Emerging citrus diseases in Europe caused by species of *Diaporthe*

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**Abstract:** Species of *Diaporthe* are considered important plant pathogens, saprobes, and endophytes on a wide range of plant hosts. Several species are well-known on citrus, either as agents of pre- or post-harvest infections, such as dieback, melanosporum and stem-end rot on fruit. In this study we explored the occurrence, diversity and pathogenicity of *Diaporthe* species associated with *Citrus* and allied genera in European orchards, nurseries, and gardens. Surveys were carried out during 2015 and 2016 in Greece, Italy, Malta, Portugal, and Spain. A total of 79 *Diaporthe* strains were isolated from symptomatic twigs, branches and trunks. A multi-locus phylogeny was established based on five genomic loci (ITS, lef1, cal, his3 and tub2), and the morphological characters of the isolates determined. Preliminary pathogenicity tests were performed on lemon, lime, and orange plants with representative isolates. The most commonly isolated species were *D. foeniculina* and *D. baccae*, while only four isolates of *D. novem* were collected. Two new *Diaporthe* species, described here as *D. limonicola* and *D. melitensis* spp. nov. were found associated with a new devastating dieback disease of lemon plants. Furthermore, one cluster of sterile *Diaporthe* isolates was renamed as *D. infertilis*. Pathogenicity tests revealed most of the *Citrus* species as susceptible to *D. baccae*, *D. foeniculina*, and *D. novem*. Moreover, *D. limonicola* and *D. melitensis* caused serious cankers affecting all the *Citrus* species tested. This study is the first report of *D. baccae* and *D. novem* on citrus in Europe, and the first detection of a new *Diaporthe* canker disease of citrus in Europe. However, no isolates of *D. citri* were found. The study improves our understanding of the species associated with several disease symptoms on citrus plants, and provides useful information for effective disease management.

**Key words:** Canker, Citrus, multi-locus sequence typing, pathogenicity

**INTRODUCTION**

*Diaporthe* species are present worldwide as plant pathogens and endophytes in healthy leaves, stems, seeds and roots, or as saprobes on decaying tissues of a wide range of hosts (Muralli et al. 2006, Garcia-Reyne et al. 2011, Udayanga et al. 2011). *Diaporthe* species are well-known as the causal agents of many important plant diseases, including root and fruit rots, dieback, stem cankers, leaf spots, leaf and pod blights, and seed decay (Uecker 1988, Mostert et al. 2001a, b, Van Rensburg et al. 2006, Rehner & Uecker 1994, Santos et al. 2011, Udayanga et al. 2011, Diaz et al. 2017). Species of *Diaporthe* have also been extensively screened in bioassays for natural products (Isaka et al. 2001, Dai et al. 2005, Kumaran & Hur 2009, Yang et al. 2010), and for the biocontrol of fungal pathogens (Santos et al. 2016).

The generic names *Diaporthe* and *Phomopsis* are no longer used to distinguish different morphs of this genus, and recent studies (Rossman et al. 2015) have recommended that *Diaporthe* be adopted as the correct generic name as it has priority over *Phomopsis*.

*Diaporthe* was historically considered monophyletic based on the typical *Phomopsis* asexual morph and diaporthalean sexual morph (Gomes et al. 2013). However, the paraphyletic nature was recently revealed by Gao et al. (2017), who demonstrated that *Ophiodiaporthe* (Fu et al. 2013), *Pustulomyces* (Dai et al. 2014), *Phaeocytostroma*, and *Stenocarpella* (Lamprecht et al. 2011), are embedded in *Diaporthe* s. lat. To address this issue, Senanayake et al. (2017) subsequently named several additional diaporthalean clades within *Diaporthales*.

The taxonomy of *Diaporthe* species has been reviewed in several major studies (Thompson et al. 2011, 2014, Gomes et al. 2013, Udayanga et al. 2014a, b, 2015). Almost 2000 species names are available for both *Diaporthe* and *Phomopsis* (Index Fungorum; http://www.indexfungorum.org). The majority of the known species in early literature were described in relation to their host association (Uecker 1988), except for about 150 species that have been described more recently supported by molecular data (Gomes et al. 2013, Lombard et al. 2014, Udayanga et al. 2014a, b, 2015). However, most *Diaporthe* species can be found on...
diverse hosts, and can co-occur on the same host or lesion in different life modes (Rehner & Uecker 1994, Mostert et al. 2001a, Guarnaccia et al. 2016). This is demonstrated by *D. foeniculina*, usually known as an opportunistic pathogen of various herbaceous weeds, ornamentals, and fruit trees including citrus (Santos & Phillips 2009, Udayanga et al. 2014b). However, it has also been isolated from tropical trees as an endophyte, and from herbaceous plants and weeds as a pathogen or saprobe (Udayanga et al. 2014a). As a consequence, identification and description of species based on host association alone is no longer tenable within *Diaporthe* (Gomes et al. 2013, Udayanga et al. 2014a, b).

Before the molecular era, morphological characters such as immersed ascomata and erumpent pseudostromata with elongated perithecial necks in the sexual morph (Udayanga et al. 2011), and black conidiomata with dimorphic conidia in the asexual morph (Rehner & Uecker 1994), was the basis on which to study the taxonomy of *Diaporthe* (Van der Aa et al. 1990). Recent studies demonstrated that these characters are not always reliable for species level identification due to their variability under changing environmental conditions (Gomes et al. 2013).

Following the adoption of DNA sequence-based methods, the polyphasic protocols for studying the genus significantly changed the classification and species concepts, resulting in a rapid increase in the description of novelties. Therefore, genealogical concordance methods, based on multi-gene DNA sequence data, provide a much clearer approach to resolving the taxonomy for *Diaporthe*.

Recent plant pathological studies have shown several *Diaporthe* species to be particularly important on a wide range of economically significant agricultural crops, such as blueberries, citrus, grapes, oats, sunflowers, soybeans, tea plants, tropical fruits, vegetables, and various trees (Van Rensburg et al. 2006, Crous et al. 2011a, b, 2016, Thompson et al. 2011, Santos & Phillips 2009, Santos et al. 2011, Grasso et al. 2012, Huang et al. 2013, Lombard et al. 2014, Gao et al. 2015, 2016, Udayanga et al. 2015, Guarnaccia et al. 2016). Furthermore, several *Citrus* species are colonized and/or affected by different *Diaporthe* species (Timmer et al. 2000, Huang et al. 2013), which are focussed on here.

**BACKGROUND**

*Citrus* represents one of the most important fruit industries worldwide. In the Mediterranean region, Greece, Italy, Portugal, and Spain especially are important producers of citrus fruits, and are the biggest fruit exporter after South Africa (FAO 2016). Therefore, recognizing the pathogens affecting these crops in these countries is imperative.

*Diaporthe citri* is a well-known pathogen causing melanose and stem-end rot disease of *Citrus* species in several regions (Timmer 2000, Mondal et al. 2007). Several additional *Diaporthe* species have been reported associated with *Citrus* (often as *Phomopsis*) and have previously been considered as synonyms of *D. citri*, such as *D. citrincola* described from the Philippines, *P. californica* from California, *P. caribaea* from Cuba, and *P. cytopsorella* from Italy (Rehm 1914, Fawcett 1922). Wehmeyer (1933) also considered *D. medusaea,* *P. citri,* and *P. citrincola* as synonyms of *Diaporthe citri*.

Polyphasic approaches in recent years have revealed many species associated with citrus. Huang et al. (2013) reported *D. citri* as the predominant species in China and described two new taxa: *D. citriasiana* and *D. citrichinensis*. In another study, Huang et al. (2015) identified several *Diaporthe* species as endophytes of citrus but which had previously been recovered from other hosts, such as *D. endophytica*, *D. eres*, *D. hongkongensis*, *D. sojae*, and the different taxa clustering in the *D. arceae* species complex. Moreover, they described *D. biconispora*, *D. biguttulata*, *D. discoidispora*, *D. multiguttulata*, *D. ovalispora*, *D. subclavata*, and *D. unshiuensis* as new species occurring on citrus.

Several strains from China, Korea, New Zealand, and the USA have been re-assessed by Udayanga et al. (2014b) within *D. citri*, which was also epitypified. In the same study, *D. cytopsorella* was recovered from specimens of *Citrus limon*, *C. limonia*, and *C. sinensis* collected respectively in Spain, Italy, and the USA, and *D. foeniculina* has also been widely associated with citrus.

*Diaporthe citri* is generally accepted as an important pathogen of citrus, causing stem-end rot and melanoese of fruits, young leaf and shoot gummosis, and blight of perennial branches and trunks (Kucharek et al. 1983, Timmer & Kucharek 2001, Mondal et al. 2007, Udayanga et al. 2014b). This species occurs in many citrus growing regions of the world on several *Citrus* species, including *C. limon*, *C. paradisi*, *C. reticulata*, and *C. sinensis* (Timmer et al. 2000).

Further infections involving twigs, perennial branches and trunks of citrus are caused by other *Diaporthe* species, such as cankers developing in woody tissues, often with a gummose exudate, generating serious blight and dieback (Huang et al. 2013, Mahadevakumar et al. 2014). Canker diseases of citrus are also caused by other fungal genera such as *Fusarium* and *Neocosmospora* (Sandoval-Denis et al. 2018), and species of *Botryosphaeriaceae* and *Diatrypaceae* (Timmer et al. 2000, Polizzi et al. 2009, Mayorquin et al. 2016).

Although the biology and epidemiology of melanose are well studied also with a robust phylogenetic relationship of the causal organisms, genetic variability and population structure (Burnett 1962, Mondal et al. 2004, 2007, Udayanga et al. 2014b), the identification of *Diaporthe* species associated with citrus cankers and dieback has not been well resolved. Moreover, Gomes et al. (2013) performed a major phylogenetic and morphological study of *Diaporthe* species and grouped three isolates, one of which was collected from *Citrus sinensis* in Suriname, under *D. citri*. However, Udayanga et al. (2014b) re-assessed *D. citri* based on molecular phylogenetic analysis of conserved ex-type and additional strains collected exclusively from symptomatic citrus tissues in different geographic locations worldwide. Furthermore, according to this latter study, *D. citri* is unknown in Europe. Because of all these findings, changes in species concepts and poor investigation of *Diaporthe* on citrus in Europe, new surveys were required to study *Diaporthe* species diversity related to citrus and their occurrence and association with diseases.

The current study aims to investigate the major citrus production areas in Europe by employing large-scale
sampling to isolate *Diaporthe* strains, and to identify the strains obtained in the light of modern taxonomic concepts via morphological characterization and multi-locus DNA sequence data. In 2015 and 2016, several surveys were conducted in commercial nurseries, citrus orchards, gardens, backyards, and plant collections to determine the occurrence of *Diaporthe* species associated with *Citrus* and allied genera (e.g. *Microcitrus*). In particular the objectives of the present study were to: (1) conduct extensive surveys for sampling symptomatic plant materials; (2) cultivate as many *Diaporthe* isolates as possible; (3) subject those isolates to DNA sequence analyses combined with morphological characterization; (4) compare the obtained results with the data from other phylogenetic studies on the genus; (5) place three strains previously named as *D. citri* in the correct taxonomic context based on DNA sequence inference; and (6) evaluate the pathogenicity of the isolated *Diaporthe* species to citrus plants.

**MATERIALS AND METHODS**

**Sampling and isolation**

During 2015 and 2016 many regions of the main citrus-producing area of Europe were surveyed (Guarnaccia et al. 2017a, b). Twig, branch and trunk portions showing cankers and dieback were collected from more than 90 sites in: Andalusia, Valencia, and the Balearic Islands (Spain); Apulia, Calabria, Sicily, and the Aeolian Islands (Italy); Algarve (Portugal); Arta, Crete, Missolonghi, and Nafplio (Greece); and Malta and Gozo (Malta). Investigated species of *Citrus* and allied genera such as *Microcitrus* (*Rutaceae*) included Australasian lime, citrons, kumquat, mandarins, oranges, pummelo, grapefruit, limes, and lemons.

Wood fragments (5 × 5 mm) were cut from the margin between affected and healthy tissues and washed in running tap water. Then, each fragment was surface sterilised by soaking in 70 % ethanol for 5 s, 4 % sodium hypochlorite for 90 s, sterile water for 60 s (Kumaresan & Suryanarayanan 2001) and then dried on sterile filter paper. The fragments were placed on malt extract agar (MEA; Crous et al. 2009) amended with 100 μg / mL penicillin and 100 μg / mL streptomycin (MEA-PS) and incubated at 25 °C until characteristic *Diaporthe* colonies were observed. In a second procedure, plant material was incubated in moist chambers at room temperature (20 ± 3 °C) for up to 10 d and inspected daily for fungal sporulation. Sporulating conidiomata obtained through both procedures were collected and crushed in a drop of sterile water and then spread over the surface of MEA-PS plates. After 24 h germinating spores were individually transferred onto MEA plates. The isolates used in this study are maintained in the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and in the working collection of Pedro Crous (CPC), housed at the Westerdijk Institute.

**DNA extraction, PCR amplification and sequencing**

Genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega, WI) following the manufacturer’s instructions. Partial regions of six loci were amplified. The primers ITS5 and ITS4 (White et al. 1990) were used to amplify the ITS region of the nuclear ribosomal RNA operon, including the 5’ end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene; the second internal transcribed spacer region and the 5’ end of the 28S rRNA gene. The primers EF1-728F and EF1-968R (Carbone & Kohn 1999) were used to amplify part of the translation elongation factor 1-a gene (*tef1*). Primers CAL-228F and CAL-737R (Carbone & Kohn 1999) or CL1/ CL2A (O’Donnell et al. 2000) were used to amplify part of the calmodulin (*cal*) gene. The partial histone H3 (*his3*) region was amplified using CYLH3F and H3-1b primer sets (Glass & Donaldson 1995, Crous et al. 2004a), and the beta-tubulin (*tub2*) region was amplified using Bt2a and Bt2b primer sets (Glass & Donaldson 1995). The PCR products were sequenced in both directions using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies, Carlsbad, CA), after which amplicons were purified through Sephadex G-50 Fine columns (GE Healthcare, Freiburg) in MultiScreen HV plates (Millipore, Billerica, MA). Purified sequence reactions were analysed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA). The DNA sequences generated were analysed and consensus sequences were computed using SeqMan Pro (DNASTAR, Madison, WI).

**Phylogenetic analyses**

New sequences generated in this study were blasted against the NCBI’s GenBank nucleotide database to determine the closest relatives for a taxonomic framework of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and sequences downloaded from GenBank, were initially performed with the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh & Standley 2013), and then manually adjusted in MEGA v. 7 (Kumar et al. 2016).

To establish the identity of the isolates at species level, phylogenetic analyses were conducted first individually for each locus (data not shown) and then as combined analyses of five loci. One analysis was performed for all the *Diaporthe* isolates recovered from samples collected during the surveys conducted for this study. Additional reference sequences were selected based on recent studies of *Diaporthe* species (Gomes et al. 2013, Huang et al. 2013, Udayangla et al. 2014a, b). Phylogenetic analyses were based on Maximum Parsimony (MP) for all the individual loci and on both MP and Bayesian Inference (BI) for the multi-locus analyses. For BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analyses. MrBayes v. 3.2.5 (Ronquist et al. 2012) was used to generate phylogenetic trees under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set to 0.2 and trees were sampled every 1000 generations. Analyses stopped once the average standard deviation of split frequencies was below 0.01. The MP analyses were done using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swoford 2003). Phylogenetic relationships were estimated by heuristic searches with 100
random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on “best trees” only with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony and the bootstrap analyses (Hillis & Bull 1993) were based on 1000 replications. Sequences generated in this study are deposited in GenBank (Table 1) and alignments and phylogenetic trees in TreeBASE (www.treebase.org).

Morphological analyses
Agar plugs (6 mm diam) were taken from the edge of actively growing cultures on MEA and transferred onto the centre of 9 cm diam Petri dishes containing 2 % tap water agar supplemented with sterile pine needles (PNA; Smith et al. 1996), potato dextrose agar (PDA), oatmeal agar (OA) and MEA (Crous et al. 2009), and incubated at 20–21 °C under a 12 h near-ultraviolet light/12 h dark cycle to induce sporulation as described in recent studies (Gomes et al. 2013, Lombard et al. 2014). Colony characters and pigment production on MEA, OA and PDA were noted after 10 d. Colony colours were rated according to Rayner (1970). Cultures were examined periodically for the development of ascomata and conidiomata. Colony diameters were measured after 7 and 10 d. The morphological characteristics were examined by mounting fungal structures in clear lactic acid and 30 measurements at ×1000 magnification were determined for each isolate using a Zeiss Axioskop 2 microscope with interference contrast (DIC) optics. Descriptions, nomenclature and illustrations of taxonomic novelties are deposited in MycoBank (www.MycoBank.org; Crous et al. 2004b).

Pathogenicity
Pathogenicity tests with five Diaporthe species isolated from the European citrus samples were performed to satisfy Koch’s postulates.

Two isolates of each of the five species (D. baccaei: CPC 26170, CPC 27831; D. foeniculina: CPC 28033, CPC 28081; D. limonicola: CPC 28200, CPC 31137; D. melitensis: CPC 27873, CPC 27875; and D. novem: CPC 26188, CPC 28165), were inoculated onto potted 2-yr-old healthy plants of lemon (Citrus limon), lime (C. aurantifolia), mandarin (C. reticulata), and two clones (‘New Hall’ and ‘Tarocco Meli’) of sweet orange (C. sinensis). Three plants per replicate for each isolate were inoculated, each having five wounds on twigs made using a sterile blade. Mycelial plugs (6 mm diam), taken from the margin of actively growing colonies on MEA, were placed on the wound sites on each plant. An equivalent number of plants and inoculation sites were inoculated with sterile MEA plugs and served as controls. The inoculation sites were covered with Parafilm® (American National Can, Chicago, IL). The inoculated plants were incubated with a 16 h photoperiod in a growth chamber at 100 % relative humidity and 25 ± 1 °C. After 2 mo external symptoms were assessed. Twigs were cut and the bark peeled off to check for any internal discolouration.

Small sections (0.5 cm) of symptomatic tissue from the edge of twig lesions were placed on MEA to re-isolate the fungal species, and were identified based on tef1 and tub2 sequencing to fulfil Koch’s postulates.

RESULTS

Isolates
Several shoot blight and canker infections on woody tissue were frequently observed on multiple Citrus species in all countries investigated. Some orchards presented blight of vigorously growing branches and cankers involving both scion branches and rootstock trunks, resulting in a general dieback and tree death (Fig. 1A). Affected trunks and branches appeared cracked, darkly discoloured and/or slightly sunken. Abundant gummosis was frequently associated with the affected tissues (Fig. 1B–D). Twigs showed wilting, typical dieback and wither-tip, and occasionally gummosis (Fig. 1E–F). Under the bark, cankers were reddish brown and variable in shape. Pyridional formation on dead twig tissue was observed (Fig. 1G). A total of 79 monsporic isolates resembling those of the genus Diaporthe were collected. The Diaporthe isolates were recovered from 10 species of Citrus at 31 sites in different locations of Greece, Italy, Malta, Spain, and Portugal. Among them, 27 isolates were obtained from branch infections, 13 were associated with trunk cankers, and 39 from twig dieback (Table 1).

Phylogenetic analyses
Six alignments were analysed representing single gene analyses of ITS, tub2, his3, tef1, cal and a combined alignment of the five genes. The alignments produced topologically similar trees. The combined species phylogeny of the Diaporthe isolates consisted of 123 sequences, including the outgroup sequences of Diaporthea corylina (culture CBS 121124). A total of 3026 characters (ITS: 1–582, tef1: 589–1052, tub2: 1059–1 862, cal: 1869–2484, his3: 2 491–3026) were included in the phylogenetic analysis, 1355 characters were parsimony-informative, 468 were variable and parsimony-uninformative, and 1161 were constant. A maximum of 1000 equally most parsimonious trees were saved (Tree length = 5528, CI = 0.584, RI = 0.868 and RC = 0.507). Bootstrap support values from the parsimony analysis are plotted on the Bayesian phylogenies in Fig. 2. For the Bayesian analyses, MrModeltest suggested that all partitions should be analysed with dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: GTR+I+G for ITS, tef1 and cal, HKY+G for tub2 and GTR+G for his3. In the Bayesian analysis, the ITS partition had 188 unique site patterns, the tef1 partition had 357 unique site patterns, the tub2 partition had 510 unique site patterns, the cal partition had 364 unique site patterns, the his3 partition had 239 unique site patterns and the analysis ran for 1 880 000 generations, resulting in 3762 trees of which 2822 trees were used to calculate the posterior probabilities.

In the combined analysis, 54 Citrus isolates clustered with five reference strains and the ex-type of D. foeniculina, whilst 14 isolates clustered with the ex-type of D. baccaei. Four isolates clustered with the ex-type strain of D. novem. Moreover, five isolates identified as D. limonicola and a further two as D. melitensis, formed two highly supported subdivisions (1.00/100) embedded in the D. arecae species complex.

The individual alignments and trees of the five single loci used in the analyses, were also compared with respect to their performance in species recognition. D. novem was differentiated by each gene used. Moreover, tef1 and tub2 separated both D. limonicola and D. melitensis from the other species belonging to the D. arecae species complex.
Fig. 1. Symptoms on citrus tissues with associated *Diaporthe* species. A. Commercial lemon orchard infected by *D. limonica* and *D. melitensis* (Malta). B–C. Trunk canker with gummosis of *Citrus limon* and *C. sinensis* plants (Malta). D. Branch canker of *C. sinensis* (Portugal). E–F. Twigs dieback of lemon (Italy). G. Orange twigs wither-tip with *Diaporthe* pycnidial formation (Italy).
Table 1. Collection details and GenBank accession numbers of isolates included in this study.

| Species        | Culture no. | Host                        | Locality | Associated symptoms | GenBank no.2 | GenBank no.1 |
|----------------|-------------|-----------------------------|----------|---------------------|--------------|--------------|
| D. angelicae   | CBS 111592  | Heracleum sphondylium       | Austria  | -                   | KC343026     | KC343994     |
| D. arecae      | CBS 161.64  | Areca catechu               | India    | -                   | KC343032     | KC344000     |
| D. arengae     | CBS 114979  | Arenga engleri              | Hong Kong| -                   | KC343034     | KC344002     |
| D. baccae      | CBS 136972  | Vaccinium corymbosum        | Italy    | -                   | KJ160565     | MF418509     |
| D. discoidispora| CPC 26170  | Citrus sinensis ‘Tarocco Tapi’| Italy, Catania| Twig dieback| MF418351 | MF418510 |
| D. endophytica | ZJUD73      | Citrus unshiu               | China    | -                   | KJ490597     | KJ490418     |
| D. eres        | CBS 439.82  | Cotoneaster sp.             | Scotland | -                   | KC343090     | KC344058     |
| D. foeniculina | CBS 187.27  | Camellia sinensis           | Italy    | -                   | KC343101     | KC344069     |
| D. foeniculina | CBS 111553  | Foeniculum vulgare          | Spain    | -                   | KC343101     | KC344069     |
Table 1. (Continued).

| Species          | Culture no. | Host              | Locality           | Associated symptoms          | GenBank no.         | ITS     | tub2    | his3    | tef1    | cal    |
|------------------|-------------|-------------------|--------------------|------------------------------|---------------------|---------|---------|---------|---------|--------|
| CBS 111554       |             | Foeniculum vulgare| Portugal           | -                            |                      | KC343102| KC344070| KC343586| KC343828| KC343344|
| CBS 123208       |             | Foeniculum vulgare| Portugal           | -                            |                      | KC343104| KC344072| KC343588| KC343830| KC343346|
| CBS 123209       |             | Foeniculum vulgare| Portugal           | -                            |                      | KC343105| KC344073| KC343589| KC343831| KC343347|
| CBS 135430       |             | Citrus limon      | USA                | -                            |                      | KC843301| KC843215| MF418284| MF418444| MF418199|
| CPC 26184        |             | Citrus maxima     | Italy, Messina     | Branch canker                | MF418365 MF418366   | MF418525| MF418286| MF418445| MF418200|
| CPC 26194        |             | Citrus sinensis 'Sanguinello' | Italy, Messina   | Branch canker                | MF418365 MF418366   | MF418525| MF418286| MF418445| MF418200|
| CPC 26365        |             | Citrus limon      | Italy, Catania     | Twig dieback                 | MF418367 MF418368   | MF418527| MF418287| MF418446| MF418201|
| CPC 26439        |             | Citrus reticulata | Italy, Catania     | Twig dieback                 | MF418368 MF418369   | MF418528| MF418289| MF418447| MF418202|
| CPC 26441        |             | Citrus reticulata | Italy, Catania     | Twig dieback                 | MF418369 MF418370   | MF418529| MF418290| MF418448| MF418203|
| CPC 26461        |             | Citrus reticulata | Italy, Catania     | Twig dieback                 | MF418370 MF418371   | MF418530| MF418290| MF418449| MF418204|
| CPC 26663        |             | Citrus maxima     | Greece, Missolonghi| Branch canker                | MF418371 MF418372   | MF418531| MF418291| MF418450| MF418205|
| CPC 26873        |             | Citrus reticulata | Greece, Arta       | Twig dieback                 | MF418372 MF418373   | MF418532| MF418292| MF418451| MF418206|
| CPC 26883        |             | Citrus maxima     | Greece, Missolonghi| Branch canker                | MF418373 MF418374   | MF418533| MF418293| MF418452| MF418207|
| CPC 26885        |             | Citrus bergamia   | Greece, Missolonghi| Branch canker                | MF418374 MF418375   | MF418534| MF418294| MF418453| MF418208|
| CPC 26913        |             | Citrus limon      | Greece, Missolonghi| Branch canker                | MF418375 MF418376   | MF418535| MF418295| MF418454| MF418209|
| CPC 26923        |             | Citrus maxima     | Greece, Missolonghi| Branch canker                | MF418376 MF418377   | MF418536| MF418296| MF418455| MF418210|
| CPC 26927        |             | Citrus maxima     | Greece, Missolonghi| Branch canker                | MF418377 MF418378   | MF418537| MF418297| MF418456| MF418211|
| CPC 26953        |             | Citrus bergamia   | Greece, Missolonghi| Branch canker                | MF418378 MF418379   | MF418538| MF418298| MF418457| MF418212|
| CPC 26967        |             | Citrus mitis      | Italy, Messina     | Twig dieback                 | MF418379 MF418380   | MF418539| MF418299| MF418458| MF418213|
| CPC 26971        |             | Citrus mitis      | Italy, Messina     | Twig dieback                 | MF418380 MF418381   | MF418540| MF418300| MF418459| MF418214|
| CPC 27027        |             | Citrus limon      | Italy, Cosenza     | Branch canker                | MF418381 MF418382   | MF418541| MF418301| MF418460| MF418215|
| CPC 27033        |             | Citrus mitis      | Italy, Messina     | Twig dieback                 | MF418382 MF418383   | MF418542| MF418302| MF418461| MF418216|
| CPC 27037        |             | Citrus paradisi   | Italy, Vibo Valentia| Branch canker                | MF418383 MF418384   | MF418543| MF418303| MF418462| MF418217|
| CPC 27041        |             | Citrus sinensis   | Italy, Cosenza     | Branch canker                | MF418384 MF418385   | MF418544| MF418304| MF418463| MF418218|
| CPC 27167        |             | Citrus paradisi   | Italy, Vibo Valentia| Branch canker                | MF418385 MF418386   | MF418545| MF418305| MF418464| MF418219|
| CPC 27756        |             | Citrus limon      | Italy, Catania     | Trunk canker                 | MF418386 MF418387   | MF418546| MF418306| MF418465| MF418220|
| CPC 27832        |             | Citrus sinensis   | Italy, Catania     | Trunk canker                 | MF418387 MF418388   | MF418547| MF418307| MF418466| MF418221|
| CPC 27833        |             | Citrus sinensis   | Italy, Catania     | Trunk canker                 | MF418388 MF418389   | MF418548| MF418308| MF418467| MF418222|
| CPC 27859        |             | Citrus paradisi   | Malta, Gozo        | Trunk canker                 | MF418390 MF418391   | MF418549| MF418309| MF418468| MF418223|
| CPC 27877        |             | Citrus limon      | Malta, Gozo        | Trunk canker                 | MF418392 MF418393   | MF418550| MF418310| MF418469| MF418224|
| CPC 27895        |             | Citrus japonica   | Malta, Gozo        | Twig dieback                 | MF418394 MF418395   | MF418551| MF418311| MF418470| MF418225|
| CPC 27896        |             | Citrus japonica   | Malta, Gozo        | Twig dieback                 | MF418396 MF418397   | MF418552| MF418312| MF418471| MF418226|
| Species                  | Culture no.¹ | Host            | Locality          | Associated symptoms | GenBank no.² |
|--------------------------|--------------|-----------------|-------------------|---------------------|--------------|
|                          |              |                 |                   |                     | ITS | tub2 | his3 | tef1 | cal |
| CPC 27897                | Citrus japonica | Malta, Gozo    | Twig dieback     | MF418393, MF418553, MF418313, MF418472, MF418227 |
| CPC 27898                | Citrus japonica | Malta, Gozo    | Twig dieback     | MF418394, MF418554, MF418314, MF418473, MF418228 |
| CPC 27901                | Citrus limon   | Malta, Gozo    | Branch canker    | MF418395, MF418555, MF418315, MF418474, MF418229 |
| CPC 27903                | Citrus limon   | Malta, Gozo    | Branch canker    | MF418396, MF418556, MF418316, MF418475, MF418230 |
| CPC 27945                | Citrus paradisi | Portugal, Faro | Branch canker    | MF418397, MF418557, MF418317, MF418476, MF418231 |
| CPC 27947                | Citrus sinensis | Portugal, Faro | Branch canker    | MF418398, MF418558, MF418318, MF418477, MF418232 |
| CPC 27949                | Citrus sinensis | Portugal, Faro | Branch canker    | MF418399, MF418559, MF418319, MF418478, MF418233 |
| CPC 27950                | Citrus sinensis | Portugal, Faro | Twig dieback    | MF418400, MF418560, MF418320, MF418479, MF418234 |
| CPC 27959                | Citrus sinensis | Portugal, Faro | Twig dieback    | MF418401, MF418561, MF418321, MF418480, MF418235 |
| CPC 28033 = CBS 142547   | Citrus sinensis 'Valencia' | Portugal, Mesquita | Twig dieback | MF418402, MF418562, MF418322, MF418481, MF418236 |
| CPC 28035                | Citrus paradisi | Portugal, Faro | Twig dieback    | MF418403, MF418563, MF418323, MF418482, MF418237 |
| CPC 28039                | Citrus limon   | Portugal, Monchique | Twig dieback | MF418404, MF418564, MF418324, MF418483, MF418238 |
| CPC 28041                | Citrus limon   | Portugal, Monchique | Twig dieback | MF418405, MF418565, MF418325, MF418484, MF418239 |
| CPC 28043                | Citrus limon   | Portugal, Monchique | Twig dieback | MF418406, MF418566, MF418326, MF418485, MF418240 |
| CPC 28045                | Citrus limon   | Portugal, Monchique | Twig dieback | MF418407, MF418567, MF418327, MF418486, MF418241 |
| CPC 28047                | Citrus limon   | Portugal, Monchique | Twig dieback | MF418408, MF418568, MF418328, MF418487, MF418242 |
| CPC 28071                | Citrus limon   | Spain, Alge mesi | Twig dieback    | MF418409, MF418569, MF418329, MF418488, MF418243 |
| CPC 28072                | Citrus limon   | Spain, Alge mesi | Twig dieback    | MF418410, MF418570, MF418330, MF418489, MF418244 |
| CPC 28073                | Citrus reticulata | Spain, Alge mesi | Twig dieback | MF418411, MF418571, MF418331, MF418490, MF418245 |
| CPC 28074                | Citrus reticulata | Spain, Alge mesi | Twig dieback | MF418412, MF418572, MF418332, MF418491, MF418246 |
| CPC 28077                | Citrus limon   | Spain, Alge mesi | Twig dieback    | MF418413, MF418573, MF418333, MF418492, MF418247 |
| CPC 28079                | Citrus reticulata | Spain, Alge mesi | Twig dieback    | MF418414, MF418574, MF418334, MF418493, MF418248 |
| CPC 28081 = CBS 142548   | Citrus reticulata | Spain, Alge mesi | Twig dieback    | MF418415, MF418575, MF418335, MF418494, MF418249 |
| CPC 28163                | Microcitrus australasia | Italy, Catania | Twig dieback | MF418416, MF418576, MF418336, MF418495, MF418250 |
| CPC 31135                | Citrus limon   | Malta, Gozo    | Branch canker    | MF418417, MF418577, MF418337, MF418496, MF418251 |
| CPC 31159                | Citrus sinensis | Malta, Zurieq   | Branch canker    | MF418418, MF418578, MF418338, MF418497, MF418252 |
| D. helianthi             | CBS 344.94    | Helianthus annuus | -    | -    | KC343114, KC344082, KC343598, KC343840, KC343356 |
| D. hongkongensis         | CBS 592.81    | Helianthus annuus | Serbia | -    | KC343115, KC344083, KC343599, KC343841, JX197454 |
| D. inconspicua           | CBS 115448    | Dichroa febrifuga | China | -    | KC343119, KC344087, KC343603, KC343845, KC343361 |
| D. infertilis            | CBS 133813    | Maytenus ilicifolia | Brazil | -    | KC343123, KC344091, KC343607, KC343849, KC343365 |
| CBS 199.39               | Unknown       | Italy          | -    | -    | KC343051, KC344019, KC343535, KC343777, KC343293 |
| CBS 230.52               | Citrus sinensis | Suriname      | -    | -    | KC343052, KC344020, KC343536, KC343778, KC343294 |
| Species            | Culture no. | Host           | Locality            | Associated symptoms     | GenBank no.                  |
|--------------------|-------------|----------------|---------------------|-------------------------|------------------------------|
|                    |             |                |                     |                         | ITS | tub2   | his3   | tef1   | cal     |
| CPC 20322          | Glycine max | Brazil         | -                   |                         | KC343053 | KC344021 | KC343537 | KC343779 | KC343295 |
| D. limonicola      | CPC 27869   | Citrus limon   | Malta, Gozo         | Trunk canker            | MF418419 | MF418579 | MF418339 | MF418498 | MF418253 |
|                    | CPC 27871   | Citrus limon   | Malta, Gozo         | Trunk canker            | MF418420 | MF418580 | MF418340 | MF418499 | MF418254 |
| CPC 27879          | Citrus limon | Malta, Gozo    | Branch canker       |                         | MF418421 | MF418581 | MF418341 | MF418500 | MF418255 |
| PC28200 = CBS 142549 | Citrus limon | Malta, Gozo    | Branch canker       |                         | MF418422 | MF418582 | MF418342 | MF418501 | MF418256 |
| PC31137 = CBS 142550 | Citrus limon | Malta, Zurrieq | Branch canker       |                         | MF418423 | MF418583 | MF418343 | MF418502 | MF418257 |
| D. melitensis      | CPC 27873   | Citrus limon   | Malta, Gozo         | Branch canker            | MF418424 | MF418584 | MF418344 | MF418503 | MF418258 |
|                    | CPC 27875   | Citrus limon   | Malta, Gozo         | Branch canker            | MF418425 | MF418585 | MF418345 | MF418504 | MF418259 |
| D. multigutullata  | ICMP20656   | Citrus grandis | China               | -                       | KJ490633 | KJ490454 | KJ490575 | KJ490512 | -       |
| D. novem           | CBS 127270  | Glycine max    | Croatia             | -                       | KC343156 | KC344124 | KC343640 | KC343882 | KC343398 |
| CBS 127271         | Glycine max | Croatia        | -                   |                         | KC343157 | KC344125 | KC343641 | KC343883 | KC343399 |
| CPC 26188 = CBS 142553 | Citrus japonica | Italy, Messina | Twig dieback       |                         | MF418426 | MF418586 | MF418346 | MF418505 | MF418260 |
| CPC 28165 = CBS 142554 | Citrus aurantiifolia | Italy, Catania | Twig dieback       |                         | MF418427 | MF418587 | MF418347 | MF418506 | MF418261 |
| CPC 28167          | Citrus aurantiifolia | Italy, Catania | Twig dieback       |                         | MF418428 | MF418588 | MF418348 | MF418507 | MF418262 |
| CPC 28169          | Citrus aurantiifolia | Italy, Catania | Twig dieback       |                         | MF418429 | MF418589 | MF418349 | MF418508 | MF418263 |
| D. ovalispora      | ICMP20659   | Citrus limon   | China               | -                       | KJ490628 | KJ490449 | KJ490570 | KJ490507 | -       |
| D. pseudomangiferae CBS 101339 | Mangifera indica | Dominican Republic | -                   |                         | KC343138 | KC344149 | KC343665 | KC343907 | KC343423 |
| D. pseudophoenicicola CBS 462.69 | Phoenix dactylifera | Spain            | -                   |                         | KC343184 | KC344152 | KC343668 | KC343910 | KC343426 |
| D. rudis           | CBS 113201  | Vitis vinifera | Portugal            | -                       | KC343234 | KC344202 | KC343718 | KC343960 | KC343476 |
| D. saccarata       | CBS 116311  | Protea repens  | South Africa        | -                       | KC343190 | KC344158 | KC343674 | KC343916 | KC343432 |
| D. sojae           | FAU 635     | Glycine max    | USA                 | -                       | KJ590719 | KJ610875 | KJ659208 | KJ590762 | -       |
| D. sojae           | ZJUD68      | Citrus unshiu  | China               | -                       | KJ490603 | KJ490424 | KJ490545 | KJ490482 | -       |
| D. sterilis        | CBS 136969  | Vaccinium corymbosum | Italy        | -                       | KJ160579 | KJ160528 | MF418350 | KJ160611 | KJ160548 |
| D. subclavata      | ICMP20663   | Citrus unshiu  | China               | -                       | KJ490630 | KJ490451 | KJ490572 | KJ490509 | -       |
| D. unshiuensis     | CGMCC3.17569 | Citrus unshiu  | China               | -                       | KJ490587 | KJ490408 | KJ490529 | KJ490466 | -       |
| Diaporthea corylina CBS 121124 | Corylus sp. | China         | -                   |                         | KC343004 | KC343372 | KC343488 | KC343730 | KC343246 |

1 CPC: Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CGMCC: China, General Microbiological Culture Collection, Beijing, China; FAU: Isolates in culture collection of Systematic Mycology and Microbiology Laboratory, USDA-ARS, Beltsville, MD, USA; ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; ZJUD, Diaporthe strains in Zhejiang University, China. Ex-type and ex-epitype cultures are indicated in **bold**.

2 ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; tub2: partial beta-tubulin gene; his3: histone3; tef1: partial translation elongation factor 1-α gene; cal: partial calmodulin gene. Sequences generated in this study indicated in *italics*. 
Table 2. *Diaporthe* species associated with citrus and their morphological characteristics.

| Species                   | Conidiomata (μm) | Conidiophores (μm) | Alpha conidia (μm) | Beta conidia (μm) | References               |
|---------------------------|------------------|--------------------|--------------------|-------------------|--------------------------|
| *D. arecae*               | 15–40 × 1.5–3    | 6–10 × 2–3         | -                  | -                 | Gomes et al. (2013)      |
| *D. baccae*               | 7–9 × 2–3        | 20–24 × 1–2        | -                  | -                 | Lombard et al. (2014)    |
| *D. bicornispora*         | 12–35.5 × 1.6–2.6| 6–10.5 × 2–3.5     | -                  | -                 | Huang et al. (2015)      |
| *D. biguttulata*          | 5.8–16.9 × 1.3–2.3| 5.7–7.8 × 2.5–2.9 | 23.7–31.6 × 0.9–1.6| Huang et al. (2015) |
| *D. citri*                | 10–15 × 1–2      | 7.6–10.2 × 3–4.2   | -                  | -                 | Udayanga et al. (2014b)  |
| *D. citrijsiana*          | 3.5–10.5 × 1–2   | 10.5–15 × 4–6.5    | 24–42 × 1–2        | Huang et al. (2013)  |
| *D. citrichinensis*       | 9–19.5 × 1.5–3   | 5.5–9 × 1.5–2.5    | 27.5–40 × 1–1.5    | Huang et al. (2013)  |
| *D. cytopsorea*           | 7–18 × 1–2       | 8–9 × 2.6–3.2      | -                  | -                 | Udayanga et al. (2014b)  |
| *D. discoideispora*       | 8.9–23.4 × 1.3–2.7| 5.6–8 × 2.1–3.2    | 21.2–38.7 × 0.9–1.6| Huang et al. (2015)  |
| *D. endophytica* (sterile)| 6.6–12.7 × 1.1–1.4| 8.6–16.7 × 1.8–2.1 | -                  | -                 | Gomes et al. (2013)      |
| *D. eses*                 | 10–15 × 2–3      | 6.5–8.5 × 3–4      | 22–28 × 1–1.5      | Udayanga et al. (2014a)|
| *D. foeniculina*          | 9–15 × 1–2       | 8.5–9 × 2.3–2.5    | 22–28 × 1.4–1.6    | Udayanga et al. (2014b)|
| *D. hongkongensis*        | 5–12 × 2–4       | 6–7 × 2.5          | 18–22 × 1.5–2      | Gomes et al. (2013)   |
| *D. infertilis* (sterile) | -                | -                  | -                  | -                 | This study               |
| *D. limoncola*            | 5–20 × 1.5–4     | 5.5–8.5 × 1.5–2.5  | 15–26.5 × 1–2      | This study         |
| *D. melittensis*          | 5–15 × 1.5–5.5   | 4.5–7 × 1.5–3      | -                  | -                 | This study               |
| *D. multiguttulata*       | 9.8–14.8 × 1.3–3.6| 8–12.6 × 4.2–6     | -                  | -                 | Huang et al. (2015)      |
| *D. novem*                | 5.3–10.4 × 1.9–3.2| 6.3–8.9 × 1.9–2.5  | 26.4–37.7 × 1–1.3  | Santos et al. (2011) |
| *D. ovalispora*           | 9.5–21.6 × 1.6–3.6| 6.1–7.9 × 2.7–3.8  | -                  | -                 | Huang et al. (2015)      |
| *D. sojae*                | 12–16 × 2–4      | 5.3–7.3 × 2–3      | -                  | -                 | Udayanga et al. (2015a)  |
| *D. subclavata*           | 14.2–27.3 × 1.6–2.6| 5.5–7.2 × 2.2–2.9  | -                  | -                 | Huang et al. (2015)      |
| *D. unshiuensis*          | 14.3–24.2 × 1.4–2.6| 5.2–7.5 × 2–3.9    | -                  | -                 | Huang et al. (2015)      |

**TAXONOMY**

Morphological observations, supported by phylogenetic inference, were used to identify three known species (*D. baccae*, *D. foeniculina*, and *D. novem*), and to recognize three new species described here (Table 2). One species (represented by three isolates) was sterile in culture, and is therefore characterized by DNA sequence data (Gomes et al. 2013).

**Diaporthe infertilis** Guaraccia & Crous, sp. nov.
MycoBank MB821727 (Fig. 3)

**Etymology:** Named after its sterile growth in culture.

**Diagnosis:** *Diaporthe infertilis* differs from its closest phylogenetic neighbour, *D. ovalispora*, in 26 unique fixed alleles in ITS locus, 68 in *tef1*, 30 in *tub2* and 48 in *his3* based on the alignments deposited in TreeBASE.

**Type:** Suriname: Paramaribo, from decaying fruit of *Citrus sinensis*, Apr. 1932, N.J. van Suchtelen (CBS H-23179 – holotype; CBS 230.52 – culture ex-type).

**Description:** Culture characteristics: Colony on MEA covering the entire plate after 10 d, pale luteous with abundant white compact aerial mycelium in fluctuating rings. On OA and PDA at first white, becoming cream to yellowish, flat, with dense and felted mycelium, reverse pale brown with brownish dots with age. Cultures sterile.

**Notes:** Three isolates clustered in a clade distinct from species of *Diaporthe* known from DNA sequence data. One strain (CPC 20322) was differentiated from the other two (CBS 199.39, CBS 230.52) by unique fixed alleles in four loci based on alignments of the separate loci deposited in TreeBASE: *tef1* positions 115 (C), 261 (indel), 314 (G), 438 (G); *tub2* positions 123 (C), 631 (G); *his3* positions 201 (A), 364 (G), 395 (C); *cal* positions 312 (T), 207 (A), 210 (T), 256 (T), 259 (T), 262 (A), 364 (G), 366 (A), 438 (G), 439 (G), 448 (C); *his3* positions 201 (A), 438 (A), 448 (T), 450 (A). Gomes et al. (2013) tentatively referred to this clade as *D. citri*. However, after a molecular re-assessment of many *Diaporthe* species, *D. citri* is restricted to a different clade of citrus isolates (Udayanga et al. 2014b). We therefore describe *D. infertilis* as a new species for this clade.

Fig. 2. Consensus phylogram of 3762 trees resulting from a Bayesian analysis of the combined ITS, *tub2*, *his3*, *tef1* and *cal* sequence. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. The asterisk symbol (*) represents full support (1/100).

Substrate and country of origin are listed next to the strain numbers. The newly recognized species are in red. The tree was rooted to *Diaporthella corylina* (CBS 121124).
Additional material examined: Brazil: from seeds of Glycine max, A. Almeida (culture LGMF946 = CPC 20322). – Italy: from unknown host, G. Goidanich (CBS 199.39).

*Diaporthe limonicola* Guarnaccia & Crous, sp. nov.
MycoBank MB821731
(Fig. 4)

*Etymology:* In reference to the occurrence on *Citrus limon*.

*Diagnosis:* *Diaporthe limonicola* can be distinguished from the closely related *D. pseudomangiferae* based on *tef1*, *tub2*, *his3* and *cal* loci (96% in *tef1*, 96% in *tub2*, 97% in *his3*, and 96% in *cal*). *Diaporthe limonicola* differs from *D. pseudomangiferae* in the shorter alpha conidia and in producing beta and gamma conidia.

*Type:* Malta: Gozo, from branch canker of *Citrus limon*, 11 Jul. 2016, V. Guarnaccia (CBS H-23126 – holotype; CBS 142549 = CPC 28200 – culture ex-type).

*Description:* Conidiomata pycnidial in culture on PNA, PDA, OA and MEA, solitary or aggregated, deeply embedded in
PDA, erumpent, dark brown to black, 250–670 μm diam, whitish translucent to cream conidial drops exuded from the ostioles. Conidiophores hyaline, smooth, 1-septate, densely aggregated, cylindrical, straight, 5–20 × 1.5–4 μm. Conidiogenous cells phialidic, hyaline, terminal, cylindrical, 5–12 × 1–2 μm, tapered towards the apex. Paraphyses intermingled among conidiophores, hyaline, smooth, 1–3-septate, to 90 μm long, apex 1–2 μm diam. Alpha conidia unicellular, aseptate, fusiform, hyaline, mono- to biguttulate and acute at both ends, 5.5–8.5 × 1.5–2.5 μm, mean ± SD = 6.8 ± 0.6 × 2.1 ± 0.3 μm, L/B ratio = 2.8. Beta conidia hyaline, aseptate, eguttulate, filiform, curved, tapering towards both ends, 15–26.5 × 1–2 μm, mean ± SD = 22.7 ± 2.6 ± 1.4 ± 0.3 μm, L/B ratio = 16.2. Gamma conidia hyaline, multiguttulate, fusiform to subcylindrical with an acute or rounded apex, 9–15.5 × 1–2 μm, mean ± SD = 10.7 ± 1.6 ×1.4 ± 0.2 μm, L/B ratio 7.6.

Culture characteristics: Colonies covering the medium within 1 wk at 21 °C, surface mycelium flattened, dense and felt-like. Colony on MEA and OA at first white, becoming cream to yellowish, flat, with dense and felted mycelium, reverse pale brown with brownish dots with age, with visible solitary or aggregated conidiomata at maturity. On PDA cream to smoke-grey, reverse pale brown.

Notes: Diaporthe limonica was isolated from Citrus limon trunk cankers in two different islands of the Malta archipelago, where all the plants were affected. Five strains representing D. limonica cluster in a well-supported clade, and appear most closely related to D. pseudomangiferae and D. arengea. Diaporthe limonica can be distinguished based on tef1, tub2, his3 and cal loci from D. pseudomangiferae (96 % in tef1, 96 % in tub2, 97 % in his3, and 96 % in cal), and from D. arengea (97 % in tef1, 98 % in tub2, 98 % in his3, and 96 % in cal). This species is phylogenetically close to but clearly differentiated from D. melitensis (described below) by 22 unique fixed alleles in ITS locus, 2 in tef1 and 47 in tub2.

Morphologically, D. limonica differs from D. pseudomangiferae in the shorter alpha conidia (5.5–8.5 vs. 7–9 μm) (Gomes et al. 2013) and the production of beta and gamma conidia, which are not known in D. pseudomangiferae (Gomes et al. 2013).

Additional material examined: Malta: Zurrieq, from branch canker of Citrus limon, 11 Jul. 2016, V. Guarnaccia (culture CBS 142550 = CPC 31137).

Diaporthe melitensis Guarnaccia & Crous, sp. nov. MycoBank MB821732 (Fig. 5)

Etymology: Named after the country where it was collected, Malta (ancient Latin name, Melita).

Diagnosis: Diaporthe melitensis can be distinguished from the closely related D. pseudomangiferae by the ITS, tef1, tub2, his3 and cal loci (98 % in ITS, 96 % in tef1, 97 % in tub2, 97 % in his3, and 96 % in cal). Diaporthe melitensis also differs from D. pseudomangiferae in the shorter alpha conidia.

Type: Malta: Gozo, from branch canker of Citrus limon, 22 Sep. 2015, V. Guarnaccia (CBS H-23127 – holotype; CBS 142551 = CPC 27873 – culture ex-type).

Description: Conidiomata pycnidial in culture on PNA, PDA, OA and MEA, solitary or aggregated, deeply embedded in the PDA, erumpent, dark brown to black, 250–650 μm diam, whitish translucent to yellowish conidial drops exuded from the ostioles. Conidiophores hyaline, smooth, 1-septate, densely aggregated, cylindrical, straight, 5–15 × 1.5–5.5 μm. Conidiogenous cells phialidic, hyaline, terminal, cylindrical, 6–12 × 1–3 μm, tapered towards the apex. Paraphyses not observed. Alpha conidia unicellular, aseptate, fusiform, hyaline, 1–4-guttulate with acute ends, 4.5–7 × 1.5–3 μm, mean ± SD = 5.9 ± 0.6 × 2.2 ± 0.4 μm, L/B ratio = 2.7. Beta conidia and Gamma conidia not observed.

Culture characteristics: Colonies covering the dish within 1 wk at 21 °C, surface mycelium flattened, dense and felt-like. Colony on MEA and OA at first white, becoming yellowish, flat, with dense and felted mycelium, reverse pale sepiat with brownish dots with age, with visible solitary or aggregated conidiomata at maturity. On PDA cream to smoke-grey, reverse pale brown.

![Fig. 5. Diaporthe melitensis (CBS 142551). A. Conidiomata sporulating on PNA. B. Conidiogenous cells. C. Alpha conidia. Bars = 10 μm.](image-url)
Notes: *Diaporthe melitensis* was isolated from trunk samples of *Citrus limon* showing serious cankers in Gozo (Malta). The two strains representing *D. melitensis* cluster in a well-supported clade, and appear closely related to *D. pseudomangiferae* and *D. arengae*. This species is phylogenetically closely related to, but clearly differentiated from, *D. limonicola* (described above) by 22 different unique fixed alleles in ITS, *tef1* and *tub2* loci (22, 2, and 47 respectively) based on the alignments deposited in TreeBASE.

Morphologically *D. melitensis* differs from *D. pseudomangiferae* in the shorter alpha conidia (4.5–7 vs. 7–9 μm) (Gomes et al. 2013).

Additional material examined: **Malta**: Gozo, from branch canker of *Citrus limon*, 22 Sep. 2015, V. Guarnaccia (culture CBS 142552 = CPC 27875).

**PATHOGENICITY**

After 30 d all the isolates of the inoculated species induced lesions on most of the *Citrus* species tested. The inoculated twigs developed cankers similar to those detected in the field, and the fungi were successfully re-isolated, fulfilling Koch’s postulates (Fig. 6). Cankers and internal discoloration were observed in correspondence to inoculation points. On the contrary, no symptoms were observed on the control plants. Clear differences in aggressiveness among the isolates and susceptibility of the *Citrus* species were observed: *D. limonicola* and *D. melitensis* caused the most serious symptoms with no difference among the hosts. *Diaporthe foeniculina* was weakly aggressive to each *Citrus* species. Similarly, *D. novem* was weakly aggressive on all the hosts except the orange clones, whilst *D. baccae* caused disease symptoms only on mandarin.

**DISCUSSION**

After a major screening of fungal diseases of citrus in Europe (Guarnaccia et al. 2017a, b, Sandoval-Denis et al. 2018), molecular phylogenetic and morphological analyses were used to evaluate the diversity of *Diaporthe* species in the Mediterranean basin, focusing on symptomatic plants.

Fig. 6. Pathogenicity test of selected *Diaporthe* isolates on citrus plants after 30 d. **A.** Shoot blight of lime plants inoculated with *D. novem* (CPC 26188). **B–C.** Cankers with gummosis of lemon plants caused by *D. limonicola* and *D. melitensis* (CPC 28200, CPC 27873). **D–E.** Internal discoloration of mandarin twigs inoculated respectively with *D. melitensis* and *D. baccae* (CPC 27873, CPC 26170). **F.** Internal lesion of orange branch caused by *D. foeniculina* (CPC 28081).
Several Diaporthe species are well established in Europe (Thomidis & Michailides 2009, Santos et al. 2011, Lombard et al. 2014, Guarinacca et al. 2016). Diaporthe species are also frequently associated with citrus diseases worldwide (Timmer et al. 2000, Huang et al. 2013), such as melanose and stem-end rot. Since the late 18th century these diseases have affected different citrus organs and also cause a sort of wood gummosis (Fawcett 1936, Timmer et al. 2000, Mondal et al. 2007). Diaporthe citri is considered a key pathogen of citrus species and has been confirmed from Brazil, China, Korea, and New Zealand, and is also reported as widely spread throughout Asia, Australasia, and South America (Timmer et al. 2000, Mondal et al. 2007, Udayanga et al. 2014b). However, D. citi has never been reported from Europe, whilst D. cytopsorella and D. foeniculina have been recently isolated from citrus in Spain (Udayanga et al. 2014b).

DNA sequence data are essential in resolving taxonomic questions, redefining species boundaries, and the accurate naming of species required for effective communication about plant pathogens. Thus, during the past decade, a polyphasic approach was used in several Diaporthe studies, revealing new species involved with citrus diseases and as endophytes and plant pathogens (Huang et al. 2013, 2015). Santos et al. (2017a) showed that species separation is better when five loci (ITS, tef1, tub2, his3, and cal) are simultaneously used to build the phylogeny of Diaporthe isolates.

Citrus crops are already compromised by a range of fungal pathogens other than Diaporthe (Vicent et al. 2007, Aiello et al. 2015, Guarinacca et al. 2017a, Sandoval-Denis et al. 2018). Considering that no surveys for citrus diseases caused by Diaporthe had been performed in Europe, a large-scale investigation of Diaporthe species associated with citrus infections in Europe was needed. This study provides the first molecular characterization of Diaporthe diversity related to citrus production in Europe, combined with morphological characterisation.

Several citrus orchards, plant nurseries, private gardens and collections in five Mediterranean European countries were investigated. We further investigated different host plants in Citrus-allied genera such as Microcitrus, which is also economically important for fruit production.

Canker symptoms were frequently observed on several Citrus species in all countries investigated. Twigs showed wilting, dieback, wither-tip, and gummosis. Some orchards presented branch blight and trunk cankers associated with abundant gummosis. The most critical situation seen was in different lemon orchards in Malta, where the infections led to tree death. Melanose and stem-end rot were never observed.

We collected 79 Diaporthe strains. Phylogenetic analyses based on single and the combined five loci (ITS, tef1, tub2, his3, and cal), as well as morphological characters, revealed five Diaporthe species associated with infections on several Citrus species in Europe. We included in the analysis the closest taxa to the five Diaporthe species recovered in this study, based on BLAST searches of NCBI’s GenBank nucleotide database. The final phylogenetic tree distinguished two newly described species (D. limonicola and D. melitensis) and three known species (D. baccae, D. foeniculina, and D. novem). Moreover, a known clade represented by three strains (CBS 199.39, CBS 230.52, CPC 20322), previously named D. citi, appeared in our final tree. However, this clade also required a separate name as D. citi s. str. is restricted to the pathogen causing melanose and stem-end rot of citrus fruit (Udayanga et al. 2014b). Thus, in this study we have described these three isolates as D. infertilis. Based on sampling in this study, D. citi appears to be absent in Europe as previously reported by Udayanga et al. (2014b).

Huang et al. (2015) obtained two separate groups of citrus isolates within the D. arecae complex, which were either not well supported or non-monophyletic based on a four-locus phylogenetic analysis. However, our analysis based on five loci, combined with morphological observations, clearly separated both D. limonicola and D. melitensis from D. pseudomangiferae and D. areangae, the most closely related species, and from other species in the D. arecae complex such as D. podocarp-macrophylli and D. xishuangbanica (Gao et al. 2017). Morphologically, D. limonicola and D. melitensis differ from D. pseudomangiferae in the shorter alpha conidia. Moreover, D. limonicola is the only taxon among these species to produces beta and gamma conidia.

Diaporthe foeniculina was the predominant species found in all the Mediterranean countries sampled, but its pathogenicity on Citrus was unknown (Udayanga et al. 2014b). Recently, Lombard et al. (2014) described D. baccae as a new species associated with Vaccinium corymbosum cankers in Italy. Similarly, we found this species associated with twig, branch and trunk cankers of citrus in Italy. Diaporthe novem was isolated for the first time from infected citrus plants in our study, where it was found associated with twig dieback of C. japonica (kumquat) and C. aurantiifolia (lime) in Italy. Moreover, the newly described species were isolated from devastated lemon plants in several orchards on Malta: D. limonicola was recovered from symptomatic trunks and branches, whilst D. melitensis was isolated only from branches. They were isolated separately and from the same affected sample. Colonization of the same host plant by diverse Diaporthe species appears to be frequent as previously reported (Crous & Groenewald 2005, Van Niekerk et al. 2005, Thompson et al. 2011).

Our results reveal a large diversity of Diaporthe species spanning several clades and species complexes, associated with citrus wood cankers in European countries. These include D. baccae, D. infertilis, D. novem, and the two newly described species. In total, 22 Diaporthe species are now confirmed as associated with citrus.

Pathogenicity of the species isolated from citrus samples collected in Europe was tested on healthy plants of lemon, lime, mandarin, and two clones of Citrus sinensis (‘New Hall’ and ‘Tarocco Meil’). All of the Diaporthe species tested caused lesions to develop on twigs. Recently, D. foeniculina (syn. D. neothecolida) has been reported as causing disease in many other hosts: shoot blight of persimmon in Australia (Golzar et al. 2012), kiwi-fruit disease in Greece (Thomidis et al. 2013), and avocado branch cankers (Guarinchia et al. 2016). This species evidently has the ability to infect a wide range of fruits and plant hosts as an opportunistic pathogen. Diaporthe foeniculina (as “D. foeniculacea” in Gomes et al. 2013) proves to be a pathogen with a broad host range amongst temperate woody plants and fruit trees. In our study, D. foeniculina was
isolated from symptomatic plants of eight Citrus species (C. berganiana, C. japonica, C. limon, C. maxima, C. mitis, C. paradisi, C. reticulata, and C. sinensis) and also Microcitrus australasica. In the pathogenicity tests, it was weakly aggressive, but produced lesions on each species tested.

These results demonstrate a cross-infection potential of multiple Diaporthe species on different Citrus species, as previously reported (Lombard et al. 2014, Guarnaccia et al. 2016). Diaporthe limonica and D. melitensis caused prominent symptoms in all the citrus species inoculated, and because they were isolated from plants with severe disease symptoms, these species can be considered as potentially major new pathogens of Citrus limon. Diaporthe baccae caused symptoms only on mandarin, while D. novem infected lime, lemon, and mandarin plants. Both of these species seemed to be weakly aggressive, with different host susceptibility and known distribution. These fungi merit adding to the list of fungal taxa causing citrus cankers worldwide (Adesemoye et al. 2014, Mayorquin et al. 2016, Sandoval-Denis et al. 2018).

This study provides the first overview of Diaporthe diversity associated with cankers of citrus plants in Europe, and includes information on their pathogenicity. Two of the new species described were established as causal agents of a devastating disease of lemon plants, inducing branch and trunk cankers that lead to plant death. The present study also appears to represent the first reports of D. baccae and D. novem associated with citrus disease in Europe. Despite the worldwide distribution and economical importance of citrus, knowledge of the fungal species associated with Citrus species is still incomplete. Further studies are required in order to fully elucidate the host range, specificity, and global distribution of Diaporthe species, as well as other fungi causing cankers of citrus plants.

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