Vol. 21(1), pp.6-15, January 2022
DOI: 10.5897/AJB2020.17297
Article Number: 179EED968576
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Molecular and morphological identification of fungi causing canker and dieback diseases on *Vangueria infausta* (Burch) *subsp. rotundata* (Robyns) and *Berchemia discolor* (Klotzsch) Hemsl in lower Eastern Kenya

Susan Karani[1*], Jane Njuguna[1], Steven Runo[2], Alice Muchugi[1,4], Joseph Machua[1*] and Phoebe Mwaniki[3]

[1]Department of Biosciences and Biotechnology, Kenya Forestry Research Institute, P. O. Box 20412-00200, Nairobi, Kenya.
[2]Department of Biochemistry, Microbiology and Biotechnology, Kenyatta University, P. O. Box 43844-00100, Nairobi, Kenya.
[3]Genetic Resources Unit, World Agroforestry (ICRAF), United Nations Avenue, Gigiri P. O. Box 30677, Nairobi, 00100, Kenya.
[4]Forage Genebank, International Livestock Research Institute (ILRI), P.O. Box 5689, Addis Ababa Ethiopia.

Received 10 December, 2020; Accepted 29 November, 2021

Drought-tolerant multipurpose fruit trees *Vangueria infausta* (Burch) subsp. *rotundata* (Robyns) and *Berchemia discolor* (Klotzsch) Hemsl are native to Kenya. These fruit tree species are suitable for dryland agroforestry and support local communities with food, medicine, fodder and other necessities. Reports by the local communities indicate that the two species suffer from diebacks and cankers. The aim of this study was to identify the fungi associated with *V. rotundata* and *B. discolor* and determine the cause of diebacks and cankers symptoms observed. Samples were collected from two sites (Tiva and Ikanga) in Kitui County and one site (Mkange) in Makueni County. Fungal isolations were carried out by incubating the samples on malt extract agar media supplemented with Streptomycin Sulphate. Morphological identification grouped the fungal isolates into 7 clusters. *Botryosphaeriaceae* and *Nectriaceae* had the highest frequency of occurrence (32.7 and 30.5%) respectively. DNA was extracted from pure fungal cultures, amplified and sequenced. Phylogenetic analysis of DNA sequences clustered the fungal isolates into seven families; *Botryosphaeriaceae, Sporocadaceae, Nectriaceae, Trichosphaeriaceae, Pleosporaceae, Diaporthaceae* and *Glomerellaceae.* Using Koch's postulates, this study showed that isolates of *Botryosphaeriaceae* within the genera *Lasiodiplodia, Alanphillipsia* and *Dothiorella* are pathogenic to *B. discolor* and other indigenous agroforestry species due to their ability to cause similar symptoms to those observed in the field. This is the first study to investigate the fungal flora linked to *V. rotundata* and *B. discolor* dieback and canker diseases.

Key words: *Vangueria rotundata*, *Berchemia discolor*, *Botryosphaeriaceae, Nectriaceae* canker, dieback, DNA and ITS primers.

INTRODUCTION

Vangueria rotundata and *Berchemia discolor* are drought tolerant multipurpose trees with potential of providing medicine, food and other commodities to drylands communities of Kenya. They are candidates for dry land agroforestry due to their ability to withstand a wide range of temperature and rainfall regimes. *Berchemia discolor* can withstand a temperature range between 14-30°C and an annual rainfall of between 200-1400 mm. On the other hand, *V. rotundata* can withstand temperatures between 12-36°C and an annual rainfall of between 700-1500 mm (Maundu 1999). The two fruit trees are of great importance during the famine and crop failure as their fruits provide a wide range of nutrients such as carbohydrates, vitamins and proteins for people residing in the arid and semi-arid areas (Feyssa et al., 2012; Eulalia et al, 2015). Cheikhyoussef et al. (2010), Maroyi (2018) and Ramavhale et al. (2018) pointed out the medicinal and nutritional value of the two species as well as their importance as food, feed and source for construction material. However, local communities have reported decreased fruit production and tree death on trees with diebacks and cankers. Moreover, Njuguna et al. (2011) had previously reported that canker and dieback were threatening the cultivation of *Grevillea robusta* in the arid and semi-arid areas.

The World Agroforestry Centre (ICRAF), in conjunction with the Kenya Forestry Research Institute (KEFRI), have initiated domestication of wild fruit trees over the years. Tree Genebanks have been established in order to promote the conservation of key indigenous tree species such as *V. rotundata* and *B. discolor* across the country (Muok et al, 2000). These Genebanks also provide healthy germplasm (Kitonga et al., 2020) for utilization in breeding programs so that high-quality tree varieties with desired traits such as drought tolerance and disease resistance can be developed for improved productivity. Moreover, domestication enhances ecosystem sustainability, improved livelihood, nutrition security and poverty reduction. (Jamnadass et al., 2019; Miller et al., 2020). However, domestication and cultivation of indigenous fruit trees face many challenges, including diseases and pests, overexploitation, low acceptance and insufficient research on their growing (Gachie et al., 2020; Omotayo and Aremu, 2020). Plant diseases play a crucial role in agriculture, horticulture and forest ecosystems and have become a worldwide concern on food security and climate change (Agrios, 2005). Diseases of plants are caused by a wide range of biotic and abiotic factors (Nazarov et al., 2020), however, disease will only manifest if the host is in an intimate relationship with a virulent pathogen and in favourable environmental conditions (Agrios, 2005). Diseases affect the productivity and vigor of the trees leading to reduction in their health, quality and quantity of tree production and causing losses that may amount to billions of US dollars (Jeger et al., 2021; Thambugala et al., 2020). However, diseases caused by biotic factors may overlap with those caused by abiotic factors (Pernezny et al., 2008) and it is therefore essential to correctly determine the actual cause based on an appropriate observation of signs and symptoms present in the field and to finally carry out laboratory isolations and diagnostics.

Major biotic factors that cause plant diseases include fungi and fungal like organisms, bacteria, viruses, nematodes and parasitic higher plants. However, fungi have been described as the most dominant causal agents of plant diseases globally (Hariharan and Prasannath, 2021). Fungal pathogens are ecologically, morphologically and genetically diverse, thus making their identification to species level quit challenging (Lücking et al., 2020; Raja et al*.,* 2021; Tekpinar and 2019) Monitoring the health of plants and diagnosing diseases of plants is crucial in controlling diseases (Nalla and Kalmer, 2020).

Fungi are usually identified both morphologically and by the use of molecular techniques. Although morphological identification is important, it can sometimes be problematic, especially for untrained mycologists and closely related genera (Raja et al*.,* 2017). The use of molecular techniques with morphological traits offers a better fungal identification (Bernreiter, 2017, Dayarathne et al., 2020). Moreover, molecular identification is a standardized method that is fast and accurate to species-level identification based on gene phylogenies (Das et al., 2015; Dulla et al., 2016). Subsequently, the ITS operon has been identified by many studies as a potential primary DNA barcode marker for most fungi due to its ease in amplification and availability of a large number of Genbank sequences (Schoch et al., 2012; Badotti et al., 2017). This study employed morphological methods to cluster fungal pathogens associated with *V. infausta subsp. rotundata* and *B. discolor* into fungal families and used molecular phylogenetics to identify species within the families.

MATERIALS AND METHODS

Study area

This study was carried out in Kitui County at the Tiva ICRAF field gene bank site (1.506° S: 38.011° E) where a selection of indigenous fruit tree species had been established in 1990 and also

*Corresponding author. E-mail: suzieshiku@gmail.com. Tel: +254790968602.

at two farmer's fields in Ikanga (2.356° S: 39.172° E) and Mkange (2.697° S: 38.817° E) within Kitui and Makueni counties respectively. The sites are located in semi-arid zones and receive a mean annual rainfall being less than 500 mm. Mean annual temperatures range between a minimum of 17°C and a maximum of 31°C. The study sites experience a bimodal type of rainfall with long rains in March to May, whereas the short rains fall between October and December. Rainfall in these areas is low, unreliable and poorly distributed. According to Jaetzold et al. (2012), these areas are mainly characterized by luvisols which are fertile soils with high cation exchange capacities and high base saturation. The major economic activities in such areas include subsistence farming, livestock rearing, and apiculture. Over the past decades, the study areas have been experiencing frequent droughts, which affect the availability of water, pasture and food for humans (Ngaina et al., 2014).

Sample collection

The general symptoms in the field in the Tiva, Ikanga and Kibwezi were characterized by dieback, canker, for both *V. rotundata* and *B. discolor* trees. Dieback symptoms were characterized by dead branches and twigs that began from the tip and progressed downwards. On the other hand, canker was characterized by cracks, dead and sunken areas on either stems or branches and when the bark was removed, the area appeared discolored (Bush, 2018). Tissue samples were collected from bark, branches, and leaves in a zigzag pattern, with a zigzag transect covering the entire farm or plantation and multiple locations placed along the transect. Samples were then collected from the front trees that were closest to the points. This was repeated for all the sites. There were a total of 71 trees that were diseased and 30 healthy trees were sampled. To conserve moisture, samples were placed in separate paper bags and enclosed in larger plastic bags until isolation was completed within 24 h. Samples that were not processed right away were maintained in a cool, dry environment or in a refrigerator at 4°C. To prevent reinfection by other pathogens, the cut piece of the tree was treated with a broad-spectrum fungicide (Bavistin) after each sampling. After each sampling, the sampling equipments were disinfected with 70% ethanol.

Tissue preparation

Leaves, branches and parts of stems showing symptoms of the disease and from healthy trees were collected and separately placed in khaki bags, sealed in larger plastic bags to retain moisture until isolation was done within 24 h. Samples were transported to KEFRI laboratories, where following standard laboratory techniques, small pieces were cut from the disease edges of the trees showing cankers on branches and stems and symptoms of dieback on shoots and branches. Samples from woody tissues and the inner bark of healthy trees were also used for fungal isolation using a modified procotol by Njuguna et al. (2011). The pieces were surface sterilized by immersing them for 1 min in 70% ethanol; they were then immersed in 33% hydrogen peroxide and rinsed three times in sterile distilled water for about 1 min for every rinse. They were then blotted dry using sterile filters paper in aseptic conditions. An antibiotic, Streptomycin sulphate (Duchefa Biochemie), was incorporated in the media to inhibit bacterial contamination. Samples from leaves, branches and bark were cut into tiny pieces and placed into plates containing MEA media in a laminar flow hood and incubated at 25ºC for fungal growth. Subculturing was done onto fresh media to obtain pure cultures. Isolated fungi were grouped based on the texture of the mycelia and the color of the colony (Jacobs and Rehner, 1998).

Morphological identification

The emergence of a young fungal colony was noted and given different numbers then isolated onto fresh MEA media. Fungal isolates were grouped based on mycelia texture and colony color. The isolates were purified through single hyphal tip isolations as described by Brown (1924). Spores in aniline blue were placed on microscope slides and examined using an Olympus SZ61 stereomicroscope to identify and group the fungi using protocols described by Jacobs and Rehner (1998) and Slippers et al. (2004). Isolation of single hyphae for DNA analysis was done according to the method described by Machua et al. (2016).

Molecular identification

DNA extraction, amplification and sequencing

Fungal isolates were sub-cultured on MEA media for 48 h at 25ºC. As detailed by Machua et al. (2016), mycelium was scraped from actively developing cultures with a sterilized surgical blade, placed into 2-ml Eppendorf tubes, freeze-dried, and ground to a powder using a Retsch Mixer MM 301. DNA was extracted using the CTAB (CetylTrimethylAmmonium Bromide) method described by Gardes and Bruns (1993). The variable internal transcribed spacer regions (ITS1, ITS2), including the complete 5.8S gene of the nuclear rDNA, were amplified using ITS1 (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) fungal primers. The ITS1 and ITS4 sequences were given as 5'-CTTGGTCATTTAGAGGAAGTAA-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively. The PCR amplicons were purified and sequenced in both forward and reverse (Inqaba biotec^{TM} SA). The sequences were modified in Bioedit 7.2 (Biological Sequence Alignment Editor) and Blast searches in GenBank [National Centre for Biotechnology Information (NCBI), of the National Institute of Health Bethesda (http://www.ncbi.nlm.nih.gov/BLAST), USA]. The sequences were then compared to sequences from closely similar species that had previously been published. Sequences with a similarity of 98-100% were chosen for further alignment using MAFFT version 7 online (https://mafft.cbrc.jp/alignment/software/). The model parameters were utilized to create a phylogenetic tree using the Maximum likelihood technique in MEGA X after the MAFFT alignment was subjected to a nucleotide substitution model test.

Phylogenetic analysis by Maximum Likelihood method

With 1000 bootstrap support, the phylogeny of the fungal isolates was inferred using the Maximum Likelihood technique and the Tamura-Nei model (Tamura and Nei., 1993). The highest log-likelihood tree (-4400.73) is shown. Next to the branches is the proportion of tree(s) in which the associated taxa clustered together. The initial tree(s) for the heuristic search were generated automatically by applying the Neighbor-Join and BioNJ algorithms on a matrix of pairwise distances calculated using the Tamura-Nei model and selecting the topology with the highest log-likelihood value. To describe evolutionary rate variations between sites (2 categories (+G, parameter = 0.3796), a discrete Gamma distribution was utilized. The branch lengths are measured in the number of substitutions per site, and the tree is depicted to scale. There were 47 nucleotide sequences in this study. In the end, there were 608 positions in the dataset. MEGA X was used to undertake evolutionary analysis (Molecular Evolutionary Genetics Analysis, Kumar et al., 2018).

Pathogenicity of the three pathogenic fungal families

Isolate pathogenicity was assessed using isolates from known

(a) (b)

Figure 1. Symptoms of canker and dieback (a) Stem canker on *B. discolor* (b) Severe shoot dieback on *V. rotundata.*

pathogenic fungal families *Botryosphaeriaceae* (3 species) and *Diaporthaceae* (1 species) inoculated on four indigenous agroforestry tree species; *B. discolor, Croton. megalocarpus*, *Tamarindus indica* and *Olea europaea.* A negative control (a sterile MEA plug) was included as a fungal treatment. *T. indica* and *C. megalocarpus are indigenous trees found in the drylands and* usually intercropped with *V. rotundata* and *B. discolor*. A highlands agroforestry tree species *O. europaea* was included in the pathogenicity test to examine the promiscuity of the test fungal pathogens. *V. rotundata* was not included in the pathogenicity test for lack of seeds or seedlings at the study time. The pathogenicity test was conducted using a method described by Njuguna et al. (2011) by inoculating healthy seedlings of approximately 30-35 cm in height and with a root collar diameter of approximately 8-10 cm. A total of 320 seedlings were used for the pathogenicity test (4 tree species × 4 fungal treatments × 20 seedlings per treatment). Inoculated and control treatments were arranged in 4 replicate blocks using a complete randomized block design containing 5 seedlings of each fungal treatment per block. The isolates were grown in MEA at 25°C for 5 days before inoculation. The stems were injured using a sterilized scalpel blade, and mycelia plugs of approximately 6 mm in diameter were placed on the wound and wrapped using Parafilm tape. Another set of seedlings were inoculated with sterile MEA plugs of 6 mm diameter as controls. All the inoculated seedlings were placed in a glasshouse and watered in the mornings and evenings. The seedlings were observed daily for 5 months and any disease symptoms were recorded. Fungal pathogens from the infected seedlings were re-isolated using the same procedure and the isolates used for molecular identification.

Statistical analysis

Data on the internal lesion sizes were transformed by square root prior to analysis. Data were analyzed using Minitab Version 10 (Minitab Inc 2010). Descriptive statistics were used to test the normality of the data set before the analysis of variance. The data were found to have a normal distribution. Significance differences in the level of virulence of the fungal isolates determined were inferred by one-way analysis of variance (ANOVA).

RESULTS

Disease symptoms in the field

The general symptoms observed in Tiva, Ikanga and Kibwezi were characterized by dieback and canker. It was noted that scattered branches of *V. rotundata* were also dying from the tip suggestive of dieback (Figure 1b). Canker was however easily identified in both *V. rotundata* and *B. discolor* trees on the stem and branches with visible discoloration on the affected areas but no resin flow (Figure 1a). In all the trees that were assessed, 54% of *V. rotundata* and 18% *B. discolor* trees had dieback and canker infections (Table 1).

Fungi associated with *B. discolor* and *V. rotundata* and their characterization

According to morphological characteristics, 7 fungal groups were identified through observation of the colony color, namely: *Botryosphaeriaceae*, *Sporocadaceae*, *Glomerellaceae, Diaporthaceae, Pleosporaceae, Trichosphaeriaceae* and *Nectriaceae* (Table 1). The most dominant fungi isolated were *Botryosphaereacea* followed by *Nectriaceae*. Since the same fungi that were isolated from dieback symptoms were also isolated from canker, there was a high probability of a connection between the two symptoms.

Molecular identification

Phylogenetic and sequence analysis of the ITS rDNA data grouped the fungal isolates into 7 families; *Botryosphaeriaceae, Diaporthaceae, Glomerellaceae, Nectriaceae, Pleosporacea, Sporocadaceae* and *Trichosphaeriaceae* (Figure 2).

Botryosphaeriaceae isolates clustered into three genera; *Diothiorella, Alanphillipsia* and *Lasiodiplodia.* Isolate MW940855 was identified as *Lasiodiplodia lignicola,* while isolate MW931778 was identified as *Alanphillipsia aloegena*. Although isolates OK036579 and OK036778 were identified with the genus *Diothiorella,* the two had strong bootstrap support for a separate species Isolate MW931851 had a very strong bootstrap suppor

Table 1. Fungal frequency Mean ± SE.

Variable	Family	Mean ± SE	Perecnt
Frequency	*Botryosphaeriaceae*	606.50 ± 6.50	32.7
	Nectriaceae	567.0 ± 17.0	30.5
	Sporocadaceae	338.0 ± 35.0	18.2
	Pleosporaceae	310.5 ± 11.5	16.7
	Glomerellaceae	19.00 ± 1.00	1.0
	Trichosphaeriaceae	10.00 ± 1.00	0.5
	Diaporthaceae	6.50 ± 1.50	0.3
Total		1857.50	100

(100) within *Diaportheceae* and was identified as *Diaporthe ganjae*. Isolate MW931878 was identified as *Colletotrichum gloeosporioides* within the *Glomerellaceae* (100 bootstrap support).

The *Nectriaceae* family formed a polyphyletic clade with *Fusarium chlamydosporum* and *Fusarium lateritium* in one group and *Fusarium proliferatum* and *Fusarium equiseti* in another group. Isolate MW931873 was identified as F. chlamydosporum (95 bootstrap support), while isolate OK036782 was identified as *F. lateritium* (99 bootstrap support). Under the *F. proliferatum* and *F. equiseti* group, isolate OK036780 was identified as *F. proliferatum* (99 bootstrap support) while isolates OK036779, OK036582, OK036583 and OK036781 were identified as *F. equiseti* (99 bootstrap support.

Pleosporaceae isolates MW931855 was identified as *Culvularia pseudoclavata* (100 bootstrap support) within the family, while MW931858 was identified as *Culvularia pseudointermedia* (92 bootstrap support). Isolates OK036580 and OK036581 were identified as *Alternaria species* (99 bootstrap support) since the ITS phylogeny could not singularly distinguish between *Alternaria alternata* and *Alternaria tenuissima* within the same clade.

Within the *Sporocadaceae*, isolates OK036684, OK036685, OK036682, OK036683, OK036593 and OK036594 were identified as belonging to the genus *Neopestalotiopsis*. However, the ITS phylogeny did not have strong bootstrap support to identify the isolates to species level within the *Neopestalotiopsis*.

The *Trichosphaeriaceae* group had two isolates OK036783 and OK036588 which were identified as *Nigrospora sphaerica* (100 bootstrap support) and *Nigrospora oryzae* (73 bootstrap support), respectively. All the identified isolates, their host species and sites of origin are presented in Table 2.

Pathogenicity test

Results showed different levels of susceptibility to the fungi. Internal lesions were defined by inner tissue discoloration and decay. The internal lesion size differed significantly ($P \leq 0.05$) among the host species and between the host species and the control species. The lesion length caused by *Lasiodiplodia lignicola* was significantly ($P \leq 0.05$) longer in all the host species. This trend was followed by *Alanphillipsia aloegena, Dothiorella* sp and *Diaporthe ganjae* was least virulent.

Croton megalocarpus and *T. indica* resulted in the longest internal lesions among the host species. *O. europaea* was the least susceptible ($P \leq 0.05$) to the test pathogens (Table 3).

DISCUSSION

This is the first report of identifying fungi causing canker and dieback on *V. infausta,* and *B. discolor* in Kenya using molecular and morphological techniques. Morphological features observed suggested that the isolates belonged to the 7 ascomycetous fungal families: *Botryosphaeriaceae, Nectriaceae, Sporocadaceae, Pleosporaceae, Glomerellaceae, Trichosphaeriaceae* and *Diaporthaceae.* However, morphological characteristics alone were insufficient for species identification of the isolates. The use of morphological features alone in fungal identification is challenging due to the limited number of phenotypic characters usually confusing within related taxa. This often leads to inaccurate identification of the fungal isolates especially below the genus level. This study incorporated the use ITS phylogeny for molecular identification since the method has been largely accepted and used as a barcode marker for identification of fungi to the species level (Kõljalg et al., 2013). The internal transcribed spacer (ITS) is found in the chromosome between the small subunit ribosomal RNA (rRNA) and large subunit rRNA genes, or in the polycistronic rRNA precursor transcript's corresponding transcribed region. Because of various advantageous qualities, such as the high degree of variation between closely related species, sequence comparison of the eukaryotic ITS regions is commonly employed in taxonomy and molecular phylogeny (Bußkamp et al., 2020). The spacer sequences are non-coding and hence have very low evolutionary pressure acting on them, thus

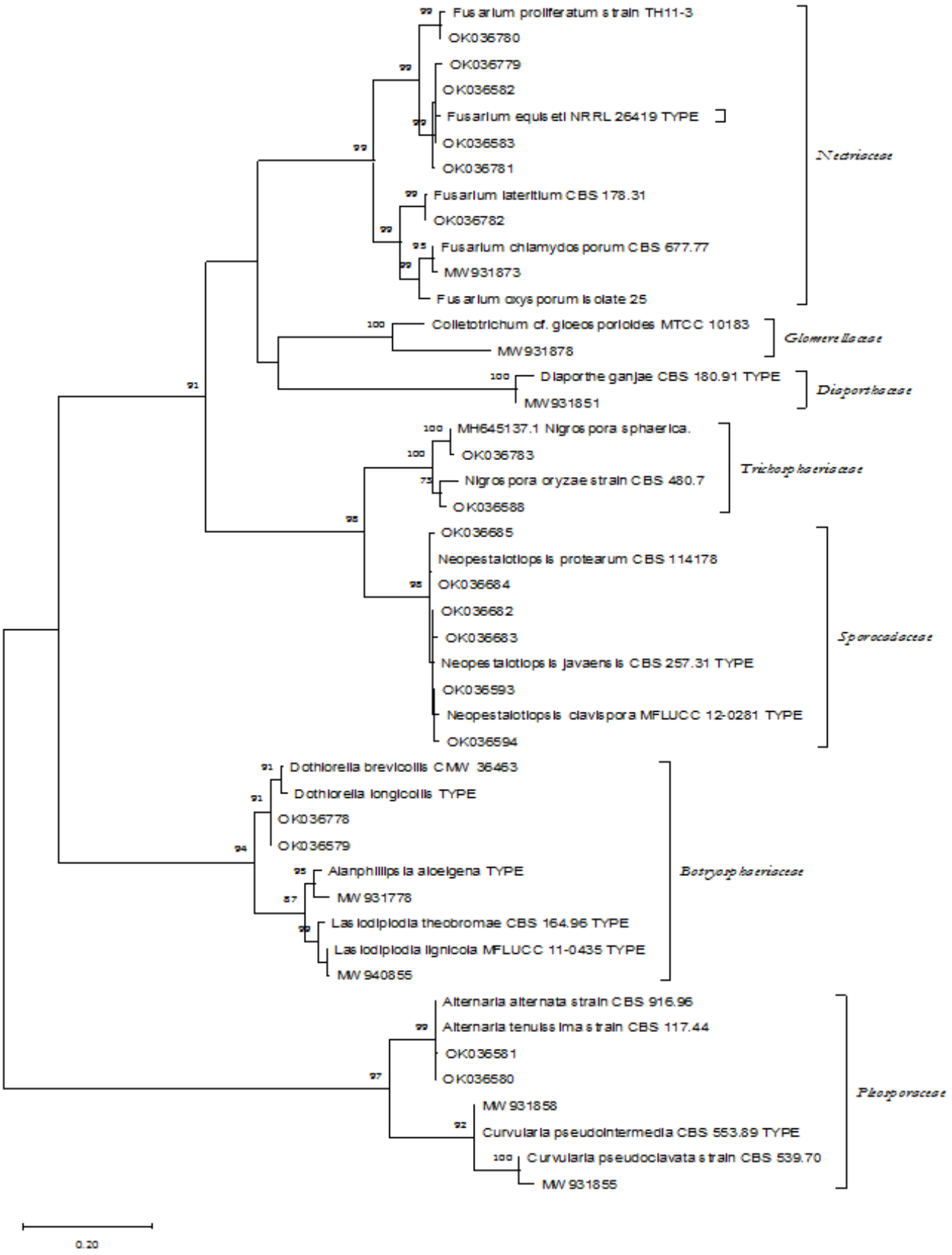

Figure 2. Molecular phylogenetic analysis by maximum likelihood method (bootstrap values less than 70 are not shown).

Table 2. Identified isolates and their host species.

Site	Host species	Isolate no.	Identification	Genbank acc. no.
Mukange	*Vangueria infausta* sub sp *rotundata*	VMT28	*Lasiodiplodia lignicola*	MW940855
Mukange	*Vangueria infausta* sub sp *rotundata*	VMT29	*Alanphillipsia aloeigena*	MW931778
Mukange	*Vangueria infausta* sub sp*. rotundata*	VML43	*Nigrospora* oryzae	OK036588
Mukange	*Vangueria infausta* sub sp*. rotundata*	VMB88	*Fusarium equiseti*	OK036583
Mukange	*Vangueria infausta* sub sp. *rotundata*	VMB11	*Neopestalotiopsis* sp	OK036594
Mukange	*Berchemia discolor*	BMB52	*Alternaria* sp	OK036580
Mukange	*Berchemia discolor*	BML53	*Curvularia pseudoclavata*	MW931855
Mukange	*Berchemia discolor*	BMT26	*Neopestalotiopsis* sp	OK036684
Mukange	*Berchemia discolor*	BMT25	*Neopestalotiopsis* sp	OK036683
Mukange	*Berchemia discolor*	BML27	*Neopestalotiopsis* sp	OK036685
Tiva	*Berchemia discolor*	BTB100	*Dothiorella* sp	OK036579
Tiva	*Berchemia discolor*	BTL82	*Diaporthe ganjae*	MW931851
Tiva	*Berchemia discolor*	BTB57	*Colletotrichum gloeosporioides*	MW931878
Tiva	*Berchemia Discolor*	BTL57	*Fusarium equiseti*	OK036582
Tiva	*Berchemia Discolor*	BTT58	*Fusarium chlamydosporum*	MW931873
Tiva	*Berchemia Discolor*	BTL33	*Nigrospora sphaerica*	OK036783
Tiva	*Vangueria infausta* sub sp. *rotundata*	BTL60	*Fusarium equiseti*	OK036779
Tiva	*Vangueria infausta sub* sp*. rotundata*	VTB66	*Fusarium lateritium*	OK036782
Tiva	*Vangueria infausta* sub sp. *rotundata*	VTT7	*Neopestalotiopsis* sp	OK036682
Ikanga	*Vangueria infausta* sub sp. *rotundata*	VIT30	*Dothiorella* sp	OK036778
Ikanga	*Vangueria infausta* sub sp. *rotundata*	VIL54	*Curvularia pseudointermedia*	MW931858
Ikanga	*Vangueria infausta* sub sp*. rotundata*	VIB70	*Fusarium equiseti*	OK036781
Ikanga	*Vangueria infausta* sub sp*. rotundata*	VIB78	*Fusarium proliferatum*	OK036780
Ikanga	*Berchemia discolor*	BIL18	*Neopestalotiopsis* sp	OK036593

Table 3. Mean internal lesion length (cm) caused by the *Botryosphariaceae* isolates.

Fungal species	Mean internal lesion length (Mean±SE)			
	C. megalocarpus	***T. indica***	***B.discolor***	***O.europaea***
Lasiodiplodia lignicola	5.39 ± 1.24^{aA}	4.773 ± 0.81^{abA}	2.260 ± 0.30^{bA}	2.01 ± 0.26^{bA}
Alanphillipsia aloegena	4.41 ± 0.71^{aAB}	3.020 ± 0.52^{abAB}	2.19 ± 0.14^{bcA}	0.95 ± 0.37^{bB}
Dothiorella sp	2.93 ± 0.47^{aA}	2.55 ± 0.38^{aBC}	1.89 ± 0.21^{abAB}	1.07 ± 0.07^{cB}
Diaporthe ganjae	1.92 ± 0.34^{aBC}	2.03 ± 0.33^{aBC}	1.82 ± 0.23^{aAB}	0.59 ± 0.11^{bB}
Control	1.37 ± 0.27^{abC}	1.040 ± 0.09^{bC}	1.11 ± 0.19^{bB}	0.83 ± 0.15bB

Means with lowercase superscripts represent significance differences among the tree species (across) while uppercase superscripts represent significance differences between the fungal species (vertically). Means with the same superscript were not significant.

helping to maintain evolutionary diversity (Baldwin et al., 1995). However, it was impossible to identify some of the fungal isolates from this study to the species level as the ITS region alone may not always provide adequate variation for fungal differentiation within a genus (Raja et al., 2017).

Botryosphaeriaceae family was most frequent on all the sites, and apparently, its isolates were the most pathogenic to the hosts tested. This family consists of saprobes, endophytes and plant pathogens (Dissanayake et al., 2016). Moreover, as endophytes, they can remain latent for an indefinitely and become pathogenic when the host suffers from physiological stress resulting in large cankers on trunks and branches of the affected trees (Moricca et al., 2010). In addition, *Botryosphaeriaceae* species have been known as the principal cause of canker and dieback in woody plants (Burgess et al., 2019).

The ability of the *Botryosphaeriaceae* isolated in this study to cause dieback and canker symptoms observed

Table 4. Description of the reference isolates used in this study.

Species	Culture no.	Genbank acc. No.	Host	Origin	Collector
Lasiodiplodia lignicola	MFLUCC 11-0435	MFLUCC 11-0435	Wood	Thailand	A.D Ariyawansa
Lasiodiplodia theobromae	CBS 164.96	NR_111174.1	Fruit along coral reef coast	Papua New Guinea	Phillips et al. (2005)
Alanphillipsia aloeigena	TYPE MATERIAL	NR_137121.1	*Aloe melanacantha*	South Africa	M.J. Wingfield
Dothiorella brevicollis	CMW 36463	NR_111703	*Acacia karroo*	South Africa	Jami F
Dothiorella longicollis	TYPE MATERIAL	NR_136999.1	*Lysiphyllum cunninghamii*	Australia	Pavlic et al. (2008)
Alternaria alternate	MH237955.1	CBS 916.96	Apple	China	Unknown
Alternaria tenuissima	CBS 117.44	MH856117.1	Unknown	Denmark	Vu, D
Curvularia pseudointermedia	CBS 188.61	MN688820.1	Decaying grass	Guadeloupe	Marin-Felix et al. (2020)
Curvularia pseudoclavata	CBS 539.70	MN688817.1	*Oryza sativa*	Denmark	S.B. Mathur
Diaporthe ganjae	CBS 180.91	NR_120259.1	*Cannabis sativa*	USA: Illinois	J.M. McPartland
Colletotrichum gloeosporioides	JF908919.1	MTCC 10183	Unknown	India	Mansoor Alam
Fusarium equiseti	CBS 307.94	NG_068575.1	*Gibberella intricans*	Germany	Vu et al. (2019)
Fusarium lateritium	CBS 178.31	MH855172.1	Unknown	unknown	Vu,D
Fusarium chlamydosporum	CBS 677.77	MH861111.1		Solomon Islands	Vu et al. (2019)
Nigrospora sphaerica	isolate COL8	MH645137.1	*Citrus reticulata*	Pakistan	Naeem, I
Nigrospora oryzae	CBS 480.7	MH860749.1	Unknown	Kazakhstan	Redet. W. Gams
Neopestalotiopsis javaensis	CBS 257.31	NR_145241.1	*Cocos nucifera*	Indonesia	R.L. Steyaert
Neopestalotiopsis clavispora	MFLUCC 12-0281	NR_111782.1	Dead plant material	China	Unknown
Neopestalotiopsis protearum	CBS 114178	LT853103.1	*Leucospermum cuneiforme*	Zimbabwe	Unknown

in the field in healthy seedlings of *B. discolor, C. megalocarpus*, *T. indica* and *O. europaea* and their re-isolation in culture media, confirmed them as the probable causal agent of symptoms that were observed in the field.

Occurrence of a pathogen with a wide range of host will pose a threat to *V. rotundata* and *B. discolor* and other agroforestry trees and crops growing in close proximity. Additionally, as Slippers and Winfield reported in 2007, the latent phase of the pathogenic fungi may sometimes be overlooked hence underestimating their ability to cause disease. The latent phase of *Botryosphaeriaceae* endophytes (Luo et al., 2019), makes them a significant threat to the two agroforestry fruit trees, farm and commercial forestry at large.

Conclusion

This is the first report of canker and dieback on *V. rotundata* and *B. discolor*. Although research on the fungi infecting these tree species is scanty, there is no doubt that these indigenous fruit trees are a host to several fungal species of economic importance. Of particular importance, it is clear that canker and dieback are widespread in the drylands of Eastern Kenya indicating that agroforestry in these regions is under serious threat if measures are not taken to mitigate the disease. Further studies should therefore be conducted on the pathogenic nature of these fungi on agricultural crops.

Morphological identification coupled with molecular phylogeny is important for accurate identification of fungal species in this study. However, Internal subscribed spacer (ITS) phylogeny alone may present some drawbacks for some fungal families and for species level identification of some isolates (Table 4). It is therefore, highly recommended that multiple gene phylogeny using genes such as elongation factor 1-α and β-tubulin be utilized for conclusive identification.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors appreciate World Agroforestry (ICRAF) for the financial support through the Genebank Platform funding (GCDT-1213) and Kenya Forestry Research Institute (KEFRI) for providing materials necessary for the success of this study. The staffs at ICRAF and KEFRI are also acknowledged for their inputs in making this study a success.

REFERENCES

Agrios GN (2005). Plant pathology 5th Edition. Elsevier Academic Press pp. 79-103.

Badotti F, de Oliveira FS, Garcia CF, Vaz ABM, Fonseca PLC, Nahum LA, Góes-Neto, A (2017). Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi). BMC Microbiology 17(1):1-12.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell

CS, Donoghue MJ (1995). The ITS Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny. Annals of the Missouri Botanical Garden 82(2):247-277.
Bernreiter A (2017). Molecular diagnostics to identify fungal plant pathogens–a review of current methods. ECUADOR ES CALIDAD-Revista Científica Ecuatoriana.
Brown W (1924). II. A method of isolating single strains of fungi by cutting out a hyphal tip. Annals of Botany 38(150):402-404.
Burgess TI, Tan YP, Garnas J, Edwards J, Scarlett KA, Shuttleworth LA, Jami F (2019). Current status of the *Botryosphaeriaceae* in Australia. Australasian Plant Pathology 48(1):35-44.
Bußkamp J, Langer GJ, Langer EJ (2020). Sphaeropsis sapinea and fungal endophyte diversity in twigs of Scots pine (Pinus sylvestris) in Germany. Mycological Progress 19(9):985-999.
Bush EA (2018). Botryosphaeria canker and dieback of trees and shrubs in the landscape.
Cheikhyoussef A, Naomab E, Potgieter S, Kahaka GK, Raidron C, MuAshekele H (2010). Phytochemical properties of a Namibian indigenous plant; Eembe (*Berchemia discolor*).
Dayarathne MC, Mridha AU, Wang Y (2020). Diagnosis of Fungal Plant Pathogens Using Conventional and Molecular Approaches. In Diagnostics of Plant Diseases. Intech Open.
Das S, Deb B (2015). DNA barcoding of fungi using ribosomal ITS marker for genetic diversity analysis: A review. International Journal of Pure and Applied Bioscience 3(3):160-167.
Dissanayake AJ, Phillips AJL, Li X H, Hyde KD (2016). Botryosphaeriaceae: Current status of genera and species. Mycosphere *7*(7):1001-1073.
Dulla EL, Kathera C, Gurijala HK, Mallakuntla TR, Srinivasan P, Prasad V, Jasti PK. (2016). Highlights of DNA Barcoding in identification of salient microorganisms like fungi. Journal de Mycologie Medicale 26(4):291-297.
Eulalia C (2015). Utilization of Wild Fruit in Mozambique – Drying of *Vangueria infausta* (African medlar). Food Technology, Engineering and Nutrition, Lund University.
Thambugala KM, Daranagama DA, Phillips AJ, Kannangara SD, Promputtha I (2020). Fungi vs. fungi in biocontrol: an overview of fungal antagonists applied against fungal plant pathogens. Frontiers in Cellular and Infection Microbiology 10:604923.
Hariharan G, Prasannath K (2021). Recent advances in molecular diagnostics of fungal plant pathogens: a mini review. Frontiers in Cellular and Infection Microbiology 10:829.
Feyssa DH, Njoka JT, Asfaw Z, Nyangito MM (2012). Nutritional Value of *Berchemia discolor*: A Potential to Food and Nutrition Security of Households. Journal of Biological Sciences 12(5):263-271.
Gachie P, Kipsat J, Cheboiwo J, Esitubi M, Mwaura J, Wairimu P, Gathogo M (2020). On-farm tree growing opportunities and constraints in Murang'a County, Kenya. East African Agricultural and Forestry Journal 84(1):53-65
Gardes M, Bruns TD (1993). ITS primers with enhanced specificity for Basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology 2(2):113-118.
Jacobs KA, Rehner SA (1998). Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia 90(4):601-610.
Jaetzold R, Hornetz B, Shisanya CA, Schmidt H (2012). Farm management handbook of Kenya Volume I-IV (Western Central Eastern Nyanza Southern Rift Valley Northern Rift Valley Coast). Nairobi: Government Printers.
Jamnadass R, Ofori DA, Dawson I.K, Tchoundjeu Z, McMullin S, Hendre PS, Theme T (2019). Enhancing agroforestry systems through tree domestication. Sustainable development through trees on farms: agroforestry in its fifth decade. Bogor, Indonesia: World Agroforestry (ICRAF) Southeast Asia Regional Program pp. 45-59.
Jeger M, Beresford R, Bock C, Brown N, Fox A, Newton A, Yuen J (2021). Global challenges facing plant pathology: multidisciplinary approaches to meet the food security and environmental challenges in the mid-twenty-first century. CABI Agriculture and Bioscience 2(1):1-18.
Kitonga K, Jamora N, Smale M, Muchugi A (2020). Use and benefits of tree germplasm from the World Agroforestry genebank for smallholder farmers in Kenya. Food Security 12:993-1003.
Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AF, Bahram M, Larsson KH (2013). Towards a unified paradigm for sequence based identification of fungi. Molecular Ecology 22(21):5271-5277.
Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35(6):1547-1549.
Luo Y, Lichtemberg PS, Niederholzer FJ, Lightle DM, Felts DG, Michailides TJ (2019). Understanding the process of latent infection of canker-causing pathogens in stone fruit and nut crops in California. Plant Disease 103(9):2374-2384.
Machua J, Jimu L, Njuguna J, Wingfield MJ, Mwenje E, Roux J (2016). First report of *Teratosphaeria gauchensis* causing stem canker of *Eucalyptus* in Kenya. Forest Pathology 46(2):168-170.
Maundu MP (1999). Traditional food plants of Kenya. National Museum of Kenya.
Maroyi A (2018). Nutraceutical and ethnopharmacological properties of Vangueria infausta subsp. infausta. Molecules 23(5):1089
Miller DC, Muñoz-Mora JC, Rasmussen LV, Zezza A (2020). Do trees on farms improve household wellbeing? Evidence from national panel data in Uganda. Frontiers in Forests and Global Change 3:101.
Minitab INC (2010). Minitab 16 statistical software. URL: [Computer software]. State College, PA: Minitab, Inc. (www. minitab. com).
Moricca S, Uccello A, Ragazzi A (2010). Endophytic mycocenoses in urban forests under environmental stress. Micologia Italiana 39(1):47-56.
Muok BO, Owuor B, Dawson I, Were J (2000). The potential of indigenous fruit trees: results of a survey in Kitui District, Kenya. Agroforestry Today 12(1):13-16.
Nalla K, Pothabathula SV, Kumar S (2020). Applications of Computational Methods in Plant Pathology. In Natural Remedies for Pest, Disease and Weed Control X Academic Press.
Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD, Wingfield MJ (2004). Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia 96(1):83-101.
Nazarov PA, Baleev DN, Ivanova MI, Sokolova LM, Karakozova MV (2020). Infectious plant diseases: Etiology, current status, problems and prospects in plant protection. Acta Naturae 12(3):46-59.
Ngaina JN, Mutua FM, Muthama NJ, Kirui JW, Sabiiti G, Mukhala E, Maingi NW, Mutai BK (2014). Drought monitoring in Kenya: A case of Tana River County.International Journal of Agricultural Science Research 3(7):126-135.
Njuguna JW, Barklund P, Ihrmark K, Stenlid J (2011). A canker and dieback disease is threatening the cultivation of Grevillea robusta on small-scale farms in Kenya. African Journal of Agricultural Research 6(3):748-756.
Lücking R, Aime MC, Robbertse B, Miller AN, Ariyawansa HA, Aoki T, Schoch CL (2020). Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? IMA fungus 11(1):1-32.
Omotayo AO, Aremu AO (2020). Underutilized African indigenous fruit trees and food–nutrition security: Opportunities, challenges, and prospects. Food and Energy Security 9(3):e220.
Pernezny K, Elliott M, Palmateer A, Havranek N (2008). Guidelines for identification and management of plant disease problems: part II. Diagnosing plant diseases caused by fungi, bacteria and viruses. EDIS 2008(2).
Ramavhale RP (2018). Seasonal abundance and diversity of insects on *Sclerocarya birrea* and *Berchemia discolor* in Tshikundamalema, Limpopo Province, South Africa (Doctoral dissertation).
Raja HA, Miller AN, Pearce CJ, Oberlies NH (2017). Fungal identification using molecular tools: a primer for the natural products research community. Journal of Natural Products 80(3):756-770.
Raja HA, Oberlies NH, Stadler M (2021). Occasional comment: Fungal identification to species-level can be challenging. Phytochemistry 190:112855.
Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Fungal Barcoding Consortium. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences 109(16):6241-6246.
Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD,

Wingfield MJ (2004). Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia 96(1):83-101.
Slippers B, Winfield MJ (2007). *Botryosphaericeae* as endophytes and latent pathogens of woody plants: diversity, ecology and impact. Fungal Biology Reviews 21(2-3):90-106.
Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10(3):512-526.
Tekpinar AD, Kalmer A (2019). Utility of various molecular markers in fungal identification and phylogeny. Nova Hedwigia 109(1-2):187-224.
White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal rRNA genes for phylogenetics. PCR protocols: a guide to methods and applications. Academic Press pp. 315-322.