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Identification and characterization of *Diaporthe vaccinii* Shear causing upright dieback and viscid rot of cranberry in Poland

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Abstract During the summers of 2013–2014, symptoms similar to viscid rot and upright disease were observed on cranberries (*Vaccinium macrocarpon*) on one plantation in central Poland. The associated fungi were isolated from symptomatic plant tissue. On the basis of morphological and cultural characteristics and the ability of isolated fungi to elicit viscid rot symptoms on cranberry fruits, they were classified as the genus *Diaporthe*. Further analysis of ITS sequence data allowed for the classification of the newly obtained isolates as *D. vaccinii*. Additional analysis of genetic diversity using five RAPD and eight ISSR primers constituted additional confirmation of genetic distance existing between closely related *D. vaccinii* and *D. eres* species, enabling their differentiation.

Keywords Filamentous fungi · Fruit rot · ITS rDNA analysis · Morphological characteristics · Stem cankers

Introduction

Plants of *Vaccinium macrocarpon* (large cranberry or American cranberry) are commercially cultivated in Europe (Prodorutti et al. 2007) and are becoming more popular also in Poland. However, their cultivation is limited by fungal diseases including those caused by species from the genus *Diaporthe* Nitschke (anamorph *Phomopsis*) (Sacc.) Bubák, comprising many important plant pathogenic species with wide host ranges and geographic distributions (Udayanga et al. 2012; Gomes et al. 2013). Several species from the genus *Diaporthe* were reported as pathogenic to *Vaccinium* spp. plants in Europe, causing twig blight, stem cankers and fruit rot (Farr et al. 2002b; Tadych et al. 2012; Elfar et al. 2013; Lombard et al. 2014). On the basis of multi-locus DNA sequences analysis, including beta-tubulin, calmodulin, translation elongation factor 1-alpha and the internal transcribed spacer (ITS) regions of the nuclear rDNA, and morphological characteristics, Lombard et al. (2014) classified the collection of European isolates from *Vaccinium* spp., initially identified as *D. vaccinii* based on host association, to six species, including *D. vaccinii*, *D. viticola*, *D. eres* and three newly described as *D. asheicola*, *D. baccae* and *D. sterilis*. Among these, *D. vaccinii* Shear is globally regarded as the dominant species of *Diaporthe* on *Vaccinium* spp. (Farr et al. 2002a; Lombard et al. 2014), causing stem cankers, twig blight, leafspots and soft rot of fruit, mainly on highbush blueberry and cranberry, recognized as 'upright dieback and viscid rot of cranberry' (Farr et al. 2002a, b; Polashock and Kramer 2006; Farr and Rossman 2012; Tadych et al. 2012). *D. vaccinii* overwinters on the previous year's infected dead twigs and possibly on plant debris (twigs, leaves, fruits) lying on the soil surface (Shear et al. 1931; Wilcox 1939). In the infested areas, the primary inoculum seems to be conidia that are produced in pycnidia of the anamorph *Phomopsis vaccinii*. Pycnidia are found on dead cankered stems and leaf lesions (Wilcox

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1939; Weingartner and Klos 1975; Parker and Ramsdell 1977). This species, indigenous to North America, is categorised as a quarantine pest for the European Union as indicated in European Food Safety Authority report (EFSA 2014). *D. vaccinii* is currently present in Latvia (with restricted distribution) and the Netherlands (transient, incidental findings, under surveillance; EFSA 2014). In Germany, the pest has been eradicated (EFSA 2014). In Romania and the United Kingdom, the pest is no longer present, while there are some reports of the pathogen on wild host plants in Lithuania (Kačergius and Jovaišiene 2010).

In addition to the fungi from the genus *Diaporthe*, showing similar cultural and morphological characteristics between species, other fungal species have been reported to cause stem blight symptoms on *Vaccinium* spp. similar to those caused by *D. vaccinii* (e.g. *Pestalotiopsis* spp., *Botryosphaeria dothidea, Godronia cassandrae* and *Colletotrichum* spp.) (MacKenzie et al. 2009; Stromeng and Stensvand 2011; Wright and Harmon 2010; Espinoza et al. 2008). Due to the presence of latent infections (Friend and Boone 1968; Milholland and Daykin 1983), as well as coexistence of some fungal species on one host, diagnosis of the disease based only on symptoms or fungal morphological traits is not reliable. Confirmation of the pathogen identity is required at least by analysing the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) region in axenic cultures of *D. vaccinii* (OEPP/EPPO 2009). A higher level of confidence in the identification can be obtained by a multigene approach to phylogenetic analyses (Udayanga et al. 2012; Elfar et al. 2013; Lombard et al. 2014).

During the survey conducted in the late summers of 2013 and 2014, symptoms similar to those described as viscid rot (soft rot of fruit) and upright dieback on cranberry were observed on one of the few plantations of American cranberry (*Vaccinium macrocarpon*) located in central Poland. The objective of this study is to identify and characterize the causal agent of the new disease affecting cranberry in Poland based on morphological and genomic features.

Material and methods

Sampling and isolation of fungi

During the summer of 2013, early leaf discoloration was observed on the 2-year Pilgrim cultivar on one cranberry plantation located in central Poland. The symptoms were scarce, scattered, and similar to those described as 'upright dieback'. In the subsequent year (2014), changes in the appearance of ripening fruits were noted on the same plantation and cultivar. Some of the changes included clearly brighter, reddish-brown, more soft and squashy than healthy-appearing fruits, causing leakage of juice when gently pressed (Fig. 1). Symptomatic cranberry shoots and fruits were collected and disinfected by submerging in 70% ethanol for 30 s. The small part of the fruit flesh or a fragment of the shoot from the border of diseased and healthy tissue were cut out and placed onto PDA medium in Petri dishes. Plates were incubated at 24 °C until the production of conidiomata (pycnidia) with spore mass eruptions were observed. Then, a water conidial suspension was prepared and spread evenly onto the PDA surface. After 24 h, single germinating conidial cells were picked up and monoconidial cultures were established on fresh PDA. Finally, seven isolates were obtained including ZA, ZB derived from shoots and A1, A2, P1, D and B obtained from fruits. These isolates were subjected to further morphological and molecular examinations. For comparison purposes, reference strains derived from Centraalbureau voor Schimmelcultures in the Netherlands: CBS 160.32 (type culture of *D. vaccinii* Shear, derived from *V. macrocarpon*) and CBS 524.82, derived from *V. vitis-idaea* and reported by Lombard et al. (2014) as *D. eres*, and one strain Lat4 (*Diaporthe* sp., from cranberry shoots cv. Pilgrim, Latvia; 2014) from the collection of Research Institute of Horticulture (RIH), Skierniewice, were included to analyses.

Fig. 1 Symptoms of viscid rot caused by *D. vaccinii* on cranberry fruit

Morphology and growth examination

For the examination of morphology of all 10 isolates and strains cultured in vitro, four types of microbiological media were tested. Three of the media: PDA (Potato Dextrose Agar; Becton Dickinson, Sparks, MD, USA), MEA (Malt Extract Agar; Difco Detroit, USA) and CMA (Corn Meal Agar; Difco, Detroit, USA) were prepared according to the manufacturer's instruction. The fourth medium was MSM prepared from dried stems of sweet clover according to the procedure in Appendix 2 of the EPPO standard diagnostic protocol (OEPP/EPPO 2009). Agar plugs (6 mm in diameter) from the edge of 10-day-old cultures grown on PDA medium were transferred onto newly prepared media, and cultures were incubated at 24 °C in 8-h light/16-h darkness photoperiod. Colony morphology and growth were observed and measured for eight consecutive days. Mean growth rates per 24 h were compared between isolates using one-way ANOVA followed by Tukey's post-hoc honestly significance difference test (STATISTICA). Each experiment was performed twice with five replicates for each isolate.

For each isolate and strain, the morphology and dimensions of conidia obtained in in vitro cultures were determined under an optical light microscope (Eclipse 80*i*, Nikon, Chiyodaku, Tokyo, Japan) combined with digital camera. For each isolate, 100 alpha and – if present - 50 beta conidia were observed and measured at 600 x magnification. For isolates and strains where no conidia production was observed on any used medium, mycelia were transplanted onto PDA in Petri dishes supplemented with carnation leaf fragments that were previously disinfected with 96% ethanol and washed with sterile water. After mycelial plug transfer, the plates were incubated in the same conditions as plates containing only media.

DNA extraction, PCR and sequencing

Approximately 100 mg of a mycelium was cut out from 10-day-old cultures grown on PDA medium and ground using a glass rod in an Eppendorf tube. The total DNA was extracted using the GeneMatrix Plant & Fungi DNA Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer's instructions.

The ITS region of nuclear rDNA of each examined isolate was amplified using the universal fungal primers ITS1 and ITS4 (White et al. 1990) using the PCR conditions described by Chen et al. (2006). Reaction mixtures consisted of 2.5 μl of extracted DNA, 1 μM of each primer, 0.2 mM of each dNTP, 1.5 U of DreamTaq Green DNA Polymerase (Thermo Scientific, Vilnius, Lithuania), 1x optimised DreamTaq Green Buffer and double distilled water up to 50 μl. PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced in both directions using the same primer pair. Obtained sequences were compared with the 20 sequences of *Diaporthe* spp. and *Phomopsis* spp. strains, originating from or reported previously on *Vaccinium* plants (Elfar et al. 2013; Lombard et al. 2014) and with three sequences from other hosts, all available in GenBank database (National Center for Biotechnology Information). Comparisons were performed by cluster analysis conducted with MEGA v. 6.0 software (Tamura et al. 2013; available at www.megasoftware.net/mega.php). Phylogenetic relationships between sequences were inferred using the neighbor-joining and maximum likelihood method and the heuristic search. Bootstrap support values based on 1,000 replications were calculated for tree branches in both phylogenetic methods. From all ITS1-5.8S-ITS2 rDNA sequences obtained in this study, three representatives were selected (from isolates A1, D and Lat4) and deposited in GenBank and were assigned with the accession numbers. The sequences of eight examined isolates were aligned with the corresponding sequences of reference strains CBS 160.32 and CBS 524.82, and additionally with the sequences of CBS 138594 strain (ex-epitype of *D. eres*, derived from *Ulmus laevis*, Germany), CBS 134742 (*D. eres* from *V. oxycoccus*, Lithuania) and CPC 23806 (*D. eres* from *Vaccinium sp.*, Germany) all from the GenBank, and the percentage of similarity between them was calculated using MEGALIGN software (DNASTAR Inc.).

Pathogenicity test on cranberry fruits

Conidia of eight *Diaporthe* spp. isolates were suspended in sterile double-distilled water, counted using a haemocytometer and adjusted to a final concentration of 1×10^5–10^6 CFU (colony-forming units)/ml. Cranberry fruits were inoculated by introducing 100 μl of conidial suspension into a natural opening formed after the removal of the stalk. In cases when the production of conidia was not observed (CBS 524.82, CBS 160.32), fruits were inoculated with 3-mm diameter mycelial plugs of that isolate. Fruits with plugs from sterile

PDA agar or injected with water droplets were used as a negative control. Inoculated fruits were incubated for 14 days at room temperature in a sterilized glass container filled with water-soaked cotton wool, maintaining 90–95% relative humidity. The experiment was repeated twice with 10 fruits per each isolate. After completion of the experiment, the suspected causal agent was re-isolated from symptomatic fruits onto PDA and its identity was confirmed in order to fulfil Koch's postulates.

Genetic diversity evaluation based on RAPD and ISSR markers

DNA extracted from all eight examined isolates and two reference strains was tested in two types of fingerprinting PCR assays. Amplification patterns were generated in separate reactions with eight ISSR primers as follows: $(GACA)_4$ (Weising et al. 1989); $(GACG)_4$, $(GCA)_5$ (Talhinhas et al. 2005); $(ACTG)_4$ (Urena-Padilla et al. 2002); Mf-10$(GTG)_5$ (Zhou et al. 2001); MF-8$(AG)_8$C (Ma et al. 2003); $(GTC)_6$, $(AC)_8$T (Fan et al. 2010) and five RAPD primers: OPT-07, OPC-05, OPU-19, OPAR-03 and OPA-11 (Operon Technologies Inc., CA, USA). Conditions of amplification for ISSR-PCR assays were as follows: 3 min of denaturation at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at an appropriate annealing temperature: 48 °C for $(GACA)_4$, $(ACTG)_4$, and $(AG)_8$C, 50 °C for $(AC)_8$T, 52 °C for $(GACG)_4$, 55 °C for $(GCA)_5$ or 60 °C for $(GTG)_5$ and $(GTC)_6$, 90 s at 72 °C, ending with one cycle of 5 min at 72 °C. For amplifications with RADP primers, 40 cycles in similar conditions were performed, with the exceptions of an annealing step for 45 s at 36 °C and polymerization for 1 min at 62 °C for OPT-07, OPC-05, OPU-19 primers, and an annealing step for 90 s at 38 °C and polymerization for 2 min at 72 °C for the OPAR-03 and OPA-11 (Operon Technologies) primers. Each reaction contained 1 μM of ISSR or 0.8 μM of RAPD primer, 0.2 mM of each dNTP, 0.55 U of DreamTaq Green DNA Polymerase, 1x optimised DreamTaq Green Buffer and double-distilled water up to 20 μl. All RAPD and ISSR-PCR assays were performed twice.

Products obtained in all PCR-based experiments were separated on 1.5% (w/v) agarose gels and stained with ethidium bromide, visualised on a UV transilluminator and documented. Product sizes were estimated based on O'GeneRuler™ 100-bp DNA Ladder Plus (Thermo Scientific, Vilnius, Lithuania).

Results

Morphology and growth examination

For all ten fungal isolates and strains cultured on four different media, a consistent radiate growth pattern was observed. The highest mean growth rates for A1, A2, D, B, P1, ZA, ZB, and CBS 160.32 isolates and strains were observed on MEA medium (9.52–11.85 mm/day), while the lowest rates of all strains were observed on MSM medium (5.82–7.62 mm/day) (Table 1). For the Lat4 and CBS 524.82 strains, the highest growth rates were observed on PDA and on MEA, respectively. Colony morphology was similar for each isolate cultured on PDA and MEA, comprising zonation and pigmentation of aerial mycelia, visible also on the reverse side (Fig. 2a and b). Colonies on PDA and MEA observed after 8 days of growth were white, with a brownish-grey colouration around the agar plug and clear zonation, surface mycelium cottony, margin feathery. On MSM medium, mycelia were downy with zonation, but without the pigmentation and stromata (Fig. 2d), while mycelia cultured on CMA medium were very soft, almost transparent, with zonation but without pigmentation, and growth was almost entirely inside the agar (Fig. 2c). The growth rates for Lat4 and CBS 524.82 strains were always the highest on all media (Table 1). The fastest conidiomata formation, scattered in concentric circles on the mycelium surface, was observed for A1, A2, P1, B, D, ZA and ZB isolates during their growth on PDA and MEA medium, clearly visible from 14 day of incubation (d.o.i.). On MSM and CMA media, conidiomata formation was observed after 27 d.o.i. only for single strains. Within these structures, the conidia production was recorded on PDA and MEA (Fig. 2g), beginning from 16 d.o.i. for isolates A1, A2, P1, B, D, ZA and ZB. Alpha conidia were observed in the following size ranges: length of 6.5–8.5 μm, width of 2.9–4.3 μm (Table 2), hyaline, unicellular, fusiform, straight, mostly biguttulate, aseptate (Fig. 2f). Besides alpha conidia, the presence of filiform, unicellular, uncinated and eguttulate beta conidia, less abundant, were observed only for the D isolate (length of 16.2 μm ± 0.47 SEM; width of 1.9 μm ± 0.08 SEM; Fig. 2e). Strains CBS 524.82, CBS 160.32 and Lat4 did not produce conidia on any medium; however, strain Lat4 was able to produce pycnidia and release spore masses in the presence of carnation leaves on PDA only when mycelium was growing directly on plant tissue. Here, alpha and beta conidia were observed from the

Table 1 Mean growth rate of mycelium *D. vaccinii* and *D. eres* strains, growing on four different media: PDA, MEA, CMA and MSM

strain	Mean mycelium growth rate per 24 h in mm, ±SEM							
	PDA	Tukey HSD test for means	MEA	Tukey HSD test for means	CMA	Tukey HSD test for means	MSM	Tukey HSD test for means
A1	9,38 ± 0,38	9.27 ± 0.17 e*	10,33 ± 0,37	10.71 ± 0.12 fgh	8,38 ± 0,31	8.52 ± 0.14 d	7,62 ± 0,32	6.90 ± 0.11 ab
A2	9,58 ± 0,35		10,15 ± 0,35		8,68 ± 0,40		6,82 ± 0,27	
P1	9,20 ± 0,33		10,15 ± 0,29		8,60 ± 0,38		6,65 ± 0,34	
B	9,23 ± 0,45		11,13 ± 0,25		8,50 ± 0,40		6,92 ± 0,34	
D	8,77 ± 0,50		11,85 ± 0,37		7,87 ± 0,25		6,20 ± 0,25	
ZA	9,56 ± 0,51		10,75 ± 0,21		8,87 ± 0,36		6,98 ± 0,26	
ZB	9,13 ± 0,63		10,62 ± 0,26		8,77 ± 0,52		7,10 ± 0,29	
CBS 160.32 (*D. vaccinii*)	8,23 ± 0.35 cde		9.52 ± 0.31 defg		8.07 ± 0.28 bcde		5.82 ± 0.22 a	
Lat4	15.52 ± 0.42 k		13.7 ± 0.26 j		11.93 ± 0.43 hi		11.23 ± 0.30 ghi	
CBS 524.82 (*D. eres*)	9.47 ± 0.26 def		12.03 ± 0.26 ij		9.60 ± 0.33 defg		7.02 ± 0.28 abc	

SEM - standard error of the *mean*

n/o – not observed in this study

* the same letters indicate lack of statistically significant differences between values in columns, according to the Tukey HSD test

16th d.o.i., alpha were aseptate, hyaline, fusiform, straight, bi- or multiguttulate (length of 7.3 μm ± 0.08 SEM; width of 2.8 μm ± 0.04 SEM, Table 2), while beta were aseptate, hyaline, fusiform to hooked, eguttulate, smooth and uncinated (in size 22.5 × 1.7 μm). On the basis of examined morphological features of fungi cultured on agar media, the isolates A1, A2, P1, B, D, ZA and ZB were preliminary classified as the genus *Diaporthe,* and the genus affiliation of Lat4 strain was confirmed.

ITS rDNA analysis

The newly obtained nucleotide sequences of isolates A1, A2, P1, B, D, ZA and ZB, comprising the ITS1–5.8S rDNA–ITS2 region showed high similarity (99.6%) with the corresponding DNA region of reference strain CBS 160.32 of *D. vaccinii*, while the sequence of Lat4 strain showed 100% similarity with the sequence of *D. eres* CBS 134742 from Lithuania, 99.4% with sequence of *D. eres* CPC 23806 from Germany and 97.4% with sequences of *D. eres* CBS 524.82 from Poland and of ex-epitype *D. eres* CBS 138594 from Germany. The resulting dendrogram, comprising sequences of the analysed isolates and strains together with 23 sequences of other strains, reported on *Vaccinium* or other hosts, clustered the seven analysed isolates together with reference strains of *D. vaccinii* CBS 160.32 (Fig. 3), enabling classification of these isolates as *D. vaccinii* species. The strain Lat4 was clustered together with two strains of *D. eres*, which was supported by high bootstrap values (Fig. 3). However, other *D. eres* strains: CBS 524.82 from Poland, CBS 138594 from Germany and CPC 23809 from Lithuania segregated into another separate group, although in one clade. Based on this segregation, Lat4 was classified as *D. eres* species. Maximum likelihood analysis resulted in a dendrogram with the same topology and very similar bootstrap values as those obtained by neighbor-joining analysis. Therefore, the NJ-tree was chosen based on these two graphic presentations and is presented here.

Pathogenicity test

Leaking juice was observed on the cranberry fruits from the 8th day of inoculation, but typical swollen-appearing symptoms occurred on 13–16 day after inoculation (Fig. 2h and i). All ten examined *Diaporthe* strains were associated with the above described symptoms. Symptomatic fruits inoculated with *D. vaccinii* strains released viscid juice when touched. The identity of re-isolated fungi with those used for inoculation was confirmed.

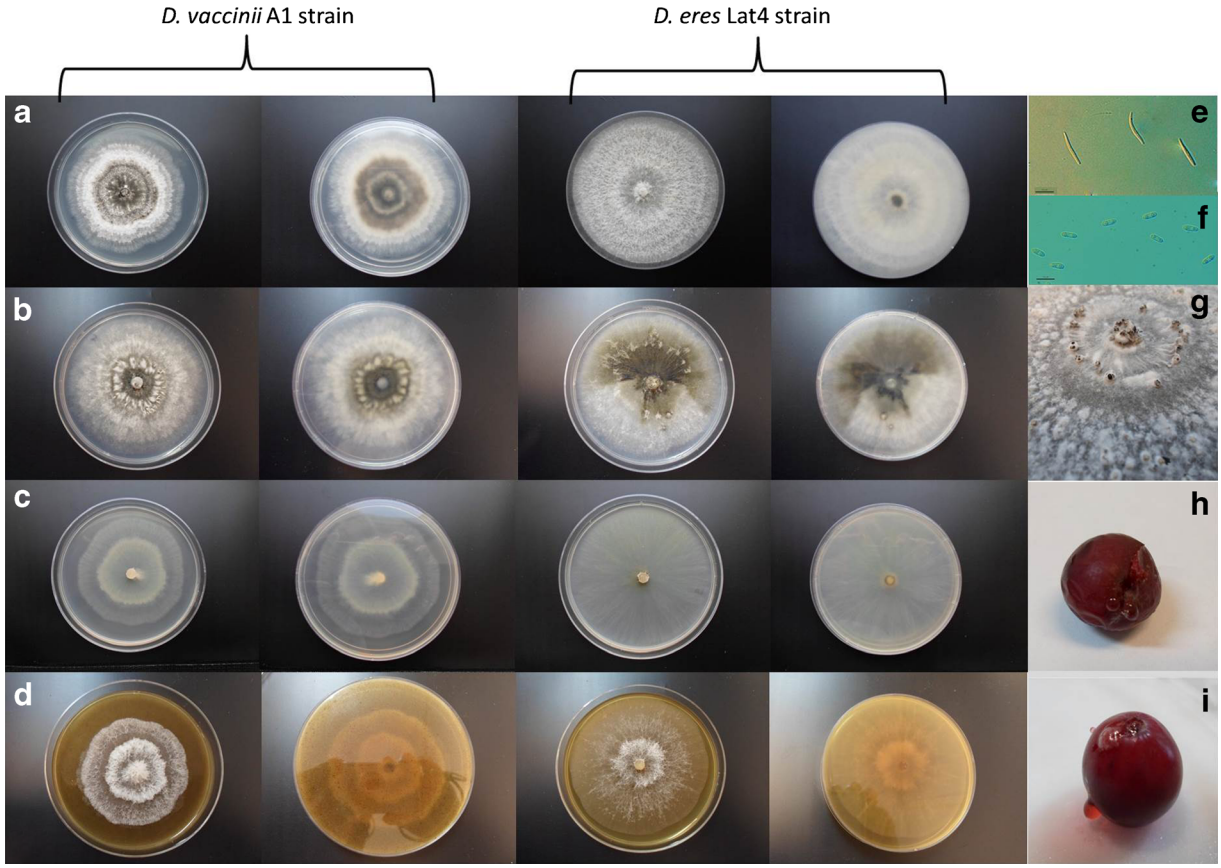

Fig. 2 Morphology of 8-day-old mycelia of *D. vaccinii* (A1) and *D. eres* (Lat4) strains growing on (**a**) PDA, (**b**) MEA, (**c**) CMA and (**d**) MSM media. (**e**) Beta and (**f**) alpha conidia of *D. vaccinii* strain D. (**g**) Conidiomata sporulating on PDA. (**h** and **i**) Symptoms of viscid rot on cranberry fruit after artificial infection with *D. vaccinii* spores, 8 days post inoculation

ISSR and RAPD PCR assays

A total of 105 reproducible bands were obtained in the ISSR-PCR assay, ranging in size from 300–4,000 bp, and 63 bands of 200–4,000 bp were obtained in the RAPD PCR assay. In both assays, a low variability was observed between eight *D. vaccinii* strains, while the amplification patterns obtained for *D. eres* strains (Lat4 and CBS 524.82) were clearly different from those obtained for *D. vaccinii* strains but similar to each other (Fig. 4).

Discussion

Although various fungal species can be associated with similar stem blight symptoms or fruit rot on *Vaccinium* spp. plants (Olatinwo et al. 2003; Espinoza et al. 2008; MacKenzie et al. 2009; Stromeng and Stensvand 2011; Wright and Harmon 2010; Lombard et al. 2014), in our study the causal agent on cranberry growing in one location in central Poland was classified as *D. vaccinii* Shear. Due to intensive plant material exchange and possible latent fungal infections, as well as close proximity of other, non-cultivated host plants, we should be aware of the possibility of introducing new and particularly harmful pathogenic fungal species to cultivated *Vaccinium* plants in our country. Cranberry fruits are infected at all stages of their development, but infections remain latent until fruit maturity. Infected berries become reddish-brown, soft, mushy, often splitting with leakage of juice (viscid rot) at harvest (Milholland and Daykin 1983). Similarly, in our study the first typical symptoms of viscid rot were observed on naturally affected mature fruits, sampled at harvest time, and they were also found in artificial infections.

Fungi from the genus *Phomopsis* are characterized by ostiolate, black conidiomata containing elongate, cylindrical phialides that may produce two types of hyaline, non-

Table 2 Mean conidia size of *D. vaccinii* and *D. eres* strains growing on PDA or PDA with carnation leaves (details in the text)

strain	Mean conidia size in μm, ±SEM			
	Alpha		Beta	
	length	width	length	width
A1	7,59 ± 0,12	4,28 ± 0,11	n/o	n/o
A2	7,60 ± 0,11	3,73 ± 0,09	n/o	n/o
P1	7,64 ± 0,13	3,77 ± 0,11	n/o	n/o
B	7,89 ± 0,17	2,97 ± 0,08	n/o	n/o
D	6,49 ± 0,12	2,93 ± 0,09	16,22 ± 0,47	1,91 ± 0,08
ZA	8,32 ± 0,11	3,11 ± 0,11	n/o	n/o
ZB	8,53 ± 0,10	3,17 ± 0,10	n/o	n/o
CBS 160.32 (*D. vaccinii*)	n/o	n/o	n/o	n/o
Lat4	7,33 ± 0,08	2,79 ± 0,04	22,46 ± 0,57	1,73 ± 0,05
CBS 524.82 (*D. eres*)	n/o	n/o	n/o	n/o

SEM - standard error of the *mean*

n/o – not observed in this study

septate conidia, named alpha and beta (Rehner and Uecker 1994). Beta conidia are not always produced, thus are not used for identification, but when present, they usually are unicellular, filiform and uncinate, 16.0–24.0 μm long and 1.0–1.5 μm wide (OEPP/EPPO 2009). Beta conidia, observed among our *D. vaccinii* strains only for D strain, showed similar dimensions as those previously reported, but the mean width was slightly out of the range. Both morphology in culture and dimensions of alpha conidia of examined *D. vaccinii* strains were similar to those observed by Farr et al. (2002a) who reported alpha conidia in ranges of 5.9–11.3 μm long x 2.1–3.9 μm wide and by OEPP/EPPO (2009) reporting alpha sizes of 6–11 × 2.5–4 μm, except from alpha conidia of strain A1, whose mean width was 4.28 μm. As reported by Udayanga et al. (2014), colonies of *D. eres* strains growing on PDA in the dark at 25 °C for 1 week showed growth rates of 5.5 ± 0.2 mm/day, and white, aerial, fluffy mycelium with dark pigmentation developing in the centre, producing abundant black stromata at maturity. Dimensions of conidia of the Lat4 *D. eres* strain were similar to those reported by Udayanga et al. (2014): alpha 6–9 μm long x 3–4 μm wide and beta: 18–29 μm long × 1–1.5 μm wide, with the exception of beta conidia width– slightly out of this range, while the mean growth rate on PDA was three times higher than reported, although Lat4 mycelium grown in light/dark regime, without producing any pycnidia. According to OEPP/EPPO (2009), *P. vaccinii* grows well on PDA, MEA and MSM up to 10–12 mm in diameter/day, where MSM medium is considered preferable for pycnidia and conidia production. In our study, newly obtained isolates of *D. vaccinii* showed fast growth on four examined media, with mean growth rates ranging from 6.9 ± 0.11 (MSM) to 10.7 ± 0.12 (MEA) mm/day, but no reproductive structures were observed on MSM medium. The 8h/16h light/dark regime was suitable to induce sporulation from the 16th day of growth on PDA and MEA. On the basis of colony colour growing on PDA, Kanematsu et al. (1999) designated two groups: W-type, producing white aerial hyphae with scattered stroma and irregular pycnidial locules, producing both alpha and beta conidia, and G-type, with few aerial hyphae, white to grey and forming abundant small pycnidial stroma with irregular pycnidial locules, producing only alpha conidia from the 16th day of incubation. Based on the whitish appearance with zonation, the *D. vaccinii* strains analysed in the study could be classified as the W-type colonies, although most of them only produced alpha conidia. The colony morphology of Lat4 and CBS 524.82 strains was clearly distinct from that of *D. vaccinii* strains, producing brownish inclusions within flat mycelia; however, because neither conidiomata nor conidia were produced during their growth on PDA, it is hard to reliably classify them based on colony type.

Since some of the morphological features depend on the cultural conditions and media used, including the zonation and pigmentation of aerial mycelia which may

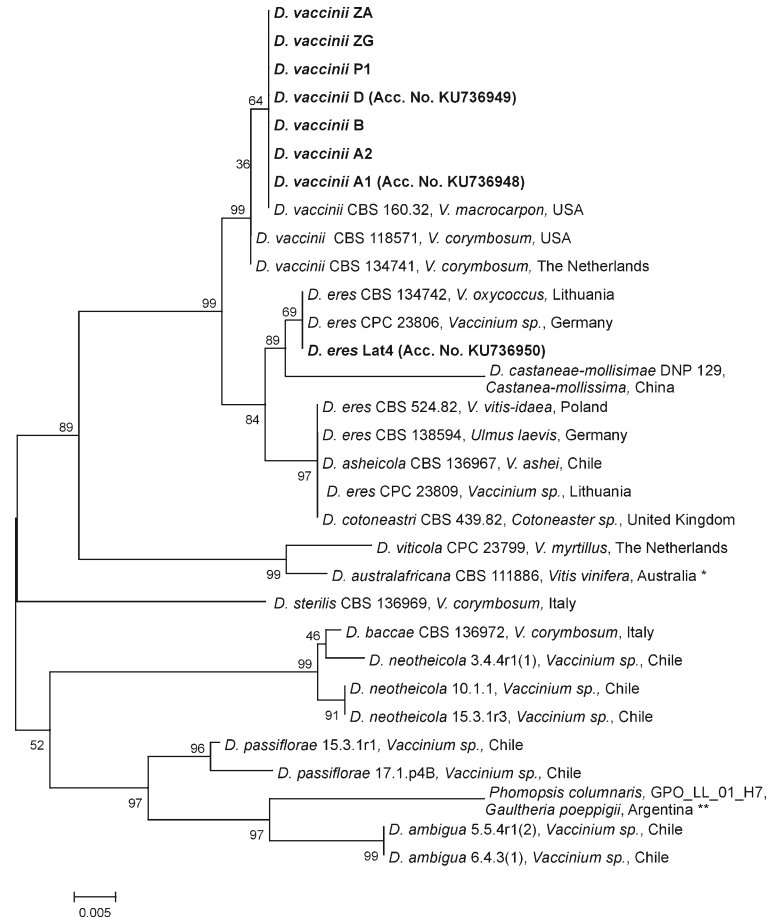

Fig. 3 The evolutionary history of 30 *Diaporthe* and 1 *Phomopsis* strains was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 0.271 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site (scale). The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 467 positions analysed in the final dataset. Among the sequences of strains originating from non-*Vaccinium* hosts, those with asterisks were reported on *Vaccinium* spp. elsewhere (* - Elfar et al. 2013, ** - Farr et al. 2002b)

be influenced by light (Brayford 1990), and the overlap in conidial size and features, as well as similar colours and structures of mycelia produced by different *Diaporthe* species were reported (Rehner and Uecker

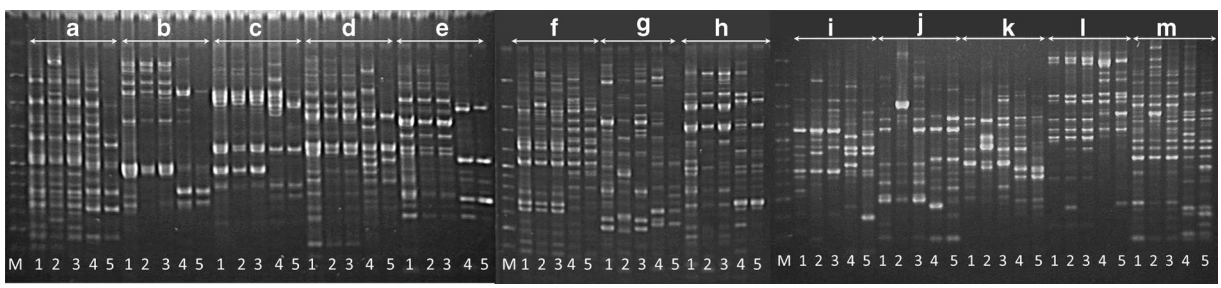

Fig. 4 Amplification patterns obtained by PCR with: (**a**) $(GCA)_5$, (**b**) $(AC)_8T$, (**c**) $(ACTG)_4$, (**d**) MF-8$(AG)_8C$, (**e**) $(GACA)_4$, (**f**) $(GACG)_4$, (**g**) Mf-10$(GTG)_5$ and (**h**) $(GTC)_6$ ISSR primers and (**i**) OPU-19, (**j**) OPT-07, (**k**) OPC-05, (**l**) OPA-11 and (**m**) OPAR-03 RAPD primers for representative strains of *Diaporthe* spp. used in the study. Lanes 1–5 contain DNA of: 1 – strain CBS 160.32, 2 – strain A1, 3 – strain D, 4- strain CBS 524.82, 5 – strain Lat4. M - O'GeneRuler™ 100 bp Plus DNA Ladder

1994; Santos and Philips 2009; Gomes et al. 2013; Udayanga et al. 2014), this makes it no longer possible to precisely distinguish the species of *Diaporthe* based on morphological features alone. The results of comparative study conducted here also indicate that both analysed *Diaporthe* species have similar culture morphologies and overlapping conidial dimensions, making differentiation ineffective.

A preliminary approach toward species recognition and characterization is usually based not only on morphology and culture characteristics, but also on host affiliation (Rehner and Uecker 1994; Santos and Philips 2009). However, recent studies of the *Diaporthe* genus show that since the same species can be found on different hosts and several species can occur on the same host, host association cannot be the determinant for species definition (Gomes et al. 2013). Our finding of strains belonging to two different *Diaporthe* species and derived from *Vaccinium macrocarpon* plants is congruent with this statement. Recent recommendations are to validate conventional diagnostic methods with molecular techniques. Those based on single- or multi-locus genetic information, applying among others ITS rDNA, partial sequence of EF 1-α or actin, DNA-lyase, mating type, beta-tubulin or calmodulin genes (Udayanga et al. 2014) are particularly helpful in redefining species in this genus, especially when cryptic species need to be resolved. The ITS region of ribosomal gene clusters, although commonly used to infer phylogenies in fungi (Nilsson et al. 2008), was recently demonstrated to show high within-species variation in some *Diaporthe/Phomopsis* species (Santos et al. 2010). However, in the study performed by Diogo et al. (2010) it was still possible to distinguish species in this genus using ITS data, due to little species variation of *P. amygdali*. The phylogenetic analysis performed by Udayanga and others (2014) showed that, beside several other analysed DNA regions, clear (99-100% bootstrap value) distinction of *D. vaccinii* strain CBS 160.32 from the closely related *D. alleghaniensis* strain, based on rDNA ITS sequence only. A similar trend was observed in our phylogenetic analysis: seven examined strains formed a uniform clade with the CBS 160.32 strain and the same phylogenetic group with other *D. vaccinii* strains, and were clearly distinct from other clades and groups of species containing *D. eres* strains. Thus, in our study, it was possible to classify those strains as *D. vaccinii* species.

The species grouping based on ITS sequences obtained by Diogo et al. (2010) and Santos and Phillips (2009) was consistent with the groups revealed using the ISSR-PCR assay. In our study, the ISSR-PCR and RAPD assays provided additional confirmation of genetic distance existing between *D. vaccinii* and another closely related *Diaporthe* species (occurring on cranberry) and justifies their affiliation to distinct species. The reciprocal similarity of all *D. vaccinii* strains, including the reference CBS 160.32 strain, was clear in both analyses. Although the Lat4 strain showed similar amplification patterns as those of CBS 524.82 strain belonging to *D. eres* (Lombard et al. 2014), was not directly grouped according to ITS sequence with this strain, but was clustered with *D. eres* strains from Lithuania and Germany. These results may derive from the sequence variability within the ITS1-5.8S-ITS2 rDNA region within the *D. eres* species, what is consistent with the finding of Udayanga et al. (2014), where sequence heterogeneity of ITS within the species was observed. Although morphological and genetic characteristics of the Lat4 strain indicated its affiliation to the *Diaporthe* genus, and, the most probably, to *D. eres* species, a multi-locus data analysis involving at least EF1-α, Apn2 and HIS genes, as proposed by Udayanga et al. 2014, is required for better resolving the genetic distinction between Lat4 and some *D. eres* strains.

Conclusions

Preliminary species-recognition criteria of the *Diaporthe* genus has been based on morphological features, colony characteristics and host affiliation. However, the current status of the taxonomic position of *Diaporthe* spp. primarily focuses on molecular data obtained from phylogenetic species recognition (Udayanga et al. 2012, 2014). On the basis of studies by conventional and molecular biology methods, seven fungal isolates obtained from cranberry plants showing viscid rot and upright dieback symptoms, were identified as *Diaporthe vaccinii* Shear. This is the first study and confirmed identification of this species in Poland, which is a quarantine organism from A2 list (EPPO 2014). This pathogen was detected with restricted distribution on one cranberry plantation in the center of the country, and the entire affected plant material was destroyed. This is also the second after Lombard et al. (2014) report of the occurrence of *D. eres* on *Vaccinium* plant.

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