

RESEARCH PAPER

## ***Diaporthe* species associated with *Vaccinium*, with specific reference to Europe**

LORENZO LOMBARD<sup>1</sup>, GERARD C.M. VAN LEEUWEN<sup>2</sup>, VLADIMIRO GUARNACCIA<sup>3</sup>, GIANCARLO POLIZZI<sup>3</sup>, PATRICIA C.J. VAN RIJSWICK<sup>2</sup>, KARIN C.H.M. ROSENDAHL<sup>2</sup>, JUTTA GABLER<sup>4</sup> and PEDRO W. CROUS<sup>1,5,6</sup>

<sup>1</sup> CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584CT Utrecht, The Netherlands

<sup>2</sup> Dutch National Plant Protection Organization (NPPO-NL), PO Box 9102, 6700 HC Wageningen, The Netherlands

<sup>3</sup> Dipartimento di Gestione dei Sistemi Agroalimentari e Ambientali Sez. Patologia Vegetale, 95123 Catania, Italy

<sup>4</sup> Institute for Epidemiology and Pathogen Diagnostics of the Julius Kühn-Institute (JKI) – Federal Research Centre of Cultivated Plants, Quedlinburg, Germany

<sup>5</sup> Laboratory of Phytopathology, Wageningen University and Research Centre (WUR), Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

<sup>6</sup> Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

**Summary.** Species of the genus *Vaccinium* are commercially cultivated in Europe for their berries, which are highly valued for dietary and pharmaceutical properties. Cultivation is severely limited due to a range of fungal diseases, especially those caused by species of *Diaporthe*. A number of *Diaporthe* isolates have been collected from *Vaccinium* growing regions in Europe, and initially identified as *D. vaccinii* based on host association. Using DNA sequence inference of the combined  $\beta$ -tubulin, calmodulin, translation elongation factor 1-alpha and the internal transcribed spacer region of the nuclear rDNA, along with morphological characteristics, six species were characterised. *Diaporthe eres*, *D. vaccinii* and *D. viticola* are known species and three novel taxa are described here as *D. asheicola*, *D. baccae* and *D. sterilis*. This study is the first confirmed report of *D. vaccinii* in Latvia and the Netherlands.

**Key words:** *Diaporthe vaccinii*, quarantine.

### **Introduction**

The genus *Vaccinium* contains approximately 450 species of woody, perennial shrubs belonging to the family *Ericaceae* (heath) (Dierking *et al.*, 1993; Nestby *et al.*, 2012). Four *Vaccinium* spp. are regarded as economically important and indigenous to Europe, namely *V. myrtillus* (bilberry), *V. oxycoccus* (cranberry), *V. uliginosum* (bog bilberry) and *V. vitis-idaeae* (lingonberry) (Dierking *et al.*, 1993). *Vaccinium macrocarpon* (large cranberry or American cranberry) and *V. corymbosum* (highbush blueberry), which were introduced from North America, are also commercial-

ly cultivated in Europe (Naumann, 1993; Caruso and Ramsdell, 1995; Prodorutti *et al.*, 2007; Nestby *et al.*, 2012). The berries of these plants are greatly prized in the food industry based on their high content of beneficial nutrients (Nestby *et al.*, 2012), and in the pharmaceutical industry for their bioactive phytochemicals such as naturally occurring antioxidants (Faria *et al.*, 2005). However, commercial cultivation of *Vaccinium* is severely limited due to a range of fungal pathogens (Caruso and Ramsdell, 1995).

With the abolition of dual nomenclature from the International Code of Nomenclature for algae, fungi and plants (ICN), asexual and sexual names of fungi received equal status (Hawksworth *et al.*, 2011; Wingfield *et al.*, 2012), with *Phomopsis* being treated as synonym of *Diaporthe* (Santos *et al.*, 2010; 2011; Crous *et al.*, 2011; Udayanga *et al.*, 2012; Gomes

Corresponding author: L. Lombard  
Fax: +31 30 212 26 00  
E-mail: l.lombard@cbs.knaw.nl

et al., 2013; Kirk et al., 2013). Species of *Diaporthe* are known to cause several diseases of *Vaccinium*, including twig blight, stem cankers and fruit rot (Caruso and Ramsdell, 1995; Farr et al., 2002a,b; Miller et al., 2006; Polashock, 2006; Latorre et al., 2012; Tadych et al., 2012; Elfar et al., 2013). Several *Diaporthe* and two *Phomopsis* spp. have been reported from *Vaccinium* spp., including *D. ambigua* (Elfar et al., 2013), *D. australafricana* (Latorre et al., 2012; Elfar et al., 2013), *D. neotheicola*, *D. passiflorae* (Elfar et al., 2013), and *D. vaccinii* (Caruso and Ramsdell, 1995; Farr et al., 2002a,b; Miller et al., 2006; Polashock, 2006; Tadych et al., 2012), as well as *P. columnaris* and *P. myrtilli* (Farr et al., 2002b). One of these species, *D. vaccinii*, is listed as a quarantine pest for the European Union (European Union 2000). Extensive surveys to collect and identify fungal pathogens of *Vaccinium* spp. have been conducted recently in Chile (Elfar et al., 2013), New Zealand (Miller et al., 2006) and the USA (Tadych et al., 2012). However only a few reports have been made of *Diaporthe* and *Phomopsis* spp. isolated from *Vaccinium* spp. grown in Europe (Petraik, 1924; Wilcox and Falconer, 1961; Baker, 1972; Teodorescu et al., 1985; Farr et al., 2002b; Gabler et al., 2004).

Although *D. vaccinii* is globally regarded as the prominent species of *Diaporthe* on *Vaccinium* spp. (Caruso and Ramsdell, 1995; Farr et al., 2002a), this fungal pathogen has only been reported from Romania (Teodorescu et al., 1985), the United Kingdom (Wilcox and Falconer, 1961; Baker, 1972) and Lithuania (Gabler et al., 2004; Kačergius et al., 2004) in Europe, apparently introduced with American *Vaccinium* cultivars being exploited for commercial cultivation. This fungus was, however, unable to establish and spread from both these regions (Teodorescu et al., 1985). Generally, *D. vaccinii* is regarded as indigenous to North America as it has been reported from all *Vaccinium* growing regions in the USA and Canada (Shear et al., 1931; Weingartner and Klos, 1975; Chao and Glawe, 1985; Farr et al., 2002a,b; Tadych et al., 2012).

With the increase in commercial cultivation of *Vaccinium* spp. in Europe, a number of *Diaporthe* isolates have been collected from these regions and maintained in culture collections (Table 1). The majority of these isolates are treated as *D. vaccinii*, based only on host association, as they are similar to *D. phaseolorum*, but differ in host specificity (Wehmeyer, 1933; Chao and Glawe, 1985). The present study therefore aimed to place these European *Diaporthe*

isolates from *Vaccinium* plants in the correct taxonomic context based on DNA sequence inference, supported by morphological and cultural data.

## Materials and methods

### Isolates

Strains of *D. vaccinii* and other *Diaporthe* spp. isolated from *Vaccinium* spp. were obtained from various culture collections, as indicated in Table 1. Several strains were also freshly isolated from symptomatic and asymptomatic *Vaccinium* spp. collected in the Netherlands and Italy (Table 1), as explained by Elfar et al. (2013). Representative strains were deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre (CBS), in Utrecht, the Netherlands, and the working collection of Pedro Crous (CPC) maintained at the CBS.

### DNA phylogeny

Total genomic DNA was extracted from cultures grown on potato dextrose agar (PDA) for 7 d, using UltraClean™ Microbial DNA isolation kits (Mo Bio Laboratories, Inc.) according to the manufacturer's protocol. Partial gene sequences were determined for  $\beta$ -tubulin (BTUB), calmodulin (CAL), translation elongation factor 1-alpha (TEF), and the internal transcribed spacer region (ITS) of the nuclear rDNA using the primers and protocols described by Gomes et al. (2013). Integrity of the sequences was checked by sequencing the amplicons in both directions using the same primer pairs used for amplification. Consensus sequences for loci were assembled in MEGA v. 5.1 (Tamura et al., 2011), then compared and added to representative sequences from Udayanga et al. (2012) and Gomes et al. (2013) (Table 1). Alignments for each locus was generated in MAFFT v. 6 (Katoh and Toh, 2010) and manually corrected where necessary.

The 70% reciprocal bootstrap method of Mason-Gamer and Kellogg (1996) was used to determine whether the four loci sequenced were congruent. Evolutionary models were estimated in MrModeltest (Nylander, 2004) using the Akaike Information Criterion (AIC) for each locus, and implemented in the bootstrap analysis runs in PAUP (Phylogeny Analysis Using Parsimony, v. 4.0b10; Swofford, 2003) for 10,000 replicates. The inferred tree topologies

were visually compared for conflicts between the separate loci.

Phylogenetic analyses were based both on Bayesian inference (BI) and Maximum Likelihood (ML). For both methods, the best evolutionary models for each partition were determined using MrModeltest (Nylander, 2004) and incorporated into the analyses. For the BI analysis, MrBayes v. 3.1.1 (Ronquist and Huelsenbeck, 2003) was used to generate phylogenetic trees under optimal criteria per partition. A Markov Chain Monte Carlo (MCMC) algorithm of four chains was started in parallel from a random tree topology with the heating parameter set at 0.3. The MCMC analysis lasted until the average standard deviation of split frequencies came below 0.01 with trees saved each 1,000 generations. The first 25% of saved trees were discarded as the “burn-in” phase and posterior probabilities (PP) determined from the remaining trees.

The ML analysis was carried out for the same partitions using RAxML software (Stamatakis *et al.*, 2005) through the CIPRES Website (<http://www.phylo.org>), to obtain a second measure of branch support. The robustness of the analysis was evaluated by bootstrap support (BS) with the bootstrap replicates automatically determined by the software (Stamatakis *et al.*, 2008). Novel sequences generated in this study were deposited in GenBank (Table 1), and the alignments and phylogenetic tree were deposited in TreeBASE (S15189).

Sterile species (see species notes) were characterised using unique fixed single nucleotide polymorphisms (SNP's). For each sterile species treated, the closest phylogenetic neighbour(s) were selected and subjected to SNP analyses using DnaSP v.5.00.07 (Librado and Rozas, 2009).

## Taxonomy

Axenic cultures were sub-cultured onto 2% tap water agar supplemented with sterile pine needles (PNA; Smith *et al.*, 1996), PDA, oatmeal agar (OA) and 2% malt extract agar (MEA) (Crous *et al.*, 2009), and incubated at 18–20°C under a 12 h near-ultraviolet light/12 h dark cycle to induce sporulation. Fungal structures were mounted in clear lactic acid, and 30 measurements were made for all structures including the conidia. The 95% confidence levels were determined, and extreme dimensions determined (recorded here in parentheses). Colony characteris-

tics were noted after 10 d of growth on PDA, OA and MEA at 24°C in the dark, and colony colours were determined using the colour charts of Rayner (1970). Descriptions, nomenclature and illustrations were deposited in MycoBank (Crous *et al.*, 2004).

## Results

### Isolates

A total of 38 isolates representing *Diaporthe* spp. isolated from *Vaccinium* spp. were obtained from various culture collections, of which 28 isolates originated from Europe (Table 1). Seventeen of these were obtained from the symptomatic and asymptomatic plant material collected in Italy and the Netherlands (Table 1). Typical symptoms observed on infected *Vaccinium* plants included dark red-brown cankers on the stems with internal discolouration of the vascular tissues beneath these cankers radiating up and down the stems. Red-brown leaf spots were also observed.

### DNA phylogeny

The 70% reciprocal bootstrap tree topologies showed no conflicts for the BTUB, CAL and TEF gene regions. However, the ITS gene region revealed a conflicting tree topology to the other three gene regions included. This was ignored, based on the argument of Cunningham (1997) that combining incongruent partitions could increase phylogenetic accuracy. Therefore, the four gene regions were combined, as was done by Gomes *et al.*, (2013) for the same loci.

The combined alignment of BTUB, CAL, ITS and TEF used for both Bayesian and ML analyses contained 1621 characters from 76 isolates (including outgroup). Of the ingroup isolates, 37 were selected based on comparisons to representative sequences from Udayanga *et al.* (2012) and Gomes *et al.* (2013), and subsequently retrieved from GenBank (Table 1). The numbers of unique site patterns per data partition were 286 from 537 for BTUB, 165 from 334 for CAL, 136 from 474 for ITS and 199 from 276 for TEF which included alignment gaps. Based on the results of MrModeltest all partitions had dirichlet base frequencies and a GTR+I+G model with inverse gamma-distributed rates was used for ITS and HKY+I+G with inverse gamma-distributed rates were implemented for BTUB, CAL and TEF. The Bayesian anal-

**Table 1.** Diaporthe isolates used in this study.

| Species                         | Strain <sup>1</sup>                 | Host                         | Country      | Collector               | Genbank accession numbers <sup>2</sup> |          |          |          |          |
|---------------------------------|-------------------------------------|------------------------------|--------------|-------------------------|--|----------|----------|----------|----------|
|                                 |                                     |                              |              |                         | BTUB                                   | CAL      | ITS      | TEF      | TEF      |
| <i>Diaporthe alleghaniensis</i> | CBS 495.72 = ATCC 24097             | <i>Acer saccharum</i>        | –            | R.H. Arnold             | KC343975                               | KC343249 | KC343007 | KC343733 | KC343733 |
| <i>D. ampelina</i>              | CBS 114016 = CPC 2660 = PV F98-1    | <i>Vitis vinifera</i>        | France       | P. Larignon             | JX275452                               | AY745026 | AF230751 | AY745056 | AY745056 |
| <i>D. amygdali</i>              | CBS 114867 = CPC 4708               | <i>V. vinifera</i>           | Turkey       | M. Erkan                | KC343985                               | KC343259 | KC343017 | KC343743 | KC343743 |
|                                 | CBS 115620 = FAU 1005               | <i>Prunus persica</i>        | USA          | W. Uddin                | KC343988                               | KC343262 | KC343020 | KC343746 | KC343746 |
|                                 | CBS 126679                          | <i>P. dulcis</i>             | Portugal     | E. Diogo                | KC343990                               | KC343264 | KC343022 | KC343748 | KC343748 |
| <i>D. angelicae</i>             | CBS 111591 = AR 3724                | <i>Heracleum sphondylium</i> | Austria      | A.Y. Rossman            | KC343994                               | KC343268 | KC343026 | KC343752 | KC343752 |
| <i>D. arctii</i>                | CBS 136.25                          | <i>Arctium</i> sp.           | –            | A.W. Archer             | KC343999                               | KC343273 | KC343031 | KC343757 | KC343757 |
| <i>D. asheicola</i>             | CBS 136967 = CPC 16508              | <i>Vaccinium ashei</i>       | Chile        | A. Schilder             | KJ160518                               | KJ160542 | KJ160562 | KJ160594 | KJ160594 |
|                                 | CBS 136968 = CPC 16511              | <i>V. ashei</i>              | Chile        | A. Schilder             | KJ160519                               | KJ160543 | KJ160563 | KJ160595 | KJ160595 |
| <i>D. australafriicana</i>      | CBS 111886 = CPC 2676               | <i>Vitis vinifera</i>        | Australia    | R.W.A. Schepers         | KC344006                               | KC343280 | KC343038 | KC343764 | KC343764 |
|                                 | CBS 113487 = CPC 2655               | <i>V. vinifera</i>           | South Africa | L. Mostert              | KC344007                               | KC343281 | KC343039 | KC343765 | KC343765 |
| <i>D. baccae</i>                | CBS 136971 = CPC 20587 <sup>a</sup> | <i>Vaccinium corymbosum</i>  | Italy        | G. Polizzi              | –                                      | –        | KJ160564 | KJ160596 | KJ160596 |
|                                 | CBS 136972 = CPC 20584 <sup>a</sup> | <i>V. corymbosum</i>         | Italy        | G. Polizzi              | –                                      | –        | KJ160565 | KJ160597 | KJ160597 |
|                                 | CPC 20583 <sup>a</sup>              | <i>V. corymbosum</i>         | Italy        | G. Polizzi              | –                                      | –        | KJ160566 | KJ160598 | KJ160598 |
|                                 | CPC 20585 <sup>a</sup>              | <i>V. corymbosum</i>         | Italy        | G. Polizzi              | –                                      | –        | KJ160567 | KJ160599 | KJ160599 |
|                                 | CPC 20586 <sup>a</sup>              | <i>V. corymbosum</i>         | Italy        | G. Polizzi              | –                                      | –        | KJ160568 | KJ160600 | KJ160600 |
| <i>D. celastrina</i>            | CBS 139.27                          | <i>Celastrus scandens</i>    | –            | L.E. Wehmeyer           | KC344015                               | KC343289 | KC343047 | KC343773 | KC343773 |
| <i>D. chamaeropsis</i>          | CBS 753.70                          | <i>Spartium junceum</i>      | Croatia      | J.A. von Arx            | KC344017                               | KC343291 | KC343049 | KC343775 | KC343775 |
| <i>D. cinerascens</i>           | CBS 719.96                          | <i>Ficus carica</i>          | Bulgaria     | E. Ilieva               | KC344018                               | KC343292 | KC343050 | KC343776 | KC343776 |
| <i>D. cuppatea</i>              | CBS 117499 = CPC 5431               | <i>Aspalathus linearis</i>   | South Africa | J.C. Janse van Rensburg | KC344025                               | KC343299 | KC343057 | KC343783 | KC343783 |
| <i>D. cynaroidis</i>            | CBS 122676 = CPC 13180 = CMW 22190  | <i>Protea cynaroides</i>     | South Africa | S. Marincowitz          | KC344026                               | KC343300 | KC343058 | KC343784 | KC343784 |

(Continued)

Table 1. Continued

| Species                | Strain <sup>1</sup>                   | Host                       | Country         | Collector               | Genbank accession numbers <sup>2</sup> |          |          |          |     |
|------------------------|---------------------------------------|----------------------------|-----------------|-------------------------|--|----------|----------|----------|-----|
|                        |                                       |                            |                 |                         | BTUB                                   | CAL      | ITS      | TEF      | TEF |
| <i>D. eres</i>         | CBS 287.74                            | <i>Sorbus aucuparia</i>    | The Netherlands | W.M. Loerakker          | KC344052                               | KC343326 | KC343084 | KC343810 |     |
|                        | CBS 439.82 = BBA P-407 =IMI 162181a   | <i>Cotoneaster</i> sp.     | Scotland        | H. Butin                | KC344058                               | KC343332 | KC343090 | KC343816 |     |
|                        | CBS 524.82                            | <i>V. vitis-idea</i>       | Poland          | H.A. van der Aa         | KJ160520                               | –        | JQ807448 | JQ807377 |     |
|                        | CBS 109767 = AR 3538 = WJ 1643        | <i>Acer campestre</i>      | Austria         | W. Jaklitsch            | KC344043                               | KC343317 | KC343075 | KC343801 |     |
|                        | CBS 134736 = PD02125472               | <i>Vaccinium</i> sp.       | The Netherlands | –                       | –                                      | KJ160544 | KJ160569 | KJ160601 |     |
|                        | CBS 134739 = PD05035464               | <i>V. corymbosum</i>       | The Netherlands | L. Colon                | –                                      | –        | KJ160570 | KJ160602 |     |
|                        | CBS 134742 = PD02026319               | <i>V. oxycoccus</i>        | Lithuania       | Z. Jovaišienė           | KJ160521                               | –        | KJ160571 | KJ160603 |     |
|                        | CPC 16510                             | <i>V. corymbosum</i>       | Chile           | A. Schilder             | KJ160522                               | KJ160545 | KJ160572 | KJ160604 |     |
|                        | CPC 23802 = PD04995578-2 <sup>a</sup> | <i>V. myrtilillus</i>      | The Netherlands | G.C.M van Leeuwen       | KJ160523                               | KJ160546 | KJ160573 | KJ160605 |     |
|                        | CPC 23803 = PD04995551 <sup>a</sup>   | <i>V. myrtilillus</i>      | The Netherlands | G.C.M van Leeuwen       | KJ160524                               | –        | KJ160574 | KJ160606 |     |
|                        | CPC 23804 = PD04328898 <sup>a</sup>   | <i>V. corymbosum</i>       | The Netherlands | G.C.M van Leeuwen       | KJ160525                               | –        | KJ160575 | KJ160607 |     |
|                        | CPC 23806                             | <i>Vaccinium</i> sp.       | Germany         | –                       | KJ160526                               | –        | KJ160576 | KJ160608 |     |
|                        | CPC 23808 = PD03173021                | <i>V. corymbosum</i>       | USA             | –                       | KJ160527                               | KJ160547 | KJ160577 | KJ160609 |     |
|                        | CPC 23809                             | <i>Vaccinium</i> sp.       | Lithuania       | –                       | –                                      | –        | KJ160578 | KJ160610 |     |
| <i>D. foeniculacea</i> | CBS 187.27                            | <i>Camellia sinensis</i>   | Italy           | M. Cruz                 | KC344075                               | KC343349 | KC343107 | KC343833 |     |
|                        | CBS 117501 = CPC 5422                 | <i>Aspalathus linearis</i> | South Africa    | J.C. Janse van Rensburg | –                                      | –        | DQ286287 | DQ286261 |     |
|                        | CBS 123208 = Di-C004/5                | <i>Foeniculum vulgare</i>  | Portugal        | A.J.L. Phillips         | KC344072                               | KC343346 | KC343104 | KC343830 |     |
|                        | CBS 123209 = Di-C004/4                | <i>F. vulgare</i>          | Portugal        | A.J.L. Phillips         | KC344073                               | KC343347 | KC343105 | KC343831 |     |
| <i>D. juglandina</i>   | CBS 121004 = DP 0659                  | <i>Juglans</i> sp.         | USA             | L. Vasilyeva            | KC344102                               | KC343376 | KC343134 | KC343860 |     |
| <i>D. lusitanicae</i>  | <b>CBS 123212</b> = Di-C001/5         | <i>F. vulgare</i>          | Portugal        | J.M. Santos             | KC344104                               | KC343378 | KC343136 | KC343862 |     |
|                        | CBS 123213 = Di-C001/3                | <i>F. vulgare</i>          | Portugal        | J.M. Santos             | KC344105                               | KC343379 | KC343137 | KC343863 |     |
| <i>D. nobilis</i>      | CBS 200.39                            | <i>Laurus nobilis</i>      | Germany         | Kotthoff                | KC344119                               | KC343393 | KC343151 | KC343877 |     |

(Continued)

Table 1. Continued

| Species                | Strain <sup>1</sup>                   | Host                       | Country         | Collector               | Genbank accession numbers <sup>2</sup> |          |          |          |          |
|------------------------|---------------------------------------|----------------------------|-----------------|-------------------------|--|----------|----------|----------|----------|
|                        |                                       |                            |                 |                         | BTUB                                   | CAL      | ITS      | TEF      | TEF      |
| <i>D. novem</i>        | CBS 127270 = 3-27/3-1                 | <i>Glycine max</i>         | Croatia         | T. Duvnjak              | KC344124                               | KC343398 | KC343156 | KC343882 | KC343882 |
|                        | CBS 127271 = 5/27/3-3                 | <i>G. max</i>              | Croatia         | T. Duvnjak              | KC344125                               | KC343399 | KC343157 | KC343883 | KC343883 |
| <i>D. pernicioso</i>   | CBS 124030 = GJS 77-49                | <i>Malus pumila</i>        | New Zealand     | G.J. Samuels            | KC344117                               | KC343391 | KC343149 | KC343875 | KC343875 |
| <i>D. pulla</i>        | CBS 338.89                            | <i>Hedera helix</i>        | Yugoslavia      | M. Muntanaola-Cvetkovic | KC344120                               | KC343394 | KC343152 | KC343878 | KC343878 |
| <i>D. sterilis</i>     | CBS 136969 = CPC 20563 <sup>a</sup>   | <i>V. corymbosum</i>       | Italy           | G. Polizzi              | KJ160528                               | KJ160548 | KJ160579 | KJ160611 | KJ160611 |
|                        | CBS 136970 = CPC 20577 <sup>a</sup>   | <i>V. corymbosum</i>       | Italy           | G. Polizzi              | KJ160529                               | KJ160549 | KJ160580 | KJ160612 | KJ160612 |
|                        | CPC 20575 <sup>a</sup>                | <i>V. corymbosum</i>       | Italy           | G. Polizzi              | KJ160530                               | KJ160550 | KJ160581 | KJ160613 | KJ160613 |
|                        | CPC 20580 <sup>a</sup>                | <i>V. corymbosum</i>       | Italy           | G. Polizzi              | KJ160531                               | KJ160551 | KJ160582 | KJ160614 | KJ160614 |
|                        | CPC 20582 <sup>a</sup>                | <i>V. corymbosum</i>       | Italy           | G. Polizzi              | KJ160532                               | KJ160552 | KJ160583 | KJ160615 | KJ160615 |
| <i>D. subordinaria</i> | CBS 101711                            | <i>Plantago lanceolata</i> | New Zealand     | B. Alexander            | KC344181                               | KC343455 | KC343213 | KC343939 | KC343939 |
| <i>D. vaccinii</i>     | CBS 160.32 = IFO 32646                | <i>V. macrocarpon</i>      | USA             | C.L. Shear              | KC344196                               | KC343470 | KC343228 | KC343954 | KC343954 |
|                        | CBS 118571                            | <i>V. corymbosum</i>       | USA             | G.C. Adams              | KC344191                               | KC343465 | KC343223 | KC343949 | KC343949 |
|                        | CBS 122112 = FAU 474                  | <i>V. macrocarpon</i>      | USA             | L. Carris               | KC344192                               | KC343466 | KC343224 | KC343950 | KC343950 |
|                        | CBS 122113 = FAU 446                  | <i>V. macrocarpon</i>      | USA             | F. Caruso               | KJ160532                               | KJ160553 | KJ160584 | KJ160616 | KJ160616 |
|                        | CBS 122114 = FAU 634                  | <i>V. corymbosum</i>       | USA             | D.C. Ramsdell           | KC344193                               | KC343467 | KC343225 | KC343951 | KC343951 |
|                        | CBS 122115 = FAU 590                  | <i>V. corymbosum</i>       | USA             | D.C. Ramsdell           | KC344194                               | KC343468 | KC343226 | KC343952 | KC343952 |
|                        | CBS 122116 = DF 5022                  | <i>V. corymbosum</i>       | USA             | D.F. Farr               | KC344195                               | KC343469 | KC343227 | KC343953 | KC343953 |
|                        | CBS 134741 = PD03276619 <sup>a</sup>  | <i>V. corymbosum</i>       | The Netherlands | G.C.M van Leeuwen       | KJ160534                               | KJ160554 | KJ160585 | KJ160617 | KJ160617 |
|                        | CPC 23811 = PD02026335                | <i>V. oxycoccus</i>        | Lithuania       | Z. Jovaišienė           | –                                      | –        | KJ160586 | KJ160618 | KJ160618 |
|                        | CPC 23812 = PD04067579                | <i>V. macrocarpon</i>      | Lithuania       | K Paruma                | KJ160535                               | KJ160555 | KJ160587 | KJ160619 | KJ160619 |
|                        | CPC 23813 = PD05317242                | <i>V. macrocarpon</i>      | Latvia          | K Paruma                | KJ160536                               | KJ160556 | KJ160588 | KJ160620 | KJ160620 |
| <i>D. viticola</i>     | CBS 113201 = CPC 5683                 | <i>Vitis vinifera</i>      | Portugal        | A.J.L. Phillips         | KC344202                               | KC343476 | KC343234 | KC343960 | KC343960 |
|                        | CBS 114011 = CPC 2677                 | <i>V. vinifera</i>         | Portugal        | A.J.L. Phillips         | KC344203                               | KC343477 | KC343235 | KC343961 | KC343961 |
|                        | CPC 23799 = PD04995578-1 <sup>a</sup> | <i>Vaccinium myrtillus</i> | The Netherlands | G.C.M van Leeuwen       | KJ160537                               | KJ160557 | KJ160589 | KJ160621 | KJ160621 |

(Continued)

Table 1. Continued

| Species                       | Strain <sup>1</sup>                 | Host                     | Country         | Collector                  | Genbank accession numbers <sup>2</sup> |          |          |          |
|-------------------------------|-------------------------------------|--------------------------|-----------------|----------------------------|--|----------|----------|----------|
|                               |                                     |                          |                 |                            | BTUB                                   | CAL      | ITS      | TEF      |
|                               | CPC 23800 = PD03279491              | <i>V. corymbosum</i>     | The Netherlands | –                          | KJ160538                               | KJ160558 | KJ160590 | KJ160622 |
|                               | CPC 23801 = PD04995586 <sup>a</sup> | <i>V. myrtilloides</i>   | The Netherlands | G.C.M. van Leeuwen         | KJ160539                               | KJ160559 | KJ160591 | KJ160623 |
| <i>Diaporthe</i> sp.1         | CBS 134743 <sup>a</sup>             | <i>V. myrtilloides</i>   | The Netherlands | L. Lombard                 | KJ160540                               | KJ160560 | KJ160592 | KJ160624 |
| <i>Diaporthe</i> sp. 2        | CBS 101574                          | <i>Vaccinium</i> sp.     | Italy           | G. Mancini                 | KJ160541                               | KJ160561 | KJ160593 | –        |
| " <i>Phomopsis castanea</i> " | CBS 113470 = DAOM 226800            | <i>Castanea sativa</i>   | Korea           | K.A. Seifert               | KC344114                               | KC343388 | KC343146 | KC343872 |
| " <i>Phomopsis conorum</i> "  | CBS 587.79                          | <i>Pinus pentaphylla</i> | Japan           | G.H. Boerema               | KC344121                               | KC343395 | KC343153 | KC343879 |
| " <i>Phomopsis fukushii</i> " | CBS 116953 = NZ-26                  | <i>Pyrus pyrifolia</i>   | New Zealand     | W. Kandula & L. Castlebury | KC344115                               | KC343389 | KC343147 | KC343873 |
|                               | CBS 116954 = NZ-27                  | <i>P. pyrifolia</i>      | New Zealand     | W. Kandula & L. Castlebury | KC344116                               | KC343390 | KC343148 | KC343874 |
| <i>Diaportheella corylina</i> | CBS 121124 = AR 4131                | <i>Corylus</i> sp.       | China           | L.N. Vassiljeva            | KC343972                               | KC343246 | KC343004 | KC343730 |

<sup>1</sup> AR: Collection of A.Y. Rossman; ATCC: American type culture collection; BBA: Institut für Mikrobiologie culture collection, Berlin, Germany; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CMW: Collection of M.J. Wingfield housed at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CPC: Collection of P.W. Crous housed at CBS; DAOM: Canadian Collection of Fungal Cultures, Ottawa, Canada; DF: Collection of D.F. Farr, University of California, USA; Di: Collection of A.J.L. Phillips, Centro de Recursos Microbiológicos, Universidade Nova Lisboa, Lisbon, Portugal; FAU: Biological Science Department, Florida Atlantic University, Florida, USA; GJS: Collection of G.J. Samuels; IFO: Institute for Fermentation, 17-85, Jusohommachi, 2-chome, Yodogawaku, Osaka 532, Japan; IMI: International Mycological Institute, CABI-Bioscience, Egham, Basingstoke, Hampshire, UK; PD: Collection of the Dutch National Plant Protection Organization (NPPO-NL), Wageningen, The Netherlands; NZ: Collection of L. Castlebury. Ex-type cultures indicated in bold.

<sup>2</sup> BTUB: partial beta-tubulin gene; CAL: partial calmodulin gene; ITS: internal transcribed spacer regions of the nrDNA and 5.6S nrDNA; TEF: partial translation elongation factor 1-alpha gene; sequences generated in this study indicated in italics.

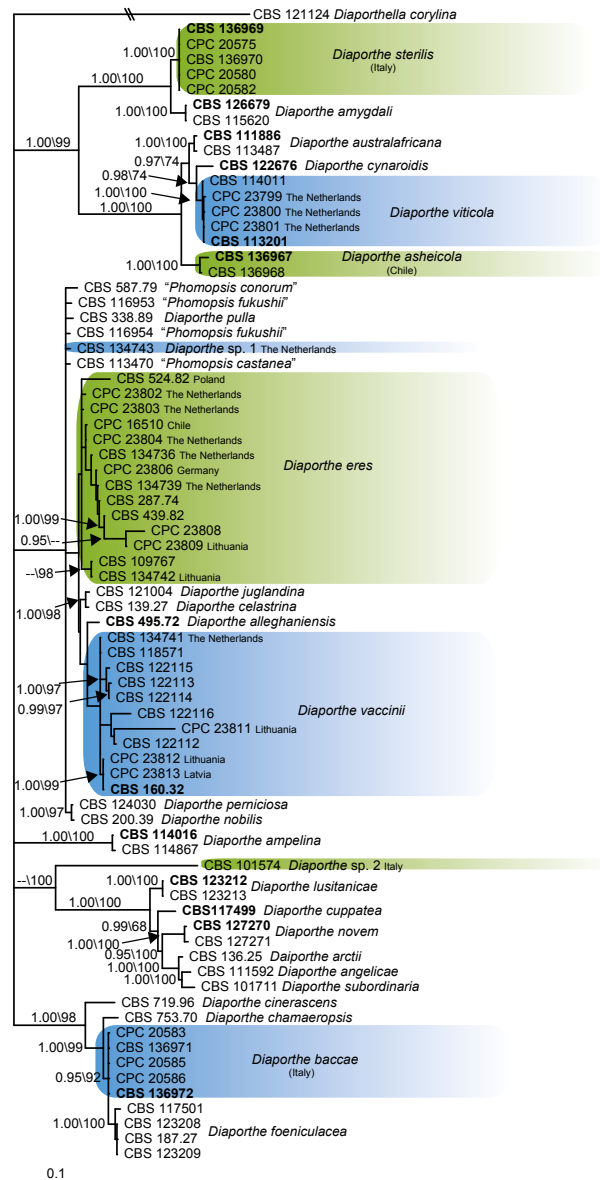
<sup>a</sup> Isolates obtained from symptomatic and asymptomatic *Vaccinium* plants during this study.

ysis lasted 10,000'000 generations, and the consensus tree with posterior probability was calculated from 15,002 trees left after 5,000 trees were discarded as the burn-in phase. The tree topology and bootstrap values of the ML supported the trees obtained from the Bayesian analysis. The tree was rooted to *Diaporthella corylina* (CBS 121124) (Figure 1).

In the phylogenetic tree the *Diaporthe* isolates from *Vaccinium* spp. clustered within eight clades, of which three clades and two single lineages represent possible new phylogenetic species (Figure 1). Two of these clades represent isolates originating from Italy. The first of these Italian clades (containing CBS 136969 and CBS 136970), with high BS (100%) and PP (1.00) values, is closely related to, but separate from, *D. amygdali* (ex-type CBS 126679). The second clade of Italian isolates (containing CBS 136971 and CBS 136972) is closely related to isolates representing *D. foeniculacea*. A third isolate originating from Italy, CBS 101574, formed a sister taxon to the clade containing *D. lusitanica* (ex-type CBS 123212) and *D. cuppatea* (ex-type CBS 117499). Isolates from Germany (CPC 23806), Poland (CBS 524.82) and Lithuania (CPC 23809 and CBS 134742) grouped within the *Diaporthe eres* (CBS 439.82, CBS 109767; Gomes *et al.*, 2013) clade along with several other isolates collected in the Netherlands (CPC 23802, CPC 23803, CPC 23804, CBS 134736 and CBS 134739). The isolates CPC 23804, CPC 23803 and CPC 23802 were initially identified as *Diaporthe vaccinii* (NPP0, 2013), however, the present study demonstrates that these isolates belong to the *D. eres* clade. The remaining isolates from Lithuania (CPC 23811 and CPC 23812) grouped within the *D. vaccinii* clade along with an isolate from Latvia (CPC 23813) and one from the Netherlands (CBS 134741). The phylogenetic position of one isolate from the Netherlands (CBS 134743) remains unresolved. Two isolates (CBS 136967 and CBS 136968) originating from Chile, grouped together in a well-supported clade (BS = 100%; PP = 1.00), closely related to, but separate from, *D. australafricana* (ex-type CBS 111886), *D. cynaroidis* (ex-type CBS 122676) and *D. viticola* (ex-type CBS 113201). Three isolates from the Netherlands (CPC 23799, CPC 23780 and CPC 23801) grouped within the *D. viticola* (ex-type CBS 113201) clade.

### Taxonomy

Based on DNA sequence analysis and morphological features, three new species of *Diaporthe* are



**Figure 1.** Consensus phylogram of 15,002 trees resulting from a Bayesian analysis of the combined four gene sequence alignment. European strains of *Diaporthe* spp. from *Vaccinium* spp. are indicated in the coloured blocks with country of origin next to the strain accession number. Accession numbers in **bold** represents ex-type strains. Bayesian posterior probabilities and Maximum Likelihood bootstrap support values are indicated at the nodes and the scale bar represents the expected changes per site. The tree was rooted to *Diaporthella corylina* (CBS 121124).



recognised. Two of these species were sterile in culture and are therefore described based on DNA sequence, following the approach of Gomes *et al.* (2013). Isolate CBS 134743 is not considered here, as its phylogenetic position could not be resolved. Isolate CBS 101574 is also excluded as this strain is sterile and possibly represents a single lineage. More isolates of this strain are needed to confirm the positions of unique fixed alleles in the four loci studied here.

***Diaporthe asheicola* L. Lombard & Crous, sp. nov.**  
Mycobank MB807598

*Etymology.* Named after the host species from which it was isolated, *Vaccinium ashei*.

Cultures sterile. *Diaporthe asheicola* differs from its closest phylogenetic neighbours, *D. australafricana*, *D. cynaroidis* and *D. viticola* by unique fixed alleles in two loci based on alignments of the separate loci deposited in TreeBASE (S15189): BTUB positions 1 (T) and 12 (A); ITS positions 8 (indel), 25 (indel), 27 (T), 28 (A), 47 (T), 49 (G), 50 (indel), 51 (indel), 52 (indel), 56 (C), 57 (C), 60 (T), 61 (C), 62 (A), 63 (C), 65 (indel), 66 (indel), 67 (C), 68 (T), 69 (T), 70 (G), 75 (T), 76 (T), 80 (A), 81 (C), 99 (C), 100 (C), 150 (T), 364 (C), 365 (C), 449 (C), 451 (C), 457 (C), 459 (G) and 460 (T).

*Culture characteristics.* Colonies covering the medium within 2 weeks at 24°C, with sparse aerial mycelium. On PDA, honey (19'') to isabelline (19''i) with salmon (9'd) patches; reverse buff (19d) to honey (19''). On OA, buff (19d) to smoke-grey (21'''d). On MEA, white to buff (19d); reverse buff (19d).

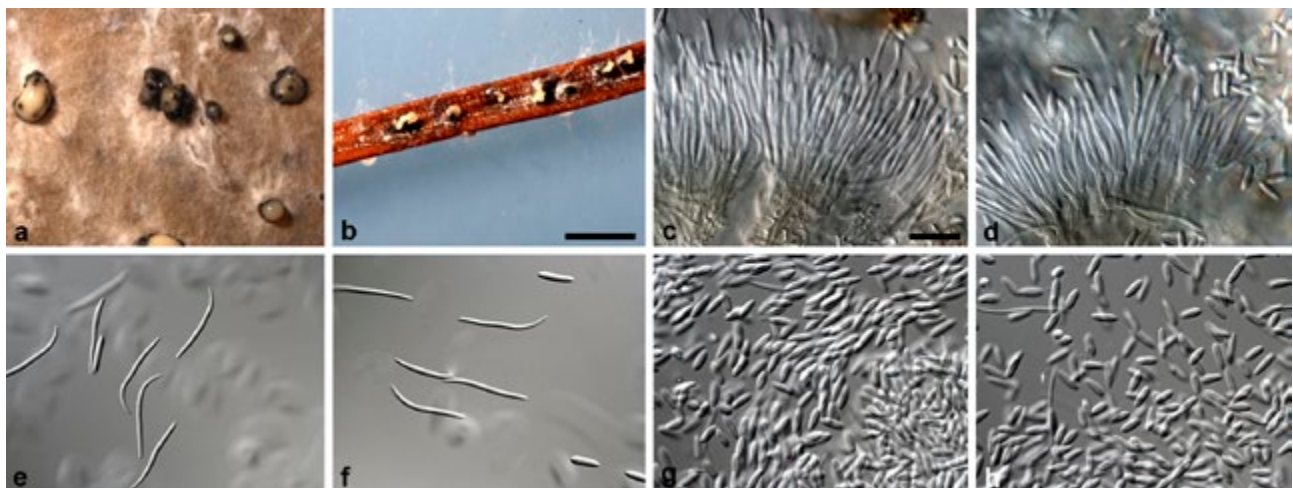
*Specimens examined:* **Chile:** near Gorbea, on *Vaccinium ashei*, Feb. 2009, A. Schilder, holotype CBS H-21513, culture ex-type CBS 136967 = CPC 16508; CBS 136968 = CPC 16511.

*Notes.* Both isolates representing *D. asheicola* could not be induced to sporulate on any of the media used in this study, nor on sterilised *V. myrtillus* tissue placed on WA.

***Diaporthe baccae* L. Lombard, G. Polizzi & Crous, sp. nov.**  
Mycobank MB807599  
(Figure 2)

*Etymology.* Name refers to the berries of the host plant, *Vaccinium corymbosum*, from which this fungus was isolated.

*Conidiomata* pycnidial in culture on PDA, PNA and OA, globose to conical, eustromatic, multilocular, occasionally with ostiolate necks, scattered or ag-



**Figure 2.** *Diaporthe baccae* (ex-type CBS 136972). a. Conidiomata sporulating on OA; b. Conidiomata sporulating on PNA; c, d. Conidiogenous cells. e, f. beta conidia; g, h. alpha conidia. Scale bars b = 500 µm; c = 10 µm (apply to d-h).

gregated, up to 650 µm diam, brown to black, surface covered with hyphae; cream to pale luteous conidial droplets exuding from the central ostioles; walls consisting of light brown, thick walled *textura angularis*. *Conidiophores* hyaline, smooth, 1–3-septate, branched, densely aggregated, cylindrical, straight to sinuous, 20–57 × 2–3 µm. *Conidiogenous cells* phialidic, cylindrical, terminal and lateral, 9–23 × 1–2 µm, slightly tapering towards the apex, with visible periclinal thickening; collarette not observed. *Paraphyses* not observed. *Alpha conidia* aseptate, hyaline smooth, guttulate, fusoid to ellipsoid, straight, tapering towards both ends, apex subobtuse, base subtruncate, (6–)7–9 × 2–3 µm (av. 8 × 2 µm). *Gamma conidia* not observed. *Beta conidia* spindle-shaped, aseptate, smooth, hyaline, apex acutely rounded, base truncate, tapering from lower third towards apex, curved, (17–)20–24(–26) × 1–2 µm (av. 22 × 2 µm).

**Culture characteristics.** Colonies covering the medium within 2 weeks at 24°C, with surface mycelium flattened, dense and felty. On PDA, cream (19'f) to smoke-grey (21'''d); reverse greyish sepia (17''m). On OA, white to greyish sepia (17''m).

**Specimens examined. Italy:** Sicily, Catania Province, Valverde, on *Vaccinium corymbosum*, June 2012, G. Polizzi, holotype CBS H-21514, cultures ex-type CBS 136972 = CPC 20584; CPC 20583; CPC 20585; CPC 20586; CPC 20587.

**Notes.** Based on the phylogenetic inference in this study, *D. baccae* is closely related to *D. chamaeropsis* and *D. foeniculacea*. The beta conidia of *D. baccae* [(17–)20–24(–26) × 1–2 µm (av. 22 × 2 µm)] are smaller than those of *D. chamaeropsis* [(20–)22–27(–30) × 1.5(–2) µm; Gomes et al., 2013] and *D. foeniculacea* [(26–)28–32(–34) × 1(–2) µm; Gomes et al., 2013]. Moreover, the conidiophores of *D. baccae* are longer (up to 57 µm) than those of *D. chamaeropsis* (up to 50 µm; Gomes et al., 2013) and *D. foeniculacea* (up to 32 µm; Gomes et al., 2013).

***Diaporthe sterilis* L. Lombard, G. Polizzi & Crous, sp. nov.**  
Mycobank MB807600

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

Cultures sterile. *Diaporthe sterilis* differs from its closest phylogenetic neighbour, *D. amygdali*, by unique fixed alleles in three loci based on alignments of the separate loci deposited in TreeBASE (S15189): BTUB positions 12 (A), 254 (G), 272 (G), 273 (A), 283 (A), 377 (A), 382 (A), 384 (T), 393 (G), 400 (T), 406 (G), 409 (A), 413 (T), 427 (G), 441 (G), 442 (A), 474 (indel), 479 (T) and 500 (T); ITS positions 44 (T), 48 (C), 50 (T), 51 (G), 68 (C), 159 (T), 358 (indel), 370 (indel), 371 (G) and 372 (G); TEF positions 16 (G), 178 (C), 179 (A), 180 (C), 181 (T), 182 (A), 183 (C), 184 (A), 185 (T), 186 (A), 187 (C), 194 (C), 195 (A), 196 (C), 197 (C), 198 (A), 199 (C), 236 (C), 238 (C) and 270 (A).

**Culture characteristics.** Colonies covering the medium within 2 weeks at 24°C, with sparse aerial mycelium. On PDA, buff (19d), honey (19'') to isabelline (19''i); reverse greyish sepia (17''m). On OA, white to greyish sepia (17''m). On MEA, white to vinaceous buff (17'''d); reverse olivaceous buff (21'''d).

**Specimens examined. Italy:** Sicily, Catania Province, Valverde, on *Vaccinium corymbosum*, June 2012, G. Polizzi, holotype CBS H-21515, cultures ex-type CBS 136969 = CPC 20563; CBS 136970 = CPC 20577; CPC 20575; CPC 20580; CPC 20582.

**Notes.** All five isolates representing *D. sterilis* could not be induced to sporulate on any of the media used in this study, nor on sterilised *V. myrtilus* tissue placed on WA.

## Discussion

Three new *Diaporthe* spp. are here described as *D. asheicola*, *D. baccae*, and *D. sterilis*, based on phylogenetic inference, morphological and cultural features. *Diaporthe baccae* and *D. sterilis* originated from Europe and *D. asheicola* originated from Chile. Both *D. asheicola* and *D. sterilis* were found to be sterile, and are therefore described based on single nucleotide polymorphisms.

Although several *Diaporthe* spp. have been recorded on *Vaccinium* spp. in various countries, *D. vaccinii* remains the main concern in Europe. This fungal pathogen is listed in both the EPPO A2 (<http://www.eppo.org>) and EU Council Directive 2000/29/EC (annex IIAI) (<http://eur-lex.europa.eu>) as an organism recommended for regulation as a quarantine pest, therefore restricting movement of infected

plants and plant products into and within Europe. To date, *D. vaccinii* has only been reported sporadically in Europe, occurring in Lithuania (Gabler *et al.*, 2004; Kačergius *et al.*, 2004), Romania (Teodorescu *et al.*, 1985), and the United Kingdom (Wilcox and Falconer, 1961; Baker, 1972). In the present study, isolates originating from Latvia, Lithuania and the Netherlands were identified as *D. vaccinii*. The study therefore confirms an earlier report of the presence of *D. vaccinii* in the Netherlands (NPPO, 2009), and represents the first report of this pathogen in Latvia.

Several isolates from the Netherlands, Germany, Lithuania and Poland were identified as *D. eres*. Although *Diaporthe eres*, the type species of the genus *Diaporthe*, has been reported on a large number of plant hosts (Wehmeyer, 1933; Gomes *et al.*, 2013), no record could be found on *Vaccinium* spp. The present record therefore also represents the first report of *D. eres* on *V. corymbosum*, *V. myrtillus* and *V. vitis-idea* (Table 1). The phylogenetic position of a single isolate from the Netherlands (CBS 134743) could not be resolved. The closest phylogenetic neighbours to *D. eres* belong to the *D. nobilis* complex (Gomes *et al.*, 2013), which is poorly resolved at present, awaiting examination of further collections.

A disease survey of *V. corymbosum* in Sicily, Italy in 2012, yielded several *Diaporthe* isolates, resulting in the identification of two new *Diaporthe* spp., *D. baccae* and *D. sterilis* in the present study. Infected plants displayed characteristic cankers at their bases, with brown lesions developing on the green stems and twigs, which resulted in twig blight. Plant mortality was observed when these cankers were present in the crowns of these plants. Preliminary pathogenicity tests on four *V. corymbosum* cultivars (Darrow, Berkeley, Legacy, Elliot) induced identical symptoms to those observed in the field. Host specificity of both these new *Diaporthe* spp., needs to be further investigated.

Three isolates from the Netherlands were identified as *D. viticola*. *Diaporthe viticola* is a well-known fungal pathogen in Europe (Phillips, 1999; Schepers *et al.*, 2000; Mostert *et al.*, 2001; van Niekerk *et al.*, 2005), associated with cane spot of grapevines. This fungus has also been found on several other woody hosts in the Netherlands (Gomes *et al.*, 2013). However, this is the first report of *D. viticola* on *V. corymbosum* and *V. myrtillus* in Europe. The description of *D. asheicola* from Chile was serendipitous, as these isolates were originally thought to represent *D. australafricana*. Al-

though both isolates representing *D. asheicola* were sterile, either due to age, repeated subculturing or as an intrinsic feature of this species, they could be distinguished from *D. australafricana* and *D. viticola* based on single nucleotide polymorphisms following the approach of Gomes *et al.* (2013). As with *D. baccae* and *D. sterilis*, the pathogenicity and host specificity of *D. asheicola* needs to be further investigated.

This study has resulted in the identification of several *Diaporthe* spp. associated with *Vaccinium* spp. in Europe. Both *D. eres* and *D. viticola* are known to be host non-specific in Europe (Gomes *et al.*, 2013), and therefore it is not surprising that they are also newly reported from various *Vaccinium* spp. *Diaporthe vaccinii* is known to be host-specific (Chao and Glawe, 1985) and is possibly indigenous to North America (Shear *et al.*, 1931; Weingartner and Klos, 1975; Chao and Glawe, 1985; Farr *et al.*, 2002a,b; Tadych *et al.*, 2012), but is now shown to be present in Europe on *V. corymbosum*, *V. macrocarpon* and *V. oxycoccus*. However, large scale surveys on *Vaccinium* spp. grown commercially and in public areas need to be conducted to determine the distribution of *D. vaccinii* and other *Diaporthe* spp. in Europe. This information would contribute to understanding of the distribution of these fungi, and the extent to which quarantine measures need to be applied for these pathogens occurring on *Vaccinium* plants and plant products moved into and within Europe.

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