Research Papers

First report of *Didymosphaeria rubi-ulmifolii* associated with canker and dieback of apple trees in southern Ethiopia

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Summary. Cultivation of apple trees in the highlands of Ethiopia began in 1955. In 2014, blistering of the bark due to cankers on the main stems mostly below the grafting points, followed by dieback and eventually death of apple trees, was observed in apple orchards in the Hadiya Zone in Ethiopia. This study aimed to identify the causal agent of canker and dieback symptoms on the apple trees. Symptomatic trunks from 20 trees (ten per cultivar) were sampled. Isolations were performed from ten trunks (five per cultivar). Fungus colonies with similar cultural features were obtained from all the samples, and the morphology of a representative isolate was characterized. Phylogenetic analyses of the concatenated internal transcribed spacers 1 and 2 and 5.8S rRNA gene, large subunit and actin gene regions confirmed the identity of two isolates as *Didymosphaeria rubi-ulmifolii*. Pathogenicity was confirmed for one isolate by inoculations of healthy branches of 'Anna' and 'Dorsett Golden' apple trees resulting in lesion formation, and subsequent re-isolation of the inoculated fungus. This study is the first report of *D. rubi-ulmifolii* associated with dieback of apple trees. This pathogen caused death of more than 26% of apple trees in one commercial orchard, and could cause severe losses for smallholder apple growers in Ethiopia. Future studies are required to assess the magnitude, distribution and management options of this economically important canker disease in Ethiopia.

Keywords. *Malus pumila*, *Paraconiothyrium brasiliense*, stem canker.

INTRODUCTION

Malus pumila Mill. (Rosaceae; syn. *M. domestica* Borkh.) is native to southwest Asia (Hedberg *et al.*, 1989), and apple is the fourth most important horticultural fruit crop in the world and is by far atypical temperate fruit tree that can reach 8–12 m high (Hedberg *et al.*, 1989; Tromp, 2005; Bekele-

Tesemma, 2007; FAO, 2010). Although originating and common in temperate regions, apple trees are grown at 2,300 m above sea level in many tropical and subtropical regions, and have become an increasingly important source of income in Ethiopia (Erez, 2000; Bekele Tesemma, 2007). Commercial apple cultivars are grafted on seed grown rootstocks.

Around 1955, missionaries established apple orchards (medium and high-chilling cultivars) in the Chencha highlands of the Southern Nations and Nationalities People (SNNP) Regional state of Ethiopia (Fetena *et al.*, 2014). This region has minimum temperatures of 11 to 13°C and maxima of 18 to 23°C, 900 to 1200 mm annual rainfall, and altitudes of 2300 to 3250 m (Hailemichael, 2006). In recent decades, government and non-government organizations have engaged in propagation of low-chilling grafted apple trees and rootstocks from abroad to improve the livelihood and income of the rural communities in this region. Approximately 138, 000 plants (105,000 grafted apples and 33,000 rootstocks) were imported between 1998 to 2007 (Sisay, 2007).

As apple production has expanded, the impacts of biotic agents have increased. Assessments in Chencha and Bonke districts showed that apple scab, powdery mildew, green aphids, scale insects and green plant bugs were the most serious apple diseases and pests (Fetena and Lemma, 2014; Fetena *et al.*, 2014). In addition to significant losses in the production from 'Bond's Red Royal Gala', 'Crispin' and 'Jona Gold', ' Royal Gala' was replaced by 'Crispin' in Chencha district, due to susceptibility of 'Royal Gala' to apple scab (Fetana and Lemma, 2014). White root rot, caused by *Rosellinia necatrix* Berl. ex Prill., also caused death of mother apple trees and grafted plants. The MM106 rootstock, which was once considered as a superior rootstock for Chencha district, was found to be susceptible to crown rot on poorly drained soils (Fetena *et al.,* 2014).

In early 2014, extensive damage due to cankers and dieback was observed on apple trees at Gibagri Farm, located in SNNP Regional State in Ethiopia. The study described here was carried out to identify the causal agent of this disease.

MATERIALS AND METHODS

Sample collection, isolation and characterization of pathogenic fungus

The Foke orchard of Gibagri Farm PLC, is situated at Olewa Peasant Association in Gibe District, Hadiya Zone in SNNPR in Ethiopia, about 260 km from the capital city Addis Ababa. The district has a wet *Woina*

Dega climate, with annual rainfall of 1100 mm, with Nitosol reddish loamy and deeply structured soil (pH 5 to 6), and altitude of 2,000 m. Gibagri Farm PLC grows the apple 'Anna', 'Princesa' and 'Dorsett Golden' imported from Spain in 2009 and 2012, on 14 ha of land. Field survey was conducted in Foke Orchard in December 2015. Symptomatic trunk samples from 20 apple trees, ten each from 'Anna' and 'Dorsett Golden' grafted on rootstocks M7, MM111 and MM106, were systematically sampled and collected, as described by Cloete *et al.* (2011). These samples were separately packed in paper bags, labeled and brought to the Forest Protection Laboratory of the Central Ethiopia Environment and Forest Research Center (CEE-FRC), Addis Ababa. In the laboratory, samples were kept at 4°C in a refrigerator before further analysis.

Three to four segments of wood fragments were collected from the margins between necrotic and healthy tissues of five symptomatic apple trunks per cultivar. The fragments were excised into approx. 2×2 cm pieces. These were then surface sterilized in 70% ethanol followed by 5% sodium hypochlorite, each for 2 min., and then rinsed in sterilized distilled water. The tissue pieces were then air-dried, each aseptically halved using a sterile knife, and then placed in 90 mm diam. Petri dishes containing malt extract agar (MEA, Oxoid Ltd) amended with 100 mg of streptomycin sulphate. In addition, cross-sections cut from stems showing disease symptoms on both sides were placed in 19.5 mm diam. Petri dishes with moistened double-layered filter paper to induce pycnidium formation. The filter papers were regularly monitored and moistened with distilled water. All dishes were placed on a laboratory bench and incubated in 12 h light and 12 h dark, at 20 to 25°C room temperature. Emerging mycelia and pycnidia were transferred to potato dextrose agar (PDA; Oxoid Ltd.) amended with streptomycin sulphate.

The growth characteristics of the isolated fungus were determined based on mycelium growth rate and colony colour on PDA after 7 d incubation at 25°C in darkness. Pycnidium formation in a moist chamber was also examined under a dissecting microscope. Sporulation of the isolated fungus was induced by transferring mycelia from the margin of 7-d-old pure cultures onto 20 g L^{-1} water agar supplemented with sterilized pine needles (Su *et al.*, 2012). Based on morphological similarities, a fungus isolate obtained from 'Anna' was designated as AY-1 and an isolate from 'Dorsett Golden' was designated as AY-2. Dimensions (length and width) and shape of 50 conidia from the AY-1 isolate were measured and described at 400× magnification using an Olympus BX 63 camera-mounted microscope. Conidiomata

were sectioned (10 μm thick) with a freeze microtome and measured at 400× and 1000× magnification using a Nikon Eclipse E600 compound microscope with a Nikon DMX1200C digital camera attachment. Ten conidiomata and 30 conidiogenous cells were measured. Cultures of both isolates were deposited at the Ethiopian Biodiversity Institute and Forest Protection laboratory of CEE-FRC.

Molecular characterization and phylogenetic analysis

DNA was extracted from 4-d-old cultures of isolates AY-1) and AY-2), and the regions were amplified of the internal transcribed spacers 1 and 2 and 5.8S rRNA (ITS), 28S rRNA the large subunit (LSU) and the partial actin (ACT) genes. The primers used for these regions were V9G/LS266 for ITS, LROR/LR6 for LSU and ACT512F/ACT783R for ACT. The PCR conditions and sequencing were as described by Samson *et al*. (2010), and were carried out at the Westerdijk Fungal Biodiversity Institute (CBS), AD Utrecht, the Netherlands. Consensus sequences were made from the forward and reverse sequences, and were lodged in Gen-Bank (MK167444 to MK167448). For phylogenetic analysis, sequences generated by Ariyawansa *et al*. (2014) were added as reference sequences. *Kalmusia longisporum* CBS 582.83 was included as the out-group. Newly generated sequences of the three gene regions were aligned separately using the E-INS-i algorithm in the MAFFT plugin of Geneious R9 software (Katoh and Standley, 2013), visually inspected for obvious alignment errors, and concatenated in Geneious. Maximum likelihood analysis was performed in PhyML-mpi (Guindon *et al*., 2010) under the best-fit model (HKY+I+G). Branch support was calculated from 100 bootstrap replicates for the concatenated dataset.

Pathogenicity test

The AY-1 isolate was used in a pathogenicity test conducted on apple trees at Foke apple orchard, in November 2017. Four apple trees each of 'Anna' and 'Dorsett Golden' from different rows of the farm were randomly selected for the inoculation trial. On each tree, two healthy and oppositely situated branches with mean diameter of 11 mm (range 10 to 14 mm) were selected. One branch was inoculated with a mycelium plug of the fungus and the other branch was treated with a sterile PDA plug (as the negative control). In total eight branches were inoculated with the fungus and eight were treated with sterile PDA plugs. The methods of Luque *et al*.

(2006) and Sami *et al*. (2014) were adopted with modifications. The bark of each of the selected branches was cleaned with 70% ethanol before inoculation. Holes (4 mm diam.) were made in the branches using a cork borer. Mycelium plugs (4 mm diam.) cut from colony margins in 10-d-old PDA cultures were placed on the wounds with mycelium facing the host cambium. A 4 mm diam. sterile PDA plug was similarly placed on one of the alternative branches of each tree as the control treatment. Inoculated wounds were wrapped with Parafilm® (American National Can) to prevent contamination and desiccation of the inoculated areas. After 8 weeks, all inoculated branches were destructively sampled and brought to CEE-FRC Forest Protection laboratory. The lengths of developed lesion's development in the cambium of inoculated branches were measured after removing the bark of each branch surrounding the inoculation point. Re-isolations from symptomatic cambium tissues beyond the areas of inoculation were performed onto PDA. ANOVA analysis of the lesion length data was done using XLStat. Differences in mean lesion lengths formed on the two cultivars were assessed with Fishers least significant differences (LSD) at $P \leq 0.05$.

RESULTS

Disease symptoms and severity in the field

Based on the documents available in Gibagri Farm PLC, 31,500 grafted apple trees were imported from Spain between May 2009 and July 2012. Of these, 384 trees were rejected due to their poor rooting and dry wood. As well, 8,186 apple trees were eradicated from the nursery and orchard sites at the farm up to November 2017, due to development of canker symptoms. The trees showed blistering of the bark of the main stems mostly below the grafting points, and eventually produced exudates, lanceolate leaf development, abscission of blossom and fruits, and dieback that progressively led to the death of the trees (Figure 1a). Cross-sectional cuts of stems of symptomatic trees showed either circular or triangular discolorations in the wood tissues (Figure 1b and 1c).

Morphological characteristics of fungi isolated fungi on PDA

Morphology of the fungi isolated from symptomatic samples of the two host cultivars was the same. Colonies on PDA were whitish with short woolly texture (Figure 2a). Black conidiomata formed on the incubated wood
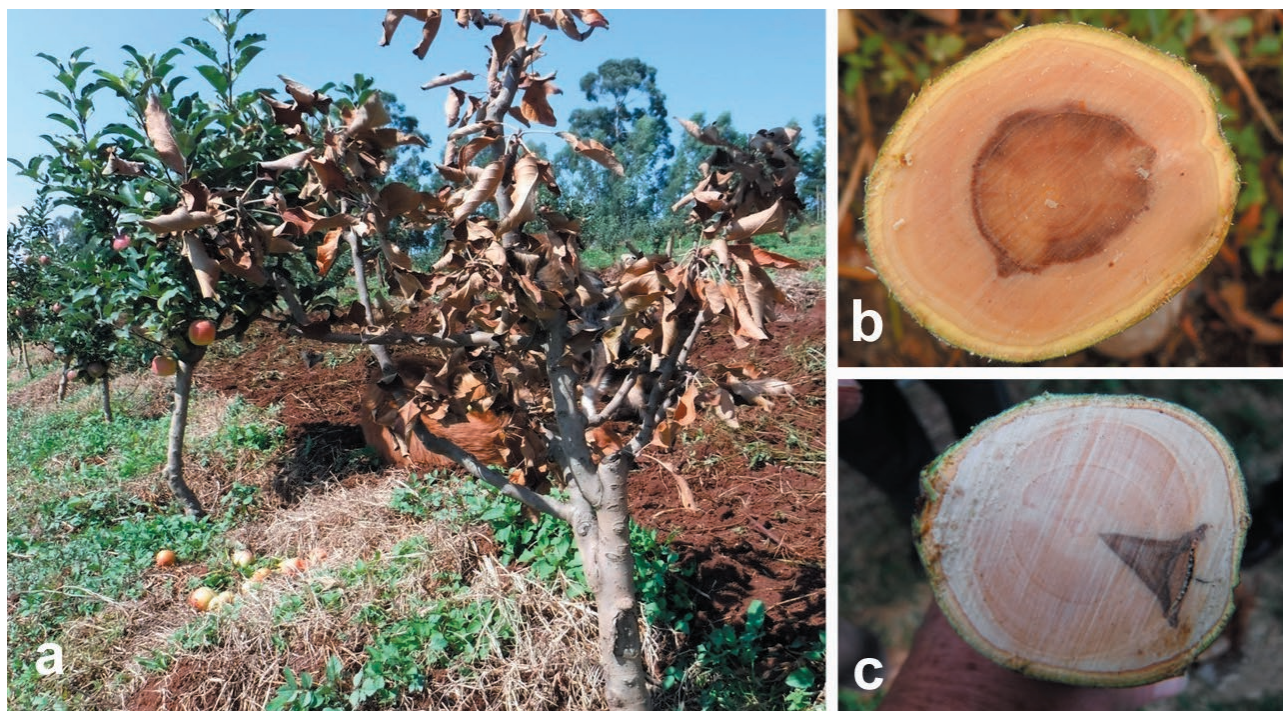
Figure 1. Apple tree at Foke Farm showing dieback symptoms (a) and main stem cross section symptoms (b and c).

samples, and fungus structures are illustrated in Figure 2. Conidiomata on PDA were superficial or immersed in the agar, were dark brown to black, and were (-316)414-659(-673) μm wide and (-303)494-584(-776) μm tall. They were mostly single (sometimes multiple) and clumped together (Figure 2b), with short necked ostioles. And were 75–180 μm long and 87-256 μm wide at the base. The conidiomata walls had textured outer layers 21–42 μm thick with thin, dark brown cells, and were lined with inner layers of hyaline globose cells 16–32 μm thick (Figure 2c to e). The surfaces of conidiomata walls were covered with dark brown hyphae. Conidiogenous cells were discrete or assembled into protruding masses, and were indeterminate and phialidic, formed from the inner cells all over the conidiomata walls, and were hyaline to pale yellow, and broadly ampulliform to globose (Figure 2f), with distinct periclinal thickening (Figure 2g). Phialide collarettes mostly absent, occasionally with visible annelations, and measured (-3)4-6(-8) μm × (-1.5)2-2.5(-3) μm (Figure 2h). Conidia were ellipsoidal to short-cylindrical, rounded at both ends, 1-celled, olivaceous, and 3-4(-4.8) μm × (-1)1.5-2(-2.8) μm (Figure 2i). The morphological characteristics were similar to those described for *Paraconiothyrium brasiliense* Verkley (Verkley *et al.*, 2004; Paul and Lee, 2014). This species was synonymised with *Didymosphaeria rubi-ulmifolii* Ariyawansa, Erio Camporesi & K.D. Hydeby

Ariyawansa*et al.* (2014b), based on phylogenetic analyses. The sexual stage as described by Ariyawansa*et al.* (2014a) was not observed in the present study.

Molecular characterization and phylogenetic analysis of fungus isolate

Phylogenetic analyses of the concatenated ITS, LSU and ACT gene regions confirmed the identity of isolates AY-1 and AY-2 as *Didymosphaeria rubi-ulmifolii* (Figure 3). Both isolates grouped together with *D. rubi-ulmifolii sensu stricto*, with a 99% bootstrap support (CBS 100299, the type strain of *P. brasiliense*). The *D. rubi-ulmifolii sensu lato* subclade did not have significant support, only an internal cluster of three *D. rubi-ulmifolii sensu lato* isolates were associated with the present study's two isolates (bootstrap support of 68%).

Pathogenicity test

Prominent brown lesions caused by the fungus after inoculation were observed on the branches of 'Anna' and 'Dorsett Golden' apple trees (Figure 4). For 'Anna', the lesions lengths were from 23 to 86 mm (mean = 54 mm; *n* = 4). For 'Dorsett Golden', the lesion lengths were from 26 to 46 mm (mean =32 mm; *n* = 4). Mean lesion
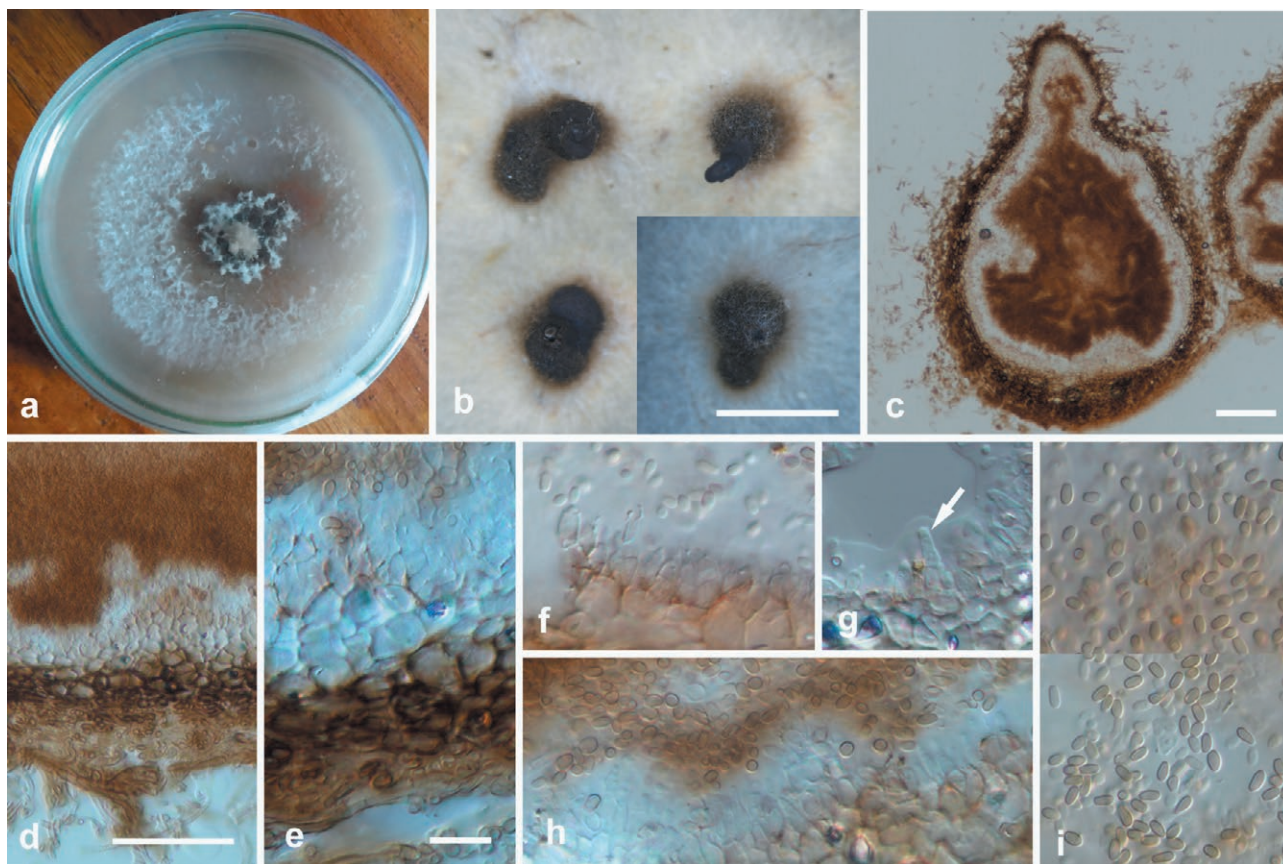
Figure 2. Cultural and morphological structures of *Didymosphaeria rubi-ulmifolii* (isolate AY-1). After 7 d growth on PDA (a), conidiomata with oozing conidia (b), cross section through a conidioma illustrating the neck (c), cross sections through conidioma wall (d and e), conidiogenous cells (f to h), arrow indicating periclinal thickening and conidia (i). Scale bars: in b = 1000 μm; c = 100 μm; d = 50 μm; e to i = 10 μm.

lengths for the two cultivars were not significantly different ($P = 0.175$). Two of the control branches (one branch each from the two cultivars) had slightly pale brown discolorations at the inoculation sites, which did not exceed 4 mm in length, and no *D. rubi-ulmifolii* was isolated from these lesions. Re-isolation of the same fungus (colony characteristics described above) from each of the pathogen inoculated branches confirmed the pathogenicity of *D. rubi-ulmifolii* on apple trees.

DISCUSSION

This study has confirmed that the trunks of *M. pumila* trees at Foke farm were infected by *D. rubi-ulmifolii* (syn. *Paraconiothyrium brasiliense*). This pathogen caused gradual decline and dieback of apple trees and decreased growth and production of fruit in the affected orchards. The dieback caused by this pathogen was so severe that the orchard owner decided to remove all of the apple trees and plant a different crop.

Previous studies showed that *D. rubi-ulmifolii* has been isolated from a number of plant species, including: fruits of *Coffea arabica* in Brazil; *Ginkgo biloba*, *Pinus tabulaeformis* and leaves of *Pinus glauca* in Canada; *Alliaria petiolata* in the USA; marine fish in China; wetland surface water in Japan; discoloured wood of a living *Platanus acerifolia* tree in Italy; South African peach, nectarine, and plum trees; and Chinese Maple leaves in Korea (reviewed in Paul and Lee, 2014). *Didymosphaeria rubi-ulmifolii* was reported to cause disease on one-year-old commercial apple trees and was also found causing latent infections in certified nursery trees in South Africa (Havenga *et al.*, 2019). The present paper is, therefore, the second report of canker disease on apple trees caused by *D.rubi-ulmifolii*, and the first report in Ethiopia. The potential pathogenicity of *D. rubi-ulmifolii* on detached apple shoots was shown by Cloete *et al.* (2011), who inoculated the fungus isolated from pears onto apple shoots, and showed it to be pathogenic causing significant lesions.
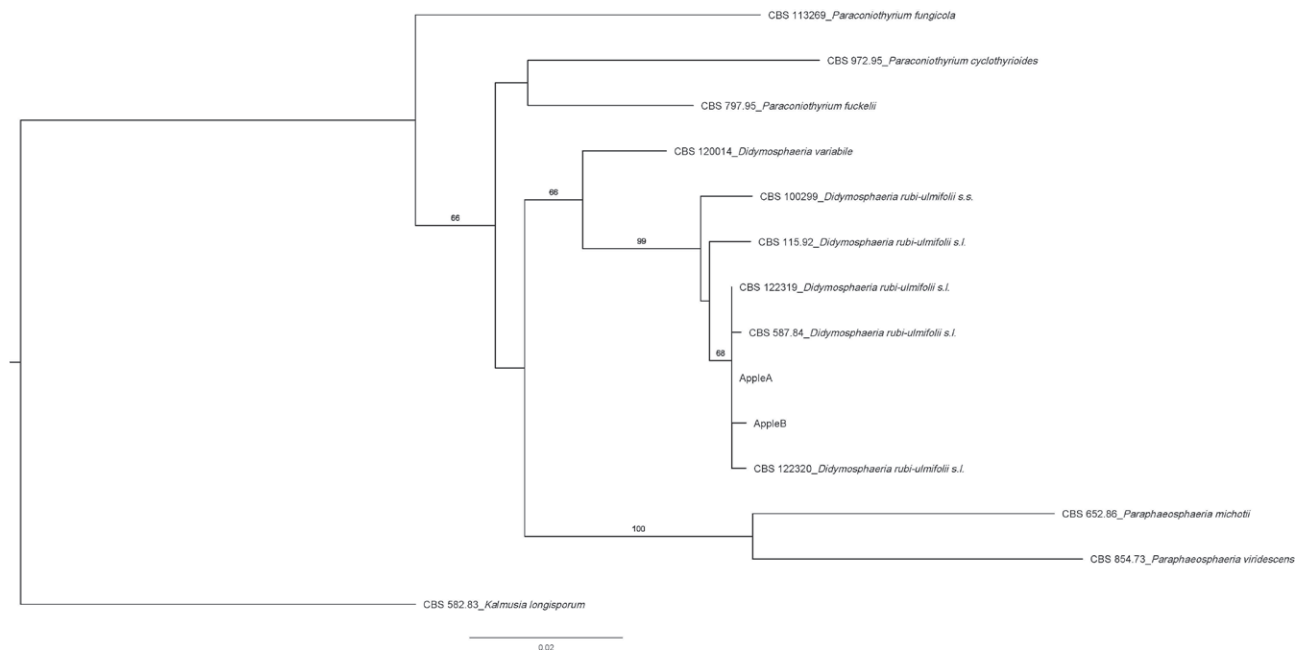
Figure 3. Maximum likelihood phylogeny of the selected genera within *Didymosphaeriaceae*, as estimated from concatenated alignments of the internal transcribed spacers 1 and 2 and 5.8S rRNA gene (ITS), the 28S rRNA large subunit gene (LSU) and the partial actin (ACT) gene regions. Maximum likelihood bootstrap percentages are indicated at the nodes. Support values less than 50% bootstrap were omitted. *Kalmusia longisporum* CBS 582.83 was included as the outgroup.

The main reasons behind the serious damage from *D. rubi-ulmifolii* on apple trees may be a combination of cultivar susceptibility and suitable environmental conditions (high air humidity and low soil pH) for the pathogen. Low soil pH causes stress to apple trees. Due to the slow development of symptoms, the cause of the disease was not initially attributed to fungal canker. *Didymosphaeria rubi-ulmifolii* was isolated from one farm and the incidence of the fungus across other apple-growing districts of Ethiopia has not been established.

In conclusion, in addition to the updating of quarantine measures required for the importation of trees harbouring latent quarantine pathogen infections, future studies are required to determine the magnitude, distribution and consequences of this pathogen on the cultivation and production of apple trees in Ethiopia. This information will assist the livelihoods of the rural communities in different apple production of this country.

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Figure 4. Brown lesion caused by *Didymosphaeria rubi-ulmifolii* (isolate AY-1) on a branch of apple 'Anna', and non-inoculated (control) branch, 8 weeks post inoculation.

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