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Dwiroopa punicae sp. nov. (*Dwiroopaceae fam. nov., Diaporthales*), associated with leaf spot and fruit rot of pomegranate (*Punica granatum*)

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Abstract: During a survey of diseases affecting pomegranate in the southeastern USA we identified a unique species of *Diaporthales* causing leaf spotting and fruit rot. Objectives of this study were to provide a morphological description of the putative new species, use DNA sequence data of three gene loci (LSU, ITS and *rpb2*) to accurately place the fungus within the *Diaporthales*, and to prove Koch's postulates. Morphological and phylogenetic comparisons confirmed the fungus to represent a new species and family, for which the names *Dwiroopa punicae sp. nov.* and *Dwiroopaceae fam. nov*. are proposed. This is the first report of a species of *Dwiroopa* being pathogenic to *Punica granatum.*

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INTRODUCTION

Pomegranate (*Punica granatum*) is a perennial fruit crop, which has been cultivated for over 5 000 years. It is believed that the origin of pomegranate is in Iran, Western Asia, from where it dispersed to India, China and the Mediterranean region. Pomegranate was introduced to North America by the Spanish in the 1700's (Chandra *et al*. 2010). It is widely accepted that pomegranate first arrived in the southeastern USA (Florida and Georgia) and was then brought to California by missionaries in the 1770's (Stover & Mercure 2007). Currently, the major pomegranate-producing countries of India, Iran, China, USA, and Turkey make up 76 % of the total worldwide production (Jain & Desai 2018).

California is currently the largest USA producer with almost 33 000 acres planted (NASS 2012). Although pomegranate was introduced into the southeastern USA more than two centuries ago, it has only recently emerged as an important fruit crop in the region. In Florida, the production area is estimated at 100 acres and probably about the same for Georgia, Alabama, and South Carolina, combined. In this region, the area under cultivation remains mostly experimental. However, it is expected that pomegranate production in the southeastern USA will grow in the near future, as producers seek alternative fruit crops. Pomegranate provides an additional fruit crop compatible with cultivation practices and equipment currently used in avocado, blueberry and citrus production in the region.

In California, the low relative humidity in the San Joaquin Valley reduces the risk of foliar diseases. Therefore, in California post-harvest diseases caused by *Botrytis cinerea* and *Alternaria alternata* are the main concern (Day & Wilkins 2009, Tedford *et al*. 2005). However, the high relative humidity and warm weather in the southeastern USA are more conducive for foliar and fruit diseases caused by *Colletotrichum* spp. (Xavier *et al.* 2017), *Coniella granati* (KC & Vallad 2016b), *Lasiodiplodia theobromae* (KC & Vallad 2015), *Neofusicoccum parvum* (KC & Vallad 2016a), and *Pseudocercospora punicae* (Day & Wilkins 2009). In addition, an unknown pathogen, which proved to be a member of the *Diaporthales*, was also found causing leaf spots, stem canker and fruit rot. Beyond its association with pomegranate in the southeastern USA, little is known about this species, and whether it can affect other hosts.

Here we describe a new species of *Diaporthales* from diseased pomegranate in the southeastern USA, and confirm its pathogenicity on pomegranate fruits, leaves, and stems to complete Koch's postulates. Furthermore, we use DNA sequence data of three loci to accurately place this species as the family level within the *Diaporthales*.

MATERIALS AND METHODS

Isolates

The 10 isolates used in this study were recovered from symptomatic leaves, flowers, buds, stems, and fruits of pomegranate during orchard surveys conducted in 2014–2015 in Florida and Georgia (Table 1). Symptomatic tissues were

Table 1. Details of the strains included for molecular and/or morphological study. Novel sequences are indicated in bold font.

			GenBank accession numbers[2]		
Fungal species	**Culture accession no.[1]**	**Host/substrate**	**ITS**	**LSU**	***rpb2***
Apoharknessia insueta	CBS 111377^{ET} = CPC 1451	*Eucalyptus pellita*	JQ706083	AY720814	–
	CBS 114575 = CPC 10947	*Eucalyptus pellita*	JQ706082	AY720813	–
Aurapex penicillata	CMW10032	*Miconia theaezans*	AY214312	AY194104	–
Aurifilum marmelostoma	CBS 124929^{PT} = CMW28285	*Terminalia mantaly*	FJ882855	HQ730873	–
Celoporthe dispersa	CBS 118782^{T} = CMW9976	*Syzygium cordatum*	DQ267130.2	HQ730853	–
Celoporthe eucalypti	CMW26913	*Eucalyptus* EC48 clone	HQ730839	HQ730865	–
Chromendothia citrina	CBS 109758 = AR3446	*Quercus mongolica*	**MK510674**	AF408335	DQ862015
Coniella africana	CBS 114133^{T} = CPC 405	*Eucalyptus nitens*	AY339344	AY339293	KX833421
Coniella fragariae	CBS 172.49^{NT} = STE-U 3930	*Fragaria* sp.	AY339317	AY339282	KX833472
Coniella granati	CBS 152.33	*Punica granatum*	MH855389	MH866839	KX833485
Coniella koreana	CBS 143.97^{T}	–	KX833584	AF408378	KX833490
Coniella obovata	CBS 111025^{T} = CPC 4196 = IMI 261318	Leaf litter	AY339313	KX833409	KX833497
Coniella peruensis	CBS 110394^{T} = RMF 74.01	Soil in rain forest	KJ710463	KJ710441.2	KX833499
Coniella straminea	CBS 149.22^{AC} = STE-U 3932	*Fragaria* sp.	AY339348	AY339296	KX833506
Coniella tibouchinae	CBS 131594^{T} = CPC 18511 = BECM1 = VIC 31443	*Tibouchina granulosa*	JQ281774	JQ281776.2	KX833507
Coniella wangiensis	CBS 132530^{T} = CPC 19397	*Eucalyptus* sp.	JX069873	JX069857.2	KX833509
Cryphonectria parasitica	AFTOL-ID 2123 = ATCC 38755	*Castanea dentata*	AY141856	EU199123	DQ862017
Cryptometrion aestuescens	CBS 124008 = CMW18790	*Eucalyptus grandis*	GQ369458	HQ730869	–
Disculoides eucalypti	CBS 132183^{T} = CPC 17650	*Eucalyptus* sp.	JQ685517	JQ685523	–
Disculoides eucalyptorum	CBS 132184^{T} = CPC 17648	*Eucalyptus viminalis*	JQ685518	JQ685524	–
Dwiroopa lythri	CBS 109755^{T} = AR3383	*Lythrum salicaria*	**MK510675**	AF408364	**MK510691**
Dwiroopa punicae sp. nov.	CBS 143163^{T} = A1 = CPC 30194	*Punica granatum*, leaf	**MK510676**	**MK510686**	**MK510692**
	A2	*Punica granatum*, pedicel	**MK510677**	–	**MK510693**
	A3 = CPC 30195	*Punica granatum*, fruit	**MK510678**	–	**MK510694**
	A4	*Punica granatum*, fruit	**MK510679**	–	**MK510695**
	A6	*Punica granatum*, leaf	**MK510680**	–	**MK510696**
	A7	*Punica granatum*, stem	**MK510681**	**MK510687**	**MK510697**
	A12	*Punica granatum*, buds	**MK510682**	–	**MK510698**
	A15	*Punica granatum*, flower	–	**MK510688**	**MK510699**
	A16	*Punica granatum*, flower	**MK510683**	–	**MK510700**
	A18	*Punica granatum*, flower	**MK510684**	**MK510689**	–
Endothiella gyrosa	AR3396	*Quercus* sp.	–	AF362555	–
Erythrogloeum hymenaeae	CBS 132185^{ET} = CPC 18819	*Hymenaea courbaril*	JQ685519	JQ685525	–
Greeneria saprophytica	MFLUCC 12-0298	*Syzygium cumini*	KJ021933	KJ021935	–
Harknessia eucalypti	CBS 342.97	*Eucalyptus regnans*	AY720745	AF408363	–
Harknessia karwarrae	CBS 115648 = CPC 10928	*Eucalyptus botryoides*	AY720748	AY720841	–
Harknessia weresubiae	CBS 113075	*Eucalyptus* sp., leaf litter	AY720741	AY720835	–
Holocryphia eucalypti	CBS 115852 = CMW14545	*Eucalyptus* sp.	JQ862840	JQ862797	–
Immersiporthe knoxdaviesiana	CBS 132862^{T} = CMW37314	*Rapanea melanophloeos*	JQ862770	JQ862760	–
Latruncellus aurorae	CMW28274	*Galpinia transvaalica*	GU726946	HQ730871	–
Luteocirrhus shearii	CBS 130775^{PT} = Bb16HGW 133/175	*Banksia baxteri*	KC197024	KC197018	–
Mastigosporella anisophylleae	CBS 136421^{T} = CPC 22462	*Anisophyllea* sp.	**MK510685**	**MK510690**	–
	CPC 22461	*Anisophyllea* sp.	KF779492	KF777221	–
Melanconiella ellisii	BPI 878343	*Carpinus caroliniana*	JQ926271	JQ926271	JQ926339

Table 1. (Continued).

Fungal species	Culture accession no.[1]	Host/substrate	GenBank accession numbers[2]		
			ITS	LSU	*rpb2*
Melanconiella spodiaea	SPOD1	*Carpinus betulus*	JQ926301	JQ926301	JQ926367
Oblongisporothyrium castanopsidis	CBS 189.71^{T} = A686 = ATCC 22470 = IFO 9263 = IMI 157598 = MUCC2289 = NBRC 9263	*Castanopsis cuspidata*	MG591850	MG591943	MG976454
Paratubakia subglobosa	CBS 193.71^{T} = A685 = ATCC 22474 = IFO 8931 = IMI 157596 = MUCC2310 = NBRC 8931	*Quercus glauca*	MG591914	MG592009	MG976490
Paratubakia subglobosoides	MUCC2293^{T} = NBRC 9343	*Quercus glauca*	MG591915	MG592010	MG976491
Sordaria fimicola	CBS 508.50 = TRTC 12135	Dung	AY681188	AY681160	DQ368647
Tubakia dryinoides	MUCC2292^{T} = NBRC 9267	*Quercus phillyraeoides*	MG591878	MG591970	MG976461
Tubakia japonica	MUCC2296^{ET} = NBRC 9268 = ATCC 22472	*Castanea crenata*	MG591886	MG591978	MG976465
Tubakia suttoniana	CBS 639.93^{IT}	*Quercus rubra*	MG591921	MG592016	MG976493
Wuestneia molokaiensis	CPC 19269	*Eucalyptus cypellocarpa*	JQ706127	JQ706248	–

[1] AFTOL: Assembling the Fungal Tree of Life culture collection; AR: Personal collection of Amy Rossman; ATCC: American Type Culture Collection, Virginia, USA; BPI: U.S. National Fungus Collections, Systematic Botany and Mycology Laboratory, USA; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMW: Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CPC: Culture collection of Pedro Crous, The Netherlands; IFO: Institute for Fermentation, Osaka, Yodogawa-ku, Osaka, Japan (collection transferred to NBRC); IMI: International Mycological Institute, Kew, UK; MFLUCC: Mae Fah Luang University Culture Collection, Thailand; MUCC (Japan): Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie Prefecture, Japan; NBRC: NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan; RMF: Rocky Mountain Herbarium, University of Wyoming, USA; STE-U: University of Stellenbosch, Plant Pathology Department, South Africa; TRTC: Royal Ontario Museum, Cryptogamic Herbarium, Canada. T: ex-type culture; EI: ex-isotype culture; EN: ex-neotype culture; EP: ex-paratype culture; ET: ex-epitype culture; AC: Authentic culture.

[2] ITS: internal transcribed spacers and intervening 5.8S nrDNA; LSU: large subunit (28S) of the nrRNA gene operon; *rpb2*: partial DNA-directed RNA polymerase II second largest subunit gene.

surface-disinfested with 1 % chlorine for 1 min, washed three times with sterile water, blotted dry on sterile paper towels, and then placed on V8 juice agar containing rifampicin (0.01 mg/L) and ampicillin (0.25 mg/L). Pure cultures from all isolates were obtained by single-spore and preserved on glycerol at -80 °C (Kitamoto *et al*. 2002).

Morphological characterisation

Samples from colonies sporulating on 2 % potato dextrose agar (PDA), oatmeal agar (OA), 2 % malt extract agar (MEA) (Crous *et al*. 2019b), and autoclaved pine needles on 2 % tap water agar (PNA) (Smith *et al.* 1996), were mounted in clear lactic acid. Observations were made with a Nikon SMZ25 dissection-microscope, and with a Zeiss Axio Imager 2 light microscope using differential interference contrast (DIC) illumination and images recorded on a Nikon DS-Ri2 camera with associated software. Colony characters and pigment production were noted after 2 wk of growth on MEA, PDA and OA incubated at 25 °C. Colony colours (surface and reverse) were scored using the colour charts of Rayner (1970). Sequences derived in this study were deposited in GenBank (Table 1; calmodulin and beta-tubulin sequences with accession numbers MK510701–MK510709 and MK510710–MK510716, respectively), the alignment in TreeBASE (www.treebase.org; study number 23965), and taxonomic novelties in MycoBank (www.MycoBank.org; Crous *et al*. 2004).

Pathogenicity assays

Pathogenicity was tested on detached pomegranate fruits, 3-mo-old rooted pomegranate cuttings, and 2-yr-old pomegranate saplings. Four fruits of pomegranate cultivar Wonderful were surface disinfested with 1 % aqueous solution of chlorine. Two wounds were created on the surface of each fruit to a depth of 3 mm with a 7 mm cork borer. A 7-mm-diam colonised mycelial plug was excised from the growing edge of 2-wk-old colonies and placed in each wound on the fruit and immediately wrapped with a plastic paraffin film (Parafilm M, Sigma). Control fruits were wounded and treated in a similar way using a sterile plug of V8 agar. The fruits were assessed for disease incidence every day until two weeks after inoculation when the entire fruits were rotten. The entire test was repeated.

The ability of the pathogen to produce leaf spot symptoms were tested on three replicate sets of 2-yr-old saplings of cultivar Azadi. The spore suspension was adjusted to a concentration of 1×10^6 spores/mL using a bright line haemocytometer counting chamber (Model 3900; Hausser Scientific Company, Horsham, Pa.). Three branches per tree were sprayed with the conidial suspension until runoff. The branches were covered with plastic bags for 24 h to maintain humidity. Control treatment included sterile distilled water sprayed in another three branches of the same tree and covered with plastic bags for the same amount of time as spore inoculated branches. The leaves were examined every other day for the symptom development until 20 d after inoculation.

For the pomegranate cuttings assay, one pomegranate cutting of cultivar Wonderful was planted per pot (15 cm) in Pro-

Mix BX (Premiere Horticulture, Ltd, Riviere du Loup, PQ, Canada). Each treatment replicate included one pot containing one plant. The spore suspension was adjusted to a concentration of 1 × 10^6 spores/mL using a bright line haemocytometer counting chamber (Model 3900; Hausser Scientific Company, Horsham, Pa.). Cuttings were inoculated with a spore suspension using 1 L plastic spray bottles. Inoculated cuttings were incubated for 72 h in box containers with 100 % humidity under 12 h photoperiod at 24 °C until the appearance of first symptoms on inoculated leaves. Three cuttings were sprayed with water and used as a negative control. This experiment was performed once.

Two-year-old saplings (n = 2) cultivar Wonderful were used to evaluate the ability of the fungus to cause stem canker. The five primary trunks of two saplings were cut with a sterilised scalpel to open a wound (1 cm × 1 cm) and place the mycelial plug. Controls (n = 2) consisted of wounded trunks covered similarly with a non-colonised piece of V8 agar. The inoculated trees were incubated in a growth chamber for 10 d under 12 h light and 95 % humidity. After 10 d the trees were moved to an outdoor protected, high-tunnel and maintained until the symptoms appeared on inoculated saplings for approximately 6 mo.

Phylogenetic analyses

A multi-locus sequence analysis (MLSA) including two representative strains of the fungus was performed. The primer pairs used were: (i) ITS1 and ITS4 (White *et al*. 1990) to amplify the internal transcribed spacer regions and included 5.8S nrRNA gene (ITS) sequence of the nrDNA operon; (ii) ITS1 (White *et al.* 1990) and LR5 (Vilgalys & Hester 1990) to amplify the ITS region and approximately 850 bp of the 5' end of the 28S rRNA gene (LSU) ; (iii) fRPB2-5F and fRPB2-7cR (Liu *et al*. 1999) were used for the amplification of partial DNA-directed RNA polymerase II second largest subunit gene (*rpb2*), (iv) T1 (O'Donnell & Cigelnik 1997) and Bt-2b (Glass & Donaldson 1995) primers were used to amplify part of the β-tubulin gene (*tub2*) and (v) CAL-228F and CAL-737R (Carbone & Kohn 1999) were used to amplify part of the calmodulin gene (*cmdA*). Each amplification reaction was performed in a final volume of 25 µL containing: 25 ng of genomic DNA, 12.5 µL GoTaq® Green Master Mix 2× (Promega Corporation, Madison, WI, USA) and 2 µL of each primer (10 µM). All PCR were conducted in an Eppendorf thermocycler (Eppendorf AG, Hamburg, Germany) with conditions as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at (i) 62.9 °C for ITS, (ii) 62.9 °C for *tub2,* (iii) 55 °C for *cmdA* or (iv) 47.5 °C for LSU; extension at 72 °C for 1 min; with a final extension at 72 °C for 10 min. The PCR thermal cycle conditions for partial *rpb2* gene were 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 50 s and elongation at 72 °C for 1.5 min, with a final extension step of 72 °C for 7 min (Liu *et al.* 1999). The PCR amplicons were purified with ExoSap and submitted to Elim Biopharmaceuticals, Inc. (25495 Whitesell St., Hayward, CA 94545) for sequencing. Forward and reverse primers were used to sequence the amplicons with the BigDye Terminator cycle-sequencing chemistry (Applied Biosystems, Foster City, CA) on an ABI 3730 DNA Capillary Sequencer following the manufacturer's protocol in a 10 µL reaction volume.

The generated sequences for each gene were subjected to megablast searches (Zhang *et al*. 2000) to identify closely related sequences in the NCBIs GenBank nucleotide database. Based on these searches, the novel sequences belonged to *Diaporthales*. Therefore, the resulting ITS, LSU and *rpb2* sequences were added to the alignment of Senanayake *et al*. (2017) and analysed under the same conditions used by those authors with MrBayes v. 3.2.6 (Ronquist *et al*. 2012). This tree showed an affiliation of the novel sequences with *Harknessiaceae* and *Cryphonectriaceae* (data not shown, see TreeBASE). A final Bayesian analysis was performed using a subset of the data from Senanayake *et al*. (2017) and supplemented with data from Braun *et al*. (2018); trees were sampled every 100 generations, the temperature parameter was set to 0.15 and the stop value to 0.01.

RESULTS

Field sampling and fungal isolation

Symptoms of leaf and fruit lesions attributable to the new genus were detected in pomegranate orchards in Florida and Georgia (Fig. 1A–D). The disease was reported on 2–8-yr-old trees in open fields. Symptoms were first observed in April and new symptoms continued to be observed throughout the season until harvest. Disease symptoms in several orchards during

Fig. 1. Leaf spot (**A, C**) and fruit rot (**B, D**) symptoms observed on pomegranate trees, naturally infected with the novel fungal species, in Central Florida during the summer of 2017. Pycnidia can be visualised as black dots in the centre of the leaf (C) fruit (D) lesions.

2014 and 2017 ranged between 10–30 % leaf spot severity and more than 30 % fruit rot incidence. Symptoms on pomegranate leaves consisted of oval, brown leaf spots ranging from 0.1 to 1.5 cm diam, which were scattered on the leaf surface (Fig. 1C). Black, melanised pycnidia were observed in the centre of developing lesions (Fig. 1C, D). Dark brown, round conidia were observed erupting from the pycnidial conidiomata. Fruit symptoms started as small brown lesions mostly on the fruit calyx at different flower or fruit stages (Fig. 1B, D). Similar to lesions on the leaves, fruit lesions increased in size and became black due to the abundance of pycnidia on the fruit surface (Fig. 1D). Fruit infection often led to premature fruit drop and caused an internal rot of the arils.

DNA phylogeny

All sequences obtained from the different loci for the novel isolates were identical. Blast searches with the calmodulin and beta-tubulin sequences had distant similarity (approx. 80 % similarity on average) to species of *Harknessia*. The final combined LSU/ITS/*rpb2* alignment contained 45 isolates representing families around, and including members of *Harknessiaceae* and *Cryphonectriaceae*. A strain of *Sordaria fimicola* (CBS 508.50; *Sordariales*) was used as outgroup. The final alignment contained a total of 2 163 characters (LSU: 772, ITS: 500, *rpb2*: 891) which were used for the phylogenetic analyses, including alignment gaps and missing data for incomplete genes. These characters represented 808 unique site patterns (LSU: 152, ITS: 234, *rpb2*: 422). The Bayesian analyses generated 153 202 trees from which 114 902 trees were sampled after 25 % of the trees were discarded as burn-in. The posterior probability values (PP) were calculated from these 114 902 trees (Fig. 2; PP > 0.69 shown, PP values > 0.94 were considered as credible). The sequences of the novel isolates clustered sister to *Dwiroopa lythri* and a new species is introduced below to accommodate them. In addition, the *Dwiroopa* and *Harknessia* clades were

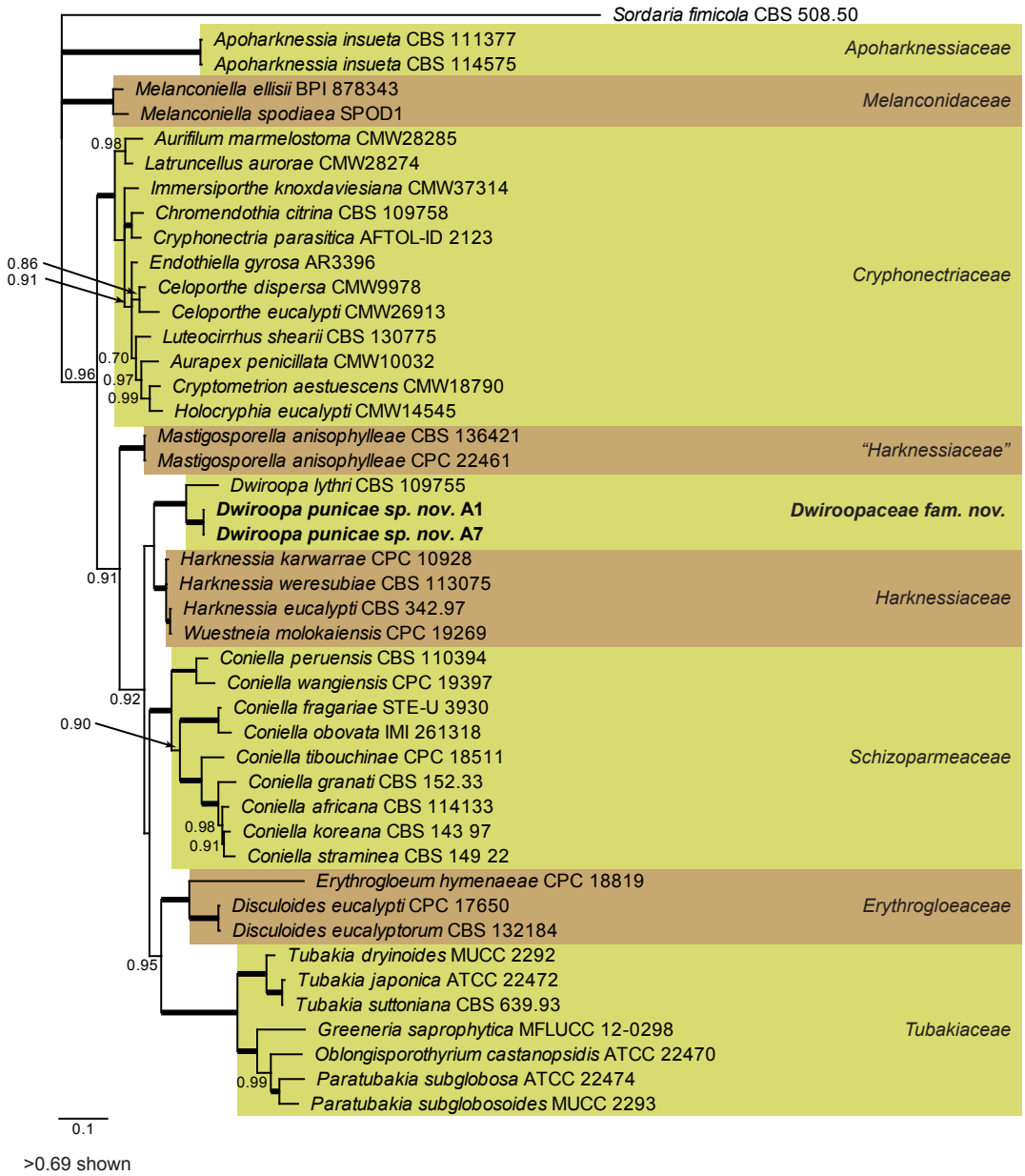

Fig. 2. Consensus phylogram (50 % majority rule) resulting from a Bayesian analysis of the combined LSU/ITS/*rpb2* sequence alignment. Bayesian posterior probabilities (PP) > 0.69 are shown at the nodes and thickened lines represent nodes with PP = 1.00. The scale bar represents the expected changes per site. Families are indicated with coloured blocks to the right of the tree. Culture accession numbers are indicated behind the species names. The tree was rooted to *Sordaria fimicola* (culture CBS 508.50) and the taxonomic novelties described in this study are indicated in **bold** face.

fully supported; however, the node linking the two genera was poorly supported (PP = 0.62) and therefore a new family is introduced below to accommodate *Dwiroopa*.

Morphology

Dwiroopaceae K.V. Xavier, KC, J.Z. Groenew., Vallad & Crous, ***fam. nov.*** MycoBank MB830873.

Etymology: Name refers to the genus *Dwiroopa*.

Conidiomata pycnidial, erumpent, globose, black, with central ostiole, oozing a slimy black conidial mass; wall of 3–6 layers of pale brown *textura angularis*. *Macroconidiophores* reduced to conidiogenous cells, ampulliform to subcylindrical, hyaline to pale brown, proliferating percurrently at apex. *Macroconidia* solitary, dark brown, aseptate, granular, guttulate, thick-walled, broadly ellipsoid to obovoid, conidium body with various longitudinal slits running along the entire length of the conidium body. *Mesoconidia* hyaline to pale brown, ellipsoid, aseptate. *Microconidia* ellipsoid, hyaline.

Type species: *Dwiroopa ramya* Subram. & Muthumary

Dwiroopa punicae K.V. Xavier, KC, J.Z. Groenew., Vallad & Crous, ***sp. nov.*** MycoBank MB830874. Fig. 3.

Etymology: Name refers to the host genus *Punica* from which it was isolated.

Conidiomata pycnidial, erumpent, globose, black, 150–300 µm diam, with central ostiole, oozing a slimy black conidial mass; wall of 3–6 layers of pale brown *textura angularis*. *Macroconidiophores* reduced to conidiogenous cells, ampulliform to subcylindrical, hyaline to pale brown, proliferating percurrently at apex, 5–12 × 4–6 µm. *Macroconidia* solitary, dark brown, aseptate, granular, with 1–2 large central guttules, thick-walled, broadly ellipsoid, apex obtuse, base truncate, 2–2.5 µm diam, conidium body with various longitudinal slits running along the entire length of the conidium body, (12–)13–16(–20) × (10–)12–14(–15) µm. *Meso-* and *microconidia* not seen.

Culture characteristics: Colonies erumpent, spreading, with sparse to moderate aerial mycelium covering the dish after 2 wk at 25 °C. On MEA surface and reverse isabelline. On PDA surface and reverse cinnamon. On OA surface buff, with numerous black conidiomata.

Typus: **USA**, Florida, Haines City, (28.0419, -81.5678), on leaf of *Punica granatum var.* Azadi (*Punicaceae*), Nov. 2014, A.N. KC (**holotype** CBS H-23915, culture ex-type CBS 143163 = A1 = CPC 30194).

Additional materials examined: **USA**, Florida, Haines City, on pedicel of *Punica granatum var.* Azadi, Nov. 2014, A.N. KC, culture A2; Florida, Haines City (28.0419, -81.5678), on fruit of *Punica granatum var.* Wonderful, Nov. 2014, A.N. KC, culture A3; Florida, Haines City (28.0419, -81.5678), on fruit of *Punica granatum var.* Wonderful, Nov. 2014, A.N. KC, culture A4; **USA**, Florida, Haines City (28.0419, -81.5678), on stem of *Punica granatum var.* Wonderful, Nov. 2014, A.N. KC, culture A5; USA, Florida, Odessa (28.1171, -82.6067), on leaf of *Punica granatum var.* Vikusnyi, Aug. 2014, A.N. KC, culture A6; USA, Florida, Odessa (28.1171, -82.6067), on stem of *Punica granatum var.* Borris, Aug. 2014, A.N. KC, culture A7; USA, Florida, Zolfo Springs (27.4135, -81.6425), on buds of *Punica granatum var.* Vietnam, Mar. 2015, A.N. KC, culture A8; USA, Florida, Zolfo Springs (27.4135, -81.6425), on buds of *Punica granatum var.* Shari, Mar. 2015, A.N. KC, culture A12; Georgia, Tifton (31.4789, -83.5195), on flower of *Punica granatum var.* Don Somner North, Apr. 2015, W. Lovett, culture A15; Georgia, Tifton (-31.4789, -83.5195), on flower of *Punica granatum var.* Eve, May, 2015, W. Lovett, culture A16; Georgia, Tifton (-31.4789, -83.5195), on flower of *Punica granatum var.* Kaj-Acik, Apr, 2015, W. Lovett, culture A18.

Note: *Dwiroopa punicae* adds a third species to the genus *Dwiroopa*, being distinct from *D. lythri* (on *Lythrum salicaria*; macroconidia 10.6–18.5 µm × 8.9–15.4 µm) and *D. ramya*

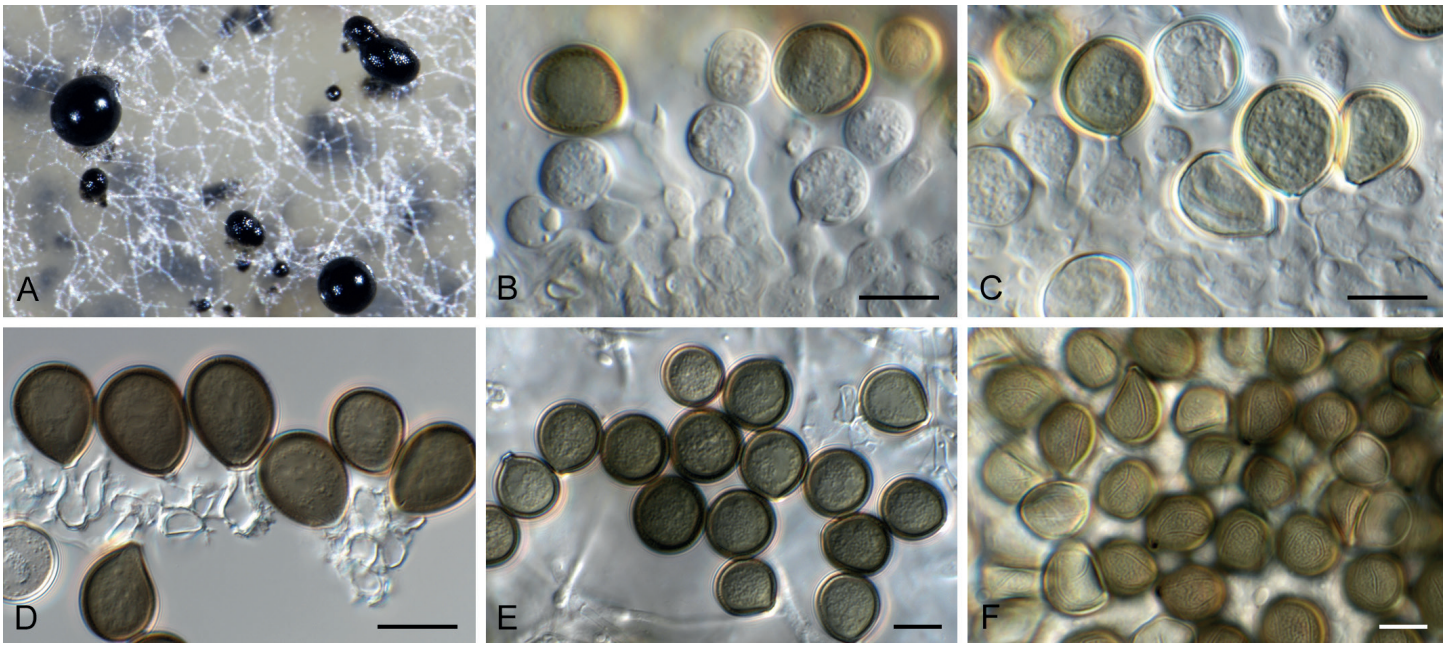

Fig. 3. *Dwiroopa punicae* (CBS 143163). **A.** Colony sporulating on OA. **B–D.** Conidiogenous cells giving rise to conidia. **E.** Thick-walled conidia. **F.** Conidia with longitudinal slits. Scale bars = 10 µm.

(on branch of unknown tree; macroconidia 21.2–26.9 × 12.0–17.2 µm; Farr & Rossman 2003). The *Dwiroopaceae* is morphologically and phylogenetically allied to *Harknessia* (*Harknessiaceae*). Lee *et al.* (2004) compared the two genera, and found them to be distinct in their conidiogenesis. Conidiogenous cells of *Dwiroopa* are thick-walled, and the point of conidial attachment forms a slightly darker scar upon dehiscence, with a minute marginal frill. In contrast, conidial appendages in species of *Harknessia* are longer, tubular, and thin-walled. Furthermore, *Dwiroopa* tends to have three conidial types (Farr & Rossman 2003), with its macroconidia having longitudinal slits running along the entire length of the conidium body, which is lacking in *Harknessia*.

Pathogenicity assays

Visible symptoms of the pathogen colonising the wounded fruits started 3 d after inoculation as soft margins and visible fungal colony. The symptoms around the wounded tissues increased as brown soft lesions and internal decay. Two weeks after inoculation 100 % of inoculated fruits exhibited soft rot (Fig. 4A, B). The symptomatic tissues were cultured again on half strength PDA and the same pathogen was re-isolated confirming Koch's postulates. Mock-inoculated fruits remained free of any fruit rot symptoms.

Symptoms on inoculated leaves consisted of discrete leaf spots, and necrosis mostly on the leaf borders 1 wk after inoculation (Fig. 4C). The dark brown conidia were visible at the surface of symptomatic leaves and the pathogen was re-isolated on half strength PDA from symptomatic tissues. The mock-inoculated control leaves remained symptomless, becoming brown and curled as they senesced.

Symptoms on pomegranate cuttings started to appear 5 d after inoculation as necrotic lesions scattered on the leaves. Disease evaluation was performed 2 wk after inoculation with 100 % of infected cuttings. Visible symptoms on inoculated saplings started as soft margins around the wound approximately 1 mo after inoculation. All inoculated wounds (100 %) resulted in canker-like symptoms. The symptoms extended in both directions from the wound with browning of internal tissues and the presence of pycnidia on the bark surrounding the wound, which produced dark brown conidia and the pathogen was re-isolated from symptomatic tissues. Approximately 60 % of inoculated branches eventually wilted and died approximately 6 mo after inoculation. Mock-inoculated pomegranate trees developed callus tissues around the wound site and remained free of disease symptoms.

DISCUSSION

In this study, we are describing a new fungal species infecting *P. granatum* across the southeastern USA that, together with *Colletotrichum* spp., was identified as the most important yield limiting factor in commercial production in the region. These two fungal pathogens are capable of causing almost 100 % fruit loss if left unmanaged (Vallad unpubl. data).

Diaporthales isolates were recovered from *P. granatum* in Florida and Georgia from 2014 to 2015. Isolates were identified based on MLSA and morphological characters to represent a unique *Diaporthales* species causing leaf spotting and fruit rot of pomegranate. These analyses revealed that the isolates represent a novel species, described here as *Dwiroopa punicae*.

The genus *Dwiroopa* is relatively obscure, being known presently from two other species that have only been found once each, namely *D. lythri* on *Lythrum salicaria* in the USA, and *D. ramya* on branches of an unknown tree in India (Subramanian & Muthumary 1986, Farr & Rossman 2003, Lee *et al*. 2004). Given that there are only these two reported species in the genus *Dwiroopa*, very little is known about the pathogenicity of *Dwiroopa* species. *Dwiroopa ramya* is considered a saprophyte and was isolated from dead twigs (Subramanian & Muthumary 1986), while *D. lythri* was demonstrated to be a pathogen on the noxious weed *L. salicaria* (Farr & Rossman 2001). In this study, pathogenicity tests with isolates of *D. punicae* confirmed that the fungus was able to cause disease on inoculated stems, leaves and fruits of *P. granatum*, thereby completing Koch's postulates.

Dwiroopa is morphologically similar to *Harknessia*, but is distinguished in lacking long basal conidia appendages, and having prominent longitudinal slits along the length of its conidia, which are absent in *Harknessia*. Although *Harknessia* was shown to represent a distinct family in *Diaporthales* (*Harknessiaceae*; Crous *et al*. 2012), the exact phylogenetic position of *Dwiroopa* remained obscure.

The first DNA phylogenetic revision of *Diaporthales* resolved six major lineages in the order, namely *Diaporthaceae*, *Gnomoniaceae*, *Melanconidaceae*, *Valsaceae* (now *Cytosporaceae*), and the *Schizoparme* and *Cryphonectria-Endothia* complexes (Castlebury *et al.* 2002). In 2007, nine families were recognised, namely *Cytosporaceae, Sydowiellaceae, Pseudovalsaceae, Togniniaceae*, and the *Cryphonectria-Endothia* and *Schizoparmeaceae* complexes, which became *Cryphonectriaceae* and *Schizoparmeaceae*, respectively (Rossman *et al*. 2007). In 2008, the family *Melogrammataceae* was added by Kirk *et al*. (2008), and in 2012, *Harknessiaceae*, *Lamproconiaceae*, *Pseudoplagiostomataceae*, and *Tirisporellaceae* were added (Cheewangkoon *et al*. 2010, Crous *et al*. 2012, Norphanphoun *et al*. 2016, Suetrong *et al*. 2015). A further eight new families were added and a total of 28 were subsequently accepted based on MLSA (Fan *et al.* 2018, Braun *et al.* 2018, Crous *et al.* 2019a), with *Dwiroopaceae* representing yet one additional family to the order.

There are some well-known fungal pathogens within the *Diaporthales* that can occur on woody plants, including *Cryphonectria parasitica* that causes chestnut blight (Gryzenhout et al., 2006), species of *Diaporthe* associated with twig, branch and trunk cankers in citrus (Guarnaccia & Crous, 2017) and grapevine (Guarnaccia *et al*. 2018). Cytospora cankers (Lawrence *et al*. 2018), and Coniella leaf spots and fruit rot (Alvarez *et al*. 2016), to name but a few. Clearly several of the diaporthalean families contain genera and species that are severe pathogens of diverse and economically important hosts. Although *Dwiroopa* has long remained obscure, the present study has resolved it to represent a distinct family within *Diaporthales*, *Dwiroopaceae*, and to include a new species, *D. punicae*, which represents a severe pathogen of pomegranate.

Although *Dwiroopa punicae* may be present in other pomegranate-growing regions of the world, the fact that it has not previously been described on pomegranate beyond the southeastern USA suggests that it may, in fact, represent a "New World" disease in which a fungus native to the southeastern USA has adapted to pomegranate as an introduced host. Further collections are now required in other pomegranate-growing regions of the world to elucidate its distribution and relative importance.

Fig. 4. Pathogenic assays on detached fruits, 2-mo-old cuttings, and 2-yr-old saplings. **A.** Soft rot development and visible fungal colony on the surface of inoculated fruits. **B.** Softening and internal decay of inoculated fruits. **C.** Leaf spots a week after incubation. **D.** Mock-inoculated control. **E–G.** Bark necrosis extending in both directions at the site of wound inoculation of 2-yr-old potted saplings. **H–I.** Internal necrosis and pycnidia on the surface of inoculated branches.

REFERENCES

Alvarez LV, Groenewald JZ, Crous PW (2016). Revising the *Schizoparmaceae*: *Coniella* and its synonyms *Pilidiella* and *Schizoparme*. *Studies in Mycology* **85**: 1–34.

Braun U, Nakashima C, Crous PW, *et al*. (2018). Phylogeny and taxonomy of the genus *Tubakia s. lat*. *Fungal Systematics and Evolution* **1**: 41–99.

Carbone I, Kohn LM (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.

Castlebury LA, Rossman AY, Jaklitsch WJ, *et al*. (2002). A preliminary overview of the *Diaporthales* based on large subunit nuclear ribosomal DNA sequences. *Mycologia* **94**: 1017–1031.

Chandra R, Babu KD, Jadhav VT, *et al*. (2010). Origin, history and domestication of pomegranate. *Fruit, Vegetable and Cereal Science and Biotechnology* **2**: 1–6.

Cheewangkoon R, Groenewald JZ, Verkley GJM, *et al*. (2010). Re-evaluation of *Cryptosporiopsis eucalypti* and *Cryptosporiopsis*-like species occurring on *Eucalyptus*. *Fungal Diversity* **44**: 89–105.

Crous PW, Gams W, Stalpers JA, *et al*. (2004). MycoBank: an online initiative to launch mycology into the 21st century. *Studies in Mycology* **50**: 19–22.

Crous PW, Schumacher RK, Akulov A, *et al.* (2019a). New and Interesting Fungi. 2. *Fungal Systematics and Evolution* **3**: 57–134.

Crous, PW, Schumacher, RK, Wingfield, MJ, *et al*. (2018). New and interesting fungi. 1. *Fungal Systematics and Evolution* **1**: 169–215.

Crous PW, Verkley GJM, Groenewald JZ, *et al.* (eds) (2019b). *Fungal Biodiversity*. *Westerdijk Laboratory Manual Series* **1**: 1–425. Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Crous PW, Summerell BA, Shivas RG, *et al*. (2012). A re-appraisal of *Harknessia* (*Diaporthales*), and the introduction of *Harknessiaceae fam. nov. Persoonia* **28**: 49–65.

Day K, Wilkins E (2009). Commercial pomegranate (*Punica granatum* L.) production in California. *Acta Horticulturae* **890**: 275–286.

Fan XL, Bezerra JD, Tian CM, *et al*. (2018). Families and genera of diaporthalean fungi associated with canker and dieback of tree hosts. *Persoonia* **40**: 119–134.

Farr DF, Rossman AY (2001). *Harknessia lythri*, a new species on purple loosestrife. *Mycologia* **93**: 997–1001.

Farr DF, Rossman AY (2003). *Dwiroopa*, a coelomycetous genus with two species. *Mycoscience* **44**: 443–446.

Guarnaccia V, Groenewald JZ, Woodhall J, *et al.* (2018). *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. *Persoonia* **40**: 135–153.

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Gryzenhout M, Myburg H, Wingfield B, *et al*. (2006). *Cryphonectriaceae* (*Diaporthales*), a new family including *Cryphonectria, Chrysoporthe, Endothia* and allied genera. *Mycologia* **98**: 239–249.

Guarnaccia V, Crous PW (2017). Emerging citrus diseases in Europe caused by species of *Diaporthe*. *IMA Fungus* **8**: 317–334.

Jain K, Desai N (2018). Pomegranate the Cash Crop of India: A Comprehensive Review on Agricultural Practices and Diseases. *International Journal of Health Sciences & Research* **8**: 315–336.

KC A, Vallad G (2015). Pomegranate (*Punica granatum* L.) in Florida: A disease snapshot. (Abstr.) *Phytopathology* **105** (Suppl. 2), S2.6.

KC A, Vallad G (2016a). First report of *Neofusicoccum parvum* causing shoot blight and stem cankers on pomegranate in Florida. *Plant Disease* **100**: 1783.

KC A, Vallad G (2016b). First report of *Pilidiella granati* causing fruit rot and leaf spots on pomegranate in Florida. *Plant Disease* **100**: 1238.

Kirk PM, Cannon PF, Minter DW, *et al.* (2008). *Ainsworth & Bisby's Dictionary of the Fungi, 10th Edition*. CABI Europe - UK.

Kitamoto Y, Suzuki A, Shimada S, *et al*. (2002). A new method for the preservation of fungus stock cultures by deep-freezing. *Mycoscience* **43**: 143–149.

Lawrence DP, Holland LA, Nouri MT, *et al*. (2018). Molecular phylogeny of *Cytospora* species associated with canker diseases of fruit and nut crops in California, with the descriptions of ten new species and one new combination. *IMA Fungus* **9**: 333–370.

Lee S, Groenewald JZ, Crous PW (2004). Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (*Diaporthales*), and the introduction of *Apoharknessia* gen. nov. *Studies in Mycology* **50**: 235–252.

Liu YJ, Whelen S, Hall BD (1999). Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* **16**: 1799–1808.

NASS (2012). *Census of Agriculture*. National Agricultural Statistics Service, USDA. United States Department of Agriculture.

Norphanphoun C, Hongsanan S, Doilom M, *et al*. (2016). *Lamproconiaceae* fam. nov. to accommodate *Lamproconium desmazieri. Phytotaxa* **270**: 89–102.

O'Donnell K, Cigelnik E (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103–116.

Rayner RW (1970). *A mycological colour chart*. CMI and British Mycological Society, Kew, UK.

Ronquist F, Teslenko M, Van der Mark P, *et al*. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539–542.

Rossman AY, Farr DF, Castlebury LA (2007). A review of the phylogeny and biology of the *Diaporthales. Mycoscience* **48**: 135–144.

Senanayake IC, Crous PW, Groenewald JZ, *et al*. (2017). Families of *Diaporthales* based on morphological and phylogenetic evidence. *Studies in Mycology* **86**: 217–296.

Smith H, Wingfield MJ, Crous PW, *et al.* (1996). *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* **62**: 86–88.

Stover E, Mercure EW (2007). The pomegranate: a new look at the fruit of paradise. *HortScience* **42**: 1088–1092.

Subramanian CV, Muthumary J (1986). *Dwiroopa*, a new genus of the Coelomycetes. *Proceedings of the Indian Academy of Science (Plant Science)* **96**: 191–197.

Suetrong S, Klaysuban A, Sakayaroj J, *et al.* (2015). *Tirisporellaceae*, a new family in the order *Diaporthales* (*Sordariomycetes, Ascomycota*). *Cryptogamie, Mycologie* **36**: 319–330.

Tedford EC, Adaskaveg JE, Ott AJ (2005). Impact of Scholar (a new post-harvest fungicide) on the California pomegranate industry. *Plant Health Progress* **10**: 94.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

White TJ, Bruns T, Lee S, *et al*. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, *et al.*, eds). Academic Press, San Diego, USA: 315–322.

Xavier K, Nepal A, Peres N, *et al*. (2017). Identification of *Colletotrichum* species causing anthracnose of pomegranate in Florida. (Abstr.) *Phytopathology* **107**: S5.114.

Zhang Z, Schwartz S, Wagner L, *et al*. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7**: 203–214.