

Key words: leaf disease, phylogenetics, pathogenicity.

Introduction

The genus *Khaya* A. Juss. includes species of trees with high commercial value due to technological characteristics and beauty of the wood they produce. The timber of *Khaya* is rated as one of the best woods for furniture. Mahogony wood is of medium density and pleasant appearance, is stable and has good working properties (Falesi and Baena, 1999; Pinheiro *et al*., 2011). *Khaya senegalensis* (Desr.) A. Juss. (African mahogany) is native to tropical Africa and Madagascar, and was first introduced into Brazil in 1976 (Falesi and Baena, 1999). The first specimen trees were planted at the Brazilian Agricultural Research Corporation (Embrapa Eastern Amazon) in Belém, Pará, and then commercial plantings were established in the states of Pará, Goiás and Minas Gerais (Falesi and Baena, 1999).

The Brazilian commercial plantings of African mahogany were initiated in Brazil due to considerable yield reductions of the native Brazilian mahogany, *Swietenia macrophylla* King (Pinheiro *et al*., 2011). The major factor that reduced production of Brazilian mahogany was the occurrence of pests, such as the mahogany shoot borer (*Hypsipyla grandella* (Zeller). Additionaly, prohibition of exploitation and marketing of native Brazilian mahogany wood in 2000 resulted in high demand for promising alternative wood sources, including African mahogany (Couto *et al*., 2004; Gasparotto *et al*., 2001).

We observed several types of leaf spots on African mahogany trees in October of 2012. The first leaf spot symptom appeared in planting areas located in Perdões, Minas Gerais, Brazil. After detailed examination of the disease symptoms and associated fungal structures, the presence of typical *Alternaria*

Corresponding author: M. Alves Ferreira
E-mail: ferreirama.ufla@gmail.com

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structure was confirmed. *Alternaria* spp., known to cause leaf spots, are a heterogeneous group of saprophytic and plant pathogenic species with wide distributions in temperate and tropical regions. The aim of the present study was to investigate which *Alternaria* species was causing leaf spot symptoms on *K. senegalensis*. To our knowledge, no *Alternaria* fungi causing disease affecting *Khaya* species has been reported to date.

Materials and methods

Symptoms, sample collection, and fungal isolations

Symptomatic *K. senegalensis* leaves were collected from commercial plantations located in Perdões, Minas Gerais, Brazil. Leaf spots containing fungal structures were examined using a dissecting microscope. Typical *Alternaria* structures on symptomatic plants were observed and were transferred to potato dextrose agar (PDA: potato 200 g, dextrose 20 g, agar 16 g, and distilled water 1 L) or potato carrot agar (PCA: potato 20 g, carrot 20 g, agar 15 g, and distilled water 1 L). For these media respectively, diced potato tubers or carrot roots were cooked for 30 min, strained through cheesecloth, the agar was added to the filtrates, and the media were autoclaved at 121°C for 15 min, After inoculation, the media were incubated at 25°C for 14 d. Monoconidial recovered isolates were transferred to 0.85% saline solution or to 15% glycerol, which were then stored at, respectively, room temperature or -80°C. All isolates were kept at the Laboratory of Forest Pathology, Department of Plant Pathology, Federal University of Lavras. Also one representative isolate was deposited in the Coleção Micológica de Lavras (CML).

Morphological characterization of the pathogen

Morphological analyses of the fungal structures were performed using two isolates, designated as PF1 and PF3. They were grown for 7 d in PCA plates, incubated at 25ºC with a 12 h daily photoperiod. Measurements of conidia and conidiophores were made after 7 d. Samples were mounted in 5% lactophenol and 1% lactic acid, and were photomicrographed using differential interference contrast (Nomarsky) microscopy. For the structures measured, approx. 50 observations were recorded per isolate. Colony pigmentation was compared to the colour chart of Rayner (1970).

Pathogenicity assay

For inoculum production, mycelial discs of colonies of *Alternaria* sp. were transferred to plates (9 cm diam.) containing PDA and kept in a growth chamber at 25°C for 15 d. After mycelial growth, the plates were maintained in a chamber at 25 ± 2°C, in black light for 60 h.

Ninety-day-old plants of *K. senegalensis* were transplanted into 3 L capacity pots containing Mec-Plant® growth substrate, supplemented with 6 kg m^{-3} of superphosphate and 3 kg m^{-3} of Osmocote® (19-6-10). Four leaves of each plant were labeled for inoculation, and wounds were made in two leaves of each plant. Three plants per isolate were each inoculated by spraying a spore suspension of 1 x 10^4 conidia mL^{-1} on the leaf surface. Plants were kept in a greenhouse at 20 to 32°C for 25 d, until the first symptoms were visible. Plants sprayed with water were used as controls. Leaves were evaluated by presence or absence of lesions, and diseased leaves were submitted to re-isolation to confirm the presence of the pathogen in infected tissues, and complete by the Koch's Postulates.

DNA extraction, PCR amplification, and sequencing

For DNA extraction from the fungal isolates PF1 and PF3, four mycelial disks of each isolate were transferred to flasks containing 200 mL of malt extract liquid medium (20 g L^{-1} of malt extract). Flasks were incubated on a shaker at 150 rpm at room temperature for 4 d. The mycelial resulting growth was filtered and stored at -20°C. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega), according to manufacturer's instructions. PCR amplification was performed for the glyceraldehyde-3-phosphate dehydrogenase (*gpd*), primers gpd1-CAA CGG CTT CGG TCG CAT TG, gpd2-GCC AAG CAG TTG GTT GTG (Berbee *et al.*, 1999), and the *Alternaria* major allergen gene (*Alt a 1*), primers Alt-for-ATGCAGTTCACCACCATCGC and Alt-rev-ACGAGGGTGAYGTAGGCGTC (Hong *et al.*, 2005). All amplification reactions were performed in a 50 µL reaction volume. PCR conditions for each gene were adjusted according to the respective references provided above. After electrophoresis in 0.8% agarose gels stained with ethidium bromide, PCR products were purified using the High Pure PCR Product Purification Kit (Roche). The purified PCR amplicons were submitted for sequencing at the

Laboratório de Genômica of the Instituto de Biotecnologia Aplicada à Agricultura, Universidade Federal de Viçosa, and Macrogen, Korea.

Phylogenetic analyses

The raw sequence data of the three gene regions were aligned against sequences deposited in GenBank (Table 1) using the BLAST search tool. Sequences of the three gene regions were aligned in MAFFT Online version v. 7.0 (Katoh and Toh, 2010), using the FFT-NS-i (Slow; iterative refinement method) alignment strategy with the 200PAM/K = 2 scoring matrix and a gap opening penalty of 1.53 with an offset value of 0.0. Aligned sequences were then manually corrected when necessary using MEGA v. 5 (Tamura *et al.*, 2011). The likelihood values were calculated, and the best model of nucleotide substitution was selected according to Akaike Information Criterion (AIC) using MrModeltest v. 2.3 (Nylander,

Table 1. Detailed information on the isolates used in this study, their sources and GenBank accession numbers for sequences used in phylogenetic analyses.

Species name	Source	Accession Number	
		Alt a1	*Gdp*
Alternaria argyroxiphii	**PF1**	**KY569271**	**KY569273**
A. argyroxiphii	**PF3**	**KY569272**	**KY569274**
A. alternata	EGS 34-016	AY563301	AY278808
A. arborescens	EGS 39-128	AY563303	AY278810
A. argyranthemi	EGS 43-033	AY563280	AY562400
A. argyroxiphii	EGS 35-122	JQ646432	JQ646350
A. argyroxiphii	PPRI 11971	JQ646434	KJ717966
A. brassicicola	EEB 2232	AY563311	AY278813
A. capsici	EGS 45-075	AY563298	AY562408
A. carotiincultae	EGS 26-010	AY563287	AY278798
A. cheiranthi	EGS 41-188	AY563290	AY278802
A. crassa	DDG Acr1	AY563293	AY278804
A. dauci	ATCC 36613	AY563292	AY278803
A. dauci	CBS 111.38	KJ718673	KJ718005
A. dauci	CBS 106.48	KJ718674	KJ718006
A. destruens	EGS 46-069	JQ646402	AY278812
A. infectoria	EGS 27-193	FJ266502	AY278793
A. japonica	ATCC 13618	AY563312	AY278814
A. longipes	EGS 30-033	AY563304	AY278811
A. macrospora	CBS 106.29	KJ718701	KJ718032
A. macrospora	EGS 50-190	KJ718702	KC584124
A. mimicula	EGS 01-056	AY563310	AY562415
A. panax	CNU086010	JX213299	JF417644

(Continued)

Table 1. (Continued).

Species name	Source	Accession Number	
		Alt a1	*Gdp*
A. petroselini	EGS 09-159	AY563288	AY278799
A. porri	EGS 17-082	KJ718725	KJ718052
A. porri	EGS 48-147	KJ718726	KC584132
A. porri	EGS 48-152	KJ718727	KJ718053
A. radicina	ATCC 96831	AY563286	AY278797
A. selini	EGS 25-198	FJ266504	AY278800
A. smyrnii	EGS 37-093	AY563289	AY278801
A. solani	ATCC 58177	AY563299	AY278807
A. solani	CBS 106.21	KJ718743	KJ718066
A. solani	CBS 111.41	KJ718744	KJ718067
A. sonchi	EGS 46-051	AY563307	AY562412
A. tagetica	CBS 297.79	KJ718759	KJ718080
A. tagetica	CBS 298.79	KJ718760	KJ718081
A. tagetica	EGS 33-081	KJ718761	KC584143
A. tenuissima	EGS 34-015	AY563302	AY278809
A. tomatophila	EGS 42.156	GQ180101	GQ180085
A. triticina	EGS 17-061	JQ646371	AY762958
A. azadirachtae	EGS 46-195	KJ718635	KJ717967
Crivellia papaveraceae	P354.8	JN383502	FJ357299

Abbreviations for sources: ATCC, American Type Culture Collection, Manassas, VA 20108; BMP, B. M. Pryor, Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721; CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands; DGG, D. G. Gilchrist, Department of Plant Pathology, University of California, Davis, CA 95616; EEB, E. E. Butler, Department of Plant Pathology, University of California, Davis, CA 95616; EGS, E. G. Simmons, Mycological Services, Crawfordsville, IN 47933. Sequences of PF1 and PF3 were generated in the present study.

2004). Bayesian Inference (BI) was conducted for the aligned data set of 42 sequences, including the two isolates from *K. senegalensis*, and the outgroup taxon *Crivellia papaveraceae*, using the MrBayes v. 3.1.1 (Ronquist and Heulsenbeck, 2003) with the algorithm of Markov chain Monte Carlo (MCMC) with two sets of four chains (one cold and three heated) and the stop rule option, stopping the analysis at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 1,000; the first 25% of trees were removed. For maximum parsimony analysis (PAUP 4.0b10), gaps were treated as a fifth base, all characters had equal weight, and the heuristic searches used simple stepwise addition and tree-bisection-reconnection. Bootstrap confidence intervals were calculated using 1,000 replicates. Sequences of the two gene regions were analyzed separately, and a combined dataset was then submitted to a partition homogeneity test (PHT) using PAUP 4.0b10 (Swofford, 2002), in order to determine whether the datasets could be combined.

Results

Leaf spot symptoms

The first symptoms of leaf spot in *K. senegalensis* were observed in a commercial plantation with

Figure 1. Leaf spot symptoms caused by *Alternaria argyroxiphii* on *Khaya senegalensis*. (A) Defoliation; (B) Leaf rolling symptoms; (C and D) Symptoms showing colonization mainly in the leaf border.

plants of approx. 1-y-old, in October of 2012. Defoliation symptoms were observed on the top of the plants with lesions on the edges of leaves (Figure 1A). In older basal leaves, a similar pattern of lesions on the edges of leaves was also observed, as well as leaf rolling in most affected leaves (Figure 1B). In young leaves, brown spots in the centres of the lesions surrounded by dark brown edges were also frequently observed (Figure 1C). Typical structures of *Alternaria* (Figure 1D) were observed in all examined lesions.

Pathogenicity assays

The two *Alternaria* isolates (PF1 and PF3) used in the pathogenicity assays caused disease on wounded leaves of *K. senegalensis*, with the first symptoms appearing 15 d after inoculation (Figure 2A). When leaves were not wounded and inoculated with either the two isolates, lesions were first noticed 30 d after inoculation (Figure 2A). After the pathogenicity experiment, the presence of *Alternaria* sp. was confirmed when the fungus was successfully re-isolated from inoculated leaves.

Morphological characterization of the pathogen

The developing colonies at 7 d on PDA (Figure 2C) produced pairs of concentric growth rings. The 15-d-old colonies covering the substrate on a 9-cm diam. plate had light olive colouration, with medium

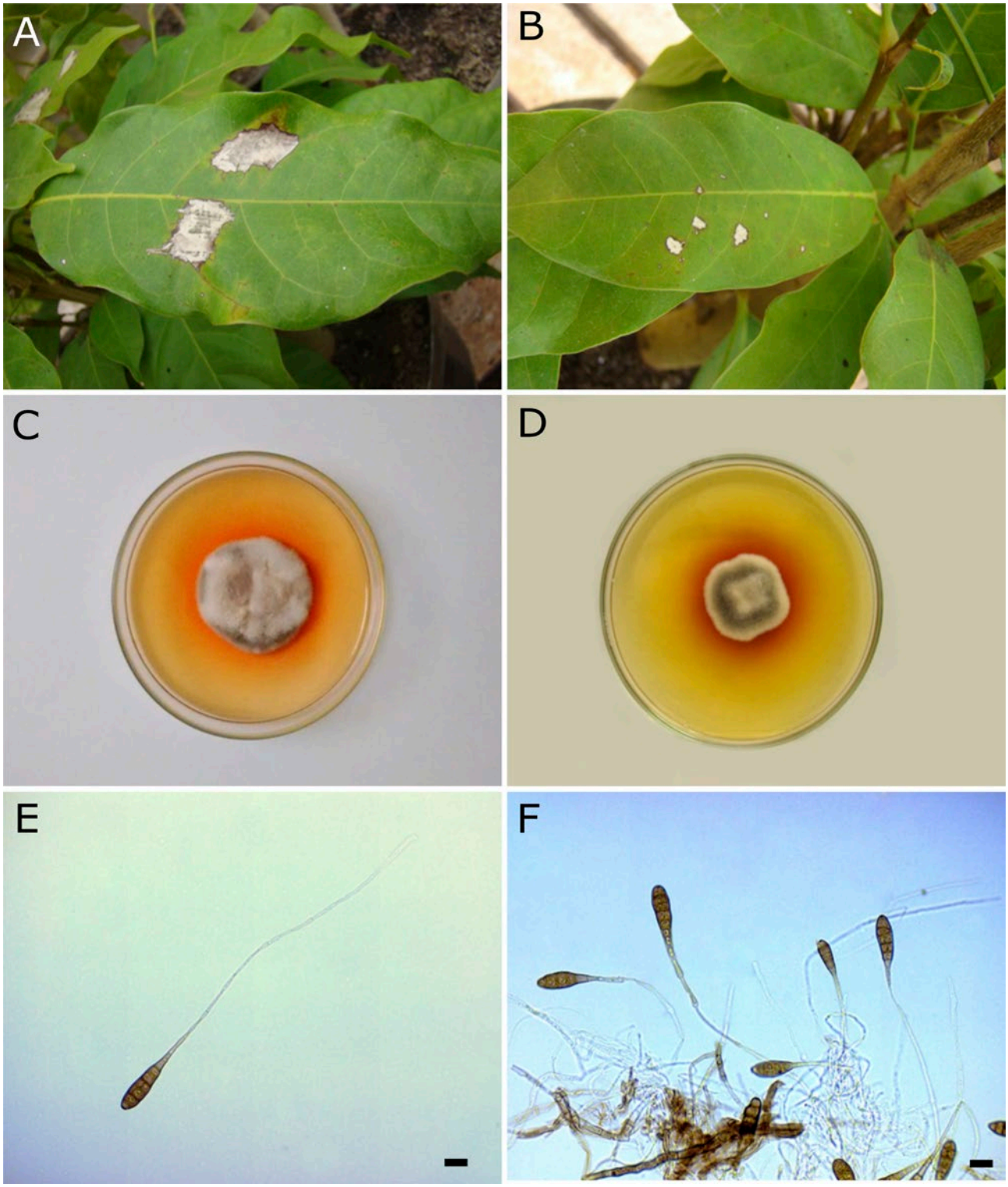

Figure 2. Symptoms of leaf spot caused by *Alternaria argyroxiphii* on *Khaya senegalensis*, and colony morphology and conidia of the pathogen. (A) Lesions on wounded and (B) unwounded inoculated leaves; (C) colony on PCA and (D) colony on PDA; (E and F) conidia. Scale bars = 10 µm.

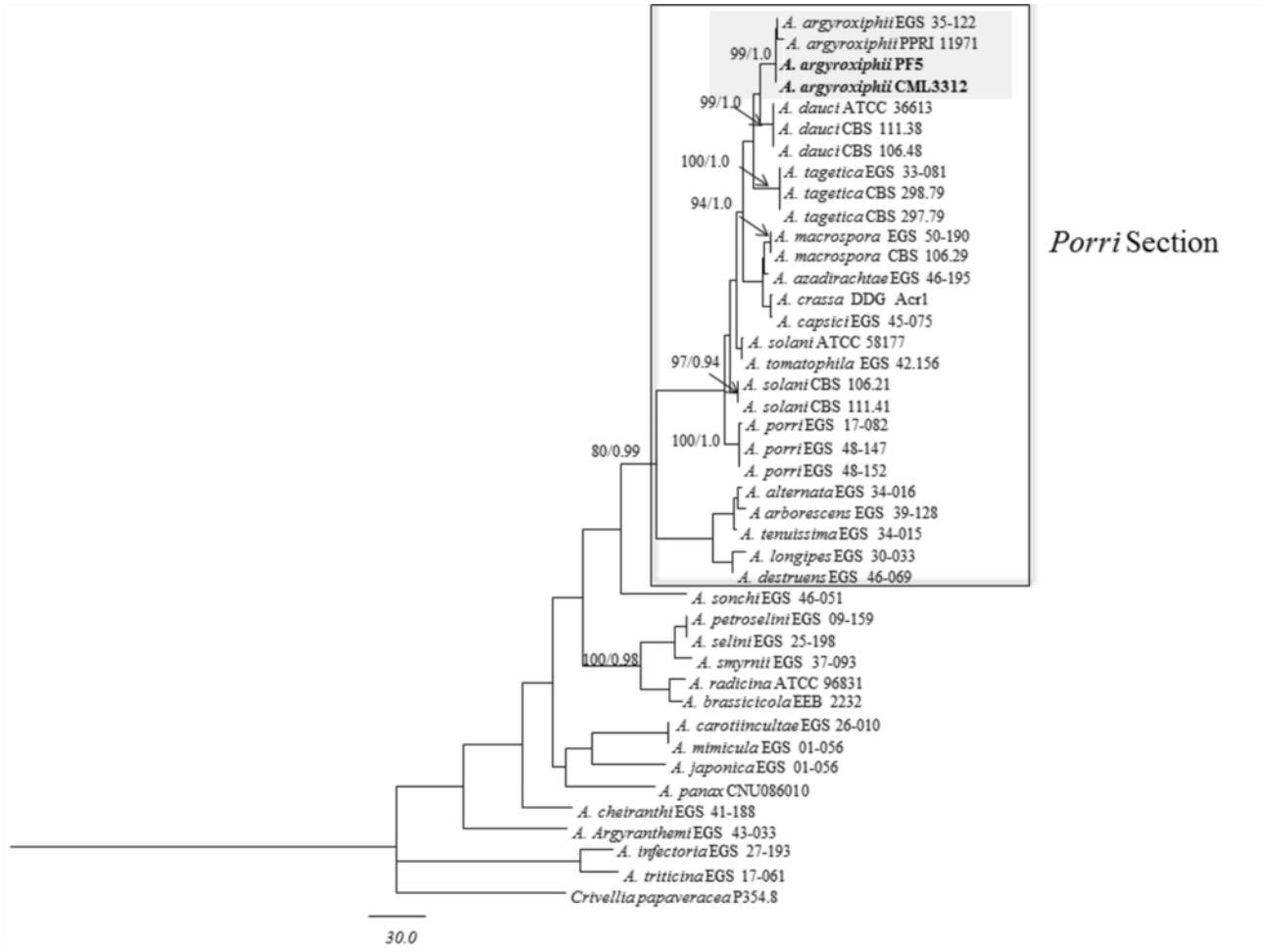

Figure 3. Most parsimonious tree based on the combined dataset of the two sequenced regions (*gpd*, and *Alt a1*) of *Alternaria* spp. Isolates in bold with gray background were isolated from *Khaya senegalensis* and sequenced in the present study. Bootstrap values greater than 70%, posterior probability values greater than 80% are indicated above the branches. *Crivellia papaveraceae* was used as an outgroup taxon. Scale bar indicates expected changes per site.

olive zones. The mycelium was superficial with few aerial hyphae in the centre of each colony, and the mycelium was light olive, with branched and septate hyphae. Conidiophores were not observed when the fungus was grown on PCA. Fully developed conidia were ellipsoid, olive brown, tapering almost abruptly into long (Figure 2D) or filiform beaks of uniform diameter. The conidia were solitary or in short chains (two to six conidia) on the host. Each conidium had five to nine transverse and zero to three longitudinal septae. Conidium dimensions were approx. 25–38 × 5–12 μm, and the conidial beaks varied from 95–302 μm in length. The beaks were of uniform diameter of 1–2 μm.

Phylogenetic analyses

For the *Alt* a1 region, an alignment of 478 characters resulted in 228 constant characters and 76 parsimony non-informative characters and 174 parsimony informative characters. For the *gdp* region, an alignment of 585 characters resulted in 391 constant characters and 54 parsimony non-informative characters and 140 parsimony informative characters.

Sequences from isolates based on morphological characters representing the sequence diversity of the genus *Alternaria* were selected for two gene phylogenetic analyses combining *gdp* and *Alt a1* sequences. The partition homogeneity test performed

for the two regions of interest resulted in *P*=0.20, which is considered strong enough to allow combination of data (Cunningham 1997). An analysis of the combined dataset containing 42 sequences produced nine trees of 872 steps (Consistency index = 0.648, Retention index = 0.813, and Composite index = 0.527), and the trees were similar in topology. One tree was therefore selected for illustration (Figure 3). For the combined data set of *gdp* and *Alt a1* regions, an alignment of 1,062 characters resulted in 618 constant characters and 130 parsimony non-informative characters, and 314 parsimony informative characters. The two *Alternaria* isolates PF1 and PF3 from *K. senegalensis* grouped together with *A. argyroxiphii* E.G. Simmons, with high bootstrap support (99%) and high values of posterior probability (1.00).

Discussion

Phylogenetic analyses of the combined dataset for *gdp* and *Alt* a1 sequences produced a robust dendrogram, which allowed the isolates investigated in this study in group to *A. argyroxiphii.* High values of bootstrap and posterior probability allowed distinction from other *Alternaria* species. *Alternaria argyroxiphii* grouped with other *Alternaria* species of the *Porri* section (Woudenberg *et al*., 2013). Phylogenetic analyses also showed that *A. argyroxiphii* is closely related with others species of the *Porri* section, and was phylogenetically distinct from other sections based on sequence analyses of the combined dataset of the *gdp* and *Alt a1* gene sequences.

The *A. argyroxiphii* isolates were distinct from other species based on morphological characteristics (Simmons, 2007). The solitary or short chains of conidia as well as conidium shape and the long conidium beaks resemble those of *A. alternata*. On the other hand, *Alternaria dauci* (J.G. Kühn) J.W. Groves & Skolko (100–350 μm), *A. solani* (80–118 μm), *A. tomatophila* E.G. Simmons (96–166 μm), *A. crassa* (320–460 μm), *A. porri* (Ellis) Cif (95–160 μm), and *A. azadirachtae* E.G. Simmons & Alcorn (200–380 μm) also have long conidium beaks (Simmons, 2007), similar to those of *A. argyroxiphii* isolates from this study (95–302 μm). According to Simmons (2007) the length range for the conidium beaks in A. *argyroxiphii* is 100 to 200 μm, in concordance with the size observed in the present study.

Woundenberg *et al.* (2013) divided the genus *Alternaria* into 24 sections based on molecular and morphological data. According to Wounderber *et al.* (2014), *Alternaria* section *Porri* is the largest section, containing almost all *Alternaria* species with medium to large conidia and long conidium beaks, and some of these species are important plant pathogens. The same authors worked with sequences of four genes from *Alternaria* isolates, and distinguised 63 species, ten of which were newly described in the section *Porri*, and 27 species names were synonymised, for example *A. crassa* and *A. capsici*. Among these species are some important plant pathogens, which have grouped along with *A. argyroxiphii*, and these include *A. porri*, *A. solani* and *A. tomatophila*. *Alternaria porri* causes purple blotch in onion which is a very destructive disease worldwide (Abo-Elyousr *et al*., 2014). *Alternaria argyroxiphii* was first described on *Argyroxiphium* sp. and later on *Ipomoea batatas* (sweet potato). *Alternaria solani* causes early blight in potato, causing typical necrotic lesions in the aerial portions of the infected plants (Lourenço *et al*., 2009), and has worldwide distribution (Horsfield *et al*., 2010). *Alternaria tomatophila* is known for causing early blight of tomato, attacking the leaves, stems and fruits. This airborne pathogen also has worldwide distribution, affecting mainly exposed field crops (Rodrigues *et al*., 2010). *Alternaria dauci* causes leaf spot of carrot, which is a common disease (Dugdale *et al*., 2000). There are, however, few reports of *Alternaria* species from commercial trees. In Meliaceae, *Alternaria* was described in *Azadirachta indica* A. Juss, and was named as *A. azadirachtae* (Simmons, 2007), which is also a member of section *Porri* (Wounderber *et al*., 2014).

Little is known about diseases on *K. senegalensis*, although commercial plantations have been widely established in Brazil. This is the first study showing that leaf spot of *K. senegalensis* is caused by *A. argyroxiphii* in plantations in Minas Gerais State in Brazil. In order to better understand the disease progress and potential economic impacts of this pathogen in the field, additional studies with other isolates are strongly recommended. Also, it is crucial to determine whether this pathogen is spreading to other mahogany plantations in other Brazilian States, as well as the potential spread to other mahogany species.

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