Cylindrocarpon Species Associated with Black-Foot of Grapevine in Northeastern United States and Southeastern Canada

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Abstract: Black-foot disease of grapevine is caused by a complex of soilborne fungi. The most common and virulent species, which are found across all major grapegrowing regions of the world, are *Cylindrocarpon liriodendri* (*C. liriodendri*) and *C. macrodidymum* (teleomorph = *Neonectria*). Other species with a more limited distribution and uncertainty regarding their pathogenicity include *C. destructans*, *C. obtusisporum*, *C. pauciseptatum*, *Campylocarpon fasciculare* (*C. fasciculare*), and *C. pseudofasciculare*. The goal was to identify the species associated with black-foot disease in vineyards of the northeastern United States (U.S.) and southeastern Canada as such regions have not previously been surveyed. Recent expansion of winegrape acreage in these regions necessitates a clear understanding of the disease risks. Eleven U.S. states and two Canadian provinces were surveyed. Genus-level identification was based preliminarily on colony morphology. Species-level identity was based on phylogenetic analysis of two nuclear loci, 5.8S rDNA and ß-tubulin, using voucher specimens and sequences with high sequence identity. We report for the first time from Canada recovery of *C. liriodendri*, *C. macrodidymum*, and *C. destructans* from symptomatic grapevines. Also reported are species not previously identified from black-foot symptomatic grapes anywhere in the world, including *C. didymum* and a *Neonectria mammoidea*-like species. Results suggest that local viticultural practices, primarily burying the vine underground during winter, may create injuries, and thus exacerbate infection by wound pathogens such as *Cylindrocarpon*. Overall this work improves the knowledge of black-foot disease in these nascent grapegrowing regions and will be helpful to growers in their decisions regarding viticultural practices, planting, and disease management.

Key words: grapevine, viticulture, wood disease, black-foot disease, *Cylindrocarpon*

Black-foot disease causes root and crown rot in grapevine with substantial economic losses because of replanting costs (Halleen et al. 2004, Petit and Gubler 2005, Scheck et al. 1998, Whitelaw-Weckert et al. 2007). The disease primarily affects young vines up to 8 years old, and symptoms include black, sunken, and necrotic lesions on roots and stunted grapevines with leaves scorched by water stress (Scheck et al. 1998). This disease is caused by several fungal taxa belonging to two genera. *Cylindrocarpon liriodendri* (*C. liriodendri*, teleomorph: *Neonectria liriodendri*) and *C. macrodidymum* (teleomorph: *Neonectria macrodidyma)* are known causal agents (Halleen et al. 2004, 2006, Petit and Gubler 2005, 2007), but other *Cylindrocarpon* and *Campylocarpon* species are also associated with this disease, including *C. destructans* (teleomorph: *Neonectria radicicola*), *C. obtusisporum*, *C. pauciseptatum*, *Campylocarpon fasciculare* (*C. fasciculare*), and *C. pseudofasciculare* (Grasso and Lio 1975, Halleen et al. 2004, Rego et al. 2000, Schroers et al. 2008).

Although the disease cycle of these pathogens on grapevine is poorly known, their behavior on other hosts has been studied in detail (Booth 1966, Brayford 1992). These fungi produce slimy spores that are dispersed in free water and chlamydospores that allow the organism to survive in the soil. After a spore comes in contact with the root surface, the hypha enters the roots and decomposes the cortex cells, eventually restricting the uptake and subsequent transport of soil-derived nutrients to the shoots and leaves and the photosynthate to the roots. Over time, vines become more and more stunted, as their capacity continually declines. In vitro screening of fungicides against mycelial growth of these pathogenic fungi demonstrated the high effectiveness of prochloraz manganese chloride and benomyl against both *Campylocarpon* and

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Acknowledgments: This research was funded by grant number 2008-51100-19334 to K. Baumgartner and P.E. Rolshausen from the USDA, National Institute of Food and Agriculture.

The authors thank the extension specialists, viticulturists, and vineyard owners for their assistance during this survey and the diagnostic lab of the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation for providing logistic support during the processing of the plant samples in Québec.

Sequence data from this article have been deposited with the GenBank Data Libraries under accession numbers HQ338494–HQ338511.

Manuscript submitted Oct 2010, revised Dec 2010, accepted Feb 2011. Publication costs of this article defrayed in part by page fees.

doi: 10.5344/ajev.2011.10112

Cylindrocarpon species and flusilazole and imazalil against *Cylindrocarpon* species alone. However, field trials testing the preventive effects of these chemicals yielded inconsistent results (Halleen et al. 2007). Hot water treatments are, yet, the most effective and consistent at eradicating the pathogens from dormant cuttings before planting (Halleen et al. 2007, Gramaje et al. 2010). But these treatments are relatively ineffective to prevent further infection in natural vineyard settings when the disease already inhabits the soil. Preventive treatments of grapevine root cuttings with arbuscular mycorrhizae before planting can lower disease severity in greenhouse (Petit and Gubler 2006), but the long-term outlook of this control strategy has not been evaluated.

Black-foot disease was first reported in France in 1961 (Maluta and Larignon 1991) and has now been identified in all major viticulture regions worldwide, including Italy (Grasso and Lio 1975), Portugal (Rego et al. 2000), Spain (Alaniz et al. 2009), South Africa and New Zealand (Halleen et al. 2004), Australia (Whitelaw-Weckert et al. 2007), Chile (Auger et al. 2007), Uruguay (Abreo et al. 2010), California (Petit and Gubler 2005, 2007, Scheck et al. 1998), and Lebanon (Choueiri et al. 2009). *Cylindrocarpon destructans* was originally identified as the causal agent of black-foot disease (Maluta and Larignon 1991), but the status of *C. destructans* as the causal agent has since been questioned. Isolates previously identified from French and Portuguese vineyards as *C. destructans* were shown to be *C. liriodendri,* based on morphological characters and sequence data (Halleen et al. 2006). *Cylindrocarpon liriodendri*, and not *C. destructans,* was later reported from grape in California (Petit and Gubler 2007), Spain (Alaniz et al. 2009), Australia (Whitelaw-Weckert et al. 2007), and Uruguay (Abreo et al. 2010). There is similar confusion in the literature with *C. obtusisporum,* which was reported to be associated with black-foot disease in Sicily (Grasso and Lio 1975) and California (Scheck et al. 1998). More detailed surveys of vineyards in California, coupled with the use of DNA-based methods of identification (Petit and Gubler 2005), later identified only *C. macrodidymum*, suggesting that the original reports of *C. obtusisporum* were mistaken. *Cylindrocarpon macrodidymum* and *C. pauciseptatum* are sister taxa of *C. destructans* (Halleen et al. 2004, Petit and Gubler 2005, Schroers et al. 2008). *Cylindrocarpon macrodidymum* is ubiquitous to all grapegrowing regions worldwide, as compared to the more restricted known range of *C. pauciseptatum* on grape in Slovenia, New Zealand, and Uruguay. Finally, *C. fasciculare* and *C. pseudofasciculare* have only been found in vineyards in South Africa, Australia (Halleen et al. 2004), and Uruguay (Abreo et al. 2010). *Campylocarpon* is a sister group to *Cylindrocarpon*. The pathogenicity of *C. fasciculare*, *C. pseudofasciculare,* and *C. pauciseptatum* to grapevine has not been demonstrated.

Studies on black-foot disease are primarily from Mediterranean regions, which differ from cold-weather grapegrowing regions in terms of climate, viticulture practices, and grapevine varieties. To our knowledge, black-foot disease has never been reported in vineyards of the northeastern U.S. or southeastern Canada. However, *C. destructans* is a serious problem in commercial production of North American ginseng, *Panax quinquefolius* L. (Seifert et al. 2003), conifers (Hamelin et al. 1996), and in fruit trees from nursery stocks (Traquair and White 1992) in this region. In view of the rising significance of *Cylindrocarpon* associated with black-foot disease in replant vineyards worldwide and the increasing interest in expansion of cold-climate viticulture, this study was initiated to identify *Cylindrocarpon* species and to evaluate their diversity in vineyards of the northeastern U.S. and southeastern Canada. This work will increase knowledge about black-foot disease in this relatively new grapevine production region, thus helping growers with decisions concerning viticultural practices and disease management.

Materials and Methods

Grapevine sampling and fungal isolation. Seventy vineyards in 11 northeastern U.S. states (Virginia, VA; Maryland, MD; New Jersey, NJ; New York, NY; Connecticut, CT; Massachusetts, MA; Rhode Island, RI; New Hampshire, NH; Vermont, VT; Ohio, OH; and Michigan, MI) and two provinces of Canada (Ontario, ON, and Québec, QC) were surveyed. The sampling included grapevines with wood cankers and dieback on spurs, cordons, and trunks (770 samples). In 14 of the 70 vineyards from three states (VA, NY, MI) and one province (QC), the diseased wood samples (n = 90) were collected from the graft union, collar, or roots because the grapevines were either too young (one to two years old) or not trained with cordons and trunks. The affected grapevines in these vineyards showed signs of low vigor and overall decline. Samples were collected from the diseased wood parts (i.e., canker, necrosis, discoloration) that were revealed by digging the roots out from the soil and cutting the trunk transversally (Figure 1). Fungal isolates were recovered from diseased wood after plating wood chips (~3 x 3 x 3 mm) sampled from the margin of the necrosis on potato dextrose agar (PDA) amended with tetracycline (100 ppm). After two weeks of growth at room

Figure 1 Cross-section at the collar of a trunk of grapevine variety Gamay in a vineyard in Québec showing sign of wood canker.

temperature in the dark, fungal isolates were subcultured on PDA to obtain pure cultures.

DNA isolation and sequencing. DNA was obtained from pure fungal cultures using a Qiagen (Valencia, CA) DNA extraction kit following manufacturer's instructions. The nuclear rDNA internal transcribed spacer region (ITS) and nuclear gene ß-tubulin were sequenced using primer pairs ITS1/ITS4 (White et al. 1990) and ßt2a/ßt2b (Glass and Donaldson 1995). Both regions were PCR-amplified in a 25-µL reaction following conditions as published elsewhere (Petit and Gubler 2005). Amplification was verified on a 1x Tris-borate EDTA 1% agarose gel stained with ethidium bromide and visualized under UV light. PCR products were cleaned using a Qiagen DNA purification kit following manufacturer's instructions and were sequenced in both forward and reverse directions at the Genomics Core sequencing facility at the University of California, Riverside.

Phylogenetic analysis. Extended contiguous sequences, obtained by joining overlapping forward and reverse sequences, were edited using Sequencher, ver. 4.1 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned with Clustal X, ver. 1.6 (Thompson et al. 1997), and corrected visually. Additional sequences representing type specimen or specimen that were closely related to the sequences of our collected samples were obtained from GenBank and added to the alignment for comparison (Table 1). Separate analyses were run for the rDNA-ITS and for the ß-tubulin data sets. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set. The bootstrap values were inferred from 1,000 replicates (Felsenstein 1985). *Fusarium solani* was used as an outgroup.

Results and Discussion

Cylindrocarpon was isolated from diseased grapevines in three vineyards in Québec on varieties Seyval blanc, Sabrevois, Chelois, Gamay, and Vidal blanc and in one vineyard in Long Island, NY, on rootstock variety 101-14 Millardet et de Grasset (Table 2). All these varieties were own-rooted. These grapevines, which were characterized by signs of low vigor, were found on soil with poor drainage. From the 90 wood samples collected from the collar, the graft union, and the roots of affected grapevines, 12 (13%) were infected with *Cylindrocarpon* species. The data presented here also indicate

Table 1 Related taxa used for comparison with field strains collected from grapevine.

Species[a]	Isolate no.	Host	Origin[b]	Collector	GenBank accession no.	
					ITS	β-tubulin
C. cylindroides	CR6	*Pseudotsuga mensiezii* (Douglas-fir)	BC, Canada	Axelrood	AY295301	AY297172
C. pauciseptatum	CBS113550	*Vitis* sp.	New Zealand	na[c]	EF607080	EF607069
C. pauciseptatum	KIS10778	*Vitis* sp.	Slovenia	na	EF607083	EF607073
Camp. fasciculare	CBS112614	*V. vinifera*	South Africa	Halleen	AY677302	AY677220
Camp. fasciculare	CBS113560	*V. vinifera*	South Africa	Halleen	AY677304	AY677217
Camp. pseudo.	CBS112592	*V. vinifera*	South Africa	Halleen	AY677305	AY677215
Camp. pseudo.	CBS112679	*V. vinifera*	South Africa	Halleen	AY677306	AY677214
Cylindrocarpon sp./ *N. mammoidea* group	CCFC226730	*Picea glauca* (white spruce)	QC, Canada	Hamelin	AY295334	na
Cylindrocarpon sp./ *N. mammoidea* group	JAT1401	*Pyrus communis* (pear)	ON, Canada	Traquair	AY295335	na
Fusarium solani	MR436	*Arachis hypogaea* (peanut)	Argentina	na	GQ121891	GQ121906
N. /C. liriodendri	FR102	*Vitis* sp.	France	Larignon	AY997533	AY997567
N./C. liriodendri	USSO150	*Vitis* sp.	CA, USA	Petit	AY997544	AY997570
N./C. macrodidymum	CBS112605	*Vitis* sp.	South Africa	Halleen	AY997549	AY677230
N./C. macrodidymum	CCFC144524	*Vitis* sp.	ON, Canada	na	AY295332	AY297198
N./C. macrodidymum	USSL152	*Vitis* sp.	CA, USA	Petit	AY997556	AY997573
N. radicola/ C. destructans	1557	*Panax quinquefolius* (North American ginseng)	ON, Canada	Reeleder	AY295329	AY297195
N. radicola/ C. destructans	CCFC139398	*Prunus cerasus* (sour cherry)	ON, Canada	na	AY295330	AY297196
N. veuillotiana/ C. candidulum	H224	na	Japan	Hirooka	na	AB237468
N. veuillotiana/ C. candidulum	H 97519I	na	China	na	EF121866	na

[a]*N.*: *Neonectria*; *C.*: *Cylindrocarpon*; *Camp.*: *Campylocarpon*; *pseudo.*: *pseudofasciculare.*
[b]BC, British Columbia; QC, Québec; ON, Ontario; CA, California.
[c]na: indicates information is not available.

that *Cylindrocarpon* was previously isolated from several cultivated and noncultivated plant hosts in Canada, including *Vitis, Pyrus, Prunus, Panax,* and *Picea* species (Table 1), but not all isolates were identified to the species level. Such taxonomic distinctions are important because virulence is variable among *Cylindrocarpon* species (Alaniz et al. 2009, Seifert et al. 2003) and growers need to know the risks of planting into infected soil.

The two main species of *Cylindrocarpon* known to cause black-foot in grapevine are *C. liriodendri* and *C. macrodidymum*. Here we report for the first time the presence of *C. liriodendri* associated with symptomatic vines in Canada (Figure 2). In this field survey, *C. macrodidymum* was not recovered, but it was identified among isolates that were previously recovered from symptomatic grapevines in Canada (Seifert et al. 2003). These findings expand the known geographic range of *C. liriodendri* and *C. macrodidymum* to southeastern Canada and support the evidence that they are ubiquitous organisms with a cosmopolitan distribution. Previous studies showed that *Cylindrocarpon* is commonly present in the wood as early as the propagation stage in nurseries studies (Dubrovsky and Fabritius 2007), which suggests that these pathogens may have been introduced to disease-free sites via infected plant material.

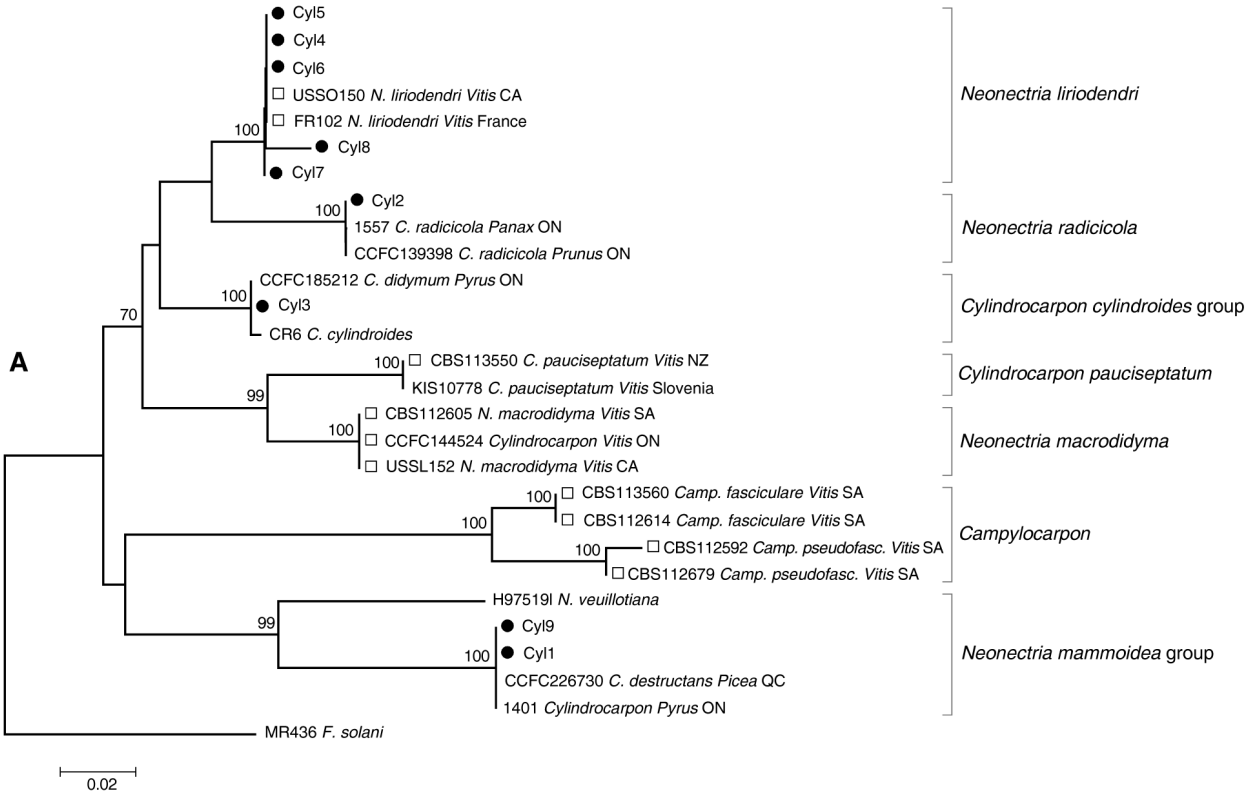
Our study confirms that *C. destructans* is associated with black-foot of grapevine and also reports two taxa (*C. didymum* and *Neonectria mammoidea* group) not previously recovered from symptomatic grapevines (http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm). *Cylindrocarpon didymum* (Cyl3) within the *C. cylindroides* group is closely related to an isolate from pear in Ontario, and this is the first report of it from grapevine (Figure 2). Two isolates (Cyl9 from QC and Cyl1 from NY) were found within the *Neonectria mammoidea* group (Booth 1966). In our analysis Cyl9 and Cyl1 clustered with two taxa isolated from *Picea* and *Pyrus* in Canada (Seifert et al. 2003), of which the former was misidentified as *C. destructans* (Figure 2A). Future studies will need to confirm the species name of these isolates. One specimen (Cyl2) belongs to the *C. destructans* complex (clade IIIb), as described elsewhere (Seifert et al. 2003) (Figure 2). This group of *C. destructans* contains isolates associated with diverse hosts, such as ginseng and hardwood tree species, and appears to be geographically restricted to Canada, based on comparison with related taxa from Korea, Japan, Indonesia, and New Zealand.

These data show the diversity of *Cylindrocarpon* species associated with black-foot disease in vineyards in southeastern Canada. The species range includes the two most common (i.e., *C. liriodendri* and *C. macrodidymum*), which have broad geographic distribution, and two not previously identified from grapevines (i.e., *C. didymum* and *Neonectria mammoidea*-like species), which have a narrow geographic range. In Québec, it was common to recover several *Cylindrocarpon* species from one vineyard. One possible explanation for this pool of diversity of fungal species is that it might be related to the wide range of *Vitis* genotypes growing in these regions. Indeed, *Vitis* species include imported commercial *V. vinifera* grafted on a rootstock, *Vitis* interspecific hybrids usually not grafted, and native grapevines such as *V. labrusca, V. riparia, V. rupestris,* and *V. aestivalis*. Some of these taxa were likely introduced with imported *V. vinifera* and/or interspecific hybrids plant materials (i.e., *C. liriodendri* and *C. macrodidymum*), while the others may be endemic to these regions and may have coevolved with their native host plant (i.e., *C. didymum* and *Neonectria mammoidea*-like species). The development of agricultural practices in these regions and/or the preadaptation of these endemic pathogens to closely related hosts such as native *Vitis* might have promoted their emergence through host jump (Stukenbrock and McDonald 2008). The finding of genetically isolated groups composed of isolates associated with various agricultural crops is supportive of the hypothesis of endemic pathogens jumping from native to cultivated crops. Unfortunately, the pathogenicity of these isolates could not be tested because permit requests for importation to the U.S. were not obtained. Pathogenicity tests of these taxa are needed. In the meantime, the finding of *C. liriodendri* and *C. macrodidymum* in southeastern Canada

Table 2 *Cylindrocarpon* species recovered from grape.

Species[a]	Isolate no.	Host	Origin[b]	Collector	GenBank accession no.	
					ITS	β-tubulin
C. cylindroides group	Cyl3	*Vitis* hybrid Seyval blanc	QC, Canada	Rolshausen	HQ338496	HQ338505
Cylindrocarpon sp./ *N. mammoidea* group	Cyl1	*Vitis* hybrid 101-14	NY, USA	Rolshausen	HQ338494	HQ338503
Cylindrocarpon sp./ *N. mammoidea* group	Cyl9	*Vitis* hybrid Sabrevoix	QC, Canada	Rolshausen	HQ338502	HQ338511
N./C. liriodendri	Cyl4	*Vitis* hybrid Seyval blanc	QC, Canada	Rolshausen	HQ338497	HQ338506
N./C. liriodendri	Cyl5	*V. vinifera* Gamay	QC, Canada	Rolshausen	HQ338498	HQ338507
N./C. liriodendri	Cyl6	*Vitis* hybrid Vidal	QC, Canada	Rolshausen	HQ338499	HQ338508
N./C. liriodendri	Cyl7	*V. vinifera* Gamay	QC, Canada	Rolshausen	HQ338500	HQ338509
N./C. liriodendri	Cyl8	*Vitis* hybrid Seyval blanc	QC, Canada	Rolshausen	HQ338501	HQ338510
N. radicola/ C. destructans	Cyl2	*Vitis* hybrid Seyval blanc	QC, Canada	Rolshausen	HQ338495	HQ338504

[a]*N.*: *Neonectria*; *C.*: *Cylindrocarpon.*
[b]QC: Québec; NY: New York.



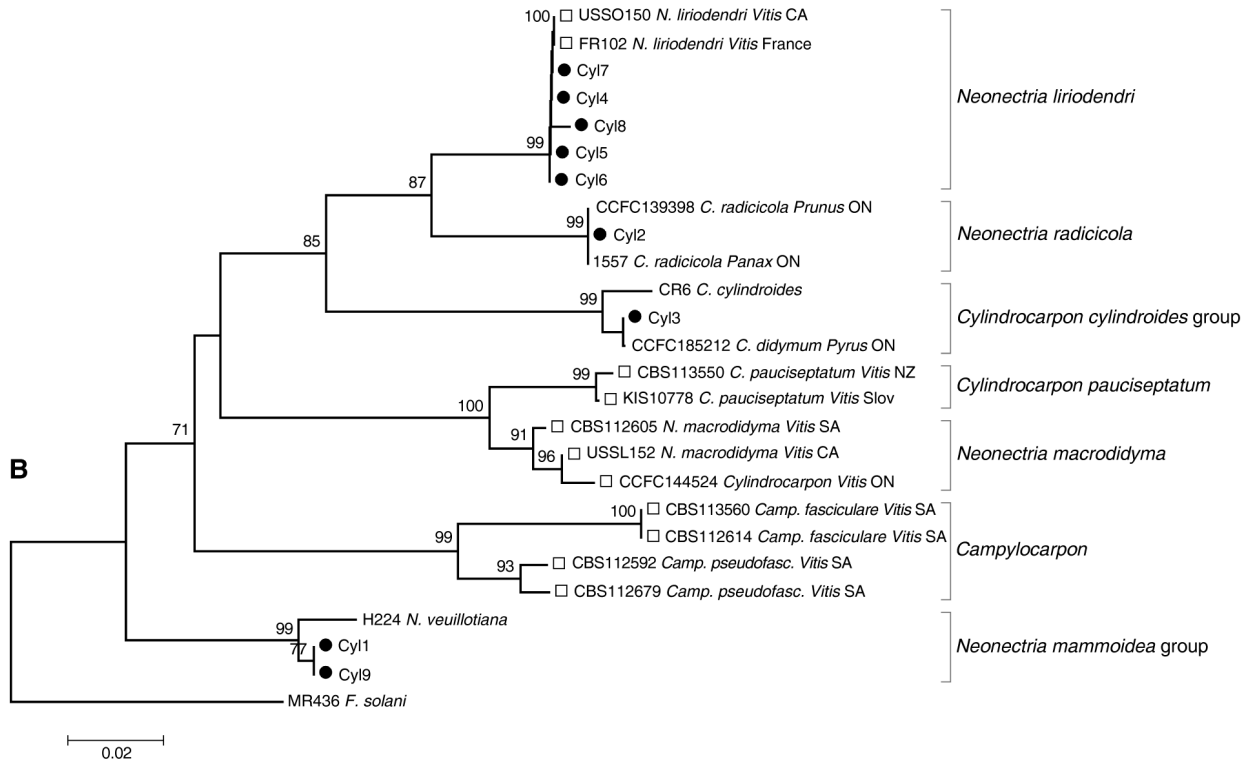


Figure 2 Evolutionary relationships of *Cylindrocarpon* derived from the present study with other *Cylindrocarpon* and *Campylocarpon* taxa based on (**A**) the internal transcribed spacer1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 DNA sequence data and (**B**) the β-tubulin DNA sequence. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown above the branches (Felsenstein 1985). *Fusarium solani* was used as an outgroup. In phylogenetic trees, isolates are indicated by their isolate numbers. Full circles designate isolates originated in the present study. Open squares indicate isolates previously isolated from grapevine plants.

represents a risk of disease emergence, as they are known virulent species on grape in other regions.

Of the 23 wood samples collected in Québec vineyards, 11 (48%) were infected with *Cylindrocarpon*, a much higher rate than any other state or province surveyed. These results suggest that the viticultural practices of these regions exacerbated the disease pressure. *Vitis* interspecific hybrids are commonly grown in cold-climate regions because they better tolerate the extremely cold winter without suffering frost injuries and they can produce a ripened crop in the relatively short growing seasons. In Québec the majority of the interspecific hybrids planted are only mildly cold tolerant (e.g., Vidal blanc, Seyval blanc, De Chaunac, Baco, Geisenheim, Lucie Kulhmann, and Maréchal Foch), and thus are commonly buried underground before winter (Figure 3). These varieties are traditionally grown because they offer good fruit yield and quality even though growing them is more challenging than cold-tolerant varieties. However, this viticulture practice entails an increased chance of mechanical injuries, which facilitates contamination with organisms residing in the soil, such as *Cylindrocarpon*, when they come into wound contact. Cold-tolerant interspecific hybrids developed at Cornell University (Geneva, NY) and the University of Minnesota (St. Paul) are now commercially available and are increasingly planted in Québec to satisfy the growing demand of winegrapes. Cold-hardy varieties (e.g., Frontenac, Traminette, Marquette, St. Croix, St. Pépin, Louise Swenson, and Sabrevois) are more suited to the local climate such that they do not need to be buried underground in the winter, thereby limiting labor and reducing the production costs. Black-foot disease caused by the *Cylindrocarpon* species complex is mostly an economic problem in replant vineyards when grapevines are under stress (Scheck et al. 1998). When grapevines are exposed to the pathogen, they get infected and die after few years into production. As the grapevine industry in Québec, and in the northeastern U.S. in general, replants new grape varieties, black-foot disease may become a more serious issue in these newly establishing vineyards.

Figure 3 Comparison of two different viticultural practices showing a cold-tolerant variety Frontenac grown aboveground (left) and a mildly cold-tolerant variety Seyval blanc buried underground (right) during winter in Québec.

Conclusion

The purpose of our study was to identify *Cylindrocarpon* species from grape in the northeastern United States and southeastern Canada. The data showed that the two main pathogens known to cause black-foot, *C. liriodendri*, and *C. macrodidymum*, were found in Canada but not in the northeastern U.S. In addition, *C. destructans*, *C. didymum* and *Neonectria mammoidea*-like species were also isolated from vineyards in Québec. The high disease incidence as observed in Québec may be due to the local viticulture practices, while the diversity of *Cylindrocarpon* species identified could be related to the range of grapevine genotypes growing in these regions. These data should reinforce the awareness of growers to ensure the planting of disease-free material at a vineyard site in order to avoid disease emergence, as none of the known pathogenic *Cylindrocarpon* species (*C. liriodendri* and *C. macrodidymum*) were found in the northeastern U.S. vineyards surveyed and in most vineyards in southeastern Canada. Also, as some of these species identified are new to grape in Canada, regulations on import of nursery plant material might have to be updated to limit the risks of disease emergence in other grapegrowing regions, upon confirmation of the pathogenicity of these fungal taxa.

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