Occurrence and diversity of endophytic, potentially pathogenic black-foot disease fungi in symptomless grapevine nursery stock in Spain

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Abstract

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In this study, 3,426 grafted plants ready to be sold to producers were surveyed from 15 grapevine nursery fields in northern Spain from 2016 to 2018. In all, 1,427 black-foot pathogen isolates were collected from the non-necrotic inner tissues of surface sterilized symptomless secondary roots and characterized based on phenotypical features and comparison of DNA sequence data of the nuclear ribosomal DNA-internal transcribed spacer region, histone H3, translation elongation factor 1-alpha and β-tubulin genes. Eleven species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* were identified, namely *Dactylonectria alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis*, *Ilyonectria liriodendri*, *I. pseudodestructans*, *I. robusta*, *Neonectria quercicola, N. sp.* 1 and *Thelonectria olida*. In addition, two species are newly described, namely *D. riojana* and *I. vivaria*. Twenty-four isolates representing the 13 black-foot species were inoculated in grapevine seedlings cultivar 'Tempranillo'. The pathogenicity tests detected virulence diversity among fungal species and between isolates within each species. The most virulent species was *D. novozelandica* isolate BV-0760, followed by *D. alcacerensis* isolate BV-1240 and *I. vivaria* sp. nov. isolate BV-2305. The present study improves our knowledge on the etiology and virulence of black-foot disease pathogens, and opens up new perspectives in the study of the endophytic role of these pathogens on grapevines.

Endophytes are defined as organisms living in internal tissues of the plants that exhibit no visible symptoms as a result of the colonization (Hallmann et al. 1997). This endophytic relationship is often unnoticed due to the lack of symptoms in the plant and is usually only discovered by examining internal tissues with a microscope, by aseptic isolations from plants, or from PCR-based methods of DNA extracted from surface-disinfested plant tissues (Stone et al. 2000). Many fungal endophytes have been sought and characterized for their ability to produce biologically active secondary metabolites with potential uses in agriculture, medicine, and other areas (Wang and Dai 2011).

In grapevine, numerous studies have been conducted to analyze the endophytic fungal communities in different plant organs (Casieri et al. 2009; Martini et al. 2009; González and Tello 2011; Hofstetter et al. 2012; Cosoveanu et al. 2014; Pinto et al. 2014; Bruez et al. 2014, 2016; Dissanayake et al. 2018; Kraus et al. 2019). However, special focus has been given on the fungal microbiome on its woody tissues, due to the problems arising from grapevine trunk diseases (GTDs) (Casieri et al. 2009; Hofstetter et al. 2012; Bruez et al. 2014, 2016; Kraus et al. 2019). These diseases, namely esca, eutypa, Diaporthe and Botryosphaeria diebacks, as well as black-foot and Petri diseases, are some of the most destructive fungal diseases of grapevine in all grape growing areas of the world. Management of GTDs has been intensively studied for decades with some great advances made in the understanding of the causal pathogens, their epidemiology, impact, and control (Gramaje et al. 2018).

Black-foot disease is a soil-borne disease caused by a broad range of *Cylindrocarpon*-like asexual morphs belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* (Gramaje et al. 2018). Among them, *Dactylonectria torresensis* is considered the most frequent species in Italy (Carlucci et al. 2017), Portugal (Reis

et al. 2013) and Spain (Berlanas et al. 2017). Internal symptoms of black-foot diseased vines usually range from black, sunken, necrotic lesions on roots to reddish brown discoloration in the base of the rootstock trunk (Fourie and Halleen 2001). Foliar symptoms associated with black-foot disease are practically indistinguishable from those observed in Petri disease affected vines and include delayed budbreak, chlorotic foliage with necrotic margins, overall stunting, and wilting of leaves or entire shoots (Agustí-Brisach and Armengol 2013). They also resemble symptomatology associated with abiotic disorders such as spring frost, winter damage, nutrient deficiency and/or water stress (Gramaje et al. 2018).

Black-foot disease is particularly important in grapevine nurseries and new plantations. *Cylindrocarpon*-like asexual morphs produce conidia and some species also chlamydospores in culture, which indicates that those propagules are likely to be produced on stem bases of infected vines and the diseased roots. The conidia are spread in soil water and the chlamydospores can allow these pathogens to survive in the soil for extended periods of time (Petit et al. 2011). Infection can occur through the small wounds made when roots break off during the planting process, through the incomplete callusing of the lower trunk or through wounds made in the grapevine propagation process, such as disbudding wounds, from which the infection progresses downward to the base of the trunk (Halleen et al. 2006a). Black-foot disease pathogens have also been detected, identified and quantified in soil samples by PCR-based methods (Damm and Fourie 2005; Probst et al. 2010; Cardoso et al. 2013; Agustí-Brisach et al. 2014; Úrbez-Torres et al., 2015; Langenhoven et al. 2018) or by dilution plating technique together with the use of a semi-selective medium (Berlanas et al. 2017).

Much of the current knowledge on black-foot disease pathogens of grapevine has been derived from research based on populations isolated from vines displaying foliar or internal

wood disease symptoms. Recent research suggested that black-foot disease fungi could act as latent pathogens in visually healthy grapevine nursery stock in South Africa (Langenhoven et al. 2018). In Spain, grapevine nursery surveys have been previously conducted for the detection of black-foot pathogens (Alaniz et al. 2007; Agustí-Brisach et al. 2013); however, this research focused on these organisms as plant pathogens causing necrotic symptoms in grapevine tissues. Therefore, the objectives of this study were to (i) conduct extensive surveys in grapevine nurseries and identify the fungal species associated with black-foot disease from non-necrotic endorhizosphere tissues of externally asymptomatic plants based on phenotypical and molecular methods, and (ii) determine the pathogenicity of these endophytic species on grapevine.

Materials and Methods

Nursery survey and fungal isolation. Isolates used in this study were collected from externally symptomless grafted plants of 110 Richter rootstock ready to be sold to producers from 15 nursery fields in northern Spain from 2016 to 2018 (Table 1). Fungal isolations were made from the non-necrotic endorhizosphere tissue of secondary roots. For this purpose, sections of externally symptomless roots (2 cm long and 1-3 mm diameter) were cut, washed under running tap water, surface sterilized in 33% sodium hypochlorite (commercial 40 g Cl/l) for 1 min and rinsed twice with sterile distilled water. Upon this treatment, bark was carefully peeled out and the endorhizosphere tissue was plated on malt extract agar (MEA) (Conda Laboratories) supplemented with 0.35 g liter^{-1} streptomycin sulphate (MEAS) (Sigma-Aldrich Laboratories). Isolation plates were incubated at 25ºC in darkness for 12 days, and colonies resembling black-foot disease pathogens were subcultured to potato dextrose agar (PDA) (Conda Laboratories).

All isolates were single-spored in order to obtain pure cultures and stored in filter paper at -20 ºC.

Morphological identification and characterization. Cultures were grown on PDA, oatmeal agar (OA) (Sigma-Aldrich laboratories), and synthetic nutrient agar (SNA) (Sifin Diagnostics) with or without bearing two 1 cm^2 pieces of sterile filter paper on the medium surface (Crous et al. 2009). Plates were incubated at 25°C under near UV light with a 12 h photoperiod. To induce perithecia of new species, heterothallic and homothallic crosses were performed according to Cabral et al. (2012a). The length and width of 40 conidia were measured at ×1,000 magnification with a compound Nikon Eclipse Ni-e microscope. Minimum, maximum, mean and standard deviation were calculated from measurements. Conidial color, shape, and the presence of septa were also recorded. Colony morphological characters were observed and colony colors were determined with the color chart of Rayner (1970).

Effect of temperature on mycelial growth. Twenty-four fungal isolates, belonging to 13 different species were randomly selected for temperature-growth assay (Table 2). Five-mm diameter mycelial plugs were transferred to the center of PDA plates, and incubated in darkness from 5 to 35°C at 5°C intervals. Colony diameter was recorded after 10 days in two orthogonal directions. Four replicate plates per isolate were used and the experiment was conducted twice. Student's t-Least Significant Difference (LSD) was calculated at the 5% significance level to compare treatment means between experiments. Regression curves were fitted to the values of temperature versus radial growth in millimeters per day for each isolate. The optimum temperature for radial growth and the maximum daily radial growth were calculated in the fitted

equation for each isolate. Data were analyzed using the Kruskal-Wallis test and mean ranks of isolates were compared at $P = 0.05$ using Dunn's test.

Molecular characterization and phylogenetic analyses. Total genomic DNA was extracted from fresh mycelium after 10 days of incubation in PDA using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Norcross, GA) following the manufacturer's instructions. The identification of black-foot pathogens was first confirmed by sequencing part of the histone H3 (*his3*) gene. In addition, the internal transcribed spacer and intervening 5.8S gene (ITS) region, and partial regions of the translation elongation factor 1-alpha (*tef1*) and the β-tubulin (*tub2*) genes were also sequenced for a better phylogenetic resolution. Each 25 µL reaction volume contained 12.5 µl of FastGene Ready Mix (NIPPON Genetics), 1 µl of each primer, 1 µl of DNA template (10 ng/ µl) and 9.5 µl sterile distilled water. PCR amplification was performed in a C1000 touch thermal cycler (Bio-Rad). For *his3*, ITS and tub2 genes amplification conditions included an initial denaturation step of 3 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 58ºC for 30 s, and elongation at 72ºC for 1 minute. A final extension was performed at 72ºC for 1 min. For *tef1* gene, amplification conditions were as follow: initial denaturation step of 3 min at 94ºC, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 51ºC for 30 s, and elongation at 72ºC for 45 s. A final extension was performed at 72ºC for 10 min. Primers used were CYLHEF and CYLH3R (Crous et al. 2004b) for *his3*, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for ITS, CylEF-1 (5'-ATGGGTAAGGAVGAVAAGAC-3'; J.Z. Groenewald, unpublished) and CylEF-R2 (Crous et al. 2004b) for *tef1*, and T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for *tub2*. PCR products were visualized in 1% agarose gels (agarose D-1 Low EEO, Conda

Laboratories). PCR products were sequenced in both directions by Eurofins GATC Biotech (Cologne, Germany)). Sequences were edited and aligned using the program MEGA v. 6 (Tamura et al. 2013). The alignments for each locus were combined in a single file using GenTool (unpublished tool).

Fungal sequences from grapevines from Spain were aligned with published GenBank sequences, including ex-type specimens from grapevines and other hosts for comparison using MAFFT sequence alignment program v. 6 (Katoh and Toh 2010) (Table 3). Alignments were corrected visually and manually edited in Sequence Alignment Editor v. 2.0a11. Firstly, a phylogenetic analysis was conducted on the *his3* single-locus alignment for representative isolates obtained in this study. In addition, a multi-locus phylogenetic analysis was performed on the combined *his3*, ITS, *tef1*, and *tub2* datasets. A partition homogeneity test was conducted in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003). The congruence among the *his3*, ITS, *tef1*, and *tub2* datasets were tested at 1,000 replicates. Maximum Likelihood (ML) analyses were performed in MEGA v. X (Kumar et al. 2018) using the best fit model as estimated with the Bayesian information criterion in jModelTest 2.1.10 (Darriba et al. 2012). Branch support was calculated from 1,000 bootstrap replicates for the single and concatenated datasets. *Campylocarpon* (*Ca.*) *fasciculare* (CBS 112613) and *Ca. pseudofasciculare* (CBS 112679) were used as outgroups in phylogenetic analyses.

Representative black-foot disease isolate sequences derived in this study were lodged at GenBank (Table 2) and the alignments in TreeBASE (http://treebase.org), and taxonomic novelties in MycoBank (www.MycoBank.org) (Crous et al. 2004a).

Pathogenicity tests. Two-month-old grapevine seedlings (cv. 'Tempranillo') were used for pathogenicity tests. Seedlings were planted individually in 250 cm^3 plastic pots containing sterilized peat moss and inoculated when one to two leaves had emerged by immersing the roots in a 10^5 mL^{-1} conidial suspension for 1 hour (Martínez-Diz et al. 2018). The viability of the conidia was tested by spreading 150 µL of each conidial suspension diluted to 100 conidia mL^{-1} onto PDA plates and counting the resulting colonies after 2-3 days incubation at 25ºC in the dark. Twenty-four fungal isolates, belonging to 13 different species were used (Table 2). Six seedlings per isolate were inoculated. Six control plants were inoculated with sterile distilled water. The experiment was repeated twice. Seedlings were maintained in a growth chamber at 25ºC in a completely randomized design. Plants were inspected every morning from day 1 to day 61 for the development of foliar symptoms. However, due to the lack of specificity of foliar symptoms in grapevine seedlings, as well as the short time occurring from foliar symptom development to plant death, the following parameters were considered: the mean time from inoculation in which the plant stayed alive (MTA) and the % of dead plants. Two months after inoculation, all plants were uprooted and washed free of soil. Roots, crown and stem were aseptically plated on MEAS to re-isolate the black-foot disease fungal species.

Statistical analysis. Homogeneity of variance across treatments was evaluated using Levene's test prior to the analysis of variance (ANOVA) (Box. et al. 1978). The percentage of dead plant and the MTA data were subjected to ANOVA with factors experiment (performed twice), pathogen and isolate nested in pathogen (two isolates for each pathogens except for *Neonectria* sp. 1 and *Thelonectria olida*). Means were compared using the LDS value at $P = 0.05$.

Results

Incidence of black-foot disease pathogens. Based on colony morphology, conidial characteristics and phylogenetic analyses (see below), 1,427 isolates associated with 13 species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* were identified. *Dactylonectria torresensis* was isolated from grafted plants in all grapevine nursery fields, and accounted for 75.05% of all isolates (Table 1). The remaining isolates were identified as *Dactylonectria* sp. (0.14%), *D. alcacerensis* (2.94%), *D. macrodidyma* (3.85%), *D. novozelandica* (3.22%), *D. pauciseptata* (0.21%), *Ilyonectria* sp. (0.21%), *I. liriodendri* (10.93%), *I. pseudodestructans* (0.8%), *I. robusta* (2.03%), *Neonectria* sp. 1 (0.06%), *N. quercicola* (0.28%), *and T. olida* (0.28%). In all nursery fields surveyed, disease incidence ranged from 4.9 to 90%, while disease severity ranged from 10 to 18%.

Morphological characterization. The color of the colonies ranged from pale buff to chestnut or cinnamon to vinaceous on PDA, and from buff to chestnut or brown to cinnamon on OA. Based on the microscopic observations, almost all the isolates produced macroconidia, microconidia and chlamydospores. This matched descriptions for *Cylindrocarpon*-like asexual morphs; therefore, it was possible to separate three different groups among black-foot disease isolates. The first consisted of isolates showing 5-septate and cylindrical, generally straight, macroconidia. Microconidia were ellipsoidal to oblong and no chlamydospores were observed. These characteristics matched those described earlier for the genus *Thelonectria* (Chaverri et al. 2011). The second group of isolates had 5-septate, fusiform and curved macroconidia. Microconidia and chlamydospores were uncommon. These matched descriptions for the genus *Neonectria* (Chaverri et al. 2011). The third consisted of isolates showing 1 to 3-septate and

cylindrical, generally straight, macroconidia. Microconidia were ellipsoidal to ovoid. These characteristics matched those described earlier for the genera *Dactylonectria* (Lombard et al. 2014) or *Ilyonectria* (Chaverri et al. 2011). According to Lombard et al. (2014), *Dactylonectria* can be distinguished from *Ilyonectria* by the characteristics of the sexual morph as well as the absence of chlamydospore production in culture.

Effect of temperature on mycelial growth. Analyses of variance indicated no significant differences of the mycelial growth between experiments (P = 0.4115), so data from both experiments were averaged. Most of the isolates studied failed to grow on PDA at 35ºC, with the exception of *N. quercicola* isolate BV-2137 and *I. vivaria* sp. nov isolate BV-1924, while some of them grew at 5ºC (*D. pauciseptata* isolate BV-1354, *I. liriodendri* isolates, *I. robusta* isolate BV-1593, *I. vivaria* sp. nov isolates, *N.* sp. 1 isolate BV-2682, *N. quercicola* isolates, and *T. olida* isolate). Optimal temperatures for mycelial growth ranged from 18.6 to 20.6ºC (Table 4). Significant differences were found in the optimal temperature between *D. pauciseptata* isolate BV-1360 and *N.* sp. 1 isolate BV-2682 ($P < 0.05$), but it was not possible to statistically differentiate the remaining isolates, because of the overlap in the optimal temperature measurements among them (Table 4). According to the Kruskal-Wallis test, maximum growth rates of isolates differed significantly ($P < 0.05$). The relationship between growth rate and temperature for all isolates was best described by a second-degree polynomial ($Y = aT^2 + bT + cT$). The three regression coefficients were highly significant in all cases ($P < 0.01$). The coefficient of determination (R^2) ranged from 0.71 to 0.93. Isolates with maximum growth rate ≤ 3 mm/day included *D. alcacerensis* isolates, *D. macrodidyma* isolate BV-0535, *D. riojana* sp. nov. isolates, *D. torresensis* isolate BV-0901, *I. pseudodestructans* isolates and *T. olida* isolate.

Isolates with maximum growth rate between 3 and 5 mm/day included *D. macrodidyma* isolate BV-1366, *D. novozelandica* isolates, *D. pauciseptata* isolates, *D. torresensis* isolate BV-0666, *I. liriodendri* isolates, *I. robusta* isolate BV-1593, *I. vivaria* sp. nov. isolates, *N. quercicola* isolates, and *N.* sp. 1 isolate. The only species that grew more than 5 mm/day was *I. robusta* (isolate BV-1654).

Molecular characterization and phylogenetic analyses. For *his*3 single-locus alignment the AIC best-fit nucleotide substitution model identified by jModelTest was Hasegawa-Kishino-Yano model (HKY) with gamma distributed with invariant sites rates (G+I). Three major clades belonging to the genera *Ilyonectria*, *Neonectria* and *Dactylonectria* were obtained (Fig. 1).

Although all the isolates were identified to the specie level, the ITS, *tub*2, and *tef*1 regions were also analyzed and their sequences concatenated with those obtained from the *his*3 region (Fig. 2). The four loci alignment contained 91 taxa (including the two outgroups). The AIC best-fit nucleotide substitution model identified by jModelTest was HKY+G+I model for *his*3 and ITS and HKY+G for *tef*1 and t*ub*2. The ML consensus trees obtained with the *his*3 locus and the four-loci alignment confirmed the existence of two novel taxa within our set of isolates.

Taxonomy. Based on the phenotypical characters previously recorded and the phylogenetic analysis, one species each of *Ilyonectria* and *Dactyonectria* are described (Figs. 3 and 4). No perithecia were observed in the heterothallic or homothallic crosses performed.

Dactylonectria riojana *C. Berlanas, M. Andrés-Sodupe, S. Ojeda & D. Gramaje, sp. nov. MycoBank MB 829945* (Fig. 3). Etymology: Name refers to the Spanish region of La Rioja, where the fungus was isolated.

Diagnosis: Morphologically *D. riojana*, can be distinguished by its shorter conidiophores when compared with *D. novozelandica*, *D. torresensis*, *D. alcacerensis* and *D. macrodidyma.* Nineteen polymorphisms can distinguish *D. riojana* from *D. alcacerensis*: two in *tub*2 locus, eleven in *his*3 locus, four in *tef*1 locus and two in ITS. Seventeen polymorphisms can distinguish *D. riojana* from *D. torresensis*: one in *tub*2 locus, four in *his*3 locus, ten in *tef*1 locus and two in ITS. Eleven polymorphisms can distinguish *D. riojana* from *D. novozelandica*: seven in *his*3 locus, two in *tef*1 locus and two in ITS. Twenty-three polymorphisms can distinguish *D. riojana* from *D. macrodidyma*: three in *tub*2 locus, thirteen in *his*3 locus and seven in *tef*1 locus.

Typus: Spain: La Rioja, Logroño, on roots of 110 Richter grapevine rootstock (*Vitis berlandieri* x *Vitis rupestris*), 2018, C. Berlanas (CBS H-23883 – holotype; CBS 145413= BV-1396 – ex-type culture).

Conidiophores simple. Complex conidiophores not observed. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or some branched with up to three phialides, 1- to 2- septate, 35 to 65 µm long; phialides monophialidic, more or less cylindrical, tapering slightly towards the apex, 12 to 29 µm long, 2.0 to 2.5 µm wide at the base, 2.5 to 3.5 µm at the widest point, 1.5 to 3 µm near the aperture.

Macroconidia (1 to) 3-septate, straight, cylindrical, but may narrow towards the tip, more or less broadly rounded, and the base appearing acute due to the presence of the hilum; 1-septate (25–) 30 to 35 (–47) × (6.5–) 7 to 8 (–8.5) µm (av. 33 × 8 µm), L/W ratio (3–) 3.8 to 4.5 (–6) (av. 4.2), 2-septate (30–) 35 to 39 (–51) × (6–) 7 to 8.5 (–9.5) µm (av. 39 × 8 µm), L/W ratio (3–) 4 to

5 (–7) (av. 4.5), and 3-septate (36–) 44 to 48 (–55) × (7–) 7.5 to 8.2 (–9) µm (av. 45 × 8 µm), L/W ratio (4–) 4.5 to 6 (–7) (av. 5.5).

Microconidia 0-1 septate, aseptate microconidia subglobose to oval, (9–) 10 to 13.5 (–16.5) × (5–) 6 to 6.5 (–7) (av. 12 × 6 µm), L/W ratio (1.5–) 1.8 to 2.2 (–2.4) (av. 2); 1-septate, mostly subcylindrical, (12.5–) 18 to 21 (–23.5) × (6–) 7 to 8 (–8.5) (av. 20.3 × 7 µm), L/W ratio (2–) 2.5 to 2.8 (–3.2) (av. 2.7). Chlamydospores rarely observed.

Culture characteristics: Mycelium felty with average density (OA) or strong density (PDA). Surface on OA straw to buff; margin buff. Surface on PDA honey to buff; margin buff. Zonation absent, transparency homogenous and margin uneven (OA) and even (PDA). Reverse similar to surface, except in color, buff to sepia on OA and sepia to chestnut on PDA. Colonies reaching a radius of 26.8 mm after 7 d at 25ºC. Minimum temperature for growth 10ºC, optimum 20ºC, maximum 30ºC.

Host and distribution: *Vitis berlandieri* x *Vitis rupestris* (roots of 110 Richter grapevine rootstock) (Spain, La Rioja).

Notes: *D. riojana* is closely related to *D. novozelandica*, *D. torresensis*, *D. alcacerensis* and *D. macrodidyma*. The morphology of these species is very similar, but *D. riojana* can be distinguished by its shorter conidiophores and the inability to grow at 5ºC. No chlamydospores were observed in *D. riojana*, while these structures rarely occur in *D. novozelandica*, *D. torresensis* and *D. alcacerensis*, or sometimes occur in *D. macrodidyma*.

Ilyonectria vivaria *C. Berlanas, B. López-Manzanares, R. Bujanda & D. Gramaje, sp. nov. MycoBank MB 829951* (Fig. 4). Etymology: Latin, from vivarium, meaning nursery. In reference to the environment it was frequently isolated from.

Diagnosis: Morphologically *I. vivaria*, can be distinguished by its slower growth at 25ºC and the ability to grow at 35ºC when compared with *I. mors-panacis*. Seventy six polymorphisms can distinguish *I. vivaria* from *I. mors-panacis*: seventeen in *tub*2 locus, thirty two in *his*3 locus, twenty five in *tef*1 locus and two in ITS.

Typus: Spain: Navarra, Larraga, on roots of 110 Richter grapevine rootstock (*Vitis berlandieri* x *Vitis rupestris*), 2018, C. Berlanas (CBS H-23884 – holotype; CBS 145414 = BV-2305 – ex-type culture).

Conidiophores simple or complex. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or some branched with up to four phialides, 1- to 4- septate, 42 to 165 µm long; phialides monophialidic, more or less cylindrical, tapering towards the apex, 21 to 47 µm long, 2.0 to 2.5 µm wide at the base, 2.5 to 3.0 µm at the widest point, 1.5 to 2.5 µm near the aperture.

Complex conidiophores aggregated in small sporodochia, repeatedly and irregularly branched; phialides cylindrical, but tapering towards the apex, 10 to 25 µm long, 1.5 to 2.5 µm wide at the base, 2.0 to 2.5 µm at the widest point, and 1.5 to 2.0 µm wide at the apex.

Macroconidia (1 to) 3-septate, straight, cylindrical, but may narrow towards the tip, more or less broadly rounded, and base sometimes with a visible, centrally hilum; 1-septate (22–) 27 to 34 (–41) × (6–) 6.5 to 8.0 (–9) µm (av. 31 × 7.5 µm), L/W ratio (3–) 3.5 to 4.2 (–6) (av. 4), 2-septate (26–) 33 to 38 (–47) × (6–) 7.0 to 8.0 (–9) µm (av. 35 × 7.5 µm), L/W ratio (3–) 4 to 5.5 (–7) (av. 4.5), and 3-septate (33–) 45 to 51 (–56) × (7–) 7.4 to 8 (–8.5) µm (av. 47 × 7.5 µm), L/W ratio (4–) 4.5 to 6.5 (–7) (av. 5).

Microconidia 0-1 septate, aseptate microconidia ellipsoidal to oval, (8–) 9 to 11.5 (–13.5) × (4.5–) 5.5 to 6.5 (–7) (av. 10.5 × 6 µm), L/W ratio (1.5–) 1.5 to 2 (–2.2) (av. 1.7); 1-septate,

mostly ellipsoidal (12–) 15 to 21 (–18.5) × (5.5–) 7 to 8 (–8.5) (av. 18 × 6.5 µm), L/W ratio (1.5) 2.5 to 3 (–3.3) (av. 2.8). Chlamydospores globose to cylindrical, 10 to 17 × 10 to 20 µm diam., smooth, but often appearing rough due to deposits, thick-walled, terminal on short lateral branches, rarely intercalary, single, in chains or in clumps, hyaline, becoming medium brown.

Culture characteristics: Mycelium cottony to felty with average density in both OA and PDA. Surface on OA honey to buff; margin buff. Surface on PDA buff, to honey to isabelline towards the centre; margin buff. Zonation concentric (PDA) or absent (OA), transparency homogenous and margin uneven (OA) and even (PDA). Reverse similar to surface, except in color, buff to cinnamon on OA and chestnut to cinnamon on PDA. Colonies reaching a radius of 27.3 mm after 7 d at 25ºC. Minimum temperature for growth 5ºC, optimum 19.4ºC, maximum 35ºC.

Host and distribution: *Vitis berlandieri* x *Vitis rupestris* (roots of 110 Richter grapevine rootstock) (Spain, Navarra).

Notes: *I. vivaria* is closely related to *I. mors-panacis* based on the *his*3 sequence alignment. The morphology of both species is very similar, but *I. vivaria* can be distinguished by its slower growth after 7 d at 25ºC and the ability to grow at 35ºC.

Pathogenicity tests. In all fungi, the viability of conidia was at least 90%. For each species, there were differences between pathogen isolates when evaluating the % of dead plants and the MTA ($P < 0.05$), so the data of isolates within species were not combined. Grapevine seedlings did not show any foliar symptoms during the experiment; however, the susceptibility to black-foot disease fungi measured as the % of dead plants and the MTA varied among fungal isolates (Table 5). *D. novozelandica* isolate BV-0760 was the most virulent species followed by *D alcacerensis* isolate BV-1240, *D. macrodidyma* isolate BV-1366 and *I. vivaria* sp. nov. isolates

when measuring the % of dead plants. Grapevine seedlings inoculated with *D. novozelandica* isolate BV-0760 stayed significantly less mean time alive than the other fungal species. *D. alcacerensis* isolates, *D. pauciseptata* isolate BV-1360, *D. riojana* sp. nov. isolate BV-1396, *I. vivaria* sp. nov. isolate BV-2305 and *T. olida* differed significantly with respect to the control when measuring the MTA. Percentage of reisolation ranged from 41.7 to 100%. *Neonectria* sp. 1 was not successfully reisolated from any inoculated seedling. No black-foot disease pathogens were reisolated from the control treatment (Table 5).

Discussion

This study is the first comprehensive effort to characterize a group of fungi associated with GTDs isolated from visually symptomless vines and non-necrotic internal wood tissue. Our results demonstrate that black-foot disease fungi can live as latent pathogens within grapevine and might become pathogenic under specific conditions. The pathogenicity of black-foot disease fungi have been so far demonstrated in tests performed under field (Sieberhagen 2016) and controlled conditions (Gubler et al. 2004; Jaspers et al. 2007; Alaniz et al. 2010; Cabral et al. 2012b; Brown et al. 2013; Probst et al. 2019). Recent studies reported the sporadic occurrence of other trunk disease fungi in grapevine as latent pathogens (Hofstetter et al., 2012, Eichmeier et al., 2018), without any disease symptoms ever becoming evident. Most of these studies employed molecular tools to decipher the grapevine fungal microbiome without the need of cultivation. However, functions such as mutualism or pathogenicity in fungi can rarely be predicted by using these tools (Brader et al. 2017).

Several factors have been reported to be determinant in influencing the effect triggering pathogenicity in an endophyte that was previously asymptomatic, such as the host genotype,

changes in plant gene expression, nutrient status, habitat or the locally occurring abiotic stress that might reduce host fitness, resulting in distortion of this delicate balance and in the occurrence of symptomatology in plants (Johnson and Oelmüller 2009). Abiotic stress factors in grapevine nursery fields and new plantations include J-rooting, waterlogging, water stress, winter-kill, nutrition deficiency, soil compaction and/or overcropping (Gramaje et al. 2018).

In the scientific literature, observations of black-foot disease fungi as endophytes colonizing asymptomatic vines (Langenhoven et al. 2018) or other plant species (Agustí-Brisach et al. 2011; Langenhoven et al. 2018) have been documented. Many of these asymptomatic plants are cereals and brassicaceous crops, used in crop rotations in grapevine nurseries (Langenhoven et al. 2018), and weeds, which may be present in field nurseries and established vineyards along with cultivated crops (Agustí-Brisach et al. 2011; Langenhoven et al. 2018). We therefore suggest that these endophytic associations among black-foot disease fungi and symptomless hosts are not unusual relationships in nature. The fact that black-foot disease fungi can be endophytes on weeds or other hosts has important implications, such as symptomless plants inadvertently serving as sources of hidden diversity of black-foot species, or serving as inoculum reservoirs. Because most research conducted on fungal trunk diseases has focused on pathogens infecting important agricultural commodities such as grapevine, almond or olive (Gramaje et al. 2016), we still lack a thorough understanding of the true nature of the associations of these fungi with other plants and their environment. In addition, the effects of weeds and/or other symptomless hosts on the genetic composition of black-foot disease pathogen populations have seldom been explored and thus remain poorly understood.

A wide diversity of black-foot disease pathogens were identified in this study, bringing the total number of fungal species associated with this disease isolated from grapevines in Spain to

17. These results confirm the richness of black-foot species in the Iberian Peninsula, with 14 species reported in Portugal (Cabral et al. 2012a, 2012c; Reis et al. 2013), compared to other countries in which black-foot disease is also prevalent, such as South Africa with 8 species (Halleen et al. 2004, 2006b; Langenhoven et al. 2018), New Zealand with 7 species (Bleach et al. 2006; Pathrose 2012), and Canada (Úrbez-Torres et al. 2014) and Italy (Carlucci et al. 2017) with 5. Our findings also confirm *Dactylonectria* as a genus commonly associated with infections in grapevine as reported previously by Lombard et al. (2014), and *D. torresensis* being the most prevalent black-foot species in Spain. This study represents the first report of *I. pseudodestructans* and *N. quercicola* on grapevine in this country. Hosts and distribution of *I. pseudodestructans* include Kentucky bluegrass (Canada), *Quercus* sp. (Austria) and grapevine (Portugal). To our knowledge, *N. quercicola* has been reported only in holm oak root seedlings showing decline aerial symptoms in forest nurseries in Spain (Mora-Sala et al. 2018).

Two novel species are newly described, namely *D. riojana* and *I. vivaria*. Morphological characteristics have been reported to play a major role in the description of fungal species (Taylor et al. 2000). However, in our study, the use of such characters alone to delimit the new species was insufficient, thus highlighting the usefulness of DNA sequences for such purpose. The *his3* region has previously shown to be the most informative locus for the correct identification of black-foot disease fungi (Cabral et al. 2012a). In our study, the use of the *his3* alone allowed us to describe these two novel species. In addition, the multilocus sequence analysis using *his3*, ITS, *tef1*, and *tub2* regions confirmed the level of polymorphism that enabled the fungal description.

Different inoculation methods, such as watering (Alaniz et al. 2009) or soaking (Martínez-Diz et al. 2018) the roots of grapevine seedlings in conidial suspensions, soaking bases of

grapevine rootstock cuttings (Alaniz et al. 2010; Cabral et al. 2012b; Probst et al. 2019) or roots (Pathrose et al. 2014) in conidial suspensions, and vacuum-inoculation of conidial suspensions throughout the vascular system of rootstock cuttings (Sieberhagen, 2016) have been used for virulence screening of black-foot disease fungi. Results of our study show that soaking roots of grapevine seedlings is a rapid and effective technique for evaluating virulence of *Cylindrocarpon*-like asexual morph isolates since most of the fungi collected from asymptomatic vines were capable of colonizing roots and causing disease under controlled conditions. Virulence varied among species and between isolates within each fungal species. High degree of virulence variability was also obtained among 14 *D. macrodidyma* isolates (Alaniz et al. 2009) and 17 *I. liriodendri* isolates (Pathrose et al. 2014) collected in Spain and New Zealand, respectively. Subsequent taxonomic studies suggested that the *D. macrodidyma* isolates used by Alaniz et al. (2009) might represent separate species within the *D. macrodidyma*-species complex (Cabral et al. 2012b). In our study, *D. novozelandica* isolate BV-0760 was considered the most virulent species, followed by *D alcacerensis* isolate BV-1240 and *I. vivaria* sp. nov. isolate BV-2305. Cabral et al. (2012b) performed pathogenicity test with 60 isolates belonging to 14 black-foot species collected from grapevine and other hosts and concluded that *I. lusitanica*, *D. estremocensis* and *I. europaea* were more virulent to grapevine than the species previously accepted as the main causal agents of black-foot disease, namely *D. macrodidyma* and *I. liriodendri*.

Pathogenicity in fungal trunk pathogens of grapevine is a complex phenomenon. The combination of many factors, such as pathogen and host genotypes and abiotic and other environmental stresses, as well as microbial interactions, seems to determine the outcome of the reaction of grapevine to the pathogen. The future direction of research on black-foot disease

needs to investigate (i) how these pathogens colonize the endorhizosphere and establish themselves inside, and (ii) what triggers latent black-foot disease fungi to transition from an endophyte to a pathogen, and cause disease symptoms in grapevine. The present study improves our knowledge on the etiology and virulence of black-foot disease pathogens, and opens up new perspectives in the study of the endophytic role of these pathogens on grapevines.

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Figure captions

Fig. 1. Maximum likelihood phylogeny of *Cylindrocarpon*-like asexual morphs as estimated from the alignment of the histone H3 gene sequences. Maximum likelihood bootstrap percentages are indicated at the nodes. Support values less than 70% bootstrap are omitted. The tree was rooted to *Campylocarpon fasciculare* (CBS 112613) and *Ca. pseudofasciculare* (CBS 112679). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. Tentative new species are indicated in red.

Fig. 2. Maximum likelihood phylogeny of *Cylindrocarpon*-like asexual morphs as estimated from concatenated alignments of four gene dataset (ITS, *tub*2, *his*3, and *tef*1). Maximum likelihood bootstrap percentages are indicated at the nodes. Support values less than 70% bootstrap are omitted. The tree was rooted to *Campylocarpon fasciculare* (CBS 112613) and *Ca.*

pseudofasciculare (CBS 112679). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. New species are indicated in red.

Fig. 3. *Dactylonectria riojana* (ex-type culture BV-1396). Ten-day-old colonies grown at 20ºC in darkness on PDA (**A**) and OA (**B**). **C-D**, Simple, sparsely branched conidiophore of the aerial mycelium. **E-H**, Micro- and macroconidia. Scale bars: C-F = 10 µm; G = 20 µm; H = 50 µm.

Fig. 4. *Ilyonectria vivaria* (ex-type culture BV-2305). Ten-day-old colonies grown at 20ºC in darkness on PDA (**A**) and OA (**B**). **C-F**, Simple, sparsely branched conidiophore of the aerial mycelium. **G**, Chlamydospores in mycelium. **H-I**, Micro- and macroconidia. Scale bars: C-I = 10 µm.

Tables

Table 1. Characteristics of the field nurseries surveyed, number of plants analyzed and the incidence of severity of black-foot disease fungi.

Location	Year of sampling	Plants analyzed	Incidence (%)[a]	Severity (%)[b]	Fungal species (No of isolates)[c]
Larraga, Navarra	2016	10	60.0	13.3	*D. torresensis* (8)
Larraga, Navarra	2016	10	90.0	11.1	*D. torresensis* (10)
Mendavia, Navarra	2016	5	80.0	10.0	*D. torresensis* (4)
Mendavia, Navarra	2017	598	23.4	15.8	*D. macrodidyma* (25)
					D. novozelandica (26)
					D. torresensis (168)
					I. liriodendri (1)
					T. olida (1)
Mendavia, Navarra	2017	614	23.0	13.7	*D. macrodidyma* (21)
					D. novozelandica (12)
					D. torresensis (154)
					I. liriodendri (5)
					I. robusta (1)
Larraga, Navarra	2017	40	12.5	10.0	*D. macrodidyma* (2)
					D. torresensis (3)
Larraga, Navarra	2017	120	60.8	13.3	*D. alcacerensis* (8)
					D. torresensis (89)
Larraga, Navarra	2017	220	14.1	11.9	*D. alcacerensis* (1)
					D. macrodidyma (2)
					D. torresensis (33)
					I. liriodendri (1)
O Barco de Valdeorras, Galicia	2018	50	26.0	14.6	*D. macrodidyma* (5)
					D. pauciseptata (3)
					D. torresensis (8)
					I. liriodendri (2)
					I. robusta (1)
O Barco de Valdeorras, Galicia	2018	243	4.9	16.7	*D. alcacerensis* (2)
					D. torresensis (6)
					I. liriodendri (12)
Larraga, Navarra	2018	136	8.8	10.8	*D. torresensis* (13)
Larraga, Navarra	2018	120	60.0	16.1	*D. alcacerensis* (25)
					D. torresensis (91)

Logroño, La Rioja	2018	260	19.2	14.0	*D. alcacerensis* (4) *D. novozelandica* (6) *D. torresensis* (54) *D. riojana* sp. nov. (2) *I. liriodendri* (3)
Larraga, Navarra	2018	960	51.8	12.3	*D. alcacerensis* (2) *D. torresensis* (425) *I. liriodendri* (132) *I. pseudodestructans* (11) *I. robusta* (27) *I. vivaria* sp. nov. (3) *N. quercicola* (2) *N.* sp. 1 (1) *T.* olida (3)
Logroño, La Rioja	2018	40	12.5	18.0	*D. novozelandica* (2) *D. torresensis* (5) *N. quercicola* (2)

[a] Disease incidence of black-foot pathogens was determined as the percentage of grafted plants that were infected by these fungi.
[b] Disease severity in infected grafted plants was determined as the percentage of root segments (10 segments per plant) that were colonized by black-foot fungi.
[c] Identification of black-foot pathogens was first confirmed by sequencing part of the *his3* gene. (*D.*): *Dactylonectria*; (*I.*): *Ilyonectria*; (*T.*): *Thelonectria*, (*N.*): *Neonectria*.

Table 2. Fungal species associated with black-foot disease recovered from grafted plants in Spain and used in the phylogenetic analyses

Species	Isolate	Location	GenBank accession number[a]			
			ITS	*tub2*	*his3*	*tef1*
Dactylonectria alcacerensis	BV-1240	Larraga, Navarra	MK602783	MK602798	MK579234	MK602813
D. alcacerensis[b]	BV-1222	Larraga, Navarra	-	-	MK579235	-
D. alcacerensis	BV-1245	Larraga, Navarra	-	-	MK579236	-
D. alcacerensis[b]	BV-1469	Larraga, Navarra	-	-	MK579237	-
Dactylonectria macrodidyma	BV-0535	Mendavia, Navarra	MK602784	MK602799	MK579238	MK602814
D. macrodidyma[b]	BV-0506	Mendavia, Navarra	-	-	MK579239	-
D. macrodidyma	BV-0560	Mendavia, Navarra	-	-	MK579240	-
D. macrodidyma	BV-0797	Mendavia, Navarra	-	-	MK579241	-
D. macrodidyma	BV-0872	Mendavia, Navarra	-	-	MK579242	-
D. macrodidyma	BV-0899	Mendavia, Navarra	-	-	MK579243	-
D. macrodidyma	BV-1359	O Barco de Valdeorras, Galicia	-	-	MK579244	-
D. macrodidyma	BV-1364	O Barco de Valdeorras, Galicia	-	-	MK579245	-
D. macrodidyma[b]	BV-1366	O Barco de Valdeorras, Galicia	-	-	MK579246	-
Dactylonectria novozelandica	BV-0760	Mendavia, Navarra	MK602785	MK602800	MK579247	MK602815
D. novozelandica	BV-0513	Mendavia, Navarra	-	-	MK579248	-
D. novozelandica[b]	BV-0428	Mendavia, Navarra	-	-	MK579249	-
D. novozelandica	BV-0777	Mendavia, Navarra	-	-	MK579250	-
D. novozelandica[b]	BV-1369	Mendavia, Navarra	-	-	MK579251	-
D. novozelandica	BV-1378	Mendavia, Navarra	-	-	MK579252	-
D. novozelandica	BV-1379	Mendavia, Navarra	-	-	MK579253	-
D. novozelandica	BV-1401	Mendavia, Navarra	-	-	MK579254	-
D. novozelandica	BV-1409	Mendavia, Navarra	-	-	MK579255	-
Dactylonectria pauciseptata[b]	BV-1354	O Barco de Valdeorras, Galicia	MK602786	MK602801	MK579256	MK602816
D. pauciseptata	BV-1358	O Barco de Valdeorras, Galicia	-	-	MK579257	-
D. pauciseptata[b]	BV-1360	O Barco de Valdeorras, Galicia	-	-	MK579258	-
Dactylonectria torresensis[b]	BV-0666	Mendavia, Navarra	MK602787	MK602802	MK579259	MK602817
D. torresensis	BV-0827	Mendavia, Navarra	-	-	MK579260	-

D. torresensis[b]	BV-0901	Mendavia, Navarra	-	-	MK579261	-
D. torresensis	BV-1076	Larraga, Navarra	-	-	MK579262	-
D. torresensis	BV-1255	Larraga, Navarra	-	-	MK579263	-
D. torresensis	BV-1256	Larraga, Navarra	-	-	MK579264	-
D. torresensis	BV-1300	Larraga, Navarra	-	-	MK579265	-
D. torresensis	BV-1313	Larraga, Navarra	-	-	MK579266	-
D. torresensis	BV-1315	Larraga, Navarra	-	-	MK579267	-
D. torresensis	BV-2103	Larraga, Navarra	-	-	MK579268	-
Dactylonectria riojana sp. nov.[b]	BV-1396	Mendavia, Navarra	MK602796	MK602811	MK602831	MK602826
D. riojana sp. nov.[b]	BV-1397	Mendavia, Navarra	MK602797	MK602812	MK602832	MK602827
Ilyonectria liriodendri[b]	BV-1591	O Barco de Valdeorras, Galicia	MK602788	MK602803	MK579269	MK602818
I. liriodendri[b]	BV-1642	Larraga, Navarra	-	-	MK579270	-
I. liriodendri	BV-1757	Larraga, Navarra	-	-	MK579271	-
I. liriodendri	BV-1762	Larraga, Navarra	-	-	MK579272	-
I. liriodendri	BV-1763	Larraga, Navarra	-	-	MK579273	-
I. liriodendri	BV-1764	Larraga, Navarra	-	-	MK579274	-
I. liriodendri	BV-1844	Larraga, Navarra	-	-	MK579275	-
I. liriodendri	BV-1923	Larraga, Navarra	-	-	MK579276	-
I. liriodendri	BV-1925	Larraga, Navarra	-	-	MK579277	-
I. liriodendri	BV-1929	Larraga, Navarra	-	-	MK579278	-
Ilyonectria pseudodestructans[b]	BV-2142	Larraga, Navarra	MK602789	MK602804	MK579279	MK602819
I. pseudodestructans	BV-2306	Larraga, Navarra	-	-	MK579280	-
I. pseudodestructans	BV-2307	Larraga, Navarra	-	-	MK579281	-
I. pseudodestructans	BV-2506	Larraga, Navarra	-	-	MK579282	-
I. pseudodestructans[b]	BV-2609	Larraga, Navarra	-	-	MK579283	-
I. pseudodestructans	BV-2652	Larraga, Navarra	-	-	MK579284	-
I. pseudodestructans	BV-2678	Larraga, Navarra	-	-	MK579285	-
Ilyonectria robusta	BV-1593	Mendavia, Navarra	MK602790	MK602805	MK579286	MK602820
I. robusta[b]	BV-0816	O Barco de Valdeorras, Galicia	-	-	MK579287	-
I. robusta[b]	BV-1654	Larraga, Navarra	-	-	MK579288	-
I. robusta	BV-2051	Larraga, Navarra	-	-	MK579289	-

I. robusta	BV-2069	Larraga, Navarra	-	-	MK579290	-
I. robusta	BV-2565	Larraga, Navarra	-	-	MK579291	-
I. robusta	BV-2649	Larraga, Navarra	-	-	MK579292	-
Ilyonectria vivaria sp. nov.[b]	BV-1924	Larraga, Navarra	MK602793	MK602808	MK602828	MK602823
I. vivaria sp. nov.[b]	BV-2276	Larraga, Navarra	MK602794	MK602809	MK602829	MK602824
I. vivaria sp. nov.	BV-2305	Larraga, Navarra	MK602795	MK602810	MK602830	MK602825
Neonectria quercicola[b]	BV-1661	Larraga, Navarra	MK602791	MK602806	MK579293	MK602821
N. quercicola[b]	BV-2137	Larraga, Navarra	-	-	MK579294	-
N. quercicola	BV-2140	Larraga, Navarra	-	-	MK579295	-
Neonectria sp. 1[b]	BV-2682	Larraga, Navarra	MK602792	MK602807	MK579296	MK602822
Thelonectria olida[b,c]	BV-0537	Mendavia, Navarra	MK602793	-	MK579297	-

[a] ITS = internal transcribed spacer, *tub2* = β-tubulin, *his3* = histone H3 and *tef1* = translation elongation factor 1- α.

[b] Isolates used for colony and conidial morphology, in the temperature growth assay and for pathogenicity tests

[c] *Thelonectria olida* was not included in the phylogenetic analyses

Table 3. Black-foot disease fungal isolates from GenBank included in the phylogenetic analyses.

Species	Strain number[a]	Host	Collector	Location	GenBank accession number			
					ITS[b]	*tub2*	*his3*	*tef1*
Campylocarpon fasciculare	**CBS 112613**	*Vitis vinifera*	F. Halleen	South Africa	AY677301	AY677221	JF735502	JF735691
C. pseudofasciculare	**CBS112679**	*V. vinifera*	F. Halleen	South Africa	AY677306	AY677214	JF735503	JF735692
Dactylonectria alcacerensis	**CBS 129087**	*V. vinifera*	A. Cabral & H. Oliveira	Portugal	JF735333	AM419111	JF735630	JF735819
	Cy134	*V. vinifera*	J. Armengol	Spain	JF735332	AM419104	JF735629	JF735818
D. amazonica	MUCL55433	*Piper* sp.	A. Gordillo & C. Decock	Ecuador	MF683707	MF683644	MF683686	MF683665
	MUCL55430	*Piper* sp.	A. Gordillo & C. Decock	Eucador	MF683706	MF683643	MF683685	MF683664
D. anthuriicola	**CBS 564.95**	*Anthurium* sp.	R. Pieters	The Netherlands	JF735302	JF735430	JF735579	JF735768
D. ecuadoriensis	MUCL55425	*Piper* sp.	A. Gordillo & C. Decock	Ecuador	MF683705	MF683642	MF683684	MF683663
	MUCL55424	*Piper* sp.	A. Gordillo & C. Decock	Ecuador	MF683704	MF683641	MF683683	MF683662
D. estremocensis	**CBS 129085**	*V. vinifera*	C. Rego & T. Nascimento	Portugal	JF735320	JF735448	JF735617	JF735806
	CPC 13539	*Picea glauca*	R. C. Hamelin	Canada	JF735330	JF735458	JF735627	JF735816
D. hispanica	**CBS 142827**	*Pinus halepensis*	B. Mora-Sala	Spain	KY676882	KY676876	KY676864	KY676870
	Cy228	*Ficus* sp.	F. Caetano	Portugal	JF735301	JF735429	JF735578	JF735767
D. hordeicola	**CBS 162.89**	*Hordeum vulgare*	M. Barth	The Netherlands	AM419060	AM419084	JF735610	JF735799
D. macrodidyma	**CBS 112615**	*V. vinifera*	F. Halleen	South Africa	AY677290	AY677233	JF735647	JF735836

	CBS 112604	*V. vinifera*	F. Halleen	South Africa	AY677284	AY677229	JF735644	JF735833
D. novozelandica	CBS 112608	*V. vinifera*	F. Halleen	South Africa	AY677288	AY677235	JF735632	JF735821
	CBS 113552	*Vitis* sp.	R. Bonfiglioli	New Zealand	JF735334	AY677237	JF735633	JF735822
D. palmicola	**MUCL55426**	*Euterpe precatoria*	A. Gordillo & C. Decock	Ecuador	MF683708	MF683645	MF683687	MF683666
D. pauciseptata	CBS 100819	*Erica melanthera*	H. M. Dance	New Zealand	EF607090	EF607067	JF735582	JF735771
	CBS 120171	*Vitis* sp.	M. Zerjav	Slovenia	EF607089	EF607066	JF735587	JF735776
D. pinicola	**CBS 173.37**	*P. laricio*	T. R. Peace	UK	JF735319	JF735447	JF735614	JF735803
	CBS 159.43	-	H. W. Wollenweber	Germany	JF735318	JF735446	JF735613	JF735802
D. polyphaga	**MUCL55209**	*Costus* sp.	A. Gordillo & C. Decock	Ecuador	MF683689	MF683626	MF683668	MF683647
	MUCL55208	*Costus* sp.	A. Gordillo & C. Decock	Ecuador	MF683699	MF683636	MF683678	MF683657
D. torresensis	**CBS 129086**	*V. vinifera*	A. Cabral	Portugal	JF735362	JF735492	JF735681	JF735870
	CBS 119.41	*Fragaria* sp.	H. C. Koning	The Netherlands	JF735349	JF735478	JF735657	JF735846
D. vitis	**CBS 129082**	*V. vinifera*	C. Rego	Portugal	JF735303	JF735431	JF735580	JF735769
D. valentina	**CBS 142826**	*Ilex aquifolium*	B. Mora-Sala	Spain	KY676881	KY676875	KY676863	KY676869
Ilyonectria capensis	**CBS 132815**	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231151	JX231103	JX231135	JX231119
	CBS 132816	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231160	JX231112	JX231144	JX231128
I. coprosmae	CBS 119606	*Metrosideros* sp.	G. J. Samuels	Canada	JF735260	JF735373	JF735505	JF735694

I. crassa	CBS 129083	*Panax quinquefolium*	S. Hong	Canada	AY295311	JF735395	JF735536	JF735725
	CBS 158.31	*Narcissus* sp.	W. F. van Hell	The Netherlands	JF735276	JF735394	JF735535	JF735724
I. cyclaminicola	**CBS 302.93**	*Cyclamen* sp.	M. Hooftman	The Netherlands	JF735304	JF735432	JF735581	JF735770
I. destructans	**CBS 264.65**	*Cyclamen persicum*	L. Nilsson	Sweden	AY677273	AY677256	JF735506	JF735695
I. europaea	CBS 102892	*Phragmites australis*	W. Leibinger	Germany	JF735295	JF735422	JF735569	JF735758
	CBS 129078	*V. vinifera*	C. Rego	Portugal	JF735294	JF735421	JF735567	JF735756
I. gamsii	**CBS 940.97**	Soil	J. T. Poll	The Netherlands	AM419065	AM419089	JF735577	JF735766
I. ilicicola	**CBS 142828**	*Ilex* sp.	B. Mora-Sala	Spain	KY676884	KY676878	KY676866	KY676872
	Cy-FO-226	*Ilex* sp.	B. Mora-Sala	Spain	KY676885	KY676879	KY676867	KY676873
I. leucospermi	**CBS 132809**	*Leucospermum* sp.	C. M. Bezuidenhout	South Africa	JX231161	JX231113	JX231145	JX231129
	CBS 132810	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231162	JX231114	JX231146	JX231130
I. liliigena	**CBS 189.49**	*Lilium regale*	M. A. A. Schippers	The Netherlands	JF735297	JF735425	JF735573	JF735762
	CBS 732.74	*Lilium* sp.	G. J. Bollen	The Netherlands	JF735298	JF735426	JF735574	JF735763
I. liriodendri	**CBS 110.81**	*Liriodendron tulipifera*	J. D. MacDonald & E.E	USA	DQ178163	DQ178170	JF735507	JF735696
	CBS 117527	*V. vinifera*	C. Rego	Portugal	DQ178165	DQ178172	JF735509	JF735698
I. lusitanica	**CBS 129080**	*V. vinifera*	N. Cruz	Portugal	JF735296	JF735423	JF735570	JF735759
I. mors-panacis	CBS 124662	*Pa. ginseng*	Y. Myazawa	Japan	JF735290	JF735416	JF735559	JF735748

	CBS 306.35	*Pa. quinquefolium*	A. A. Hildebrand	Canada	JF735288	JF735414	JF735557	JF735746
I. palmarum	CBS 135753	*Howea forsteriana*	G. Polizzi	Italy	HF937432	HF922609	HF922621	HF922615
	CBS 135754	*H. forsteriana*	G. Polizzi	Italy	HF937431	HF922608	HF922620	HF922614
I. panacis	**CBS 129079**	*Pa. quinquefolium*	K. F. Chang	Canada	AY295316	JF735424	JF735572	JF735761
I. protearum	**CBS 132811**	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231157	JX231109	JX231141	JX231125
	CBS 132812	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231165	JX231117	JX231149	JX231133
I. pseudodestructans	CBS 117824	*Quercus* sp.	E. Halmschlager	Austria	JF735292	JF735419	JF735562	JF735751
	CBS 129081	*V.vinifera*	C. Rego	Portugal	AJ875330	AM419091	JF735563	JF735752
I. robusta	CBS 129084	*V. vinifera*	N. Cruz	Portugal	JF735273	JF735391	JF735532	JF735721
	CBS 308.35	*Pa. quinquefolium*	A. A. Hildebrand	Canada	JF735264	JF735377	JF735518	JF735707
I. rufa	**CBS 153.37**	*Dune sand*	F. Moreau	France	AY677271	AY677251	JF735540	JF735729
	CBS 640.77	*A. alba*	F. Gourbière	France	JF735277	JF735399	JF735542	JF735731
I. venezuelensis	**CBS 102032**	Bark	A. Rossman	Venezuela	AM419059	AY677255	JF735571	JF735760
I. vredehoekensis	**CBS 132807**	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231155	JX231107	JX231139	JX231123
	CBS 132814	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231158	JX231110	JX231142	JX231126
Neonectria candida	CBS 182.36	*Malus sylvestris*	H. W. Wollenweber	-	JF735314	JF735439	JF735603	JF735792

N. candida, authentic strain of *C. obtusiusculum*	**CBS 151.29**	*Ma. sylvestris*	H. W. Wollenweber	UK	JF735313	JF735438	JF735602	JF735791
N. ditissima, authentic strain of *C. wilkommii*	**CBS 226.31**	*Fagus sylvatica*	H. W. Wollenweber	Germany	JF735309	DQ789869	JF735594	JF735783
N. ditissima, representative strain of *N. galligena*	CBS 835.97	*Salix cinerea*	W. Gams	Belgium	JF735310	DQ789880	JF735595	JF735784
N. major	**CBS 240.29**	*Alnus incana*	H. W. Wollenweber	Norway	JF735308	DQ789872	JF735593	JF735782
N. neomacrospora	**CBS 324.61**	*A. concolor*	J. A. von Arx	The Netherlands	JF735312	DQ789875	JF735599	JF735788
	CBS 503.67	*A. alba*	F. Roll-Hansen	Norway	AY677261	JF735436	JF735600	JF735789
N. obtusispora	CBS 183.36	*Solanum tuberosum*	H. W. Wollenweber	Germany	AM419061	AM419085	JF735607	JF735796
	CPC 13544	*Prunus armenica*	J. A. Traquair	Canada	AY295306	JF735443	JF735608	JF735797
N. quercicola	**CBS 143704**	*Q. ilex*	P. Abad-Campos	Spain	KY676880	KY676874	KY676862	KY676868
	CPC 13530	*Pyrus* sp.	J. A. Traquair	Canada	AY295302	JF735441	JF735605	JF735794
Neonectria sp. 1	CPC 13545	*Pyrus* sp.	J. A. Traquair & B. Harrison	Canada	AY295303	JF735437	JF735601	JF735790

[a] Ex-type culture indicated in bold. **CBS**: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CPC**: Culture collection of Pedro Crous, housed at CBS. **MUCL**: Mycothèque de l'Université catholique de Louvain.

[b] ITS = internal transcribed spacer, *tub2* = β-tubulin, *his3* = histone H3 and *tef1* = translation elongation factor 1- α.

Table 4. Temperature-growth relationship for black-foot disease isolates[a]

Species	Isolate	Adjusted model[b] R^2	*a*	*b*	*c*	Temperature (ºC)[c]	Growth (mm/day)[d]
Dactylonectria alcacerensis	BV-1240	0.71	-0.0157	0.6266	-3.2011	19.9 ab	3.0 bc
D. alcacerensis	BV-1469	0.82	-0.0124	0.4985	-2.4377	20.1 ab	2.6 bcd
Dactylonectria macrodidyma	BV-1366	0.80	-0.0154	0.6179	-3.0275	20.1 ab	3.2 bc
D. macrodidyma	BV-0535	0.79	-0.0141	0.5684	-2.7668	20.2 ab	3.0 bc
Dactylonectria novozelandica	BV-0760	0.78	-0.0148	0.5834	-2.6809	19.8 ab	3.1 bc
D. novozelandica	BV-1369	0.75	-0.0177	0.7107	-3.3549	20.1 ab	3.8 bc
Dactylonectria pauciseptata	BV-1354	0.83	-0.0169	0.6898	-3.2981	20.4 ab	3.7 bc
D. pauciseptata	BV-1360	0.73	-0.0215	0.8871	-4.7891	20.6 a	4.4 ab
Dactylonectria riojana sp. nov.	BV-1396	0.92	-0.0107	0.4272	-2.0464	20.0 ab	2.2 d
D. riojana sp. nov	BV-1397	0.93	-0.0109	0.4351	-2.068	20.0 ab	2.3 cd
Dactylonectria torresensis	BV-0901	0.78	-0.0135	0.5373	-2.6696	19.9 ab	2.7 bcd
D. torresensis	BV-0666	0.76	-0.0181	0.7081	-3.2878	19.6 ab	3.6 bc
Ilyonectria liriodendri	BV-1591	0.81	-0.0138	0.5362	-2.1454	19.4 ab	3.1 bc
I. liriodendri	BV-1642	0.80	-0.0173	0.668	-2.5882	19.3 ab	3.9 bc
Ilyonectria pseudodestructans	BV-2142	0.84	-0.0099	0.3956	-1.8645	20.0 ab	2.1 d
I. pseudodestructans	BV-2609	0.71	-0.0158	0.6301	-3.2205	19.9 ab	3.0 bc
Ilyonectria robusta	BV-1593	0.80	-0.0191	0.7121	-2.4659	18.6 b	4.2 ab
I. robusta	BV-1654	0.80	-0.0245	0.9523	-4.1827	19.4 ab	5.1 a
Ilyonectria vivaria sp. nov.	BV-1924	0.78	-0.0173	0.6657	-2.5358	19.4 ab	3.9 bc
I. vivaria sp. nov.	BV-2305	0.77	-0.0184	0.7116	-3.0192	19.3 ab	3.9 bc
Neonectria quercicola	BV-2137	0.82	-0.0191	0.7274	-2.6044	19.0 ab	4.3 ab
N. quercicola	BV-1661	0.85	-0.0194	0.7427	-2.6372	19.1 ab	4.5 ab
Neonectria sp. 1	BV-2682	0.78	-0.0149	0.5603	-1.7684	18.8 b	3.5 bc
Thelonectria olida	BV-0537	0.76	-0.0133	0.5287	-2.5958	19.9 ab	2.7 bcd

[a] Data are the average of eight replicates for each isolate. For each column, means with the same letter are not significantly different ($P = 0.05$) according to the Kruskal-Wallis test and the Dunn's test for multiple comparisons of mean ranks.

[b] Mycelial growth on potato dextrose agar at 5 to 35ºC was adjusted to a second-degree polynomial model: $Y = aT^2 + bT + c$, in which Y = mycelial growth (mm/day); a, b, and c are the regression coefficients; and R^2 = coefficient of determination.

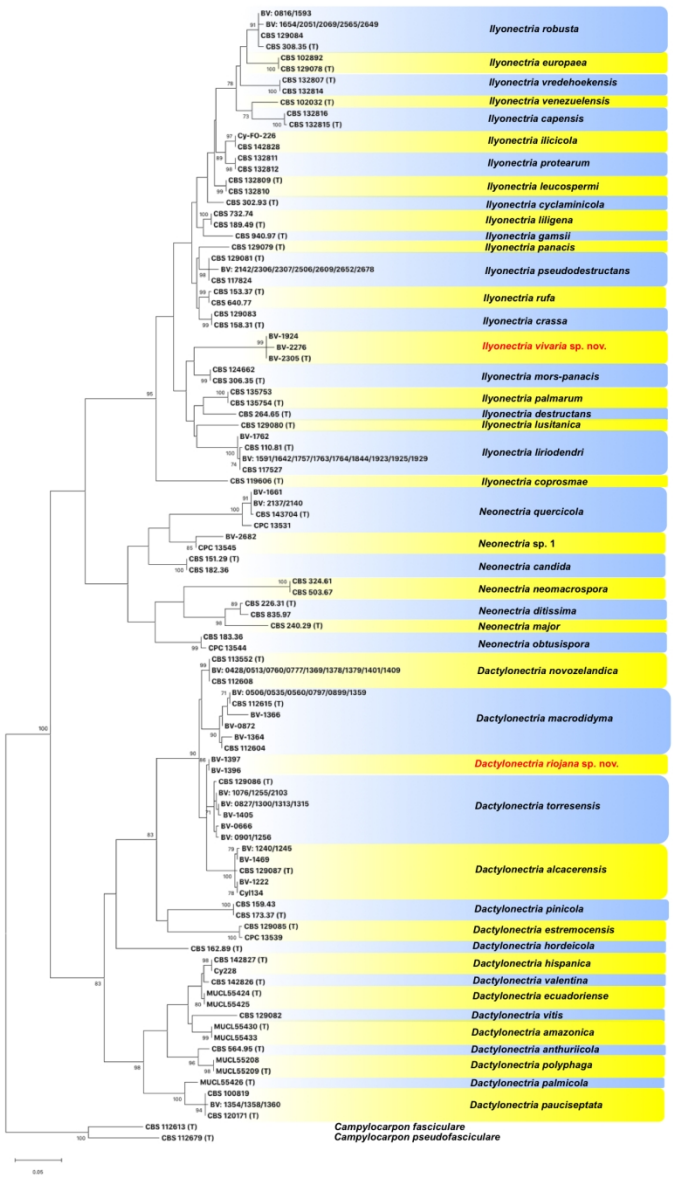
[c] Optimal temperature estimated by the adjusted model.

[d] Maximum growth rate estimated by the adjusted model

Table 5. Pathogenicity of black-foot pathogens on seedlings of *Vitis vinifera* cv. 'Tempranillo'[a]

Fungal species	Isolate	% of dead plants	MTA (days)[b]	% of reisolation[c]
Dactylonectria alcacerensis	BV-1240	75.0 b	41.2 e	100
	BV-1469	33.3 cd	49.5 bcde	75.0
Dactylonectria macrodidyma	BV-1366	66.7 bc	56.4 ab	100
	BV-0535	41.7 cd	54.9 abc	83.3
Dactylonectria novozelandica	BV-0760	100 a	12.9 f	100
	BV-1369	16.7 cde	57.4 ab	90.9
Dactylonectria pauciseptata	BV-1354	8.3 de	55.4 abc	83.3
	BV-1360	41.7 cd	47.3 cde	100
Dactylonectria riojana sp. nov.	BV-1396	33.3 cd	49.8 bcde	100
	BV-1397	41.7 cd	57.5 ab	100
Dactylonectria torresensis	BV-0901	25.0 cd	58.2 ab	100
	BV-0666	8.3 de	56.3 ab	100
Ilyonectria liriodendri	BV-1591	25.0 cd	60.3 a	100
	BV-1642	16.7 cde	54.5 abc	91.7
Ilyonectria pseudodestructans	BV-2142	25.0 cde	56.2 abc	83.3
	BV-2609	8.3 de	59.0 a	75
Ilyonectria robusta	BV-1593	8.3 de	60.5 a	66.7
	BV-1654	16.7 cde	59.3 a	83.3
Ilyonectria vivaria sp. nov.	BV-1924	50.0 c	53.3 abcd	100
	BV-2305	50.0 c	49.8 bcde	41.7
Neonectria quercicola	BV-2137	8.3 de	60.4 a	50
	BV-1661	0.0 e	61.0 a	100
Neonectria sp. 1	BV-2682	25.0 cde	55.3 abc	0
Thelonectria olida	BV-0537	58.3 c	45.4 de	100
Control		10.4 de	59.4 a	0
LSD (P<0.05)		16.4	8.5	-

[a]For each column, means with the same letter are not significantly different (P = 0.05) according to the LSD test
[b]MTA: Mean time from inoculation in which the plant stayed alive



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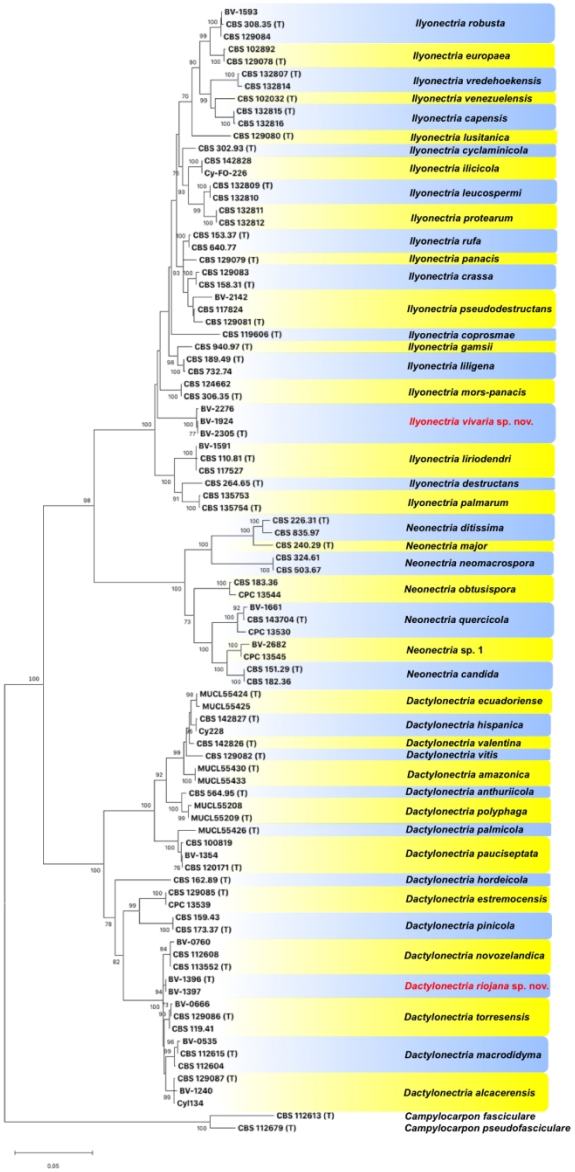

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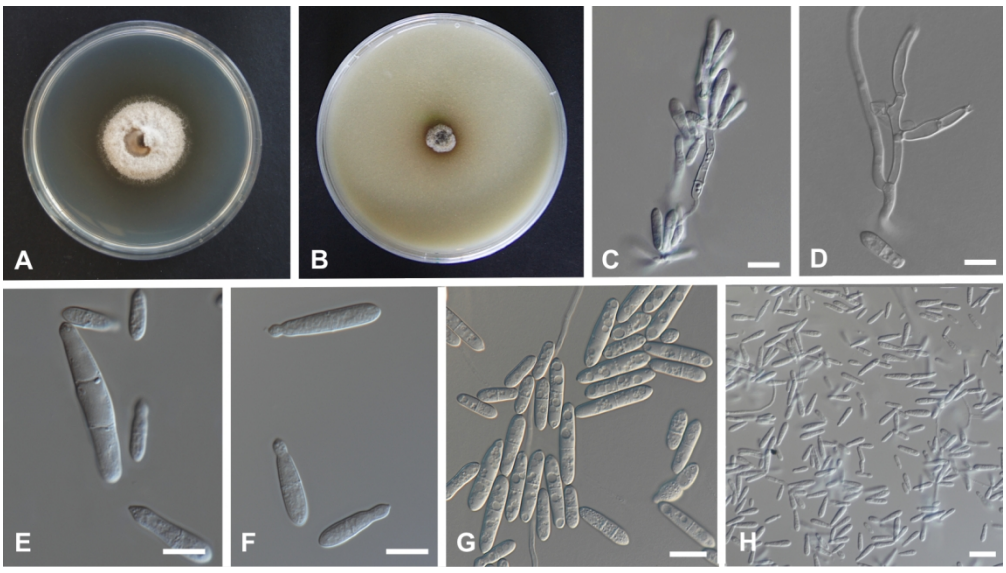

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