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Characterization of *Cytospora* isolates from wood cankers of declining grapevine in North America, with the descriptions of two new *Cytospora* species

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Cytospora species are ubiquitous pathogens of numerous woody plants, causing dieback and wood cankers in agronomic crops, timber trees and wildland trees (e.g. *Prunus*, *Eucalyptus* and *Salix*, respectively). *Cytospora chrysosperma*, *C. cincta* and *C. leucostoma* have been reported from grapevines in Iran showing symptoms of one or more recognized trunk diseases (esca, botryosphaeria-, eutypa- and phomopsis diebacks); however, only *C. chrysosperma* was shown to be pathogenic to grapevine. To understand the potential role of *Cytospora* species in the grapevine trunk-disease complex, 21 *Cytospora* isolates were examined that were recovered from dieback and wood cankers of *Vitis vinifera* and *Vitis* interspecific hybrids in seven northeastern U.S. states and two Canadian provinces. Phylogenetic analyses of ITS and translation elongation factor 1-α identified two novel species: *Cytospora vinacea* sp. nov. and *Cytospora viticola* sp. nov. Differences in culture morphology and conidial dimensions also distinguished the species. When inoculated to the woody stems of potted *V. vinifera* 'Thompson Seedless' in the greenhouse, both species were pathogenic, based on development of wood lesions and fulfilment of Koch's postulates. *Cytospora viticola* was the most virulent based on lesion length at 12 months post-inoculation. As cytospora canker shares some of the same general dieback-type symptoms as botryosphaeria-, eutypa- and phomopsis diebacks, it may be considered part of the grapevine trunk-disease complex in eastern North America.

Keywords: cytospora canker, grapevine trunk disease, systematic taxonomy, Valsaceae, *Vitis*

Introduction

The genus *Cytospora*, described in 1818, encompasses approximately 110 species (Kirk *et al.*, 2008) with diverse ecological habits, including endophytes isolated from the bark, xylem and leaves of symptomless plants (Bills, 1996; Gonzalez & Tello, 2011), saprobes colonizing the wood of dying trees (Christensen, 1940), and destructive canker pathogens causing dieback (known as cytospora-, valsa-, leucostoma- or perennial canker) of more than 85 woody plant species (Sinclair *et al.*, 1987; Adams *et al.*, 2005, 2006). Pathogenic species of *Cytospora* colonize the periderm and underlying sapwood of angiosperms, causing brown/black discolouration of the wood and loss of hydraulic conductivity within the xylem. Many pathogenic *Cytospora* species are thought to be opportunistic, whereby the pathogens are considered as facultative wound parasites that attack weakened hosts (Christensen, 1940).

Cytospora canker diseases can be devastating to perennial crops, such as *Prunus persica*, *Prunus armeniaca*, *Prunus avium*, *Juglans regia* and *Malus* spp. (Farr & Rossman, 2015). Infections are generally initiated through cracks and wounds to the bark created by wind, pruning wounds and freeze damage, and breakage of shade-weakened twigs and branches (Tekauz & Patrick, 1974; Biggs, 1989). The disease mainly impacts branches, but can cause more destructive infections in the larger scaffolds, thus limiting the longevity and productivity of orchards (Chang *et al.*, 1991). Tree mortality in affected orchards may reach up to 5% per year, with compounding effects due to the perennial nature of the wood infections (Grove & Biggs, 2006). Cytospora canker also affects forest and shade trees, such as *Alnus incana* subsp. *tenuifolia* (Worrall *et al.*, 2010), *Sophora japonica* (Fan *et al.*, 2014), *Eucalyptus*, *Populus* and *Salix* (Adams *et al.*, 2005, 2006).

Species differentiation in *Cytospora* has relied mainly on conidiomata/ascomata morphological characters, including locule shape/organization and spore

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dimensions, but this approach is confounded by many examples of overlap (Adams *et al.*, 2002; Wang *et al.*, 2011). Defago (1935) was the first to detail the futility of morphological characters in delimiting species of *Cytospora*. Additionally, the monograph by Spielman (1985) asserted that the asexual state of *Cytospora leucosperma* was indistinguishable from that of many other species of *Cytospora*, supporting the need for molecular data to properly distinguish taxa that share similar morphologies. Traditionally, sexual states of *Cytospora* have been classified into several genera including *Valsa*, *Leucostoma*, *Valsella* and *Valseutypella*. Tulasne & Tulasne (1863) were first to postulate that the sexual state *Valsa* and asexual state *Cytospora* are two morphs of the same organism. All sexual states have since been synonymized under the name *Cytospora* via molecular phylogenetic analyses (Adams *et al.*, 2006; Rossman *et al.*, 2015).

Grapevine trunk diseases, which consist of a complex of several distinct diseases (e.g. esca, botryosphaeria-, eutypa- and phomopsis dieback), are caused by various wood-infecting fungi that span four classes within the Ascomycota. These diseases are widespread and cause substantial yield losses (Munkvold *et al.*, 1994), with important economic impacts (Sipiora & Cuellar, 2014). Numerous other fungi also isolated from the wood of vines with symptoms of trunk diseases have been shown to be pathogenic, such as: *Cadophora luteo-olivacea, Cadophora melinii* and *Cadophora novi-eboraci* (Travadon *et al.*, 2015); *Diaporthe benedicti*, *Diaporthe eres/nobilis* and *Diaporthe novem* (Lawrence *et al.*, 2015); *Cryptosphaeria pullmanensis*, *Cryptovalsa ampelina*, *Diatrype oregonensis*, *Diatrypella verrucaeformis* and *Eutypa leptoplaca* (Trouillas *et al.*, 2010). However, it is not clear if they are aggressive trunk pathogens or if they are as widespread as the recognized trunk pathogens.

Defining the type of interactions (synergistic, competitive, antagonistic) in trunk-pathogen communities is a research question of critical importance, given the chronic nature of the wood infections, the typical mixed infections of trunk pathogens and numerous other fungi of unknown ecology (Úrbez-Torres *et al.*, 2006; Gonzalez & Tello, 2011; Bruez *et al.*, 2016), and the possibilities for priority effects on wood colonization and degradation (Fukami *et al.*, 2010). As such, there is a need to resolve species concepts in wood-colonizing fungi and to determine the pathogenicity of species of unknown ecology. Previous studies have reported the isolation of *Cytospora* species from grapevine (*Vitis vinifera*). Fotouhifar *et al.* (2010) reported two species, *Cytospora cincta* and *Cytospora leucostoma*, from diseased or dead grapevine wood in Iran, although the pathogenicity of these two species was not tested. The cosmopolitan species *Cytospora chrysosperma*, which has a broad host range (Adams *et al.*, 2006), has been isolated from symptomless grapevine wood and is thus classified as an endophyte by Gonzalez & Tello (2011). Since then, *C. chrysosperma* has also been isolated from grapevine wood with symptoms and pathogenicity has been demonstrated for it (Arzanlou & Narmani, 2015).

The objectives of the present study were to examine the morphological and phylogenetic diversity of *Cytospora* species isolated from grapevines with symptoms in eastern North America. Compared to the much larger grape-growing region of California, with its Mediterranean climate suitable for production of European wine grapes and seedless table grapes (cultivars of *V. vinifera*), the Continental climate of eastern North America limits production to cold-tolerant North American *Vitis* species. Such differences in climate and/or host species may contribute to differences in the species composition of the trunk-pathogen community, as has been shown for eutypa dieback (Rolshausen *et al.*, 2014) and phomopsis dieback (Baumgartner *et al.*, 2013; Lawrence *et al.*, 2015). Additionally, the pathogenicity of select isolates was investigated in order to confirm their putative role as grapevine trunk pathogens.

Materials and methods

Grapevine sampling and fungal isolation

Vineyards in North America were surveyed for general symptoms of grapevine trunk diseases (Fig. 1), namely low vigour, stunted shoots, diagnostic foliar symptoms of eutypa dieback and esca, dead spur positions, and the presence of retrained cordons and trunks, a typical management practice used to treat vines with such symptoms (Sosnowski *et al.*, 2011). In total, 860 wood samples were obtained from necrotic and discoloured wood, as revealed by cross-sections through spurs, cordons and trunks with symptoms, following Baumgartner *et al.* (2013). Fungi were isolated from 12 wood pieces (4 × 4 × 2 mm) per sample, cut from the margins of necrotic wood, surface disinfected in 0.6% sodium hypochlorite (pH 7.2) for 30 s, rinsed in two serial baths of sterile deionized water for 30 s, and plated on potato dextrose agar (PDA; Difco) plates amended with tetracycline (1 mg L^{-1}). Petri dishes were incubated at 25 °C in the dark for up to 28 days. Twenty-one isolates with morphological characters of *Cytospora*, namely colonies with uneven growth margins and thus lobate to highly lobate colony morphology, were recovered in culture, from 20 vineyards in seven states and two Canadian provinces (Table 1). These isolates were subsequently hyphal-tip purified to fresh PDA dishes for phylogenetic, morphological and pathological analyses.

Phylogenetic analyses

Total genomic DNA was isolated from mycelium scraped with a sterile scalpel from the surface of a 14-day-old culture using the DNeasy Plant kit (QIAGEN), following the manufacturer's instructions. Amplification of ribosomal DNA (rDNA), including the intervening internal transcribed spacer regions and 5.8S rDNA (ITS1–5.8S–ITS2), followed the protocol of White *et al.* (1990) using the primer set ITS1 and ITS4. Amplification of translation elongation factor 1-α (*TEF1-α*) fragments used the primer set EF1-688F and EF1-1251R (Alves *et al.*, 2008), and β-tubulin (*BT*) locus used primers T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995), with a slightly modified PCR programme consisting of initial denaturation (95 °C, 5 min); 35 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), extension (72 °C, 60 s); and a final extension (72 °C, 10 min). PCR products were visualized on a 1.5% agarose gel (120 V for 25 min) to validate presence and size of amplicons, purified via

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Figure 1 Symptoms on grapevines from which *Cytospora* was isolated: (a) dead spur positions present on the end of a live cordon, and (b) a necrotic wood canker observed in cross-section of a cordon.

exonuclease I and recombinant shrimp alkaline phosphatase (Affymetrix), and sequenced in both directions on an ABI 3730 Capillary Electrophoresis Genetic Analyzer (College of Biological Sciences Sequencing Facility, University of California, Davis).

Forward and reverse nucleotide sequences were assembled, proofread and edited in SEQUENCHER v. 5 (Gene Codes Corporation) and deposited in GenBank (Table 1). Forty-four ITS sequences with high similarity from type and non-type *Cytospora* isolates (n = 31 and 11, respectively) were included for phylogenetic reference. Multiple sequence alignments were performed in MEGA v. 6 (Tamura *et al.*, 2013) and manually adjusted where necessary in MESQUITE v. 3.10 (Maddison & Maddison, 2016). Alignments were submitted to TreeBASE under accession number S19058. The first analysis was performed on ITS sequences and a second analysis (combined dataset of ITS and *TEF1-α*, because only these data were available in GenBank) was performed on a subset of sequences, to further elucidate phylogenetic relationships among closely related *Cytospora* species/isolates as identified in the ITS analysis. The datasets were analysed using two different optimality search criteria, maximum likelihood (ML) and maximum parsimony (MP), in MEGA v. 6 (Tamura *et al.*, 2013). For ML analyses, MEGA was used to infer a model of nucleotide substitution for each dataset using the Akaike information criterion (AIC). Each ML analysis used the nearest-neighbour-interchange (NNI) heuristic method and branch stability was determined by 1000 bootstrap replicates. For MP analyses, heuristic searches with 1000 random sequence additions were implemented with the tree-bisection-reconnection algorithm, with gaps treated as missing data. Bootstrap analyses with 1000 replicates were used to estimate branch support. The ITS sequence of *Diaporthe ampelina* (Diaporthales, Diaporthaceae) isolate Wolf912 served as the out-group taxon in the ITS analysis and *Cytospora carbonacea* isolate CFCC 50056 was used as the out-group taxon in the combined analysis using a subset of *Cytospora* data.

Morphology

Mycelial plugs (5 mm diameter) were taken from the margin of an actively growing culture and transferred to 90 mm diameter Petri dishes. Optimal growth temperature was determined for a subset of isolates (Cyt5, Cyt6, Cyt17, Cyt18, Cyt19, Cyt20 and Cyt21) grown on triplicate PDA plates in darkness at 5–35 °C, in 5 °C increments. Radial growth was measured after 7 days by taking two measurements perpendicular to each other. Assessments of colony colour (Rayner, 1970) and morphology were made at 14 days. Pycnidia were induced on grapevine wood embedded in water agar (WA) medium amended with tetracycline (1 mg L^{-1}). One-year-old grapevine canes (approx. 1 cm diameter) were collected in the vineyard, the bark was peeled off, and canes were cut into 5-cm sections. Sections were placed in glass Petri dishes and autoclaved twice, 24 h apart, at 122 °C for 25 min. Autoclaved wood sections were placed in 90 mm diameter plastic Petri dishes, two sections per dish, and WA was poured to embed them. A mycelial plug from an actively growing culture was placed between the two wood sections in each dish, one isolate per dish. Petri dishes were incubated at room temperature under natural photoperiod in May 2016, and fruiting body formation was monitored weekly for 4 weeks. Morphological characterization of fruiting bodies (n = 20) included the structure and size of pycnidia, presence/absence of a conceptacle, colour of disc, diameter and number of ostioles per disc, and arrangement and number of locules for selected isolates. Fresh pycnidia were transversely sectioned by hand with a razor blade and embedded in optimal cutting temperature medium (O.C.T.; Sakura) followed by cross-sectioning (approx. 20 μm thickness) using a cryostat microtome (Ames Company) according to the manufacturer's recommendations, and observed at ×1000 magnification using a DM4000 microscope (Leica microsystems CMS GmbH). Micrographs of pycnidial structures were obtained by assembling micrographs using LAS v. 4.2 software (Leica microsystems CMS GmbH). No stain was applied, thus the native colour of each species was preserved. Conidial dimensions (n = 50 per isolate) were measured at ×1000 magnification from approximately 28-day-old cultures by producing a pycnidial squash mount that was crushed in 10 mM sterile phosphate buffer (pH 7) and observed as above. Analyses of variance (ANOVAS) were used to test the null hypotheses that colony diameters and conidial dimensions were similar amongst isolates. Homogeneity of variance was evaluated prior to ANOVA. ANOVA was performed using the MIXED procedure in SAS v. 9.2 (SAS Institute), and the main effect of isolate was treated as a

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Table 1 Fungal isolates used in this study and GenBank accession numbers

Species	Isolate[a]	Geographic origin	Host	GenBank accession[b]		
				ITS	*β-tubulin*	*TEF1-α*
Cytospora abyssinica	CBS 116819[T]	Wondo Genet, Ethiopia	*Eucalyptus globulus*	AY347353	–	–
C. atrocirrhata	CFCC 89615[T]	Xining, Qinghai, China	*Juglans regia*	KF225610	–	–
C. austromontana	CBS 116820[T]	NSW, Australia	*Eucalyptus pauciflora*	AY347361	–	–
C. berberidis	CFCC 89927[T]	Qinghai Province, China	*Berberis dasystachyum*	KP340985	–	–
C. berkeleyi	CBS 116823[T]	Palo Alto, California, USA	*Eucalyptus globulus*	AY347350	–	–
C. brevispora	CBS 116811[T]	Tchittanga, Republic of Congo	*Eucalyptus grandis* × *tereticornis*	AF192315	–	–
C. carbonacea	CFCC 50055[T]	Qiqihar, Heilongjiang, China	*Ulmus pumila*	KP281262	–	KP310851
C. cedri	CBS 196.50	Italy	Unknown host	AF192311	–	JX438575
C. chrysosperma	CFCC 89619[T]	Yinchuan, Ningxia, China	*Juglans regia*	KF225614	–	–
C. cincta	LP47	Michigan, USA	*Prunus armeniaca*	AF191169	–	–
C. cinereostroma	CMW 5700[T]	Chile	*Eucalyptus globulus*	AY347377	–	–
C. davidiana	CXY1350[T]	China	*Populus davidiana*	KM034870	–	–
C. diatrypelloidea	CBS 116826[T]	Orbost, Victoria, Australia	*Eucalyptus globulus*	AY347368	–	–
C. disciformis	CBS 116827[T]	Uruguay	*Eucalyptus grandis*	AY347374	–	–
C. elaeagni	CFCC 89632[T]	Guyuan, Ningxia, China	*Elaeagnus angustifolia*	KF765676	–	–
C. eriobotryae	CBS 116846[T]	Saharanpur, India	*Eriobotrya japonica*	AY347327	–	–
C. eucalyptina	CBS 116853[T]	Cali, Colombia	*Eucalyptus grandis*	AY347375	–	–
C. fugax	CBS 203.42[T]	Switzerland	*Salix* sp.	AY347323	–	–
C. gigalocus	HMBF155[T]	Xining, Qinghai, China	*Juglans regia*	KF225609	–	–
C. hippophaes	CFCC 89639[T]	Gannan, Gansu, China	*Hippophae rhamnoides*	KF765681	–	–
C. kantschavelii	CXY1386	Chongqing, China	*Populus* sp.	KM034866	–	–
C. multicollis	CBS 105.89[T]	Spain	*Quercus ilex* subsp. *rotundifolia*	DQ243803	–	–
C. nitschkii	CMW 10180[T]	Wondo Genet, Ethiopia	*Eucalyptus globulus*	AY347356	–	–
C. notastroma	Cottonwood16[T]	Colorado, USA	*Populus tremuloides*	JX438631	–	–
C. palm	CXY1276[T]	Beijing, Xiangshan, China	*Cotinus coggygria*	JN402990	–	–
C. pruinopsis	CFCC 50034[T]	Harbin, Heilongjiang, China	*Ulmus pumila*	KP281259	–	–
C. ribis	CFCC 50026[T]	Yulin, Shaanxi, China	*Ulmus pumila*	KP281267	–	–
C. rostrata	Ls251[T]	Gansu, China	*Salix cupularis*	KC313890	–	–
C. sacculus	CFCC 89624[T]	Gannan, Gansu, China	*Juglans regia*	KF225615	–	KP310860
C. sacculus	CFCC 89625	Gansu, China	*Juglans regia*	KR045646	–	KP310861
C. sacculus	CBS 116.21	Netherlands	*Fagus sylvatica*	AY347335	–	–
C. sacculus	CBS 192.42	Switzerland	*Taxus baccata*	AY347333	–	–
C. schulzeri	CBS 118570	Michigan, USA	*Malus domestica*	DQ243802	–	–
C. sibiraeae	CFCC 50045[T]	Gannan, Gansu, China	*Sibiraea angustata*	KP340987	–	–
C. sophoricola	CFCC 89595	Gannan, Gansu, China	*Sophora japonica* var. *pendula*	KC880148	–	–
C. translucens	CBS 152.42	St. Moritz, Switzerland	*Salix* sp.	AF191182	–	–
C. valsoidea	CBS 117003[T]	Sumatra, Indonesia	*Eucalyptus grandis*	AF192312	–	–
C. variostromatica	CBS 116858[T]	Orbost, Victoria, Australia	*Eucalyptus globulus*	AY347366	–	–
C. vinacea	CBS 141585[T]*	New Hampshire, USA	*Vitis* interspecific hybrid 'Vidal'	**KX256256**	**KX256235**	**KX256277**
C. viticola	Cyt1	Vermont, USA	*Vitis vinifera* 'Zweigelt'	**KX256246**	**KX256225**	**KX256267**
C. viticola	Cyt2	Vermont, USA	*Vitis* interspecific hybrid 'Frontenac'	**KX256238**	**KX256217**	**KX256259**
C. viticola	Cyt3	Vermont, USA	*Vitis* interspecific hybrid 'Vignoles'	**KX256240**	**KX256219**	**KX256261**
C. viticola	Cyt4	Vermont, USA	*Vitis* interspecific hybrid 'Vignoles'	**KX256241**	**KX256220**	**KX256262**
C. viticola	CBS 141586[T]*	Connecticut, USA	*Vitis vinifera* 'Cabernet Franc'	**KX256239**	**KX256218**	**KX256260**
C. viticola	Cyt7	Québec, Canada	*Vitis* interspecific hybrid 'Sabrevois'	**KX256247**	**KX256226**	**KX256268**
C. viticola	Cyt8	Québec, Canada	*Vitis* interspecific hybrid 'Seyval Blanc'	**KX256243**	**KX256222**	**KX256264**
C. viticola	Cyt9	Québec, Canada	*Vitis* interspecific hybrid 'Adalmiina'	**KX256242**	**KX256221**	**KX256263**
C. viticola	Cyt11	New York, USA	*Vitis vinifera* 'Gamay'	**KX256237**	**KX256216**	**KX256258**
C. viticola	Cyt12	New York, USA	*Vitis vinifera* 'Riesling'	**KX256236**	**KX256215**	**KX256257**
C. viticola	Cyt13	New York, USA	*Vitis vinifera*	**KX256255**	**KX256234**	**KX256276**
C. viticola	Cyt14	New York, USA	*Vitis* interspecific hybrid 'Aurore'	**KX256249**	**KX256228**	**KX256270**
C. viticola	Cyt15	Virginia, USA	*Vitis* interspecific hybrid 'Vidal'	**KX256248**	**KX256227**	**KX256269**
C. viticola	Cyt16	Québec, Canada	*Vitis vinifera* 'Gamay'	**KX256254**	**KX256233**	**KX256275**
C. viticola	Cyt17*	Michigan, USA	*Vitis vinifera* 'Riesling'	**KX256245**	**KX256224**	**KX256266**

(continued)

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Table 1 (continued)

Species	Isolate[a]	Geographic origin	Host	GenBank accession[b]		
				ITS	*β-tubulin*	*TEF1-α*
C. viticola	Cyt18	Michigan, USA	*Vitis* interspecific hybrid 'Marechal Foch'	**KX256253**	**KX256232**	**KX256274**
C. viticola	Cyt19	Ohio, USA	*Vitis vinifera* 'Chardonnay'	**KX256250**	**KX256229**	**KX256271**
C. viticola	Cyt20*	Ontario, Canada	*Vitis vinifera* 'Cabernet Franc'	**KX256252**	**KX256231**	**KX256273**
C. viticola	Cyt21	Ontario, Canada	*Vitis* interspecific hybrid 'Vidal'	**KX256244**	**KX256223**	**KX256265**
C. viticola	Cyt22	Michigan, USA	*Vitis vinifera*	**KX256251**	**KX256230**	**KX256272**
Diaporthe ampelina	Wolf912	Solano Co., CA, USA	*Vitis vinifera* 'Thompson Seedless'	KM669964	**KX256214**	KM669820
Leucostoma persoonii	CBS 116845[T]	Michigan, USA	*Prunus serotina*	AF191181	–	–
Valsa ambiens	CBS 191.42	Switzerland	*Taxus baccata*	AY347330	–	–
V. cinereostroma	CBS 117081[T]	Chile	*Eucalyptus globulus*	AY347377	–	–
V. cypri	CBS 118555	South Africa	*Olea europaea* var. *africana*	DQ243790	–	–
V. eucalypti	CBS 116815	California, USA	*Sequoia sempervirens*	AY347340	–	–
V. eugeniae	CBS 118569	Tanzania	*Eugenia* sp.	AY347344	–	–
V. fabianae	CBS 116840[T]	Tasmania, Australia	*Eucalyptus nitens*	AY347358	–	–
V. friesii	CBS 194.42	Switzerland	*Abies alba*	AY347328	–	–
V. myrtagena	HiloTib1[T]	Hilo, Hawaii, USA	*Tibouchina urvilleana*	AY347363	–	–
V. nivea	CFCC 89642[T]	Yulin, Shaanxi, China	*Salix psammophila*	KF765684	–	–
V. pini	CBS 197.42	Switzerland	*Pinus sylvestris*	AY347332	–	–
V. populina	CFCC 89644[T]	Yulin, Shaanxi, China	*Salix psammophila*	KF765686	–	–
V. sordida	CBS 197.50[T]	United Kingdom	*Populus tremula*	AY347322	–	–
V. subclypeata	CBS 117.67	Netherlands	*Rhododendron ponticum*	AY347331	–	–

[a]Bold superscript T represents type, epitype or ex-type; Bold superscript *represents isolates used for morphological characterization and pathogenicity assays.
[b]Sequences in bold were produced in this study.

fixed effect. For significant effects ($P < 0.05$), means were compared by Tukey's tests.

Pathogenicity tests

Four isolates (Cyt5, Cyt6, Cyt17 and Cyt20), representative of two of the *Cytospora* species identified by molecular analysis (Table 1), were selected for inoculation to the woody stems of potted *V. vinifera* 'Thompson Seedless' clone 2A in the greenhouse. For each isolate, mycelial suspensions were prepared as inoculum (5-day-old cultures grown in potato dextrose broth (PDB) and homogenized with a hand-held disperser), following the protocol outlined in Travadon *et al.* (2013).

Two replicate experiments were performed, starting 1 day apart, on two sets of plants propagated in two separate greenhouses. In each experiment, 10 plants were inoculated with each isolate and 10 mock-inoculated control plants (five inoculation treatments × 10 plants × two experiments = 100 plants total) were arranged in a completely randomized design. Plants were propagated from dormant cuttings according to Travadon *et al.* (2013). Briefly, starting in March 2015, cuttings were callused at 30 °C and 100% humidity in a mixture of perlite and vermiculite (1:1, vol/vol) for 21 days. Once shoot and root initials emerged from the callus tissue, a power drill was used to produce a wound (2 mm-width × 3 mm-depth) approximately 2 cm below the uppermost node. Inoculum (20 μL) was pipetted into the wound, which was then sealed with Vaseline (Unilever) and Parafilm (Bemis Co.) to prevent inoculum desiccation. Mock-inoculated controls were wounded and inoculated with sterile PDB. Cuttings were coated with melted paraffin wax (Gulf Wax; Royal Oak Enterprises), to prevent moisture loss, and potted in sterile potting mix amended with slow-release fertilizer (Osmocote Pro 24-4-9; Scotts). Plants were grown in the greenhouse at the University of California Experiment Station in Davis from April 2015 to April 2016 (natural sunlight photoperiod, 25 ± 1 °C (day), 18 ± 3 °C (night)), with some modifications to the temperature conditions (10 ± 2 °C (day), 4 ± 2 °C (night)) during dormancy (November 2015–January 2016). During the growing season, plants were watered twice per week for 15 min using a drip-irrigation system (0.5 L h^{-1}).

The length of wood discolouration (LWD) extending from the inoculation site was determined 12 months after inoculation. First, the green shoots, roots and bark of each plant were removed and discarded, and the woody stems were surface sterilized in 1% sodium hypochlorite for 2 min and rinsed with deionized water. The length of each stem was recorded and cut longitudinally to expose wood discolouration, the length of which was measured with a digital caliper. To confirm that the pathogen was responsible for wood discolouration in inoculated plants, recovery was attempted by cutting 10 pieces (2 × 5 × 5 mm) of wood from the distal margin of the lesion, followed by surface disinfection in 0.6% sodium hypochlorite (pH 7.2) for 30 s, two 30 s rinses in sterile deionized water, plating on PDA amended with tetracycline (1 mg L^{-1}), and incubation in the dark at approximately 22 °C for 14–21 days.

Length of wood discolouration was used as a measure of pathogenicity. Normality and homogeneity of variances were evaluated using normal probability plots and Levene's test, respectively. ANOVA was used to determine the effect of each individual isolate on LWD. ANOVA was performed using the MIXED procedure in SAS, with experiment considered as a random effect. Means were calculated using the LSMEANS procedure. Pairwise

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mean differences in LWD between those of inoculated and mock-inoculated plants were analysed using Dunnett's test, with mean LWD of mock-inoculated plants as controls ($P < 0.05$). Recovery from inoculated plants was a second measure of pathogenicity. Recovery rate was calculated as the percentage of plants from which the pathogen was isolated, out of the total number inoculated. To assess the main effect of isolate on recovery rate, generalized linear mixed models were performed using the GLIMMIX procedure in SAS, which uses the logit link function to accommodate binomial data. Experiment was considered a random effect. Recovery rates of the mock-inoculated controls (all of which were zero) were excluded from the analyses.

Results

Phylogenetic analyses

For ML analysis, the best-fit model of nucleotide evolution was deduced based on the AIC (K2+G for both ITS and combined analysis of ITS and *TEF1-α*). Alignment of 73 ITS sequences resulted in a 472-character dataset (300 characters were constant, 164 characters were parsimony-uninformative, and 109 characters were parsimony-informative). MP analysis produced a single parsimonious tree of 590 steps and a consistency index (CI) and retention index (RI) of 0.3707 and 0.7286, respectively. ML and MP analyses revealed that 20 of the 21 *Cytospora* isolates recovered from grapevines with symptoms in northeastern vineyards clustered into a single well-supported clade (94/98% ML and MP bootstrap values, respectively), with no apparent type or non-type association; these isolates are hereinafter identified as *Cytospora viticola* sp. nov. (Fig. 2). The remaining isolate (Cyt5) was nested with low to moderate support (<70/84%) within the clade that contains *Cytospora cedri* CBS 196.50, which originates from an unknown host in Italy, and the ex-type isolate of *Cytospora sacculus* CFCC 89624, which was isolated from walnut with symptoms in China.

Alignment of the two loci (ITS and *TEF1-α*) resulted in a 796-character dataset (657 characters were constant, 119 characters were parsimony-uninformative, and 28 characters were parsimony-informative). MP analysis produced five equally-most parsimonious trees of 138 steps, and CI and RI of 0.7209 and 0.5862, respectively. ML and MP analyses revealed that isolate Cyt5 does not cluster with *C. sacculus* as it did in the ITS analysis, albeit with low support. Furthermore, Cyt5 possesses substantial genetic distance as compared to *C. cedri* and *C. sacculus*, suggesting that this isolate represents a previously unrecognized species, which is described as *Cytospora vinacea* sp. nov. below (Fig. 3).

Morphology

Average colony growth at 25 °C differed significantly between the two species (P [$F_{(6,14)} > 56.78$] < 0.0001) (Table 2). That said, growth was slightly higher (albeit not significantly so) at 20 °C for all isolates, except for Cyt18, which grew optimally at 25 °C (data not shown). There were significant differences in conidia length (P [$F_{(5,294)} > 16.17$] < 0.0001) and width (P [$F_{(5,294)} > 29.22$] < 0.0001), with *C. vinacea* (Cyt5) having short, wide spores compared to *C. viticola* (Table 2). *Cytospora vinacea* produced a rapidly growing colony with a striking pale vinaceous colour, compared to the light-to-dark straw colour of *C. viticola* colonies (Fig. 4). Interestingly, two isolates of *C. viticola* (Cyt17 and Cyt18) produced highly divergent colony morphologies (although had similar conidial dimensions) compared to other the *C. viticola* isolates. In addition to growing much more slowly, Cyt17 colonies had an undulate, light-brown margin with a darker brown centre whereas Cyt18 colonies were light straw coloured, with an irregular and highly lobate margin both on the agar surface and embedded in the medium.

Taxonomy

Phylogenetic analyses (ML and MP) of the ITS locus identified one distinct and strongly supported clade for which no apparent species name exists. Thus the following new species name is proposed to properly circumscribe this unique taxon.

Cytospora viticola D.P. Lawr., Travadon & Pouzoulet, sp. nov

MycoBank no.: MB817120; Figs 2 and 5.

Typification: USA, Connecticut: Litchfield County, 41°42′16.9″N, 73°21′15.1″W, 223 m a.s.l. isolated from wood canker of *Vitis vinifera* 'Cabernet Franc', 2008, P.E. Rolshausen No. Cyt6 (holotype BPI 910161, dried culture; ex-type CBS 141586). GenBank accession numbers: KX256239, ITS; KX256218, *β*-tubulin; KX256260, TEF1-*α*.

Etymology: The name refers to the host (*Vitis vinifera*), from which this species was isolated.

Colony diameter of *C. viticola* isolate Cyt6 73 mm in 7 days at 25 °C on PDA, medium growing, white to off-white slightly raised colony with filiform margins with aerial mycelial tufts throughout. *Hyphae* hyaline, smooth, straight, branched and septate. *Conidiomata* pycnidial, mostly solitary some aggregate, erumpent, conical to discoid, no conceptacle, off-white to light-grey, 835–(1088)–1790 μm in diameter ($n = 20$), unilocular 191.7–(515.7)–812.5 μm ($n = 20$) with shared invaginated walls, cytosporoid rosette, single grey ostiole 69.7–(91.8)–146.5 μm diameter ($n = 20$) per black disc. *Conidiophores* reduced to filamentous conidiogenous cells 16–(21.4)–26.5 × 2.2–(3.9)–5 μm ($n = 20$). *Conidia* copious, single, hyaline, aseptate, allantoid 5.2–(6.1)–7 × 0.9–(1.2)–1.6 μm ($n = 50$). No teleomorph observed. Known distribution: Connecticut, Michigan, New York, Ohio, Vermont and Virginia, USA; and the Canadian provinces of Ontario and Québec.

Phylogenetic analysis (ML and MP) of a subset of isolates, utilizing the combined dataset of ITS and TEF1-*α*

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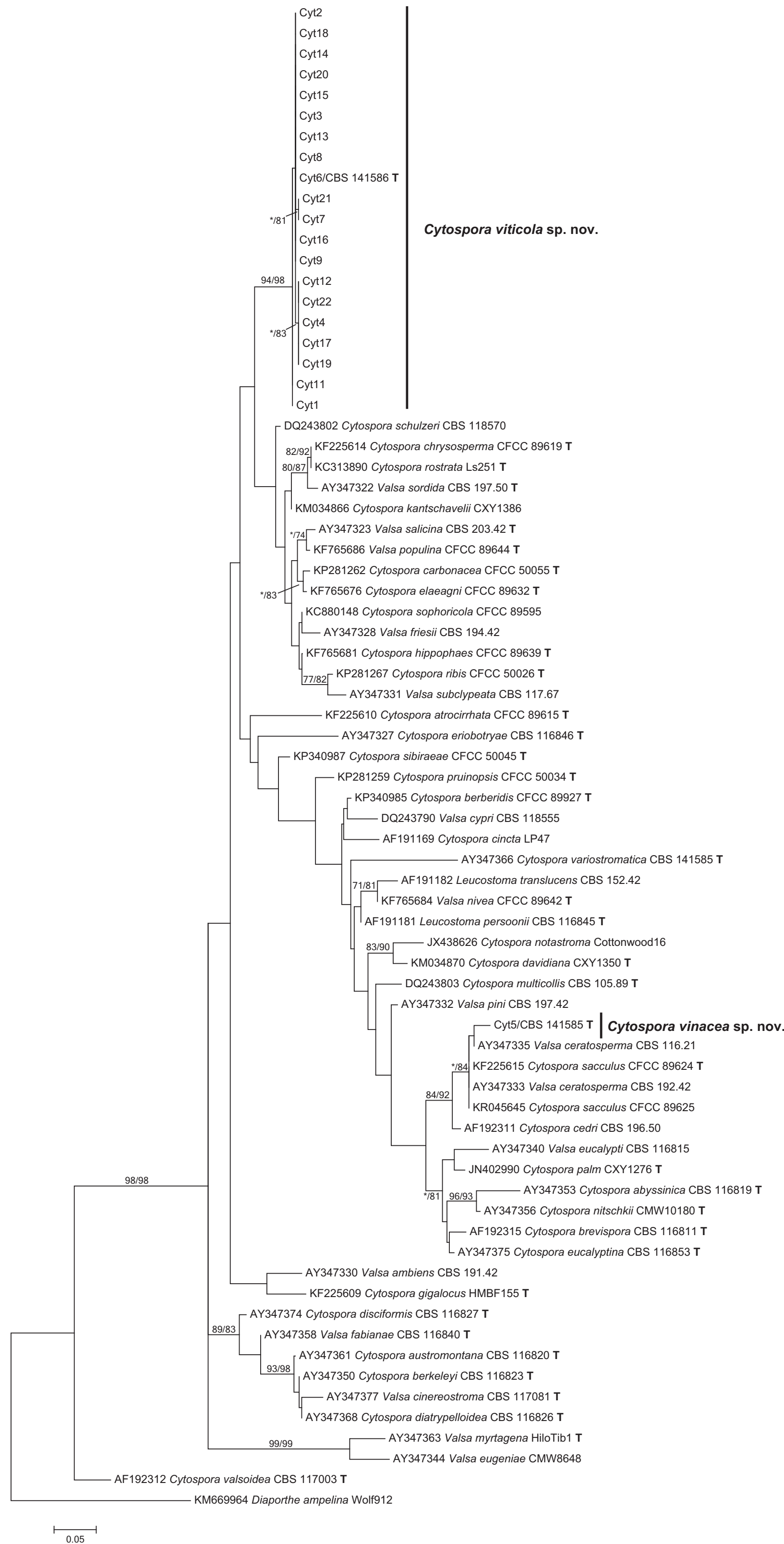

Figure 2 Single most likely tree (ln likelihood –3426.7340) resulting from the analysis of 74 *Cytospora* ITS sequences. Numbers represent maximum likelihood/maximum parsimony bootstrap values, respectively. Values represented by an asterisk were less than 70%. Scale bar represents the number of substitutions per site. Ex-type strains of *C. viticola* sp. nov. and *C. vinacea* sp. nov. are CBS 141586 (Cyt6) and CBS 141585 (Cyt5), respectively.

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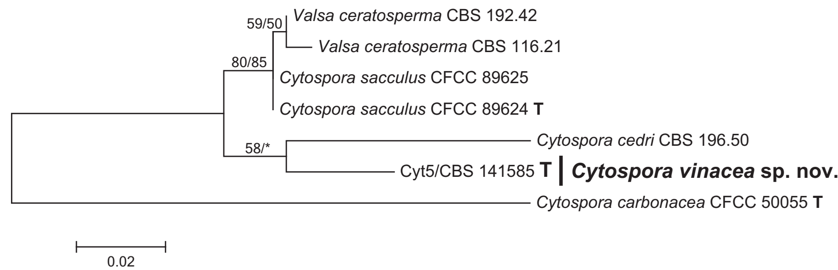

Figure 3 Single most likely tree (ln likelihood –1750.2476) resulting from the analysis of combined ITS and *TEF1-α* sequences. Numbers represent maximum likelihood/maximum parsimony bootstrap values, respectively. Values represented by an asterisk were less than 50%. Scale bar represents the number of substitutions per site.

Table 2 Colony growth and conidial dimensions of seven isolates, representing two *Cytospora* species sampled from vineyards in eastern North America

Species	Isolate	Colony diameter[a] (mm)	Conidia dimensions[b]	
			Length (μm)	Width (μm)
Cytospora vinacea	Cyt5	78.7 d	3.9–(5.2)–6.3 a	1.0–(1.6)–2.4 cd
Cytospora viticola	Cyt6	73.0 d	5.2–(6.1)–7.0 d	0.9–(1.2)–1.6 a
C. viticola	Cyt17	18.0 a	4.0–(5.7)–6.7 bc	0.9–(1.4)–2.0 b
C. viticola	Cyt18	28.6 ab	4.4–(5.8)–6.7 c	0.9–(1.2)–1.5 a
C. viticola	Cyt19	59.3 c	4.7–(5.8)–6.9 c	1.0–(1.5)–1.9 bc
C. viticola	Cyt20	56.3 c	4.0–(5.6)–7.7 c	1.2–(1.6)–2.0 c
C. viticola	Cyt21	41.3 b	4.8–(5.8)–6.9 c	1.0–(1.5)–1.9 bc

[a]Colony diameter was measured after 7 days growth at 25 °C on potato dextrose agar plates. Each value is the mean of triplicate plates. Means followed by the same letter are not significantly different ($P < 0.05$; Tukey's test).

[b]Values in parentheses represent the mean. Means followed by different letters are significantly different ($P < 0.05$; Tukey's test).

(for which data are available in GenBank), identified one genetically distinct lineage represented by isolate Cyt5, which is closely related to *C. sacculus* and *C. cedri* (Fig. 3). Additionally, the pycnidial structure and average conidial dimensions of Cyt5 differed significantly from the type isolate of *C. sacculus*, which produces torsellioid pycnidia and conidial sizes of 3.6–(4.2)–5.2 × 0.9–(1)–1.2; Cyt5 produces cytosporoid rosette pycnidia and conidia that are both longer and wider than those of *C. sacculus*, therefore the following species name is proposed to properly circumscribe this newly recognized species.

Cytospora vinacea D.P. Lawr., Travadon & Pouzoulet, sp. nov

MycoBank no.: MB817121; Figs 3 and 6.

Typification: USA, New Hampshire: Strafford County, 43°04′34.20″N, 71°01′24.00″W, 41 m a.s.l. isolated from wood canker of *Vitis vinifera* 'Vidal', 2008, P.E. Rolshausen No. Cyt5 (holotype BPI 910160, dried culture; ex-type CBS 141585). GenBank accession numbers: KX256256, ITS; KX256235, β-tubulin; KX256277, TEF1-α.

Etymology: The name refers to the distinctive pale vinaceous colony colour.

Cytospora vinacea forms a unique lineage in the combined analyses, sister to *Cytospora cedri* (Fig. 3). *Cytospora vinacea* differs from *C. cedri* by several unique

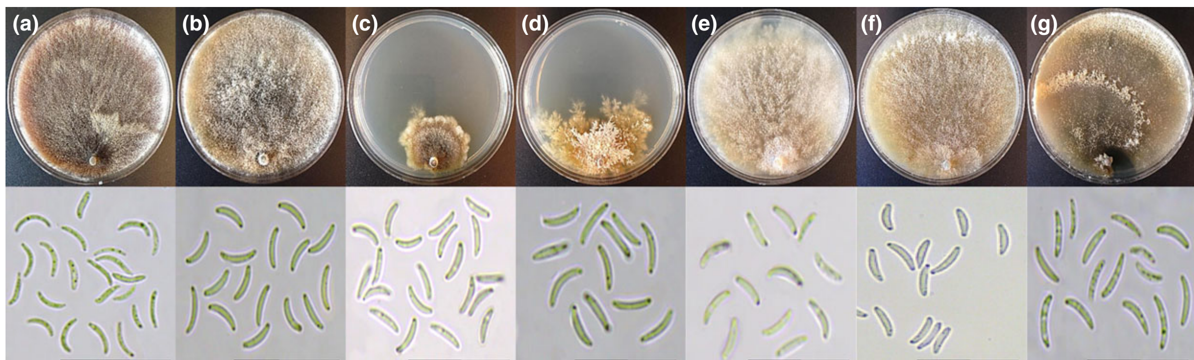

Figure 4 Culture morphology and conidia of: (a) *Cytospora vinacea* Cyt5, (b) *C. viticola* Cyt6, (c) *C. viticola* Cyt17, (d) *C. viticola* Cyt18, (e) *C. viticola* Cyt19, (f) *C. viticola* Cyt20, (g) *C. viticola* Cyt21. Descriptions were made from 14-day-old PDA cultures, incubated at 25 °C in darkness. Scale bar = 20 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

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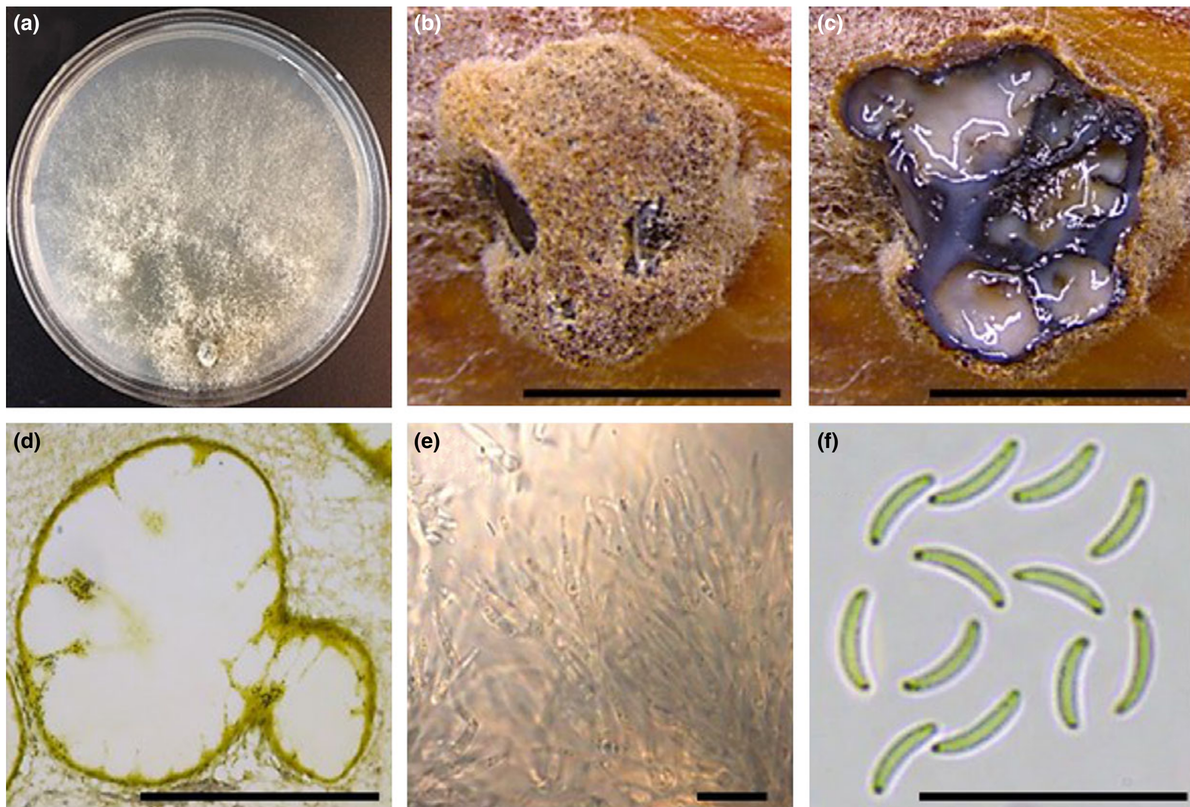

Figure 5 Morphology of *Cytospora viticola* Cyt6, ex-type CBS 141586: (a) colony cultured on PDA at 25 °C in the dark after 7 days, (b) pycnidium produced on autoclaved grapevine wood, (c) cross section of a pycnidium, (d) cross section revealing cytosporoid rosette locule arrangement, (e) conidiogenous cells, (f) conidia. Scale bars: (b) and (c) = 2 mm, (d) = 500 μm, (e) = 25 μm, (f) = 20 μm.

single nucleotide polymorphisms and/or indels: ITS positions 6(C), 9(indel), 10(indel), 13(C), 14(C), 15(T), 16 (C), 21(indel), 22(indel), 23 (indel), 24(indel), 30(G), 74 (C), 75(A), 103(A), 107(T), 116(T), 312(indel), 336(C), 365(C), and 423(C) (413/434 identical nucleotides, 95% similarity); *TEF1-α* positions 5(A), 9(indel), 16(C), 17 (G), 23(A), 40(G), 49(A), 73(indel), 96(G), 99(A), 102 (G), 112(G), 119(G), 124(A), 140(indel), 154(A), 164(T), 170(C), 178(T), 181(C), 182(A), 183(T), 186(C), 189 (C), 190(T), 194(A), 195(A), 200(A), 201(T), 216(A), 217(C), 219(G), and 234(C) (222/256 identical nucleotides, 87% similarity).

Colony diameter of *C. vinacea* isolate Cyt5 78.7 mm in 7 days at 25 °C on PDA, fast growing, pale vinaceous/magenta with short aerial tufts giving a cottony appearance, aerial hyphae becoming lighter with age. *Hyphae* hyaline to reddish, smooth, straight, branched, and septate. *Conidiomata* pycnidial, mostly solitary rarely aggregate, some with yellow conidial exudate, erumpent, discoid to conical, no conceptacle, black-grey, 1125–(1395)–2090 μm diameter ($n = 20$), unilocular 500–(762.5)–1325 μm ($n = 20$) with shared invaginated walls, cytosporoid rosette, single grey ostiole 82.9–(107.3)–134.1 μm diameter ($n = 20$) per black disc. *Conidiophores* reduced to filamentous conidiogenous cells 18.4–(23.9)–26.5 × 2.7–(4.1)–5.2 μm ($n = 20$). *Conidia* abundant, single, hyaline, eguttulate, aseptate, allantoid to variously curved, 3.9–(5.2)–6.3 × 1–(1.6)–2.4 μm ($n = 50$). No teleomorph observed. Known distribution: New Hampshire, USA.

Pathogenicity tests

The four *Cytospora* isolates inoculated to woody stems of 'Thompson Seedless' in the greenhouses caused black, vascular discolourations extending above and below the inoculation site, as observed 12 months post-inoculation (Fig. 7). Wood discolourations caused by three of four *Cytospora* isolates were significantly longer than those of mock-inoculated control plants ($P < 0.05$; Dunnett's test; Table 3), indicating that Cyt5, Cyt6 and Cyt20 were indeed pathogenic. *Cytospora viticola* isolate Cyt6 (17.3 mm) and *C. vinacea* isolate Cyt5 (14.9 mm) caused the largest discolourations (Table 3). The wood immediately surrounding the inoculation sites of mock-inoculated control plants was discoloured, but the discolouration was restricted (6.9 mm, averaged across both experiments) and no pathogenic fungi were isolated from these lesions. From lesions of the inoculated plants, recovered fungal colonies matched morphologically the isolates inoculated to the plants (Table 3). Recovery rates for the four isolates ranged from 35% to 65%; recovery

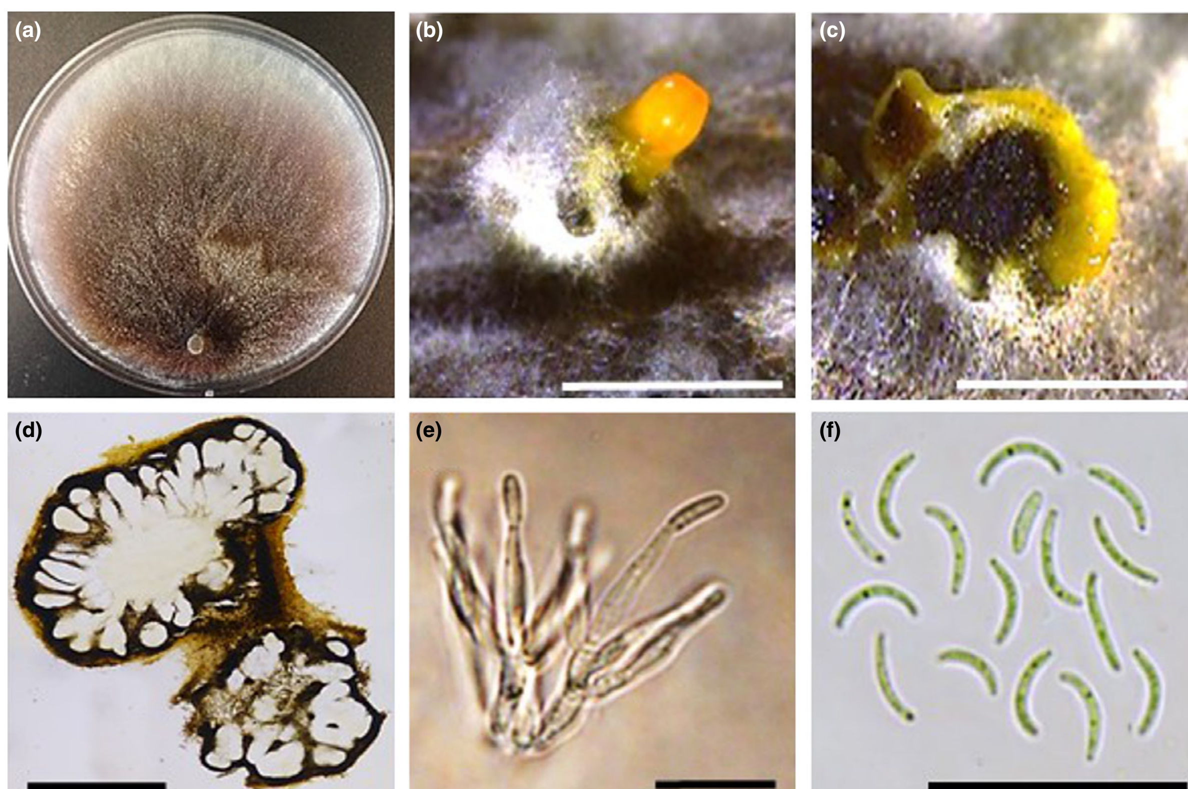

Figure 6 Morphology of *Cytospora vinacea* Cyt5, ex-type CBS 141585: (a) colony cultured on PDA at 25 °C in the dark after 7 days, (b) pycnidium produced on autoclaved grapevine wood with exudate, (c) cross section of a pycnidium, (d) cross section revealing cytosporoid rosette locule arrangement, (e) conidiogenous cells, (f) conidia. Scale bars: (b) and (c) = 2 mm, (d) = 500 μm, (e) = 25 μm, (f) = 20 μm.

of *Cytospora* on culture medium was probably reduced due to the presence of fast-growing endophytes present in the plant material, such as species of *Trichoderma*.

Discussion

This is the first study characterizing the identity and pathogenicity of *Cytospora* species recovered from grapevines with symptoms of trunk diseases in North America. Two newly described species, *C. viticola* and *C. vinacea*, are typified, described, and deposited in the publicly available U.S. National Fungus Collection Database and at the CBS-KNAW collection in the Netherlands. With sparse molecular data from 31 type specimens in GenBank, mainly restricted to a single locus, identification of the two new species from grape was limited to phylogenetic analyses of ITS and, to a lesser extent, *TEF1-α*.

Species recognition in *Cytospora* has traditionally relied on morphological characters of ascomata or conidiomata and host associations (Adams *et al.*, 2002). Morphologically, the sexual state is of a diaporthalean-like nature with perithecia that produce clavate to elongate obovoid asci that release hyaline, allantoid, aseptate ascospores (Spielman, 1985; Adams *et al.*, 2005). The asexual state is characterized by pycnidia that are composed of either a single locule or multiple locules with shared or unshared invaginated walls, with filamentous conidiogenous cells that produce allantoid, aseptate conidia released in the presence of free water (Spielman, 1985; Barakat & Johnson, 1997; Adams *et al.*, 2005). Confusing morphological variation of *Cytospora* locule types has led to misidentifications and erroneous reports. Two major locule types have been described: unilocular (locule is undivided) and locular (locules composed of shared or unshared invaginated walls). Both species identified in this study produced similar locular arrangements, rosette cytosporoid, which is characterized by a single locule with multiple shared invaginated walls. *Cytospora viticola* clearly produced this locular arrangement with fewer invaginations as compared to *C. vinacea*, which produced larger pycnidia with many more invaginations that superficially resembled the labyrinthine cytosporoid locular arrangement. In addition to the potential for misinterpretation of locular arrangements, an incomplete understanding of host ranges may hinder accurate species identification; some other *Cytospora* species are reported on numerous hosts, whereas others infect only a single family or genus of host plants (Adams *et al.*, 2005; Farr & Rossman, 2015).

Contemporary species identification couples morphological and molecular data to accurately circumscribe/identify taxa (Lawrence *et al.*, 2015). Currently, the majority of molecular data available in GenBank for type

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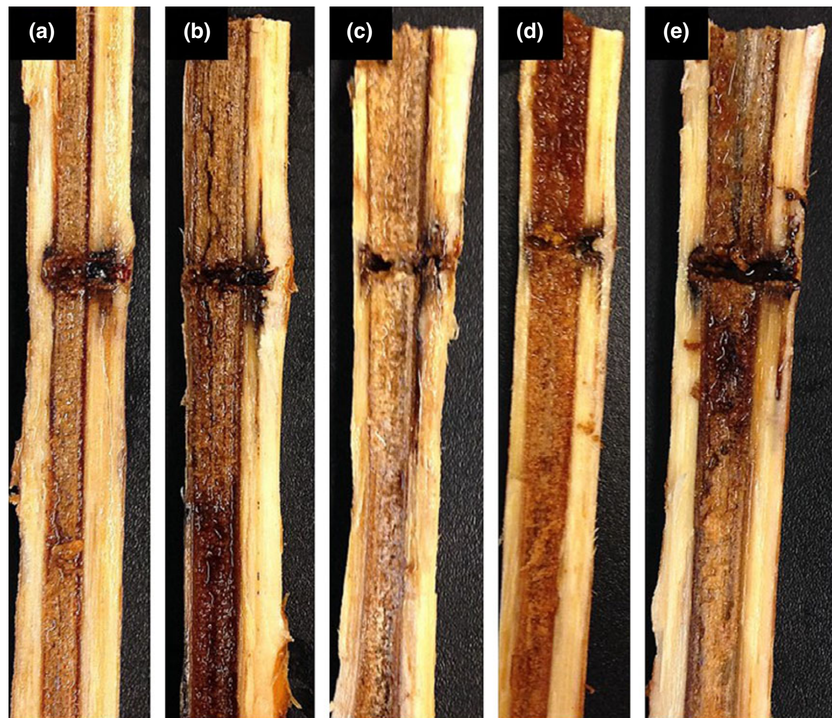

Figure 7 Representative internal wood symptoms of potted *Vitis vinifera* 'Thompson Seedless' observed after 12 months incubation: (a) mock-inoculated control, and inoculated with (b) *Cytospora vinacea* Cyt5, (c) *C. viticola* Cyt6, (d) *C. viticola* Cyt17, and (e) *C. viticola* Cyt20. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3 Mean lesion length and mean recovery rates of four *Cytospora* isolates at 12 months post-inoculation in the woody stems of *Vitis vinifera* 'Thompson Seedless'

Species	Isolate	Mean length of wood discolouration (mm)[a]	Recovery rate[b]
Cytospora vinacea	Cyt5	14.9 (8.6–29.8) b	0.35
Cytospora viticola	Cyt6	17.3 (7.4–44.9) b	0.65
C. viticola	Cyt17	9.9 (5.3–20.8) a	0.35
C. viticola	Cyt20	13.6 (8.2–22.5) b	0.45
Mock-inoculated control	–	6.9 (3.7–15.4) a	0

[a]Each value is the mean of 10 observations per experiment and two replicate experiments. Means that are significantly greater than that of the mock-inoculated control mean are followed by different letters ($P < 0.05$; Dunnett's tests).

[b]Proportion of plants from which the inoculated pathogen was recovered in culture, out of 20 plants per isolate.

specimens of *Cytospora* is only ITS. The ITS locus seems to possess sufficient signal for delimiting species, but estimates of species divergence are poor, with backbone support for species-order divergence. Future systematic studies in this unique and diverse genus would benefit greatly by including additional loci, such as TEF1-α, β-tubulin, and/or calmodulin. Moreover, a polyphasic approach where the ecology of species (e.g. pathogenic status) complements the morphological features and molecular typing, as employed in the present study, represents a more comprehensive approach to describe novel phytopathogenic fungi.

Two other *Cytospora* species, *C. cincta* and *C. leucostoma*, were isolated from the wood of diseased or dying grapevines in Iran prior to this study, but their pathogenicity was not evaluated (Fotouhifar *et al.*, 2010). Therefore the role of these two *Cytospora* species in relation to the species and symptoms observed here is unknown. Recently, *C. chrysosperma*, the type species for the genus, was also reported in Iran from grapevines displaying decline symptoms, including stunted growth, canopy chlorosis and necrosis, and black vascular streaking or discoloured wood (Arzanlou & Narmani, 2015). Pathogenicity assays conducted on excised, 1-year-old grapevine shoots revealed that two of four isolates of *C. chrysosperma* were pathogenic after 28 days' incubation. Assays using detached plant material usually allow for the production of large lesions in a limited amount of time, but relevancy of such results to the pathogenicity of an isolate on a living host is questionable. Previous studies show most trunk pathogens require up to a year to cause significant lesions within living grapevines (Lawrence *et al.*, 2015; Travadon *et al.*, 2015). Indeed, the 12 cm lesions measured by Arzanlou & Narmani (2015) after 28 days in detached canes are much larger compared to 2 cm lesions in live woody stems after 12 months in the present study.

The findings here expand the diverse fungal community involved with grapevine trunk diseases. The two newly described *Cytospora* species were isolated from wood cankers of vines showing general symptoms of decline and dieback. In the greenhouse, *C. vinacea* and *C. viticola* caused internal wood lesions in inoculated grape woody stems, suggesting that (like other trunk

pathogens) they damage the wood directly through the action of cell wall-degrading enzymes (Morales-Cruz *et al*., 2015) and/or fungal toxins (Abou-Mansour *et al*., 2015). Although wood-lesion development was slow (1-year incubation period), it was similar to that of other fungi associated with grapevine trunk diseases when assayed using a similar methodology (Lawrence *et al*., 2015; Travadon *et al*., 2015); a multi-year assay may be required for reproducing severe symptoms (e.g. larger lesions, shoot dieback). Despite their apparent low virulence, *Cytospora* strains may act in synergy with other ascomycete trunk pathogens, as has been shown in co-inoculation experiments with other genera of wood-infecting fungi (Whitelaw-Weckert *et al*., 2013).

The epidemiology of cytospora canker has been best studied in orchards, and such information may help guide future research on the spread of this disease in vineyards. As *Cytospora* species are primarily wound pathogens in tree crops (Biggs, 1989), *C. vinacea* and *C. viticola* may infect vines through the numerous pruning wounds created on grapevines every dormant season. If pruning wounds are the main infection courts for *Cytospora* in vineyards, and certainly confirmatory studies are required, recommendations for control of cytospora canker may be similar to those of the main trunk diseases of grape. These include preventative practices, such as delaying pruning until late in the dormant season, when the risk of infection is low, as demonstrated in grape against eutypa dieback (Petzoldt *et al*., 1981) and botryosphaeria dieback (Úrbez-Torres & Gubler, 2011). Also, applying pruning-wound protectants after pruning and before rain events may be an effective preventative strategy against *Cytospora* infection, assuming similar efficacy of the same materials as against the unrelated fungi that cause eutypa dieback, botryosphaeria dieback and esca (Rolshausen *et al*., 2010). To the extent that wounds from winter injury are potentially infection courts (as isolates were recovered from both cold-sensitive *V. vinifera* cultivars and cold-tolerant interspecific hybrids), standard practices to minimize such injury, including the presence of wind machines, careful selection of cultivars for the microclimate, and minimal use of nitrogen, may also aid in managing cytospora canker.

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