ITS-rDNA sequences differentiate a new lineage of *Diplodia* associated with canker disease of apple in Iran

Arzanlou M[1]* and Bakhshi M[2]

[1]Assistant Professor of Plant Pathology and Mycology, Plant Protection Department, Faculty of Agriculture, University of Tabriz, PO Box: 5166614766, Iran.[2]Etc
[3]PhD Student of Plant Pathology, Plant Protection Department, Faculty of Agriculture, University of Tabriz, PO Box: 5166614766, Iran.

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Trunk diseases have become a growing threat for apple cultivation in northwestern parts of Iran. A *Diplodia sp.* with unique morphological characteristics was recovered from symptomatic tissues. A phylogeny inferred using ITS sequence data, clustered the *Diplodia* isolates in a separate clade from other *Dilpodia* species known from apple, as well as from other hosts. Pathogenicity test using an excised shoot assay revealed that the isolates are highly pathogenic on apple. The distribution and host range of this new pathogen on apple remains to be studied.

Key words – *Botryosphaeria* – ef-1α– excised shoot assay – *Malus*

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*Corresponding author: Mahdi Arzanlou – e-mail – Arzanlou@hotmail.com

Introduction

The apple (*Malus domestica*) industry plays a major role in the economy of Iran. Apple orchards are widely distributed in almost all geographical regions of Iran, covering a total area of 130,291 hectares with an annual production of 1,662,430 tonnes (FAO 2012). In recent years, trunk disease has become a growing threat for apple cultivation in northwestern parts of Iran. The disease affects apple trees in different stages of growth in both standing and newly established orchards. The disease is more severe in old orchards experiencing abiotic stresses such as drought, nutrient deficiency or insect damage.

Members of the genus *Botryosphaeria* are known to cause canker, fruit rot and leaf spot symptoms on apple trees (Stevens 1933, Brown & Britton 1986, Brown-Rytlewski & McManus 2000, Tang et al. 2012). *Botryosphaeria* is a species-rich genus, with a wide geographical distribution, occurring on a diverse range of monocotyledonous, dicotyledonous, and gymnosperm host species, and some species possess medical relevance (Brown & Britton 1986, Brown-Rytlewski & McManus 2000, Van Niekerk et al. 2004, Alves et al. 2006, Damm et al. 2007, Lazzizera et al. 2008, Linaldeddu et al. 2011, Mehl et al. 2011). Since the description of the genus in 1863, several *Botryosphaeria* species or allied anamorphs have been described from numerous woody and non-woody plants with canker symptoms. Members of this genus are generally considered as weak or opportunistic pathogens as well as common endophytes of woody plants (Phillips 2000, Slippers et al. 2004, 2007, Van Niekerk et al. 2004, Slippers & Wingfield 2007, Pérez et al. 2010). However, some species are responsible for serious diseases on woody hosts with economic importance such as grapevines and apple (Stevens 1933, Brown & Britton 1986, Brown-Rytlewski & McManus 2000, Larignon et al.

Figs 1–**5** – Canker disease symptoms on naturally infected apple trees in orchard **1** Dieback leading to death of tree **2** Initial symptoms of the disease on stem as depressed sunken lesion **3**–**4** Disease symptoms on stems in later stages of disease development **5** Wood browning in transverse section of stem.

2001, Savocchia et al. 2007, Epstein et al. 2008, Urbez-Torrez et al. 2008). Several species of *Botryosphaeria* or related anamorphs *viz.*, *B. dothidea*, *B. malorum* (anamorph: *Diplodia malorum*), *B. obtusa* (anamorph: *Diplodia seriata*), *B. mali* and *B. rhodina* have been reported to occur on *Malus* spp. worldwide (Brown & Britton 1986, Brown-Rytlewski & McManus 2000, Tang et al. 2012). Until now, several species of the genus *Botryosphaeria* have been reported from different plant species in Iran. *B. dothidea* is the only documented *Botryosphaeria* species from apple in Iran (Ershad 2009).

In the past, taxonomy of the Botryosphaeriaceae has been based on morphological characteristics of teleomorph and host plant association criteria (Slippers et al. 2004, Crous et al. 2006, Damm et al. 2007). These criteria have proven to be inappropriate in taxonomy of the Botryosphaeriaceae and have led to much confusion both in taxonomy of the genus and understanding of the diseases caused by the members of this family (Crous et al. 2006). With the aid of sequence data, the taxonomy of the genus has undergone a major taxonomic revision and the genus *Botryosphaeria* is now restricted to just two species, *B. dothidea* and *B. corticis* (Crous et al. 2006). Several new genera have been established to accommodate the segregates, with many of these based on morphological characteristics of asexual states (Crous et al. 2006). Asexual spore-producing structures are commonly produced in culture and are also more informative for identification of '*Botryosphaeria*' species, in comparison with the sexual states, which are rare in field and not easily inducible in culture (Denman et al. 2000, Crous et al. 2006).

The present study aimed to characterize the causal agent of canker disease in apple orchards in northwestern parts of Iran by means of morphological, molecular and pathological data.

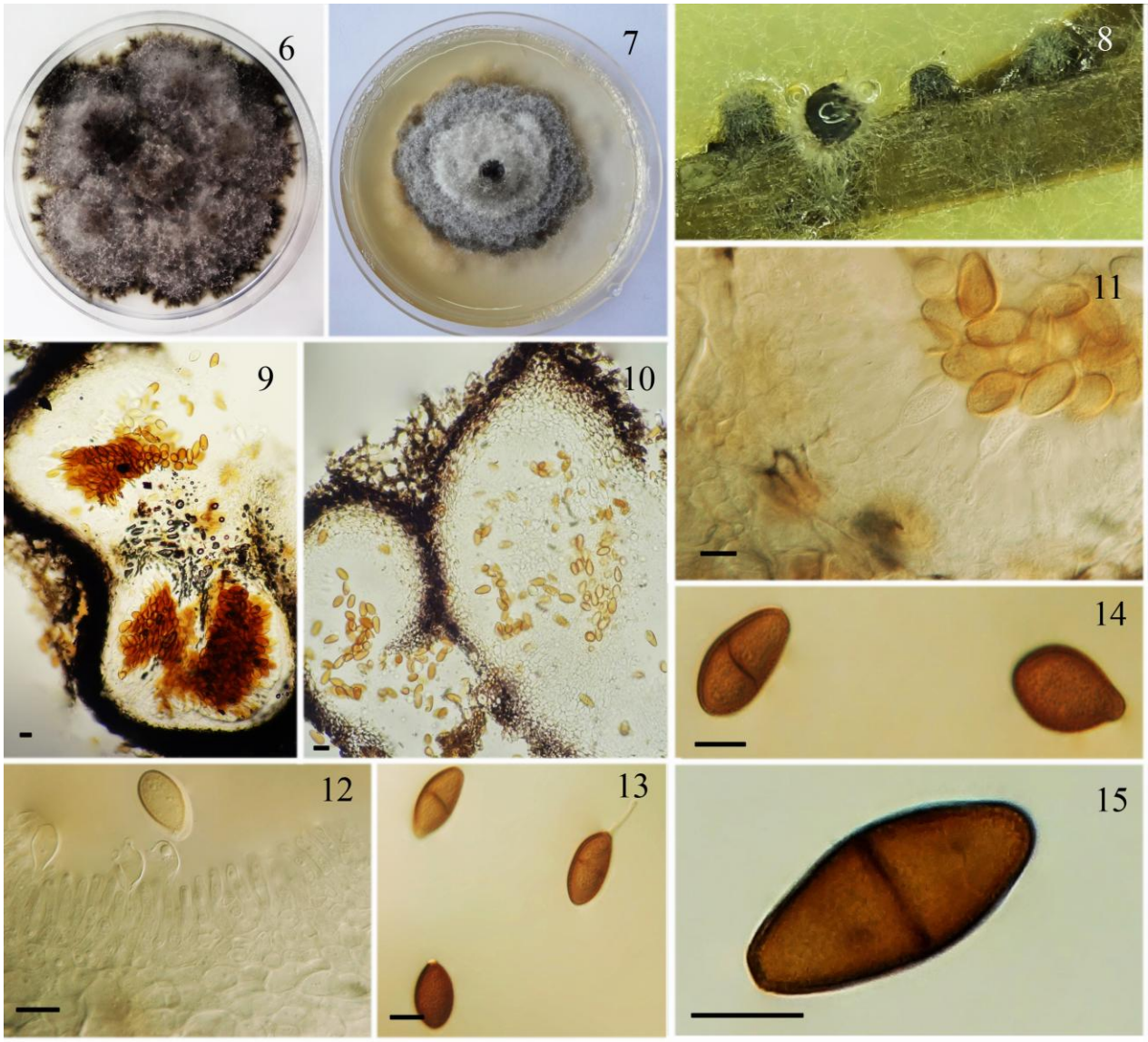
Methods

Isolates and morphology

Isolates were collected from symptomatic apple trees exhibiting canker on stems and branches and dieback from orchards in the Khoy and Salmas regions (West Azerbaijani Province, Iran). For isolation, small pieces approximately 0.5 × 0.5 × 0.5 cm were cut from the margins of infected tissues and surface-sterilized for 1 min in 70% ethanol, rinsed with sterile water for three times, dried on sterile filter paper and placed on malt extract agar (MEA, Fluka, Hamburg, Germany) plates supplemented with 100 mg/L streptomycin sulphate and 100 mg/L ampicillin. Pure cultures were established using hyphal tip technique. Isolates were characterized based on morphological features of conidiomata and colony morphology. Fungal cultures were transferred to pine needle agar medium (PA) and incubated at 25 ^{0}C under near-UV light to induce conidiomata. Colony morphology, colure and growth rate were determined on MEA plates after 7 days of incubation at 25 ^{0}C in darkness. Microscopic characters were studied based on handmade sections through pycnidia using a light microscope with a Leica camera system and software to analyse photographs. Microscopic structures were mounted in lactic acid for measurements. Dimension of microscopic structures were calculated based on 30 measurements for conidia morphology (shape, colour, and cell number), size (length and width) where possible. Cultures were deposited in the living Culture Collection of Tabriz University (CCTU), Tabriz, Iran.

Pathogenicity test

An excised shoot assay was used to evaluate the pathogenicity of the isolates. For this purpose, 1- to 2-year-old twigs were collected from healthy mature golden delicious apple. After removal of leaves, twigs were surface-sterilized with 70 % ethanol. For inoculation, twigs were wounded in the middle part by removing the cortex with a sterile 5 mm diameter metal cork borer. A 5 mm diameter mycelia plug of a 7- days-old fungal isolate on MEA was immediately placed on the wound, which was then covered with Parafilm and the control was filled with a plug of MEA. All inoculated twigs were placed in a plastic container containing moistened filter paper, in order to keep the relative humidity high and were maintained in a laboratory condition at room temperature with natural day light.



Figs 6–15 – *Diplodia sp.* **6–7** Colony morphology on PDA and MEA **8** Conidiomata developed on pine needle agar **9–10** Cross sections through pycnidia **11–12** Conidiogenous cells and conidia, which become pale brown soon after formation **13–15** Pale brown aseptate conidia, which later become one-septate.

Disease symptoms were checked daily for 2 weeks following inoculation. Longitudinal sections were made from above and below the inoculation point and wood necrosis was observed in inoculated twigs with the *Diplodia* isolates. The experiment was carried out using four fungal isolates and three replicates for each treatment.

DNA phylogeny

For DNA extraction fungal isolates were grown on MEA for 8 days. Fungal mycelia were harvested and subjected to DNA extraction using the protocol of Möller et al. (1992). Sequence data from ITS-rDNA region and parts of elongation translation (ef-1α) gene were used to infer phylogeny. The ITS region including the 3' end of the 18S rRNA gene, ITS1, 5.8S rDNA, ITS2 and the 5' end of 28S rRNA gene was amplified using the primer set V9G (Vilgalys & Hester 1990) and ITS4 (White et al. 1990). Part of elongation factor (ef-1α) gene was amplified using EF728 (Carbone & Kohn 1999) and EF2 (Jacobs et al. 2004) primer set. The reaction mixture and PCR conditions for the ITS region were the same as Arzanlou & Khodaei (2012a, b, c) and Arzanlou et al. (2012) and for ef-1α the same

as Bakhshi et al. (2012). PCR products were sequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) Cycle Sequencing Kit according to the recommendation of the vendor and analyzed on an ABI Prism 3700 (Applied Biosystems, Foster City, CA). Raw sequence files were edited manually by using SeqMan™II (DNASTAR, Madison, Wisconsin, USA) and a consensus sequence was generated for each of the sequences. Sequences were subjected to Blast search analysis at NCBI's GenBank nucleotide database for sequence similarity. The sequences obtained in this study were aligned together with the sequence data from GenBank by using ClustalW algorithm implemented in MEGA 5 (Tamura et al. 2011). A phylogentic tree was constructed using neighbor-joining method (kimura-2 as substitution model; gaps treatment as pairwise deletion). Transitions and transversions (with the equal ratio) were included in the analysis. The support of the internal nodes of the tree was evaluated by the bootstrap method with 10,000 replicates. The phylogenetic tree was rooted to *Lasiodiplodia theobromae* (GenBank accession numbers EU938329.1 and EU938330.1).

Results

Disease symptoms on naturally infected apple trees

The disease symptoms appear as shoot blights, stem cankers, brown discoloration of internal wood tissues in cross sections through the stems and trunk. Symptoms start as small depressed sunken spots on shoot, stem and the main trunk with wet appearance, expand to cover larger areas on infected tissues. At this stage, usually the skin of infected tissue separates and remains attached as a separate thin light brown layers on the infected tissue. The shoot infection eventually results in shoot blight; the cankers on stem and trunk may develop to girdle the stem and lead to death of upper parts of the branches or die back. In cross section through the infected stems and trunks the symptoms can be seen as brown discoloration of vascular tissues (Figs 1–5).

Morphological and cultural features

Pycnidia produced on pine needles on WA in 2–4 weeks, solitary or clustered, semi-immersed to erumpent, dark brown to black, globose to ovoid, up to 600 µm diam and 900 µm high, unilocular, sometimes multilocular with a short neck and a central ostiole; wall composed of three layers including a dark brown, thick-walled *textura angularis* outer layer, a middle layer with dark brown thin-walled cells, an inner layer with thin-walled hyaline cells. Conidiophores reduced to conidiogenous cells, formed from the inner wall of the picnidium. Conidiogenous cells 9−18 × 2−5 µm, hyaline, smooth, thin-walled, cylindrical, somewhat swollen at the base, discrete, indeterminate, hyaline, smooth, cylindrical, producing a single apical conidium, the first conidium holoblastic and subsequent conidia enteroblastic, proliferating internally and giving rise to periclinal thickenings, or with percurrent growth giving rise to 1−4 annellations on conidiogenous cells. Conidia aseptate, smooth to finely verruculose, thick-walled, oblong to ovoid, apex obtuse or

Table 1 Size of lesions developed on apple twigs inoculated with *Diplodia* isolates using excised shoot assay.

Isolates	Length (cm)[+]	Width (cm)[+]
CCTU 25	9.66 ± 3.5	3.5± 0.5
CCTU 25 a	12.33 ±1.52	3.33±0.76
CCTU 25 b	13 ±2	3.16±0.76
CCTU 25 c	11.16±.39	2.83± 0.76

[+] Values are means ± SD derived from three replicates for each treatment.

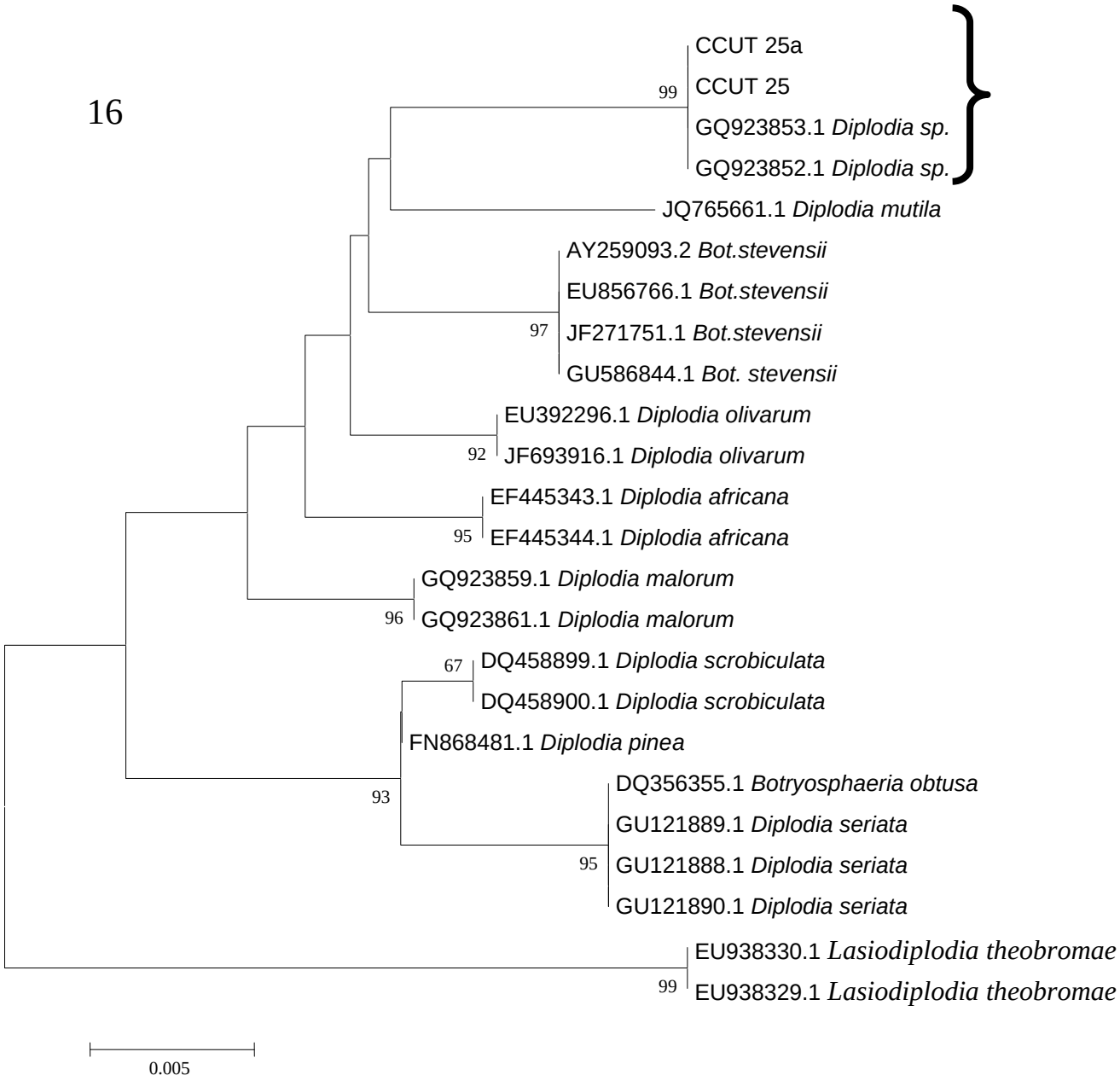


Fig 16 – A neighbor-joining phylogenetic tree obtained from the ITS region and 5.8S rDNA sequence data. Bootstrap support values from 1000 replicates are indicated on the nodes. The tree was rooted to *Lasiodiplodia theobromae*. Bootstrap values above 67 are shown above the nodes. The scale bar indicates 0.005 substitutions per site.

rounded, based truncate, (14−)24.7−27.8(−32) × (11−)14.1−15.4(−18) µm, 95% confidence limits = 25−25.7 × 16.6–17 µm (mean ± S.D. of 35 conidia = 26.22 ± 4.32 × 14.77 ± 1.7 µm, L/W ratio = 1.7 ± 0.3), initially hyaline, soon becoming pale brown, after discharge from picnidia, a few of them becoming one septate (Figs 6–15).

Cultural characteristics – colonies reaching 20 mm radius after 7 days on MEA in dark at 25^0C. Colonies fluffy with abundant aerial mycelium, irregular margin, at first white, darkening from the center of the colony after 3 days; colony olivaceous grey after 10 days; reverse dark grey. On PDA colonies reaching 35 mm radius after 7 days in dark at 25^0C, cottony with abundant aerial mycelium, lobate, at first white, darkening from the center of colony after 6 days; fully dark grey after 2 weeks.

DNA phylogeny

Phylogeny inferred using the sequence data of ITS region from the isolates obtained in

Figs 17–21– Pathogenicity assay using excided shoot method. **17** Disease symptoms developed on inoculated twigs after 14 days of inoculation a, b, c (inoculated with *Diplodia sp.*), d (control) **18** Cortex necrosis and discoloration on inoculated twigs a, b, c (inoculated with *Diplodia sp.*), d (control) **19–20** Conidiomata developed on inoculated twigs after one month (arrows indicate pycnidia) **21** Cross section through inoculated shoots a, b, c (inoculated with *Diplodia sp.*), d (control).

this study with other known *Diplodia* species occurring on apple and other hosts clustered our isolates in a separate clade together with two uncharacterized *Diplodia* spp. from apple trees (Fig. 16). The same result was obtained with ef-1α gene sequences. The sequences are available at GenBank with the accession numbers JX468096, JX468097 for ITS, and JX468098, JX468099 for ef-1α gene (Fig 16).

Pathogenicity test

All four *Diplodia* isolates tested were pathogenic on detached twigs of golden delicious apple. The disease symptoms were evaluated after 14 days of incubation at room temperature. Disease symptoms appeared as brown sunken lesions at inoculation point, which then expanded upward and downward from the inoculation point. Brown discoloration was observed in woods, whereas apple twigs in the control set remained nonsymptomatic (Figs 17–21). The average length and width of lesions developed on twigs are presented (Table 1). Fruiting structures (conidiomata) of the fungi developed in necrotic lesions. The same fungus was recovered from the inoculated twigs, while no fungal growth was observed in controls.

Discussion

The morphological characteristics of the fungal isolates recovered from apple trees with canker and dieback symptoms clearly fit with the concept of the genus *Dilpodia* (sensu Phillips et al. 2005). Conidiomata uni- or multilocular with no paraphyses; conidiogenous cells proliferating internally which give rise to periclinal thickenings, or proliferate percurrently and form two or three annellations; conidia hyaline, aseptate, thick-walled, typically remaining hyaline for a long time before they become brown and develop one-septum, but in some species, such as *D. seriata*, the conidia become coloured before discharge from the pycnidia and predominantly remain non-septate (Phillips et al. 2005).

Diolpodia is a species rich genus in the family Botryosphaeriaceae causing economically important diseases on mainly woody host plant species such as *D. pinea* on pines (Eldridge 1961, Alves et al. 2006), *D. corticola* on cork and other oaks (Alves et al. 2004). Several species of *Diplodia* have been reported to cause canker, leaf spot and fruit rot on apple worldwide namely *D. seriata*, the cause of frog-eye leaf spot, and *D. mutila* and *D. malorum* which cause black rot and canker of apples (Stevens 1933, Laundon 1973, Brown & Britton 1986, Brown-Rytlewski & McManus 2000). The *Diplodia* isolates obtained in this study are morphologically and phylogenitically distinct from the other known *Diplodia* spp. on apple. The main morphological characters to distinguish this species are the dimensions of conidia which are shorter and wider than those of *D. seriata, D. mutila* and *D. malorum* and conidial pigmentation. In the present fungus conidia become pale brown soon after they are formed, whereas in *D. seriata* conidia are initially hyaline, becoming dark brown before release from the pycnidia. In *D. mutila* and *D. malorum* conidia are aseptate, becoming dark brown and one-septate soon after release from the pycnidium.

The phylogeny inferred using sequence data from ITS sequence data further confirmed *Diplodia* isolates from apple in this study as a new lineage. The isolates clustered in a separate clade from the other *Diplodia* spp. from apple with a high bootstrap support value (99%). Our isolates clustered with two uncharacterized species of *Diplodia* originated from *Malus sylvestris* in Bulgaria with the accession numbers GQ923852 and GQ923853. The same results obtained using sequence data from ef-1α gene.

The results of pathogenicity assay using excised shoot assay revealed that the isolates are highly pathogenic on apple. The data on pathogenicity of *Diplodia* spp. on woody hosts are controversial. For example, *D. seriata* has been considered as a primary pathogen of grapevines being highly virulent (Larignon et al. 2001, Savocchia et al. 2007, Epstein et al. 2008). However, results obtained by other researchers have shown *D. seriata* as a weak or secondary pathogen on grapevine (Phillips 1998, 2000, 2005 Van Niekerk et al. 2004, Laveau et al. 2009). Similar results have been reported on the pathogenicity of *D. seriata* on apple. In the USA *D. seriata* is regarded as an important pathogen of apple which causes canker, leaf spot and fruit rot of this host (Stevens 1933, Brown & Britton 1986, Brown-Rytlewski & McManus 2000); however, it is regarded as a secondary and weak pathogen on apple in England and New Zealand (Laundon 1973). Such results might be either an artifact of overlapping morphological characters which makes species boundaries vague or difference in the virulence amongst different isolates. On apple *D. mutila* and *D. malorum* are known to cause canker and fruit rot. In present study, the fruit rot symptoms were not observed in apple orchards and no attempts were made to test the pathogenicity of *Dilpodia* isolates recovered from canker symptoms on fruits.

We have not introduced the *Diplodia* isolates from apple as a new species. We think that multiple gene genealogy with more isolates is needed to further resolve the identity of this new lineage of *Diplodia* on apple. This is mainly because of simple morphology of the genus and few distinguishing morphological features to delineate species. In the past taxonomy of *Diplodia* spp. has been based on host plant association, which has led to introduction of many names in this genus. The host plant association criterion has proven to be less important for species differentiation in Botryosphaeriaceae (Slipper et al. 2004), and possibly many of the names in *Diplodia* are synonyms. To avoid such confusions we do not introduce a new name for *Diplodia* isolates in this study. The host range and distribution of this new pathogen of apple remains to be studied.

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