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# Diversity and Pathogenicity of *Diaporthe* Species Revealed from a Survey of Blueberry Orchards in Portugal

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**Abstract:** Blueberries (*Vaccinium corymbosum*) are widely cultivated worldwide and largely consumed due to their known antioxidant and medicinal properties. Although *Diaporthe* species have been documented in Portugal as causal agents of blueberry twig blight and dieback, there is still scarce information on the species that cause these symptoms. Moreover, *Diaporthe vaccinii*, recently synonymized with *D. eres*, has been considered a concern to blueberry production worldwide. However, the current knowledge about its impact on blueberries remains unclear. The diversity of *Diaporthe* species associated with diseased blueberry plants were assessed through a national survey. A multilocus sequence analysis of the rDNA internal transcribed spacer (ITS) region, the translation elongation factor 1-alpha (*tef1-α*), β-tubulin (*tub2*), calmodulin (*cal*) and histone 3 (*his3*) genes unveiled the presence of *Diaporthe ambigua*, *D. amygdali*, *D. crousii*, *D. foeniculina*, *D. hybrida*, *D. leucospermi*, *D. malorum* and *D. rudis*. Moreover, all species were fully characterized based on a detailed morphological description. *Diaporthe amygdali*, *D. hybrida*, *D. leucospermi* and *D. malorum* are reported for the first time on diseased blueberries in Portugal. Results show that *D. eres* exhibited a high level of intraspecific variability within isolates, given that the strain CBS 160.32 might be a minor pathogen on blueberry plants, whereas CAA829 was revealed to be the most aggressive. Overall, this study also demonstrates that *Diaporthe amygdali* and *D. eres* may be two of the most aggressive species to blueberry plants. This study improves our understanding of the *Diaporthe* species and it's causing of dieback and twig blight on Portuguese blueberry orchards. Additionally, the identification of these pathogens represents crucial information for blueberry producers to apply appropriate phytosanitary measures, as well as offering new insights into the potential pathogenicity of *D. eres* on this host.

**Keywords:** *Diaporthaceae*; dieback; pathogens; phylogeny; twig blight; *Vaccinium corymbosum*



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## 1. Introduction

The genus *Vaccinium* L. includes nearly 450 species of perennial shrubs belonging to the family *Ericaceae* which have adapted to several climates [1]. The species *Vaccinium corymbosum* (known as northern highbush blueberry) is a small-fruit crop, native to North America and commercially cultivated in Europe [2]. Due to the recognized high nutritional and medicinal value of fruits [3,4], blueberry production has been rapidly increasing worldwide.

In the last 10 years the production of this crop in Portugal has increased substantially. Although production initiated intensively only in 2011 with a yield of 700 tons and a cultivated area of 75 ha, in 2019 the country was ranked as the 9th largest producer worldwide [5], with a production of 15,418 tons, occupying a total area of 2490 ha [6], making it a highly profitable crop to the economy of the country. However, due to the spread of blueberry plant material across continents, it is expected that several fungal pathogens may affect blueberry orchards, given their entry into new habitats [7]. Some of these pathogens, such as members of *Botryosphaeriaceae*, *Pestalotiopsis sensu lato* and *Diaporthe* can cause twig blight, stem cankers and dieback on blueberry plants [8–10].

The genus *Diaporthe* encompasses endophytes, saprobes and plant pathogens associated with a wide variety of agricultural crops, ornamental plants, and forest trees [11–16].

Members of this genus were previously found to colonize blueberry tissues as latent pathogens or endophytes [8] and as pathogens causing numerous diseases on this crop such as apical necrosis of twigs and stems, cankers, necrosis of the vascular tissues, wilting of leaves, and leaf spots [7,17,18]. These symptoms have been associated with an assemblage of species comprising *D. ambigua*, *D. amygdali*, *D. australafricana*, *D. baccae*, *D. crousii*, *D. eres*, *D. foeniculina*, *D. oxe*, *D. passiflorae*, *D. phillipsii*, *D. rossmaniae*, *D. rudis*, and *D. vaccinii* (syn. *D. eres*) [2,8,17,19–21].

*Diaporthe vaccinii*, formerly known as *Phomopsis vaccinii*, is native to North America [22] and it is recognized as a host-specific species on *Vaccinium* spp., causing severe losses to blueberry production worldwide [2,8]. Concerns about the damaging impacts of this pathogen on blueberry production led the European Food Safety Authority (EFSA) to recommend *D. vaccinii* as a quarantine pathogen for the European Union (Regulation (EU) 2016/2031 of 26 October 2016). Although *D. vaccinii* has been reported on blueberry plants in South America [17], North America [19,23], Europe [2,7,24] and Asia [18], the current knowledge about its pathogenicity on blueberries was barely explored. However, the most recent EU Regulation of 29 November 2919 (2019/2072), has no longer included *D. vaccinii* into the list of EU quarantine pests. Moreover, to clarify the species boundaries of the *Diaporthe eres* complex, Hilário et al. [20] implemented the Genealogical Phylogenetic Species Recognition principle (GCPSR) and the coalescent-based model Poisson Tree Processes (PTPs) and considered *D. vaccinii* to be a synonym of *D. eres*.

Nevertheless, despite the alleged presence of *D. vaccinii* in the North of Portugal based on the symptoms observed in the field [25], this species was not found in a recent survey carried out in one of the major blueberry productions in the country [8]. Given that species identification is essential to develop appropriate control measures, the focus of this study was the implementation of a large survey on *Vaccinium corymbosum* plantations across the country, aiming to understand the diversity and distribution of *Diaporthe* species associated with blueberry plants showing dieback and twig blight symptoms, as well as their pathogenic potential to different blueberry cultivars widely planted in the country.

## 2. Materials and Methods

### *2.1. Sampling and Fungal Isolates*

Sampling of plant material was carried out between April and August of 2019 in 12 Portuguese blueberry orchards (Table 1): Braga, Porto, Aveiro, Viseu, Castelo Branco and Santarém.

Plants from cultivars 'Duke', 'Ozarkblue', 'Aurora', 'Legacy', 'O'Neal', 'Liberty', 'Gupton', 'Bluecrop', 'Early Blue' and 'New Hanover' corresponding to an area of 31 ha were examined. Nearly 0.5% of plants from each plantation were checked for disease symptoms such as twig blight, stem cankers, and wilting and necrotic leaves in the apex of the plant. From the 531 plants inspected, only 95 showing these symptoms were collected. The samples were transported to the Fungal and Plant Biology Lab at University of Aveiro in paper bags, to prevent moisture, within 4–6 h. After arrival, one 10–30 cm long twig or stem per plant was taken for further fungal isolation.

Samples were first sterilized in 70% ethanol for 1 min, immersed in a 5% sodium hypochlorite solution for 1 min, and followed by rinsing two times in sterile distilled water (1 min per time). Fungal isolations were made by plating out pieces of plant tissues (2–5 mm) in potato dextrose agar plates (PDA, Merck, Germany). The plates were incubated at 25 °C and checked daily for fungal growth. Each colony was transferred to fresh PDA plates, kept at 25 °C in the dark until typical *Diaporthe* colonies were observed, and monosporic cultures were then established, as previously described by Hilário et al. [8].

**Table 1.** List of plantations used for this study.

| Region | Plantation | GPS Coordinates | | Area (ha) | Total of Plants | Inspected Plants | Sampled Plants |
|---|---|---|---|---|---|---|---|
| | | Latitude | Longitude | | | | |
| Aveiro | Águeda | 40°33′06.2″ N | 8°28′44.0″ W | 2.5 | 7000 | 35 | 10 |
| | Arouca | 40°54′35.3″ N | 8°22′49.7″ W | 1.7 | 4400 | 22 | 9 |
| Braga | Urgezes | 41°25′24.5″ N | 8°17′35.1″ W | 2 | 4000 | 20 | 9 |
| | Póvoa de Lanhoso | 41°31′35.0″ N | 8°15′08.3″ W | 1.5 | 4600 | 23 | 9 |
| Castelo Branco | Idanha-a-Nova | 39°52′58.4″ N | 7°17′23.2″ W | 2.5 | 10,416 | 52 | 4 |
| Porto | Penafiel | 41°08′07.0″ N | 8°22′42.5″ W | 0.6 | 500 | 3 | 3 |
| | Agrela | 41°08′07.0″ N | 8°28′09.7″ W | 1.8 | 6200 | 31 | 8 |
| | Arcozelo | 41°15′31.8″ N | 8°28′57.8″ W | 2.3 | 8000 | 40 | 8 |
| | Monte Córdova | 41°15′22.9″ N | 8°26′29.5″ W | 1 | 3084 | 15 | 9 |
| Santarém | Alpiarça | 39°15′04.1″ N | 8°32′39.1″ W | 6 | 20,000 | 100 | 8 |
| | Ourém | 39°39′13.5″ N | 8°35′32.0″ W | 1.7 | 8000 | 40 | 9 |
| Viseu | Mangualde | 40°37′43.2″ N | 7°51′51.8″ W | 7.4 | 31,091 | 150 | 9 |

### *2.2. Morphological Characterization*

To record the micromorphological characters of our isolates, sporulation was induced by plating 5-mm-diameter mycelium plugs on medium supplemented with sterilized fennel stems and pine needles as described by Hilário et al. [8]. Conidiomata and ascomata were observed with a Nikon SMZ1500 stereomicroscope (Nikon, Japan) and photographed with a Digital Sight DS-Fi1 camera (Nikon, Japan). The fruiting bodies were cut in a drop of sterile water in a microscope slide, mounted in 100% lactic acid and the micromorphological characters (e.g., conidia and ascospores size) were observed. All preparations were visualized with a Nikon 80i microscope (Nikon, Japan) with interference contrast equipment and captured with a Nikon Digital Sight DS-Ri1 camera (Nikon, Japan). About 50 conidia, or ascospores, were selected randomly and measured on images taken with the ×100 objective lens. Dimensions of other fungal structures (e.g., conidiophores, asci) are given as the range of a minimum of 20 measurements. Data for spores, conidiophores and asci measurements are with the minimum and maximum dimensions in parentheses and followed by mean and standard deviation (S.D.). Colony colors were analyzed according to Syme [26] after 7 days of growth on PDA in the dark at 25 °C. Cultures were maintained in the personal collection of Artur Alves (CAA), University of Aveiro (Portugal).

### *2.3. Molecular Characterization*

#### 2.3.1. DNA Extraction and PCR Amplification

The genomic DNA of all fungal isolates was extracted from fresh mycelium and grown in PDA for seven days at 25 °C according to a modified protocol by Möller [27]. Microsatellite-primed PCR (MSP-PCR) fingerprinting was performed using the $(GTG)_5$ primer and following the description of Alves et al. [28]. Isolates were clustered based on their genetic profiles in a consensus dendrogram built with GelCompar II software (Applied Maths) using Pearson's correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA). A cut-off of approximately 82.5% was chosen as the reproducibility level, since strains from a cluster above this level are deemed to belong to the same species, as previously described by Santos and Phillips [29].

The rDNA internal transcribed spacer region (ITS), translation elongation factor 1-alpha (*tef1-α*), β-tubulin (*tub2*), calmodulin (*cal*) and histone 3 (*his3*) were the loci chosen to be sequenced in this study. Primers ITS5 and NL4 were used to amplify the ITS region [30] following a description by Alves et al. [31]. The remaining loci were amplified using the

following primer sets: EF-688F/EF-1251R for *tef1-α* [32], T1/Bt2b for *tub2* [33,34], CALD-38F/CAL-737R for *cal* [8,35], and CYLH3F/H3-1bR for *his3* [33,36]. PCR conditions were as described Hilário et al. [8]. The PCR reactions, with a final volume of 25 µL, were composed of 15.75 µL of sterile pure water, 6.25 µL of NZYTaq 2× green Master Mix (Nzytech™, Lisbon, Portugal), 1 µL of each primer at 10 pmol/µL and 1 µL of DNA template and performed in a Bio-Rad C1000 touch thermal cycler (USA). PCR amplification products were checked using an electrophoresis in 1.5% agarose gels and visualised under UV light (Gel DocTM XR+, BioRad, Hercules, CA, USA). Amplicons were purified using the NZYGelPure Kit (Nzytech™, Lisbon, Portugal) and sequenced by GATC Biotech (Cologne, Germany).

#### 2.3.2. Phylogenetic Analysis

The nucleotide sequences were analyzed with FinchTV v.1.4.0 (Geospiza, Seattle, USA; www.geospiza.com/finchtv (accessed on 10 February 2021)). Sequences were aligned with ClustalX2.1 software [37] using pairwise alignment (gap opening = 10, gap extension = 0.1) and multiple alignment (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%). The alignments were optimized and edited manually using BioEdit Alignment Editor v.7.0.5. [38] and concatenated using Sequence Matrix [39].

Briefly, phylogenetic analyses were done using PAUP* v.4.0b10 [40] for Maximum Parsimony (MP) analyses, MrBayes v.3.0b4 [41] for Bayesian Inference (BI) analyses and MEGA v.7 [42] for Maximum Likelihood (ML) analyses [20]. Maximum Parsimony analyses were performed using PAUP* v. 4.0b10 [40]. For Maximum Parsimony, a branch-swapping algorithm, the heuristic search option with 100 random taxon additions, and the subtree pruning regrafting (SPR) method were applied. All characters were unordered and of equal weight, and gaps were treated as missing data. Maxtrees were set to 100 and branches of zero length were collapsed. Clade stability was assessed using a bootstrap analysis with 1000 replicates. Moreover, the following parameters were calculated: consistency index (CI), retention index (RI), tree length (TL), rescaled consistency index (RC) and homoplasy index (HI).

Bayesian analyses were performed using a Markov Chain Monte Carlo sampling (MCMC). Four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations and sampled every 100th generation for a total of 10,000 trees. The first 1000 were discarded as the burn-in phase of each analysis. Posterior probabilities (PP) were determined from a majority rule consensus tree generated with the remaining 9000 trees. The general time-reversible model of evolution assuming invariant sites and a gamma distribution was used for BI analyses [43].

Maximum likelihood analyses were carried out starting from a Neighbour-Joining tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference and 1000 bootstrap replicates were performed. MEGA v.7 was also used to determine the best nucleotide substitution model to build the ML trees. All trees were rooted to *Diaporthela corylina* and visualized with TreeView [44].

The identification of the clades containing our isolates was assessed through an initial ITS single tree containing all species currently accepted in the genus *Diaporthe*. Additionally, we also evaluated the possibility of combining all five loci (ITS, *tef1-α*, *tub2*, *cal*, and *his3*). A comparison of highly supported clades among single locus trees (including solely the phylogenetically closely related species previously selected) was performed to check phylogenetic concordance among the individual gene trees (Figures S1–S6).

Trees were edited in Inkscape Vector software v.0.92 (https://www.inkscape.org, accessed on 25 May 2021) (Adobe Systems Inc, San Jose, CA, USA). Sequences generated in this study were deposited in GenBank (Table 2) (www.ncbi.nlm.nih.gov, accessed on 17 March 2021) and all phylogenetic trees and alignments (including the ITS single tree) were deposited in TreeBASE (www.TreeBASE.org (accessed on 4 December 2021)) under accession number S28393).

**Table 2.** *Diaporthe* species used in this study.

| Species | Strain [1] | Host | Locality | GenBank Accession | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | *tef1-α* | *tub2* | *his3* | *cal* |
| *D. acaciigena* | **CBS 129521** | *Acacia retinodes* | Australia | KC343005 | KC343731 | KC343973 | KC343489 | KC343247 |
| *D. ambigua* | **CBS 114015** | *Pyrus communis* | South Africa | KC343010 | KC343736 | KC343978 | KC343494 | KC343252 |
| | CBS 123210 | *Foeniculum vulgare* | Portugal | KC343012 | KC343738 | KC343980 | KC343496 | KC343254 |
| | CBS 127746 | *Platanus acerifolia* | Italy | KC343014 | KC343740 | KC343982 | KC343498 | KC343256 |
| | CAA957 | *Vaccinium corymbosum* | Portugal | *MT073278* | *MT051928* | *MT051959* | *MT051887* | *MT051851* |
| *D. amygdali* | CBS 115620 | *Prunus persica* | USA | KC343020 | KC343746 | KC343988 | KC343504 | KC343262 |
| | **CBS 126679** | *Prunus dulcis* | Portugal | KC343022 | KC343748 | KC343990 | KC343506 | KC343264 |
| | CBS 111811 | *Vitis vinifera* | South Africa | KC343019 | KC343745 | KC343987 | KC343503 | KC343261 |
| | CAA958 | *Vaccinium corymbosum* | Portugal | MT073273 | MT051923 | MT051955 | MT051883 | MT051847 |
| | CAA959 | *Vaccinium corymbosum* | Portugal | MT073283 | MT051935 | MT051970 | MT051898 | MT051862 |
| | CFCC 52586 | *Kadsura longipeduncu-lata* | China | MH121521 | MH121563 | MH121600 | MH121479 | MH121439 |
| | CBS 136969 | *Vaccinium corymbosum* | Italy | KJ160579 | KJ160611 | KJ160528 | MF418350 | KJ160548 |
| | CBS 146754 | *Prunus dulcis* | Spain | MT007489 | MT006989 | MT006686 | MT007095 | MT006761 |
| *D. anacardii* | **CBS 720.97** | *Anacardium occidentale* | East Africa | KC343024 | KC343750 | KC343992 | KC343508 | KC343266 |
| *D. angelicae* | **CBS 111592** | *Heracleum sphondylium* | Austria | KC343027 | KC343753 | KC343995 | KC343511 | KC343269 |
| *D. aus-tralafricana* | CBS 113486 | *Vitis vinifera* | South Africa | KC343038 | KC343764 | KC344006 | KC343522 | KC343280 |
| | **CBS 113487** | *Vitis vinifera* | South Africa | KC343039 | KC343765 | KC344007 | KC343523 | KC343281 |
| *D. baccae* | **CBS 136972** | *Vaccinium corymbosum* | Italy | KJ160565 | KJ160597 | MF418509 | MF418264 | MG281695 |
| | CBS 142546 | *Citrus sinensis* | Italy | MF418358 | MF418437 | MF418517 | MF418272 | MF418192 |
| | CBS 142545 | *Citrus sinensis* | Italy | MF418351 | MF418430 | MF418510 | MF418265 | MF418185 |
| *D. beckhausii* | **CBS 138.27** | *Viburnum* sp. | - | KC343041 | KC343767 | KC344009 | KC343525 | KC343283 |
| *D. chamaeropis* | CBS 454.81 | *Chamaerops humilis* | Greece | KC343048 | KC343774 | KC344016 | KC343532 | KC343290 |
| *D. cinerascens* | CBS 719.96 | *Ficus carica* | Bulgaria | KC343050 | KC343776 | KC344018 | KC343534 | KC343292 |
| *D. crousii* | CAA821 | *Vaccinium corymbosum* | Portugal | MK792301 | MK828073 | MK837924 | MK871442 | MK883829 |
| | **MUM 19.29** | *Vaccinium corymbosum* | Portugal | MK792311 | MK828081 | MK837932 | MK871450 | MK883835 |
| | CAA828 | *Vaccinium corymbosum* | Portugal | MK792317 | MK828084 | MK837935 | MK871453 | MK883837 |
| | CAA960 | *Vaccinium corymbosum* | Portugal | *MT073274* | *MT051924* | *MT051956* | *MT051884* | *MT051848* |
| | CAA961 | *Vaccinium corymbosum* | Portugal | *MT073275* | *MT051925* | *MT051957* | *MT051885* | *MT051849* |
| | CAA962 | *Vaccinium corymbosum* | Portugal | *MT073301* | *MT051926* | *MT051962* | *MT051890* | *MT051854* |

**Table 2.** *Cont.*

| Species | Strain [1] | Host | Locality | GenBank Accession | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | *tef1-α* | *tub2* | *his3* | *cal* |
| *D. cynaroidis* | **CBS 122676** | *Protea cynaroides* | South Africa | KC343058 | KC343784 | KC344026 | KC343542 | KC343300 |
| *D. elaeagni* | CBS 504.72 | *Elaeagnus* sp. | Netherlands | KC343064 | KC343790 | KC344032 | KC343548 | KC343306 |
| *D. foeniculina* | CBS 123208 | *Foeniculum vulgare* | Portugal | KC343104 | KC343830 | KC344072 | KC343588 | KC343346 |
| | **CBS 111553** | *Foeniculum vulgare* | Spain | KC343101 | KC343827 | KC344069 | KC343585 | KC343343 |
| | CPC 28033 | *Citrus sinensis* | Portugal | MF418402 | MF418481 | MF418562 | MF418322 | MF418236 |
| | CBS 187.27 | *Camelia sinensis* | Italy | KC343107 | KC343833 | KC344075 | KC343591 | KC343349 |
| | CAA963 | *Vaccinium corymbosum* | Portugal | *MT073311* | *MT051932* | *MT051965* | *MT051893* | *MT051857* |
| | CAA967 | *Vaccinium corymbosum* | Portugal | *MT073307* | *MT051937* | *MT051973* | *MT051901* | *MT051865* |
| *D. fusicola* | PSCG 030 | *Pyrus pyrifolia* | China | MK626914 | MK654864 | MK691323 | MK726255 | MK691211 |
| | PSCG 118 | *Pyrus pyrifolia* | China | MK626910 | MK654860 | MK691317 | MK726250 | MK691204 |
| | CBS 120840 | *Prunus salicina* | South Africa | KC343021 | KC343747 | KC343989 | KC343505 | KC343263 |
| *D. infecunda* | **CBS 133812** | *Schinus terebinthifolius* | Brazil | KC343126 | KC343852 | KC344094 | KC343610 | KC343368 |
| *D. hybrida* | CAA998 | *Vaccinium corymbosum* | Portugal | MT073276 | MT051927 | MT051958 | MT051886 | MT051850 |
| | **CAA999/MUM 21.01** | *Vaccinium corymbosum* | Portugal | MT073280 | MT051929 | MT051960 | MT051888 | MT051852 |
| *D. leucospermi* | **CBS 111980** | *Leucospermum* sp. | Australia | JN712460 | KY435632 | KY435673 | KY435653 | KY435663 |
| | CAA971 | *Vaccinium corymbosum* | Portugal | *MT073313* | *MT051933* | *MT051968* | *MT051896* | *MT051860* |
| *D. longispora* | **CBS 194.36** | *Ribes* sp. | Canada | KC343135 | KC343861 | KC344103 | KC343619 | KC343377 |
| *D. malorum* | **CBS 142383** | *Malus domestica* | Portugal | KY435638 | KY435627 | KY435668 | KY435648 | KY435658 |
| | CAA740 | *Malus domestica* | Portugal | KY435642 | KY435629 | KY435670 | KY435650 | KY435660 |
| | CAA752 | *Malus domestica* | Portugal | KY435643 | KY435630 | KY435671 | KY435651 | KY435661 |
| | CAA972 | *Vaccinium corymbosum* | Portugal | MT073271 | MT051917 | MT051953 | MT051881 | MT051845 |
| | CAA973 | *Vaccinium corymbosum* | Portugal | MT073272 | MT051918 | MT051954 | MT051882 | MT051846 |
| | CAA974 | *Vaccinium corymbosum* | Portugal | MT073306 | MT051919 | MT051963 | MT051891 | MT051855 |
| | CAA975 | *Vaccinium corymbosum* | Portugal | MT073304 | MT051920 | MT051966 | MT051894 | MT051858 |
| | CAA976 | *Vaccinium corymbosum* | Portugal | MT073305 | MT051921 | MT051967 | MT051895 | MT051859 |
| | CAA977 | *Vaccinium corymbosum* | Portugal | MT073303 | MT051922 | MT051971 | MT051899 | MT051863 |
| *D. parvae* | PSCG 035 | *Pyrus* × *bretschneideri* | China | MK626920 | MK654859 | MK691249 | MK726211 | MK691169 |

**Table 2.** *Cont.*

| Species | Strain [1] | Host | Locality | GenBank Accession | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ITS | *tef1-α* | *tub2* | *his3* | *cal* |
| *D. passiflorae* | **CBS 132527** | *Passiflora edulis* | South America | JX069860 | KY435633 | KY435674 | KY435654 | KY435664 |
| *D. phillipsii* | **MUM 19.28** | *Vaccinium corymbosum* | Portugal | MK792305 | MK828076 | MN000351 | MK871445 | MK883831 |
| | CAA818 | *Vaccinium corymbosum* | Portugal | MK792307 | MK828078 | MN000352 | MK871447 | MK883833 |
| *D. portugallica* | **CBS 144228** | *Camelia sinensis* | Portugal | MH063905 | MH063911 | MH063917 | MH063899 | MH063893 |
| | CPC 34248 | *Camelia sinensis* | Portugal | MH063906 | MH063912 | MH063918 | MH063900 | MH063894 |
| *D. pyracanthae* | CAA487 | *Pyracantha coccinea* | Portugal | KY435636 | KY435626 | KY435667 | KY435647 | KY435657 |
| | **CBS142384** | *Pyracantha coccinea* | Portugal | KY435635 | KY435625 | KY435666 | KY435646 | KY435656 |
| *D. rossmaniae* | CAA763 | *Vaccinium corymbosum* | Portugal | MK792291 | MK828064 | MK837915 | MK871433 | MK883823 |
| | **MUM 19.30** | *Vaccinium corymbosum* | Portugal | MK792290 | MK828063 | MK837914 | MK871432 | MK883822 |
| *D. rudis* | CBS 114436 | *Laburnum anagyroides* | Austria | KC343232 | KC343958 | KC344200 | KC343716 | KC343474 |
| | **CBS 113201** | *Vitis vinifera* | Portugal | KC343234 | KC343960 | KC344202 | KC343718 | KC343476 |
| | CAA979 | *Vaccinium corymbosum* | Portugal | *MT073290* | *MT051930* | *MT051961* | *MT051889* | *MT051853* |
| | CAA984 | *Vaccinium corymbosum* | Portugal | *MT073282* | *MT051931* | *MT051964* | *MT051892* | *MT051856* |
| | CAA987 | *Vaccinium corymbosum* | Portugal | *MT073314* | *MT051934* | *MT051969* | *MT051897* | *MT051861* |
| | CAA991 | *Vaccinium corymbosum* | Portugal | *MT073291* | *MT051936* | *MT051972* | *MT051900* | *MT051864* |
| *D. sclerotioides* | **CBS 296.67** | *Cucumis sativus* | Netherlands | KC343193 | KC343919 | KC344161 | KC343677 | KC343435 |
| *D. sterilis* | **CBS 136969** | *Vaccinium corymbosum* | Italy | KJ160579 | KJ160611 | KJ160528 | MF418350 | KJ160548 |
| *D. stictica* | **CBS 370.54** | *Buxus sempervirens* | Italy | KC343212 | KC343938 | KC344180 | KC343696 | KC343454 |
| *Diaporthella corylina* | CBS 121124 | *Corylus* sp. | China | KC343004 | KC343730 | KC343972 | KC343488 | KC343246 |

[1] Acronyms of culture collection: CAA—Personal Culture Collection Artur Alves, University of Aveiro, Aveiro, Portugal; CBS—Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CFCC—China Forestry Culture Collection Center, Beijing, China; CPC—Personal culture Collection Pedro W. Crous, hosted at CBS; MUM—Culture Collection from Micoteca at Universidade of Minho, Center for Biological Engineering, Braga, Portugal. PSCG—Personal Culture Collection Y.S. Guo, China. Ex-type isolates are in bold face. Newly sequences generated in this study are in italics.

### 2.4. Mating-Type Assay

The mating type of the new species was determined by a PCR-based method. Primers MAT1-1-1FW and MAT1-1-1-RV at 40 pmol/µL were used to amplify part of the *MAT1-1-1* gene and the primers set MAT2 188F [8] and MAT1-2-1RV [15] at 10 pmol/µL used to amplify part of the *MAT1-2-1* gene. PCR conditions were as described by Hilário et al. [8].

### 2.5. Prevalence of Diaporthe Species

The prevalence of *Diaporthe* species in the *Vaccinium corymbosum* plants sampled was calculated as previously described by Fu et al. [45]. The Isolation Rate (RI) was calculated

for each species using Equation (1), where NS was the number of isolates from the same species, and NI was the total number of isolates collected during the survey.

$$\text{RI } \% = \left(\frac{\text{NS}}{\text{NI}}\right) \times 100 \quad (1)$$

### 2.6. Plant Material and Pathogenicity Tests

A minimum of three isolates from each species identified and from different locations, whenever possible, were selected to inoculate blueberry plants. Moreover, several other fungal isolates obtained from *V. corymbosum* plants sampled in a previous work (Table 3) [8] and the ex-type strain of *Diaporthe vaccinii* (CBS 160.32) were also selected to perform the pathogenicity tests. This latter strain was obtained from the Westerdijk Fungal Biodiversity Institute in the Netherlands.

**Table 3.** Fungal isolates used and obtained from a previous study (Hilário et al. [8]).

| Species | Isolate |
|---|---|
| *Diaporthe crousii* | CAA823 |
| *Diaporthe eres* | CAA829 |
| | CAA830 |
| *Diaporthe leucospermi* | CAA762 |
| *Diaporthe phillipsii* | CAA817 |
| | CAA818 |

The pathogenicity trials were performed from 9 June 2020 until 14 July 2020. Three cultivars of blueberry plants currently used in Portuguese plantations were selected: 'Duke'—the most cultivated in the northern area; 'Legacy'—the best suited to the climate in the southern area [46]; and 'Spartan'—alleged to be highly susceptible to infection by *Diaporthe vaccinii* [47]. Nine-month-old potted plants were obtained through the micropropagation method, thus assuring genetic homogeneity. All plants were supplied by Deifil Green Biotechnology LDA (Portugal). After arrival, plants were subjected to a 15-day acclimation period. Both acclimation period and the pathogenicity trials occurred in greenhouse conditions, with daily temperature ranging from 25 °C during the day to 15 °C at night, with a controlled photoperiod of 16/8 h (day/night) and watered regularly.

For inoculation, a wound was made on the stem 5 cm above the soil surface using a sterile scalpel, removing the bark, and exposing the cambium. A 5-mm-diameter mycelial plug was taken from 7-day-old cultures on PDA and placed in the wounded area with the mycelium in contact with the cambium. The inoculation site was sealed with Parafilm® to avoid dehydration. Plugs of sterile PDA were used to inoculate stems of control plants. External symptoms such as stem necrosis, wilting and dieback were assessed weekly and registered. Thirty-five days after inoculation, the bark was removed to measure the length of the internal lesions. Fungi were re-isolated on PDA to confirm Koch's postulates. Pieces of wood from the edges of lesions were immersed in 70% ethanol for 1 min, transferred to 5% sodium hypochlorite solution for 1 min, and then rinsed twice in sterile distilled water. These wood tissues were plated on PDA, incubated at room temperature, and checked daily for fungal growth.

The analyses were performed considering individual isolates and species. One-way analysis of variance (ANOVA) assumptions were verified using a Shapiro–Wilk test. The homogeneity of variances was also checked using the Bartletts's test. As the datasets did not meet ANOVA assumptions, the analysis was performed using the Kruskal–Wallis test. Lesion length means of the different isolates were compared with one another using the Dunn Test with $p < 0.05$. The different species were compared with one another, using the Wilcoxon rank sum test (with $p < 0.05$). Statistical analyses as well as box plots were

generated and conducted in R Statistical Software v. 4.0.1 [48], along with the GGPLOT2 package [49].

## 3. Results

### *3.1. Fungal Isolation*

Twelve plantations, harboring nearly 65,280 plants and corresponding to 31 ha, were evaluated in several blueberry growing regions in Portugal (Figure 1). From these plants, 95 plants from the cultivars 'Duke' ($n$ = 60), 'Ozarkblue' ($n$ = 2), 'Aurora' ($n$ = 7), 'O'Neal' ($n$ = 1), 'Gupton' ($n$ = 6), 'Legacy' ($n$ = 11), 'Bluecrop' ($n$ = 10), 'Liberty' ($n$ = 2) and 'New Hanover' ($n$ = 1) were collected. Symptoms such as dieback and necrosis of twigs and stems were observed in all sampled plants. The infections advanced towards the apex, developing necrosis above young shoots (Figure 2A). These symptoms were also accompanied by cankers that initially appeared as reddish to brown lesions on green twigs (Figure 2B) and later on the aged stems at the base (Figure 2C). Twig blight symptoms were also visible, developing red to rust lesion-like areas on the newer shoots, and reddish spots around buds (Figure 2D). Foliage on these diseased plants tended to redden or turn yellow, followed by leaf necrosis (Figure 2E). Additionally, in some plants, dieback led to necrosis of the vascular tissues and consequently to the twigs' death (Figure 2F). From the symptomatic blueberry samples, we obtained a collection of 116 isolates with characteristic diaporthalean colonies (white to dirty white felty mycelium spreading in a radial pattern and reverse with concentric zones) and micromorphological characteristics typical of the genus *Diaporthe* (alpha and beta conidia).

This genus was isolated from all regions surveyed although with different abundance values: Urgezes (21.6%), Arcozelo (15.5%), Ourém (11.2%), Águeda and Póvoa de Lanhoso (10.3%), Agrela (9.5%), Mangualde (6.9%), Arouca (6%), Monte Córdova (4.3%), Penafiel (2.6%), Alpiarça and Idanha-a-Nova (0.9%).

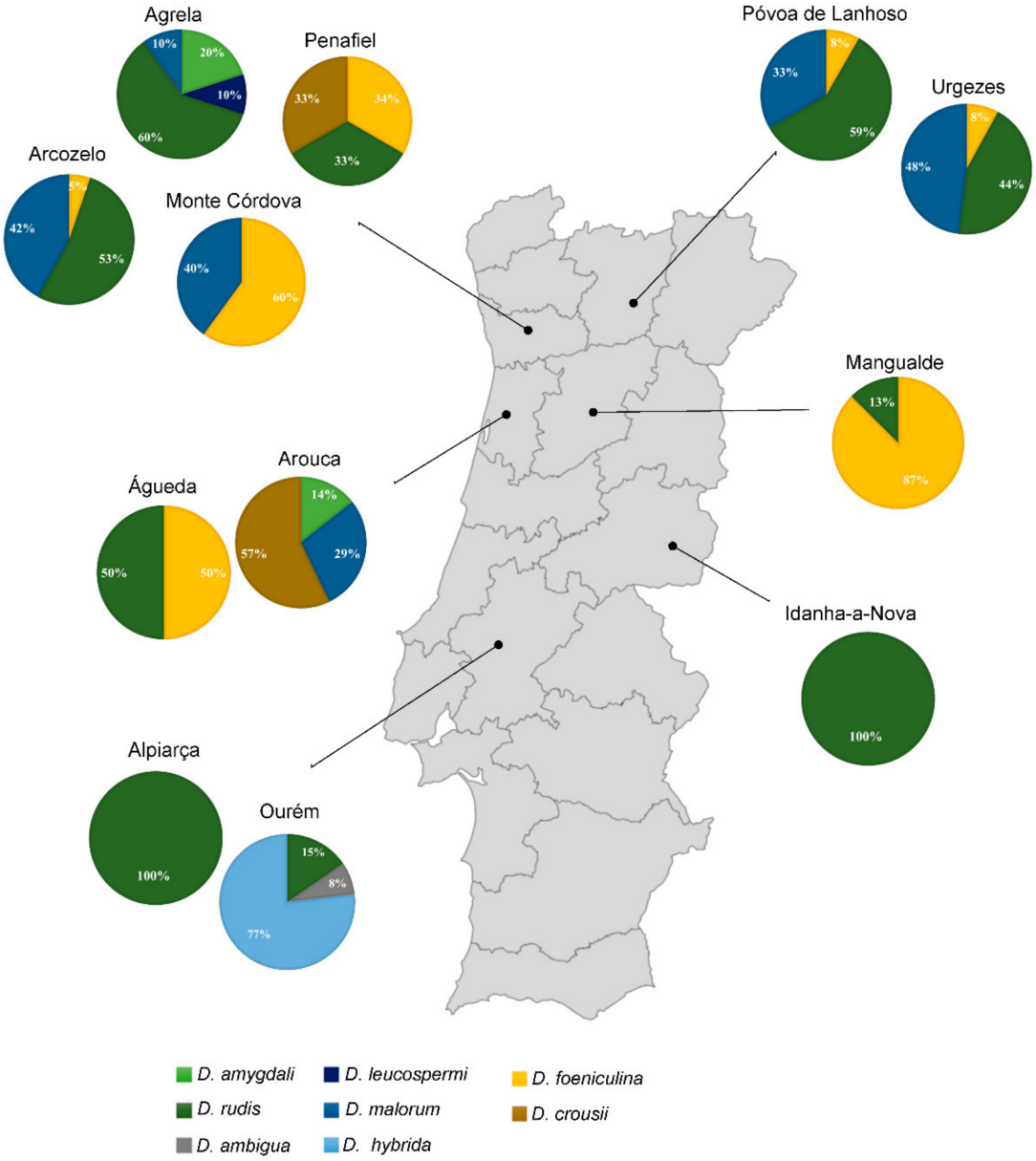


**Figure 1.** Frequency of *Diaporthe* species and distribution according to their sampling location.


**Figure 2.** Symptoms on *Vaccinium corymbosum* associated with *Diaporthe* species. (**A**)—Necrosis evolving right below a young shoot; (**B**)—Canker on a green twig. (**C**)—Canker on older stems developing at the base. (**D**)—Twig bight with visible red to rust areas. (**E**)—Leaf discoloration. (**F**)—Vascular system of a dead twig.

### 3.2. Molecular Characterization

MSP-PCR fingerprinting was performed to assess the genetic diversity of the 116 fungal isolates. A total of 21 isolates representative of each cluster, and from different geographic regions and cultivars whenever possible, were selected for further molecular characterization (Table 4).

**Table 4.** *Diaporthe* isolates collected from *Vaccinium corymbosum* plants, used in the phylogenetic analysis.

| Species | Strain [1] | Cultivar | Symptoms | Location | Mating-Type | |
|---|---|---|---|---|---|---|
| | | | | | *MAT1-1-1* | *MAT1-2-1* |
| *D. ambigua* | CAA957 | Duke | Dead stem | Ourém | (+) | (+) |
| *D. amygdali* | CAA958 | Aurora | Twig blight | Arouca | (+) | (−) |
| | CAA959 | Duke | Twig blight | Agrela | (+) | (−) |
| *D. crousii* | CAA960 | Aurora | Twig blight | Arouca | (+) | (+) |
| | CAA961 | Aurora | Twig blight | Arouca | (+) | (+) |
| | CAA962 | Legacy | Dead stem | Penafiel | (+) | (+) |
| *D. foeniculina* | CAA963 | Duke | Dead twig | Urgezes | (−) | (+) |
| | CAA967 | Aurora | Dieback | Mangualde | (−) | (+) |
| *D. hybrida* | CAA998 | Duke | Twig blight | Ourém | (+) | (−) |
| | CAA999 | Duke | Twig blight | Ourém | (+) | (−) |
| *D. leucospermi* | CAA971 | Duke | Dead twig | Agrela | (+) | (−) |
| *D. malorum* | CAA972 | Aurora | Twig blight | Arouca | (−) | (+) |
| | CAA973 | Aurora | Twig blight | Arouca | (−) | (+) |
| | CAA974 | Duke | Dieback | Póvoa de Lanhoso | (+) | (−) |
| | CAA975 | Duke | Dead twig | Urgezes | (+) | (−) |
| | CAA976 | Duke | Dead twig | Urgezes | (+) | (−) |
| | CAA977 | Duke | Dieback | Arcozelo | (+) | (−) |
| *D. rudis* | CAA979 | O'Neal | Stem blight | Ourém | (+) | (+) |
| | CAA984 | Duke | Dead twig | Urgezes | (+) | (+) |
| | CAA987 | Duke | Twig blight | Agrela | (+) | (+) |
| | CAA991 | Duke | Twig blight | Arcozelo | (+) | (+) |

[1] Acronym of collection: **CAA**—Culture Collection of Artur Alves, University of Aveiro, Portugal.

A primary identification using the ITS sequences of our isolates was done using BLASTn. The analysis revealed that our sequences matched to members of *Diaporthe* with high values of identity (98% to 100%), thus confirming the identity of our isolates. Afterwards, a multilocus analysis using ITS, *tef1-α*, *tub2*, *cal* and *his3* loci was performed, which included those species phylogenetically closely related to our isolates and whose 5 loci are available (Table 2).

The 5-loci concatenated alignment contained 2652 characters including gaps (599 from ITS, 458 from *tef1-a*, 513 from *tub2*, 546 from *cal* and 536 from *his3*). Of these 2652 characters, 1429 were constant, 344 were variable and parsimony uninformative and 879 were parsimony informative. MP analysis resulted in 100 equal, most parsimonious trees with TL = 3263 steps, CI = 0.5786, RI = 0.9049, RC = 0.5236 and HI = 0.4789. The analysis included 1 outgroup (*Diaporthella corylina* CBS 121124) and 76 taxa (22 sequences obtained in this study and 54 *Diaporthe* sequences retrieved from GenBank). The isolates from this study grouped in eight clades (Figure 3).

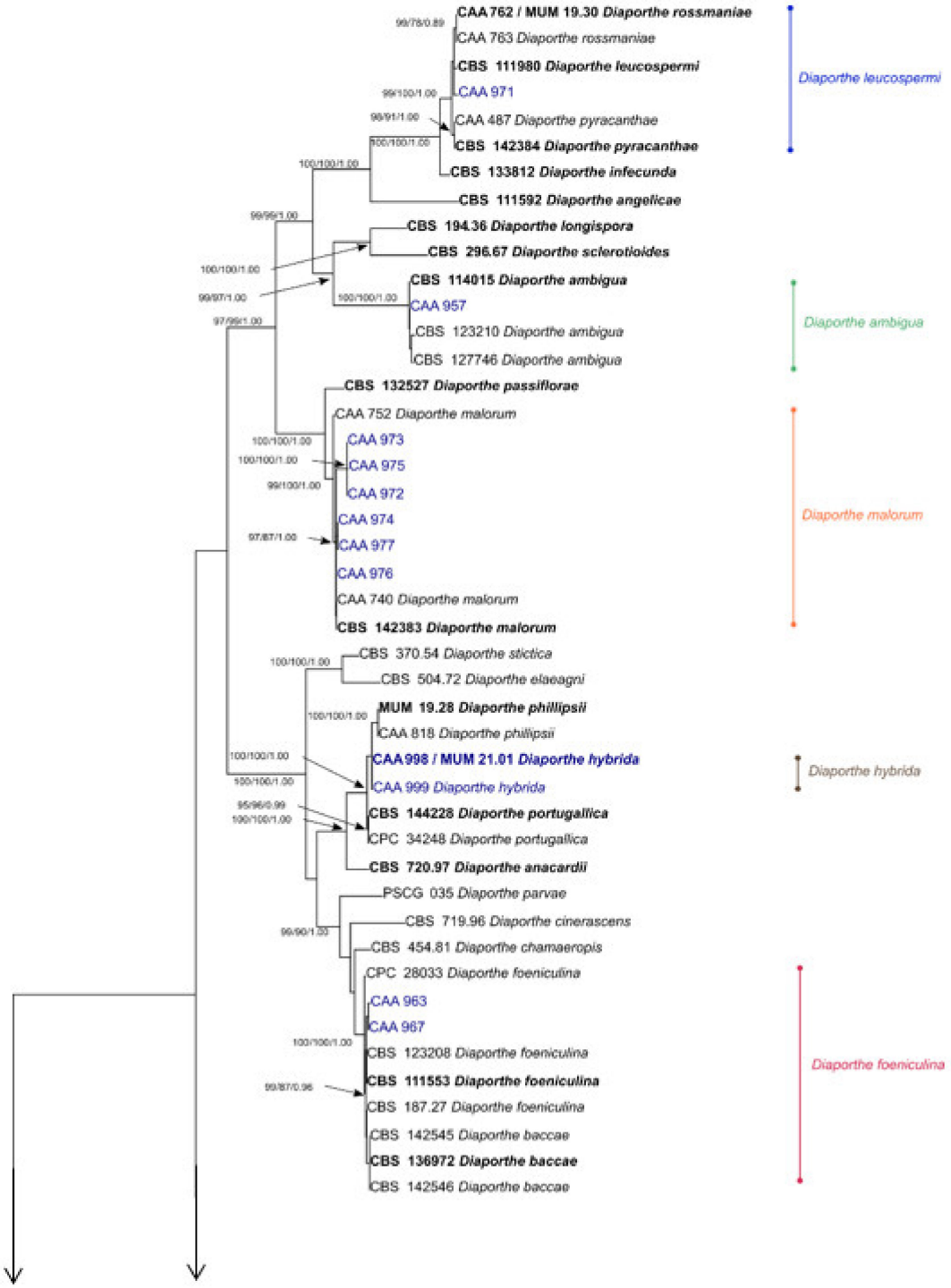


**Figure 3.** *Cont.*

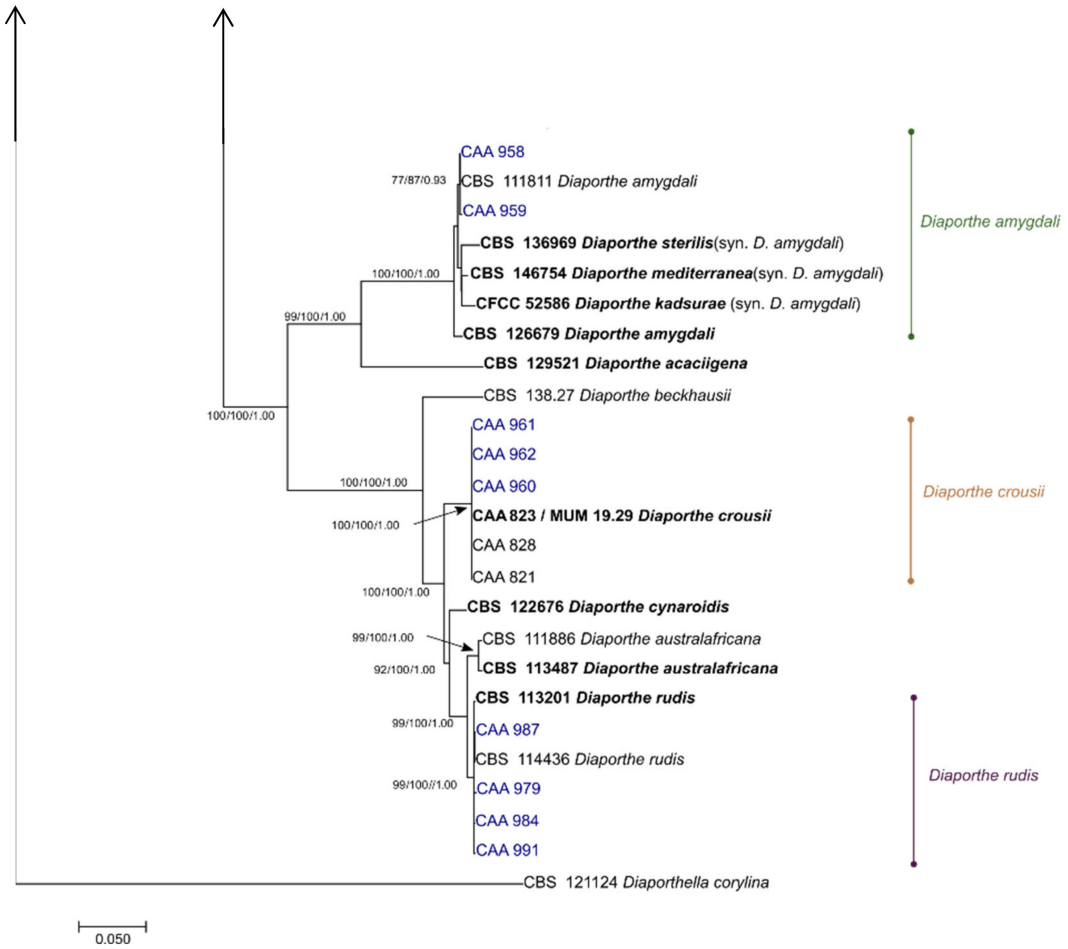


**Figure 3.** Phylogenetic relationships generated from maximum likelihood analysis based on ITS, *tef1-α*, *tub2*, *cal* and *his3* sequence data from *Diaporthe* species. The ML tree is drawn to scale with branch lengths measured in the number of substitutions per site and rooted to *Diaporthella corylina*. Maximum likelihood and maximum parsimony bootstrap values greater than 70% and posterior probabilities (PPs) inferred by Bayesian analysis greater than 0.80 are shown at the nodes. The ex-type strains are in bold.

Four isolates clustered within the well-supported *Diaporthe rudis* clade (ML/MP/PP = 99/100/1.00); three isolates clustered with *D. crousii* (ML/MP/PP = 100/100/1.00); only one isolate grouped with *D. ambigua* (ML/MP/PP = 100/100/1.00); two isolates fitted in a monophyletic clade containing the species *D. amygdali* (ML/MP/PP = 100/100/1.00); six isolates are deemed to belong to *D. malorum* (ML/MP/PP = 99/100/1.00); and two other isolates (*D. hybrida*) clustered between *D. portugallica* and *D. phillipsii*.

Isolate CAA971 clustered together with the ex-type strains of *D. rossmaniae*, *D. leucospermi* and *D. pyracanthae* forming a highly supported monophyletic clade (ML/MP/PP = 99/100/1.00). A pairwise alignment showed that CAA971 shares polymorphisms either with *D. pyracanthae* or *D. rossmaniae* in *his3*; with *D. leucospermi* in ITS, with *D. pyracanthae* in *tef1-α*; and with *D. rossmaniae* in *tub2*. Only in the *cal* locus, CAA971 differs from the remaining three species in the presence of three unique polymorphisms.

Isolates CAA963 and CAA967 fell within a highly supported clade (ML/MP/PP = 100/100/1.00) containing the type species of *D. foeniculina* and *D. baccae*. A pairwise comparison revealed that our isolates are 100% identical to *D. foeniculina* and *D. baccae* in *tef1-α* and *tub2* loci, and 100% identical to *D. foeniculina* in ITS locus. Moreover, in *cal* locus, only three unique polymorphisms separate our isolates from these species, while in *his3* locus, it was observed polymorphisms were either *D. baccae* or *D. foeniculina*.

### 3.3. Distribution and Prevalence of Diaporthe Species

Table 5 summarizes the number of isolates collected from diseased plants and the prevalence of each species found in this study. Overall, *Diaporthe* species were found in all 95 plants collected showing twig blight and dieback symptoms. From the total 116 diaporthalean isolates, *Diaporthe rudis* was the dominant species representing 38.8% of the isolates obtained, followed by *D. malorum* and *D. foeniculina* with an abundance of 25% and 19%, respectively. At least two different species were found in ten of the twelve blueberry orchards. *Diaporthe rudis*, *D. foeniculina* and *D. malorum* were found in six surveyed plantations. *Diaporthe ambigua*, *D. leucospermi* and *D. hybrida* were recovered from one orchard only, each one in different regions.

Some of the species collected were found co-existing with other species in the same plant, while others were isolated without the co-occurrence of other members of *Diaporthe*. For instance, *D. rudis* was found in association with *D. malorum*, *D. foeniculina*, *D. leucospermi*, *D. crousii* and *D. hybrida*. *Diaporthe amygdali* was also found co-existing with *D. crousii*; *D. leucospermi* was recovered from the same plant material along with *D. malorum*, while *D. ambigua* was only recovered without the co-occurrence of other species (Table S1).

**Table 5.** Number of isolates and prevalence of each *Diaporthe* species obtained from blueberry orchards.

| Species | Number of Isolates | Prevalence (%) |
|---|---|---|
| *D. ambigua* | 1 | 0.9% |
| *D. amygdali* | 3 | 2.5% |
| *D. crousii* | 5 | 4.3% |
| *D. foeniculina* | 22 | 19.0% |
| *D. leucospermi* | 1 | 0.9% |
| *D. malorum* | 29 | 25.0% |
| *D. rudis* | 45 | 38.8% |
| *Diaporthe hybrida* | 10 | 8.6% |
| TOTAL | 116 | 100% |

### 3.4. Pathogenicity Tests

#### 3.4.1. Plant Mortality

Mortality (100%) occurred only for the cultivar 'Duke' inoculated with *D. eres* (CAA829) (isolated from a previous work, Table 3) at different days post inoculation (Figure S7). Additionally, 20% mortality was observed for cultivar 'Duke' inoculated with *D. eres* (CAA830), *D. amygdali* (CAA958, CAA959), *D. malorum* (CAA975), *D. crousii* (CAA962) and *D. hybrida* (CAA998). Moreover, 25% mortality was observed also in 'Duke', after inoculation with *D. hybrida* (CAA998). The cultivar 'Legacy' showed 20% mortality after inoculations with *D. hybrida* (CAA998) and *D. amygdali* (CAA958) (Figure S8). Isolates CAA962 (*D. crousii*), CAA958 (*D. amygdali*) and CAA830 (*D. eres*) were the only ones to cause 20% mortality in the cultivar 'Spartan' (Figure S9). Besides mortality, all these plants showed symptoms such as wilting and dead leaves, dieback, extensive discoloration of the outer epidermis, necrosis of the vascular tissues, twig blight and dead twigs (Figure 4). The remaining plants that survived did not exhibit any of these symptoms, apart from the development of necrotic lesions around the inoculation point at the end of the experiment.

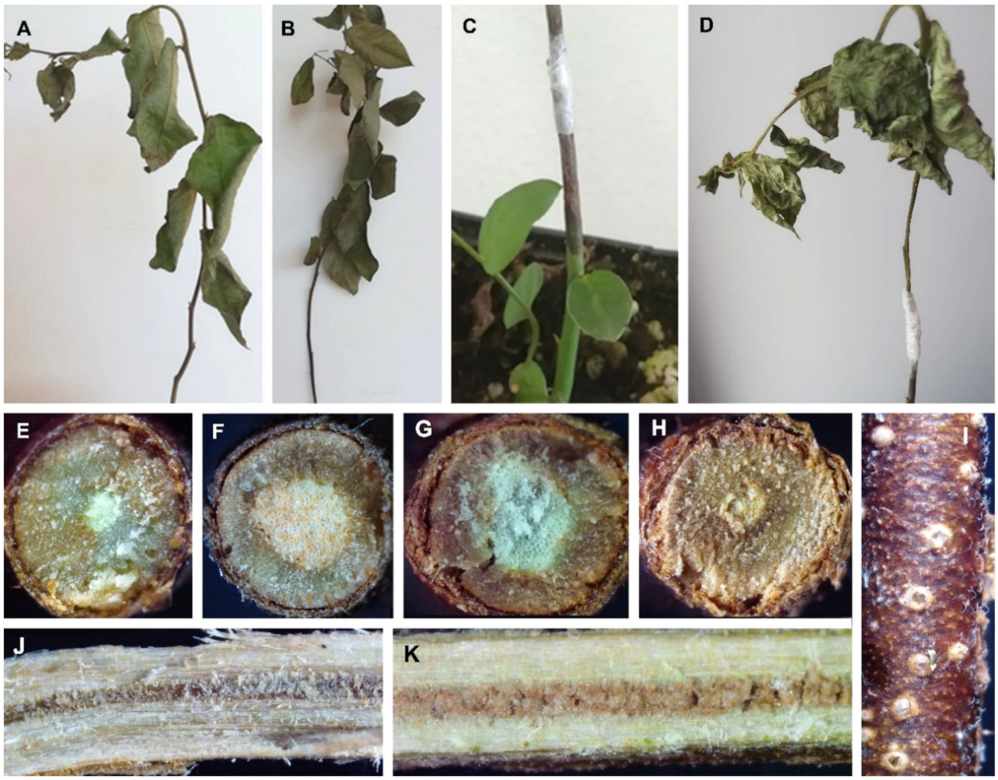


**Figure 4.** Representative symptoms of twig dieback and lesions induced by inoculation of wounded blueberry plants (cv. 'Duke', 'Spartan' and 'Legacy'). (**A**)—Death plant (cv. 'Duke') induced by CAA829. (**B**)—Death plant (cv. 'Legacy') induced by CAA958. (**C**)—Stem lesion developing from the inoculation site (cv. 'Spartan') caused by CAA958. (**D**)—Death plant (cv. 'Duke') induced by CAA999. (**E**–**H**)—Cross section of blueberry twigs showing discoloration of the vascular tissues well into the xylem, after infection by CAA830 (cv. 'Duke'), CAA975 (cv. 'Duke'), CAA998 (cv. 'Legacy'), CAA958 (cv. 'Duke') respectively. (**I**)—Pycnidia development of CAA975 on an inoculated twig. (**J**,**K**)—Necrotic internal tissues of twigs inoculated with CAA829 (cv. 'Duke') and CAA998 (cv. 'Legacy').

#### 3.4.2. Lesion Length Measurements

Lesion lengths were measured 35 days after the inoculation. The outer bark was carefully removed, and the internal lesions which correspond to the necrotic woody tissues were measured. A Shapiro-Wilk test for normality showed that the data differed significantly from a normal distribution for all three cultivars tested ($W = 0.76$, $p < 0.05$ for 'Duke'; $W = 0.65$, $p < 0.05$ for 'Legacy'; $W = 0.78$, $p < 0.05$ for 'Spartan'). Moreover, the homogeneity of variances, another assumption of linear tests (Bartlett's test) did not meet a normal distribution with $p < 0.05$ for all cultivars. Thus, the Kruskal-Wallis test was applied, indicating that there are statistical differences among isolates from all three cultivars tested, with $p < 0.05$. A Dunn's Test was used to compare all isolates, while the different species were compared with one another, using the Wilcoxon rank test.

All *Diaporthe* isolates inoculated on nine-month-year old plants of blueberry cultivars 'Duke', 'Legacy' and 'Spartan', caused necrotic lesions. Differences in aggressiveness were observed among isolates from the same species (Figure 5), and the differences varied according to the cultivar inoculated. For instance, in the cultivar 'Duke', isolate CAA975 (*D. malorum*) caused significantly larger lesions ($5.9 \pm 2.7$ cm) than isolate CAA972 ($2.4 \pm 0.6$ cm, $p = 0.0241$). Moreover, in the cultivar 'Legacy', a homogeneity in the lesion lengths was observed. Nevertheless, a statistically significant difference in the lesion length was observed between isolates of *D. foeniculina*: CAA967 ($2.0 \pm 0.5$ cm) and CAA963 ($1.2 \pm 0.4$ cm, $p = 0.0273$). Moreover, in the cultivar 'Spartan', a statistically significant lesion size was also observed between CAA967 (*D. foeniculina*) ($2.4 \pm 0.3$ cm) and CAA963 (*D. foeniculina*)

(1.4 ± 0.5 cm, $p$ = 0.0181). It is interesting to note, for instance, that lesions in cultivar 'Spartan', caused by isolate CAA998 (*D. hybrida*) (3.0 ± 1.5 cm) are significantly larger than those caused by its parental species *D. phillipsii* (CAA818) (1.5 ± 0.3 cm, $p$ = 0.0149). A similar trend was verified in the cultivar 'Duke': the isolate CAA998 (5.0 ± 3.0 cm) caused lesions significantly larger than those caused by its parental isolates CAA818 (1.9 ± 0.6 cm, $p$ = 0.0087) and CAA817 (1.8 ± 0.4 cm, $p$ = 0.0049).

When analysing the different *Diaporthe* species, differences in mean lesion length were observed (Figure S6). For instance, *D. eres* (isolates CAA829 and CAA830) was the most aggressive species in cultivar 'Duke', which caused the death of all replicates and with significantly larger internal necrotic lesions than any of the other species tested (5.9 ± 1.4 cm), except those of *D. hybrida* (3.5 ± 1.6 cm, $p$ = 0.0554). Moreover, there were no statistical differences in the cultivars 'Legacy' and 'Spartan' among lesions caused by strains CBS 160.32, CAA829 and CAA830 (*D. eres*). In general, the cultivar 'Duke' showed larger internal lesions than 'Spartan' and 'Legacy'. The cultivar 'Duke' also presented a great variability between isolates (Figure 5). Overall, the species *D. eres* and *D. amygdali* induced the longest lesion lengths in all cultivars tested. Contrarily, the smallest lesion size was observed in *D. foeniculina* and *D. leucospermi* (Figure S6). At the end of the experiment, no lesions were seen in control plants. All the fungal species inoculated were re-isolated, thus verifying Koch's postulates.

### *3.5. Taxonomy*

In this section, descriptions and illustrations of the species isolated from *Vaccinium corymbosum* plants are provided. Additionally, synonymous names for a few *Diaporthe* species are proposed, which are followed by a brief note based on phylogenetic analyses and morphological characters.

*Diaporthe ambigua* Nitschke, Pyrenomycetes Germanici: 311 (1867). Mycobank 193681 (Figure 6).

*Specimen examined*: Portugal, Santarém, Ourém, from dead stems of *Vaccinium corymbosum*, July 2019, *S. Hilário*, living culture: CAA957.

*Sexual morph*: Perithecial ascomata globose, aggregated, emerging through fennel stems. Perithecial necks black, subcylindrical, tapering towards the apex. Asci unitunicate, cylindrical to clavate, 8-spored, (48.7−)49.5−53.6(−54.7) × (6.9−)7.1−8.1(−8.2) µm, (mean ± S.D. = 51.5 ± 2.4 × 7.6 ± 0.6 µm, n = 10). Ascospores hyaline, smooth, fusoid–ellipsoidal, septate, tapering towards both ends, frequently tetraguttulate, (9.5−)10.9−11.5(−12.8) × (2.9−)3.3−3.5(−3.9) µm, (mean ± S.D. = 11.2 ± 0.9 × 3.4 ± 0.2 µm, n = 50). *Asexual morph*: Conidiomata brown, erumpent from fennel stems, extruding white conidial mass. Conidiophores subcylindrical, septate at the base, frequently unbranched, (11.8−)16.2−21.4(−24.4) × (1.2−)1.4−1.8(−2.3) µm, (mean ± S.D. = 18.8 ± 4.2 × 1.6 ± 0.3 µm, n = 30). Paraphyses not observed. Alpha conidia ellipsoidal, biguttulate, with an obtuse apex and rounded at the other end, (4.6−)6.0−6.3(−7.3) × (1.9−)2.4−2.6(−3.1) µm, (mean ± S.D. = 6.1 ± 0.6 × 2.5 ± 0.3 µm, n = 100). Beta and gamma conidia not observed.

*Sexual strategy*: homothallism.

*Culture characteristics*: on PDA at 25 °C after 7 days, mycelium flat, spreading with sparse, white to dirty white dense aerial mycelium and with solid patches of olivaceous black; reverse with brownish orange patches in the central part, surrounded by orpiment to buff orange cream areas.

*Host range*: *Aspalathus linearis*, *Malus domestica*, *Malus sylvestris*, *Prunus salicina*, *Prunus* sp., *Pyrus communis*, *Vitis vinifera*, *Foeniculum vulgare* and *Vaccinium* sp. [50].

*Known distribution*: China, Cuba, Germany, Netherlands, South Africa, United Kingdom, United States, Chile, and Portugal [50].

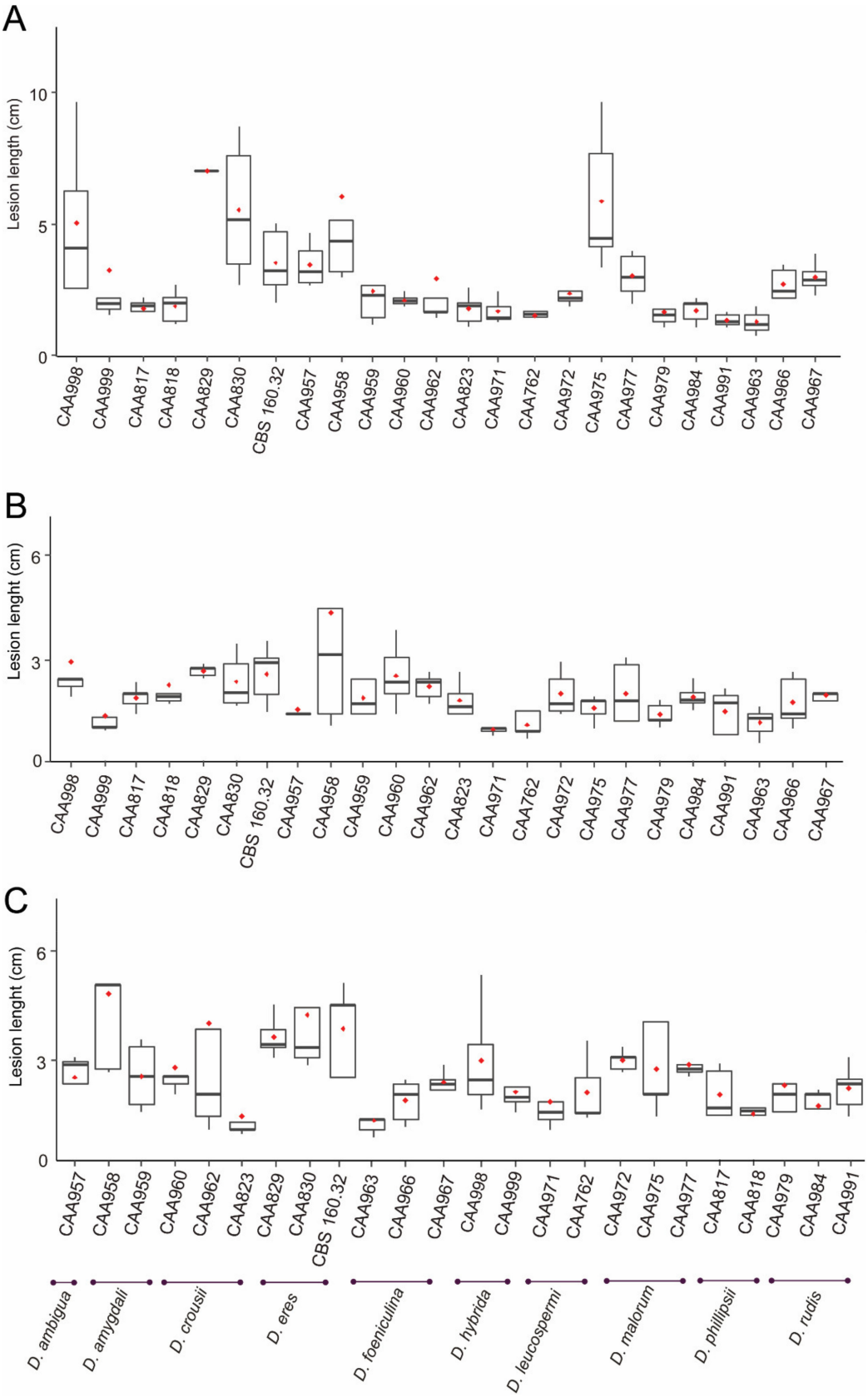


**Figure 5.** Box plot of lesion length (cm) caused by isolates of *Diaporthe* on cultivars 'Duke' (**A**), 'Legacy' (**B**) and 'Spartan' (**C**). Medians and means are represented in black lines and red dots, respectively.

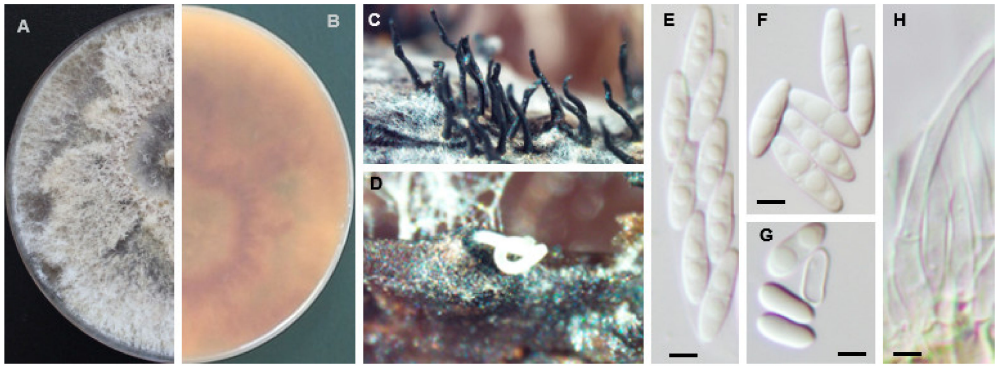


**Figure 6.** Morphology of *Diaporthe ambigua*. (**A**,**B**)—upper and reverse culture surface, respectively. (**C**)—Perithecial necks emerging from fennel twigs. (**D**)—Pycnidia oozing white conidial mass. (**E**)—8-spored ascus. (**F**)—Ascospores. (**G**)—Alpha conidia. (**H**)—Conidiophores. Bars = 2.5 µm.

*Notes*: *Diaporthe ambigua* was first described on *Pyrus communis* in Germany, and later on apple, pear and plums in South Africa [51]. Elfar et al. [17], reported for the first time the presence of this fungus on blueberry plants. This study represents the first report of this pathogen on blueberries in Portugal and Europe.

*Diaporthe amygdali* (Delacr.) Udayanga, Crous & K.D. Hyde, Fungal Diversity 56: 166 (2012). MycoBank MB 800722.

*Specimen examined*: Portugal, Aveiro, Arouca, Chave, from diseased twigs of *Vaccinium corymbosum*, 1 April 2019, *P. Pinho*, living culture CAA958. Description & Illustration [52].

*Notes*: The clade containing the strain CAA958 comprises the ex-type culture of *D. amygdali* isolated from *Prunus dulcis*, and other synonyms such us *D. sterilis* from *V. corymbosum*, *D. kadsurae* from *Kadsura longipedunculata* and *D. mediterranea* from *P. dulcis*. Our isolates clustered with a taxon isolated from *Vitis vinifera* in South Africa. As previously discussed by Hilário et al. [52], this clade represents a single species, *D. amygdali*.

*Diaporthe crousii* S. Hilário, L. Santos & A. Alves, Mycologia 55: 207 (2020). MycoBank MB831439 (Figure 7).

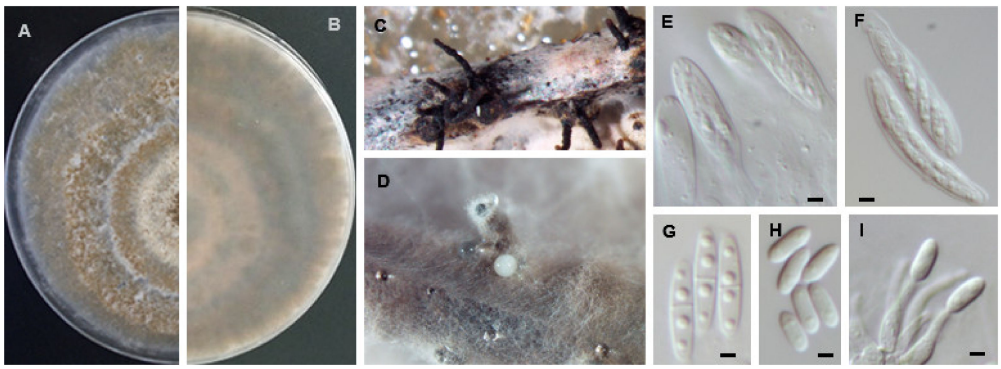


**Figure 7.** Morphology of *Diaporthe crousii*. (**A**,**B**)—upper and reverse culture surface, respectively. (**C**)—Perithecial ascomata and necks emerging through pine needles. (**D**)—White translucid cirri. (**E**)—Immature asci. (**F**)—Asci. (**G**)—Ascospores. (**H**)—Alpha conidia. (**I**)—Conidiophores with alpha conidia at their tips. Bars = 2.5 µm.

*Specimen examined*: Portugal, Porto, Penafiel, from dead stems of *Vaccinium corymbosum*, May 2019, *H. Moreira*, living culture: CAA962.

*Sexual morph*: Perithecial ascomata globose, solitary or in clusters emerging trough pine needles; brown ascomata necks, tapering towards the apex. Asci 8-spored, unitunicate, clavate to subclavate, straight to slightly curved, (31.0−)34.2−36.8(−41.5) × (4.8−)6.0−6.5(−7.7) µm, (mean ± S.D. = 35.5 ± 3.0 × 6.2 ± 0.7 µm, n = 30). Ascospores hyaline, smooth, septate, frequently tetra-guttulate, ellipsoidal, straight, (10.5−)11.0−12.5(−13.3) × (2.3−)2.5−3.3(−3.7) µm,

(mean ± S.D. = 11.7 ± 1.0 × 2.9 ± 0.6 µm, n = 50). *Asexual morph*: Conidiomata brown to black, extruding white conidial mass. Conidiophores hyaline, aggregated, reduced to conidiogenous cells (10.5−)12.8−14.4(−19.0) × (1.3−)1.8−2.2(−2.8) µm, (mean ± S.D. = 13.6 ± 1.9 × 2.0 ± 0.4 µm, n = 30). Paraphyses not observed. Alpha conidia hyaline, aseptate, ellipsoid, rounded apex and obtuse to truncate base, rarely biguttulate, (4.6−)5.5−5.7(−6.2) × (1.7−)2.0−2.2(–2.8) µm, (mean ± S.D. = 5.6 ± 0.4 × 2.1 ± 0.2 µm, n = 100). Beta conidia and gamma conidia not observed.

*Sexual strategy*: homothallism.

*Culture characteristics*: Colonies on PDA covering a Petri dish after 7 days at 25 °C, spreading with brown to grey sparse aerial mycelium, with reverse greenish to brownish concentric zone.

*Host range*: *Vaccinium corymbosum*, *Eucalyptus globulus* [53].

*Known distribution*: Portugal.

*Notes*: *Diaporthe crousii* was first described as the causal agent of twig canker of blueberries (*Vaccinium corymbosum*) and associated to *Eucalyptus globulus*.

*Diaporthe foeniculina* D. Udayanga & L. Castlebury, comb. nov. Persoonia 32: 95 (2014). MycoBank MB803929 (Figure 8).

= *Diaporthe baccae* L. Lombard, G. Polizzi & P. Crous, Phytopathologia Mediterranea 53: 295 (2014). MycoBank MB807599.

= *Diaporthe ravennica* Thambugala, Camporesi & K.D. Hyde, Fungal Diversity 82: 296 (2016). MycoBank MB552100.

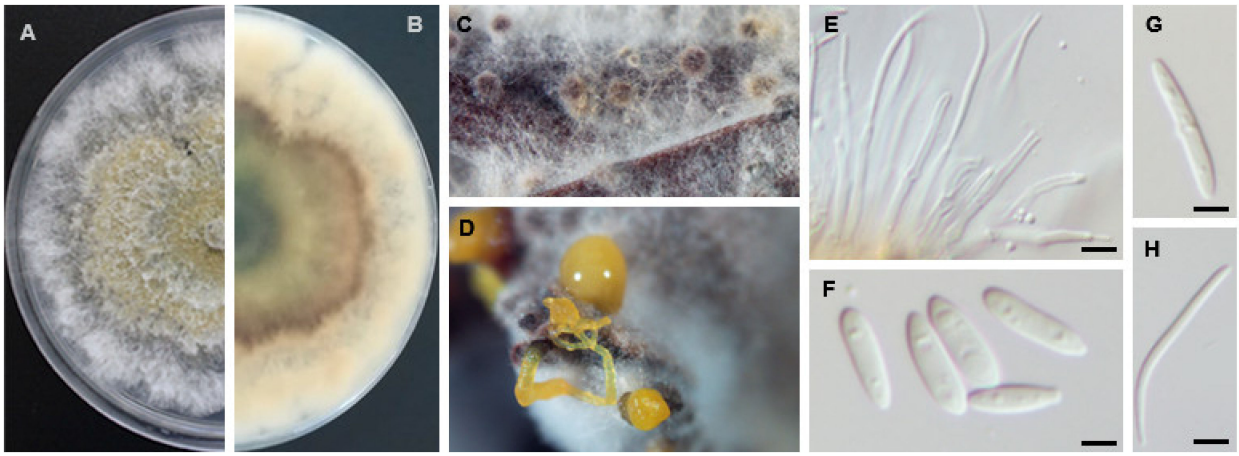


**Figure 8.** Morphology of *Diaporthe foeniculina*. (**A**,**B**)—upper and reverse culture surface, respectively. (**C**)—Conidiomata on pine needles. (**D**)—Pycnidia oozing orange cirri. (**E**)—Conidiophores with beta conidia at their tips. (**F**)—Alpha conidia. (**G**)—Gamma conidia. (**H**)—Beta conidia. Bars = 2.5 µm.

*Specimen examined*: Portugal, Viseu, Mangualde, from twig blight of *Vaccinium corymbosum*, August 2019, D. Lopes, living culture: CAA967.

*Host range*: *Acacia* sp., *Achillea millefolium*, *Ailanthus altissima*, *Arctium minus*, *Asparagus* sp., *Camelia sinensis*, *Castanea sativa*, *Citrus aurantiifolia*, *C. aurantiifolia-limon*, *C. bergamia*, *C. japonica*, *C. latifolia*, *C. limon*, *C. lamonia*, *C. maxima*, *C. medica*, *C. mitis*, *C. paradasi*, *C. paradisi-trifoliata*, *C. reticulata*, *C. sinensis*, *C. sinensis-trifoliata*, *Corylus avellana*, *Cupressus sempervirens*, *Diospyros kaki*, *Ficus benjamina*, *F. carica*, *Foeniculum vulgare*, *Fuchsia excorticata*, *Glycine max*, *Hemerocallis fulva*, *Juglans regia*, *Lunaria rediviva*, *Malus domestica*, *Melilotus officinalis*, *Microcitrus australasica*, *Paraserianthes lophantha*, *Persea americana*, *Prunus amygdalus*, *P. avium*, *Pyrus communis*, *P. pyrifolia*, *Rhus pendulina*, *Ribes nigrum*, *Rosa canina*, *Salix* sp., *Salvia* sp., *Tamarix* sp., *Vaccinium corymbosum*, *Vicia* sp., *Vitis vinifera* and *Wisteria sinensis* [50].

*Known distribution*—Chile, Croatia, France, Germany, Greece, Iran, Italy, Malta, New Zealand, Portugal, Serbia, South Africa, Spain, Turkey, Uruguay, USA [50].

*Asexual morph*: Pycnidial conidiomata, black, erumpent, aggregated or solitary, covered in white mycelium, with yellowish drop-like conidial cirrus oozing from ostiole. Conidiophores hyaline, rarely unbranched, cylindrical, straight to sinuous, reduced to conidiogenous cells, (10.5−)12.0−13.9(−15.0) × (1.4−)1.8−2.4(−3.0) µm, (mean ± S.D. = 12.9 ± 1.5 × 2.1 ± 0.5 µm, n = 30). Paraphyses not observed. Alpha conidia aseptate, hyaline, smooth, ellipsoidal, frequently with two, rounded apex and rarely with subtruncate base (6.6−)7.6−8.2(−9.6) × (1.8−)2.3−2.5(−3.0) µm, (mean ± S.D. = 7.9 ± 0.7 × 2.4 ± 0.3 µm, n = 100). Beta conidia hyaline, aseptate, eguttulate, slightly curved, abundant, apex acute, base subtruncate, (20.5−)21.5−23.4(−26.9) × (0.9−)1.2−1.4(−1.8) µm, (mean ± S.D. = 22.4 ± 1.9 × 1.3 ± 0.3 µm, n = 100). Gamma conidia rare, aseptate, hyaline, rounded apex, (11.9−)11.6−13.0(−13.1) × (1.6−)1.7−1.9(−2.0) µm, (mean ± S.D. = 12.3 ± 0.7 × 1.8 ± 0.2 µm, n = 4). *Sexual morph*: not observed.

*Sexual strategy*: heterothallism.

*Culture characteristics*: colonies on PDA at 25 °C after 7 days spreading moderate with aerial mycelium, feathery margins, pale brown zones in a radial pattern and reverse with greenish yellow pigmentation developing in the centre.

*Notes*: Phillips & Santos [29] described *Diaporthe neotheicola* in Portugal from *Foeniculum vulgare*. Later, Udayanga & Castlebury [54] synonymized *D. neotheicola* as *D. foeniculina* and that is the name currently accepted. Posterior to that, Thambugala, Camporesi & Hyde [55] described *D. ravennica* as a distinct species phylogenetically distinct from *D. baccae* and *D. foeniculina*. Here we show that *D. foeniculina* and *D. baccae* cluster in a well-supported clade and are closely related. A pairwise comparison showed that *D. foeniculina* and *D. baccae* are similar, since both species differ in 7 nt in ITS (*p*-distance = 0.012), 2 nt of *his3* (*p*-distance = 0.004) and 1 in *cal* (*p*-distance = 0.002). *Tef1-α* and *tub2* sequences from both *D. foeniculina* and *D. baccae* are 100% identical.

Moreover, in the ITS initial tree performed, we noted that *D. ravennica* was very closely related to *D. foeniculina* and *D. baccae*. Due to the absence of *cal* and *his3* sequences, we could not include the species in our phylogenetic analysis. However, we noted that *D. ravennica* is 100% identical to *D. baccae* and *D. foeniculina* based on *tef1-α* and *tub2* loci, and 9 nt (*p*-distance = 0.015) and 3 nt (*p*-distance = 0.005) in the ITS locus separate *D. ravennica* from *D. foeniculina* and *D. baccae* respectively. Morphologically, these species are virtually indistinguishable with overlapping micromorphological characters: yellow to pale luteous conidial cirrhus; alpha conidia with base subtruncate, with none, two or many guttules; beta conidia curved, base subtruncate, acute apex and with dimensions matching within the same ranges.

*Diaporthe leucospermi* P. Crous & B. Summerell, Persoonia 27: 32 (2011). MycoBank MB560561 (Figure 9).

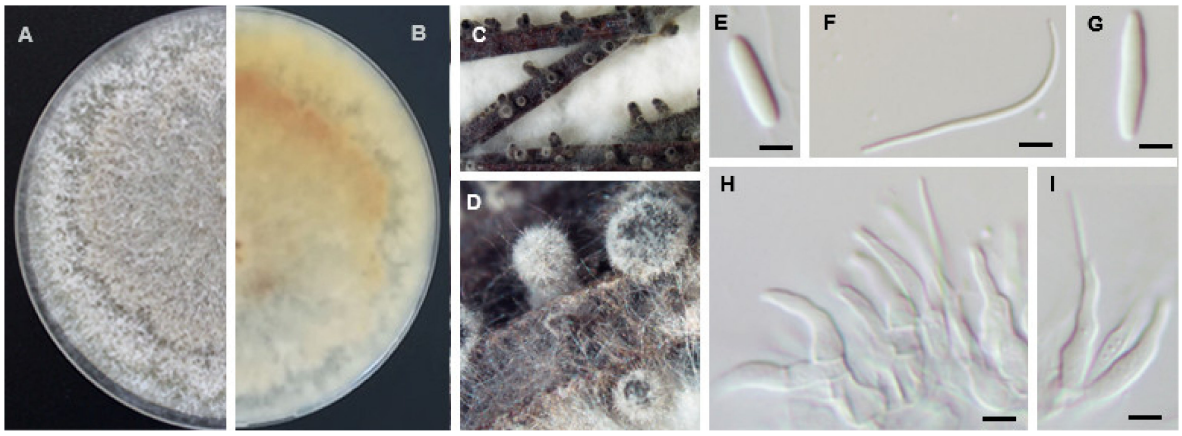


**Figure 9.** Morphology of *Diaporthe leucospermi*. (**A**,**B**)—upper and reverse culture surface, respectively. (**C**,**D**)—Conidiomata on pine needles. (**E**)—Alpha conidia. (**F**)—Beta conidia. (**G**)—Gamma conidia. (**H**)—Conidiophores arising from pseudoparenchyma. (**I**)—Conidiophores with beta conidia at their tip. Bars = 2.5 µm.

= *Diaporthe pyracanthae* L. Santos & A. Alves, Mycosphere 8: 489 (2017). MycoBank MB820224.

= *Diaporthe rossmaniae* S. Hilário, I. Amaral, L. Santos & A. Alves, Mycologia 55: 207 (2020). MycoBank MB831452.

*Specimen examined*: Portugal, Porto, Agrela, from a dead twig of *Vaccinium corymbosum*, July 2019, *S. Hilário*, living culture: CAA971.

*Host range*: *Acer negundo*, *Chamaerops humilis*, *Hydrangea macrophylla*, *Leucospermum* sp., *Pyracantha coccinea*, *Vaccinium corymbosum* [50].

*Known distribution*: Australia and Portugal [50].

*Asexual morph*: Pycnidial conidiomata, brown to black, embedded on fennel stems and pine needles, aggregated or solitary, covered in white mycelium, oozing white translucid conidial cirrhus. Conidiophores subcylindrical, aseptate, branched, reduced to conidiogenous cells, sometimes bearing beta conidia at their tips (9.9−)10.8−14.1(−15.2) × (1.5−)1.6−2.6(−3.1) µm, (mean ± S.D. = 12.5 ± 2.1 × 2.1 ± 0.6 µm, n = 30). Paraphyses not observed. Alpha conidia hyaline, ellipsoid, found infrequent, eguttulate, rounded apex and obtuse base, (4.4−)4.9−5.8(−5.9) × (1.7−)1.9−2.6(−2.3) µm, (mean ± S.D. = 5.4 ± 0.6 × 2.1 ± 0.2 µm, n = 10). Beta conidia hyaline, aseptate, smooth, filiform, hooked in apical part, apex acute, base truncate, (22.5−)26.1−28.5(−31.8) × (1.0−)1.1−1.3(−1.5) µm, (mean ± S.D. = 27.3 ± 2.9 × 1.2 ± 0.2 µm, n = 100). Gamma conidia infrequent, aseptate, hyaline, rounded apex, (8.1−)7.7−9.1(−8.8) × (1.8−)1.9−2.0(−2.1) µm, (mean ± S.D. = 8.4 ± 0.5 × 2.9 ± 0.1 µm, n = 4). *Sexual morph*: not observed

*Sexual strategy*: heterothallism.

*Culture characteristics*: colonies on PDA at 25 °C after 7 days spreading large with moderate aerial mycelium, pale olivaceous-grey to smoke-grey with patches of olivaceous black near the margins; reverse straw yellow with gallstone yellow zones.

*Notes*: Crous & Summerell [56] described *Diaporthe leucospermi* on leaves of *Leucospermum* sp. in Australia. Santos et al. [57] described *D. pyracanthae* from *Malus domestica* in Portugal as a distinct species based on multi-gene phylogenetic analyses and morphological characters. These authors have shown that despite the similarities of conidial dimensions between both species, they differ in several nucleotide positions: three nucleotides in ITS, 1 nt in *tef1-α*, 8 nt in *tub2* and 2 nt in *his3*. Later, Hilário et al. [8] described *D. rossmaniae* as a distinct but closely related species to *D. pyracanthae* and *D. leucospermi*. The authors have shown that although culture characteristics are slightly different, alpha and beta conidia dimensions are within the same ranges. Moreover, *D. rossmaniae* differs from *D. leucospermi* in 3 nt in ITS, 3 in *tef1-α*, 2 nt in *his3* and 1 in *tub2* and from *D. pyracanthae* in five nucleotides in *tub2* and 2 in *tef1-α* [8]. However, we show in this study that *D. rossmaniae* is phylogenetically indistinguishable from *D. pyracanthae* and *D. leucospermi*.

*Diaporthe malorum* L. Santos & A. Alves, Mycosphere 8: 489 (2017). MycoBank MB820226.

*Specimen examined*: Portugal, Aveiro, Arouca, on twigs of *Vaccinium corymbosum*, January 2020, *S. Hilário*, living culture: CAA972, CAA974. Description & Illustration [58].

*Host range*: *Malus domestica* [57], *Eucalyptus globulus* [53], *Vaccinium corymbosum.*

Known distribution: Portugal.

*Notes*: The clade comprising *Diaporthe malorum* firstly described as the causal agent of twig canker and shoot blight of apples (*Malus domestica*), also contains isolates from *E. globulus* and *V. corymbosum*.

*Diaporthe rudis* (Fr.) Nitschke, Pyrenomycetes Germanici 2: 282 (1870). (Figure 10).

*Specimen examined*: Portugal, Porto, Arcozelo, from twig blight symptoms of *Vaccinium corymbosum*, July 2019, *S. Hilário*, living culture: CAA991.

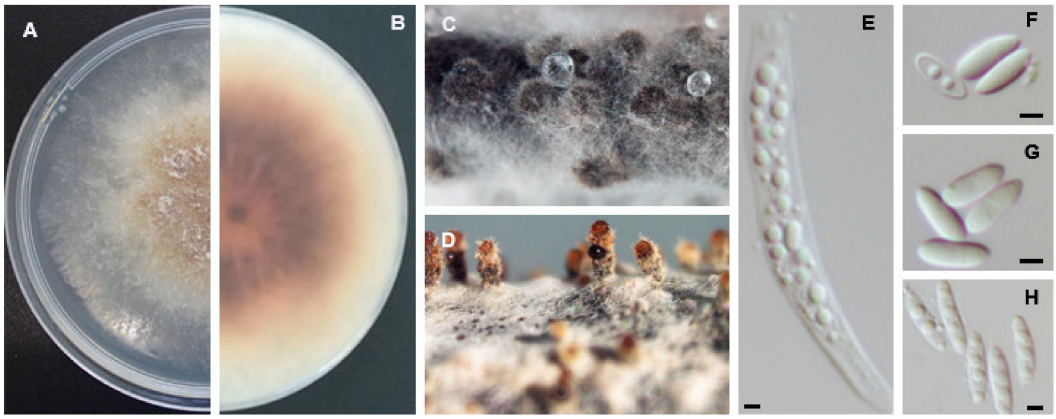


**Figure 10.** Morphology of *Diaporthe rudis*. (**A**,**B**)—upper and reverse culture surface, respectively. (**C**)—Conidiomata on pine needles. (**D**)—Perithecial ascomata on fennel twigs. (**E**)—Ascus. (**F**,**G**)—Alpha conidia. (**H**)—Ascospores. Bars = 2.5 µm.

*Sexual morph*: Perithecial ascomata globose, solitary or in clusters. Perithecial necks brown to black, covered in white mycelium. Asci unitunicate elongate, 8-spored, (48.0−)49.2−53.5(−57.6) × (5.8−)6.0−6.6(−6.9) µm, (mean ± S.D. = 51.3 ± 3.1 × 6.3 ± 0.4 µm, n = 10). Ascospores hyaline, elongated, septate, frequently tetra-guttulate with larger guttules at center, (9.7−)11.4−11.9(−13.2) × (2.1−)2.7−2.9(−3.6) µm, (mean ± S.D. = 11.6 ± 0.8 × 2.8 ± 0.3 µm, n = 50). *Asexual morph*: Conidiomata brown, erumpent from fennel stems, extruding white conidial mass. Conidiophores not observed. Paraphyses not observed. Alpha conidia ellipsoidal, biguttulate, with an obtuse apex and rounded at the other end, (5.4−)6.1−6.3(−7.0) × (2.1−)2.5−2.6(−2.9) µm, (mean ± S.D. = 6.2 ± 0.3 × 2.5 ± 0.2 µm, n = 100). Beta and gamma conidia not observed.

*Sexual strategy*: homothallism.

*Culture characteristics*: In dark at 25 °C for seven days, colonies on PDA relatively slow growing, white to pale brown zones developing in centre, aerial mycelium, reverse with reddish concentric zones.

*Host range*: *Acer* sp., *Asphodelus albus*, *Aucuba japonica*, *Brugmansia* sp., *Castanea* sp., *Corylus* sp., *Dipsacus fullonum*, *Epilobium* sp., *Eucalyptus globulus*, *Fagus* sp., *Fraxinus* sp., *Holcus* sp., *Hydrangea* sp., *Ileostylis* sp., *Laburnum* sp., *Lupinus* sp., *Malus* sp., *Protea* sp., *Pyrus* sp., *Rosa* sp., *Sambucus* sp., *Salix* sp., *Vaccinium corymbosum* and *Vitis vinifera* [50].

*Known distribution*: Australia, Canada, Chile, Austria, Germany, Italy, Latvia, Portugal, Spain, Sweden, Switzerland, New Zealand and South Africa [50].

*Notes*: *Diaporthe rudis*, commonly associated to *Vitis vinifera*, it has been reported on a wide range of hosts including blueberries. Compared with the description of Udayanga et al. [54], our isolate CAA991 has shorter and thinner ascospores (11.6 ± 0.8 × 2.8 ± 0.3 µm vs 13.2 ± 1.1 × 3.6 ± 0.1 µm), and longer conidiogenous cells. Besides that, beta conidia of isolate CAA991 were not found, whereas it was reported in the ex-type CBS 113201.

*Diaporthe hybrida* S. Hilário & A. Alves, Fungal Biology (in press) 2021. MycoBank: MB838688.

*Specimen examined*: Portugal, Santarém, Ourém, on twigs of *Vaccinium corymbosum*, January 2020, *S. Hilário*, living culture: CAA998 (MUM 21.01). Description & Illustration [58].

*Notes*: *Diaporthe hybrida* was the first hybrid described in this genus [58], from cross between *D. portugallica* [59] and *D. phillipsii* [8].

## 4. Discussion

Although a previous study identified a few species of *Diaporthe* as causal agents of twig blight and dieback diseases in the major blueberry production in central Portugal [8], the current study represents the first national survey to evaluate the distribution of *Diaporthe* on several blueberry cultivars. Additionally, this study also gives an insight into the pathogenicity potential of *Diaporthe* species, including the well-known pathogen *D. vaccinii*, to some blueberry cultivars planted in Portugal.

*Diaporthe foeniculina* (formerly known as *Phomopsis theicola* and *D. neotheicola*) was first described on *Camellia sinensis* in Italy in 1927 [29], and since then it has been isolated from several hosts such as *Vitis vinifera*, *Protea* sp., *Pyrus* sp., *Citrus* sp. [13,60,61], *Aspalathus linearis* [62], *Foeniculum vulgare* [29] and *Prunus* sp. [63]. Later, Elfar et al. [17] reported the presence of *D. foeniculina* in Chile for the first time, noting that it caused stem cankers on blueberries. Hilário et al. [8] also found *D. foeniculina* occurring as endophyte or latent pathogen in asymptomatic branches and from dead plant material co-existing with *D. rudis*. The pathogenicity testes carried out by Elfar et al. [17] and Hilário et al. [8] showed that although causing cankers, it is reasonable to consider *D. foeniculina* as a weak pathogen on *Vaccinium corymbosum* plants.

*Diaporthe rudis*, (referred to as *D. viticola*) is a well-known pathogen in Europe and New Zealand mostly associated to *Vitis vinifera* [11,54]. This fungus has also been found on a wide range of hosts such as *Aucuba japonica*, *Fraxinus excelsior*, *Citrus* sp., *Acer* sp., and *Castanea sativa* and distributed worldwide [13]. Despite the presence of *D. rudis* on blueberry plants in the Netherlands [2], it is unclear whether the species was obtained from diseased or healthy plants or if pathogenicity tests were performed. On the contrary, Cardinaals et al. [7] also isolated *D. rudis* in the Netherlands from blueberries with shoot blight, whereas Hilário et al. [8] isolated this species from diseased and asymptomatic plants. The present study records, for the second time, the occurrence of *D. rudis* on *V. corymbosum* in Portugal. Although it was the most dominant species found (38.8%), it may be claimed that the presence of *D. rudis* on blueberry just occurred as a result of high inoculum pressure, given that most of the blueberry plantations were located close to vineyards. Moreover, a recent study demonstrated that *D. rudis* caused minor symptoms on blueberry plants after artificial inoculations [8]. Taking all of this into consideration, this raises questions about the real impact of this species on blueberries, suggesting that *D. rudis* may be less prone to be a pathogen on blueberries.

*Diaporthe ambigua* was firstly described on *Pyrus communis* in Germany, and later on apple, pear and plums in South Africa [51]; In 2009, this species was isolated from *Foeniculum vulgare* in Portugal [29]; on grapevines in California and on blueberry plants in Chile around 2013 [17,64], and most recently, in 2019, *D. amygdali* was isolated from kiwi fruits in Greece [65]. Although *D. ambigua* has been claimed to be virulent in shoots, stems, and fruit of blueberries [17] the records of this pathogen on blueberries are limited and unclear. In the present study, *D. ambigua* was recovered from one orchard only. Moreover, in a recent study, Spies et al. [66] investigated twigs of 145 European olive trees in South Africa showing dieback and found that *D. ambigua* was only recovered from a single European olive tree, and that its pathogenicity to this host was unknown. This occasional occurrence of *D. ambigua* on olives in South Africa and blueberries in Portugal, allied to the fact that this species has been found on blueberry dead stems, raises the possibility of a saprophytic behaviour of *D. ambigua*. Such a conclusion was previously drawn by Santos & Phillips [29], who proposed that *D. ambigua* may be purely saprophytic, taking advantage of dead plant material to grow.

*Diaporthe leucospermi* was first described from leaves of *Leucospermum* sp. in Australia in 2012 [51] and, since then, no records of this species have been documented. However, Santos et al. [15] collected the isolates Ph-C189/1, Ph-C180/1, Ph-C174/1 and Di-C007 from *Hydrangea macrophylla* and *Acer negundo* in Portugal. It is worthy to note that based on ITS and *tef1-α* available sequences, these isolates are deemed to fall within the concept of *D. leucospermi*. A pairwise comparison revealed that the Portuguese isolates are 100% identical to *D. leucospermi* in the ITS region, whereas some polymorphisms are shared among these isolates in *tef1-α* locus. Only two nucleotide differences and the presence of a 7 bp insertion (5′ CCCCCCC 3′) in *tef1-α* region of isolates Ph-C189/1 and Di-C007 separate them from *D. leucospermi*, while no polymorphisms were observed among isolates Ph-C180/1 and Ph-C174/1 and *D. leucospermi*. However, further studies including other loci would be required to resolve the identity of the Portuguese isolates mentioned above. Later, Santos et al. [57] and Hilário et al. [8] described *D.*

*pyracanthae* and *D. rossmaniae* as two different species, but they are shown in this study to be indistinguishable from *D. leucospermi*.

The distribution of *Diaporthe malorum* is only known from Portugal, where it was found in *M. domestica* [57] and *E. globulus* [53]. The present study is the first to report the presence of this species in blueberries. *Diaporthe crousii*, firstly described as a pathogen on *V. corymbosum* in Portugal [8], was also found in *E. globulus* [53].

*Diaporthe amygdali* (=*Phomopsis amygdali*), has been reported worldwide on a wide variety of hosts [67]. In a recent study, a monophyletic clade containing *D. amygdali* and closely related species was resolved by applying the principle of Genealogical Concordance Phylogenetic Species Recognition and the coalescent-based species delimitation methods [52]. Apart from the report of *D. sterilis* (syn. *D. amygdali*) causing cankers and twig blight on *Vaccinium corymbosum* in Italy [2], the present study reports for the second time the presence of *D. amygdali* associated with twig blight of blueberry plants.

*Diaporthe hybrida*, described as the first hybrid in the genus *Diaporthe* [58] was also pathogenic to blueberry, but the prevalence of this species in the survey was low, given that its presence was restricted to one plantation only (Ourém). Therefore, the impact of this new hybrid pathogen on blueberry orchards remains unclear.

Pathogenicity trials were carried out using nine-month-old blueberry plants, unveiling the capacity of *Diaporthe* to cause lesions on this crop. All *Diaporthe* species inoculated to blueberry plants cv. 'Duke', 'Legacy' and 'Spartan' were able to cause lesions. The most severe symptoms were found on blueberry twigs inoculated with *D. eres* (CAA829) in the cultivar 'Duke', which caused the death of all replicates. *Diaporthe hybrida* was also found to cause necrosis when inoculated in cultivar Duke. It is noteworthy that this species was able to cause significantly larger lesions than its parental species (*D. phillipsii*) [58], which is corroborated by studies that state that hybrids are thought to be important for the evolution of fungal plant pathogens, increasing their virulence on the host [68]. Given that hybridization plays a crucial role in the spread of pathogenicity traits, further genetic analysis of *D. hybrida* would be essential to establish new insights into the impact of this new virulent pathogen, which may affect blueberry plants. Lombard et al. [2] have shown that *D. sterilis* (syn. *D. amygdali*) caused brown lesions developing on the green stems and twigs, resulting in twig blight on cultivar 'Legacy'. Likewise, in the present study, the isolate CAA958 (*D. amygdali*) not only caused mortality of one out of five replicates in the cultivar 'Legacy', but it also caused the longest lesions in this cultivar, when compared to the other *Diaporthe* species. Overall, *D. amygdali* and *D. eres* showed to be one of the most virulent species, while *D. ambigua*, *D. foeniculina*, *D. rudis* and *D. leucospermi* yielded the smallest lesion lengths and were thus regarded as the least aggressive species.

Although *Diaporthe vaccinii* was earlier considered as a quarantine pathogen for the European Union (EU Regulation 2016/2031), the recent EU Regulation (2019/2072) does not include the species in the current list of EU quarantine pests. However, the impact and pathogenicity potential of *D. vaccinii* was never entirely explored. [18]. It is worthy to note that *D. vaccinii* was not found during our survey across the country, and no records have been reported in Europe in the past few years [69]. Nabetani et al. [23] have shown that *D. vaccinii* might be the major causal agent of twig blight on blueberry plants in British Colombia and found that the cultivar 'Duke' was shown to be more susceptible to *D. vaccinii*. Contrarily, Cardinaals et al. [7] tested *D. vaccinii* on cultivars 'Duke' and 'Liberty' but revealed that the symptoms caused by this pathogen are insignificant, and thus it may not represent a major threat to commercial blueberry productions. Currently, it is known that it has been eradicated from all European countries where it was earlier detected [70]. This can also be a proof that *D. vaccinii* might have been wrongly identified, as also suggested by Lombard et at. [2] and Elfar et al. [17], as its identification was previously based on its *Phomopsis*-like conidia and host association. According to our pathogenicity tests, the cultivar 'Duke' was the most susceptible to *D. eres* infection. Moreover, we also demonstrated that *D. eres* strain CBS 160.32 (syn. *D. vaccinii*) caused similar lesion lengths to *D. eres* strains CAA829 and CAA830, and it was not aggressive, as has been recurrently

described in the literature. Such results corroborate the hypothesis that *D. vaccinii* is a synonym of *D. eres*, and that this species not only harbours intraspecific variability [20] but also differences in virulence among isolates. Furthermore, it is noteworthy that the pathogenicity trials carried out in this study were based solely on one strain of *D. vaccinii* (ex-type CBS 160.32). This strain has been kept in culture for over 90 years, which might have affected its virulence, leading to loss of its pathogenicity. Therefore, this study provides evidence about the capability of *D. eres* to cause disease on *V. corymbosum*, which is more aggressive than the well-known host-specific strain CBS 160.32. As previously suggested by Cardinaals et al. [7] and supported by our results, the strain CBS 160.32 might not represent a threat to blueberry orchards in Europe.

It is also worth mentioning that the *Diaporthe* species identified in this study were found to be co-existing in the same plant material with members of *Botryosphaeriaceae* and *Pestalotiopsis*. Such an occurrence is not new, as it has been previously reported [17,71–73]. This should not be overlooked, and it deserves further investigation, given that the species of *Diaporthe,* together with other pathogenic fungi [74], may contribute to cause dieback and twig blight of blueberries.

## 5. Conclusions

The present study is the first to assess *Diaporthe* species associated with blueberries from several orchards in Portugal, combining morphology and molecular data, which provide useful information to evaluate the pathogenicity potential of several species in different blueberry cultivars. To our knowledge, this study is the first to report of *D. leucospermi*, *D. amygdali* and *D. malorum* associated with blueberries, and of *D. ambigua* on this host in Europe. We have also demonstrated that strain CBS 160.32 (*D. eres*) did not cause the death of any plant inoculated, and that the symptoms caused were minor, when compared to other isolates, indicating that this strain may not be a threat to *Vaccinium* plantations. However, it is important to highlight that *D. amygdali* and with *D. eres* (strains CAA829 and CAA830) are the most aggressive species, indicating the importance of *D. eres* as a pathogen of blueberries. Moreover, pathogenicity studies coupled with morpho-physiological and biochemical parameters will be essential to clarify the pathogenic role and the impact of *Diaporthe* on the physiology of blueberry.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/agriculture11121271/s1, Figures S1–S5: Phylogram generated from Maximum Likelihood analysis based on ITS, *tef1-α*, *tub2*, *cal* and *his3* sequence data from *Diaporthe*, respectively. Figure S6: Box plot of lesion length (cm) caused by *Diaporthe* species. Figures S7–S9: Time course percentage of mortality of 'Duke', 'Legacy' and 'Spartan' cultivars, respectively. Table S1: Cases of *Diaporthe* species found alone or co-existing (+) with other species.

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