Cadophora species associated with wood-decay of grapevine in North America

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ABSTRACT

Cadophora species are reported from grapevine (Vitis vinifera L.) in California, South Africa, Spain, Uruguay, and Canada. Frequent isolation from vines co-infected with the Esca pathogens (Togninia minima and Phaeomoniella chlamydospora), and confirmation of its ability to cause wood lesions/discoloration in pathogenicity tests, suggest that C. luteo-olivacea is part of the trunk pathogen complex. In North America, little is known regarding the diversity, geographic distribution, and roles of Cadophora species as trunk pathogens. Accordingly, we characterized 37 Cadophora isolates from ten US states and two Canadian provinces, based on molecular and morphological comparisons, and pathogenicity. Phylogenetic analysis of three loci (ITS, translation elongation factor 1-alpha (TEF1-α) and beta-tubulin (BT)) distinguished two known species (C. luteo-olivacea and Cadophora melinii) and three newly-described species (Cadophora orientoamericana, Cadophora novi-eboraci, and Cadophora spadicis). C. orientoamericana, C. novi-eboraci, and C. spadicis were restricted to the northeastern US, whereas C. luteo-olivacea was only recovered from California. C. melinii was present in California and Ontario, Canada. Morphological characterization was less informative, due to significant overlap in dimensions of conidia, hyphae, conidiophores, and conidiogenous cells. Pathogenicity tests confirmed the presence of wood lesions after 24 m, suggesting that Cadophora species may have a role as grapevine trunk pathogens.

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Introduction

The genus *Cadophora* was circumscribed in 1927 by Lagerberg and Melin with *Cadophora fastigiata* Lagerb. & Melin as the type species to accommodate dematiaceous Hyphomycete fungi that produce single-phialide conidiophores with distinct flask-shaped, hyaline collarettes (Lagerberg *et al.* 1927). Interest in this group of fungi initially came from its frequent association with blue stain of lumber (Lagerberg *et al.* 1927). In 1937, Conant transferred eight *Cadophora* species to *Phialophora* Med. based on similarities of phialide morphology with the polymorphic species *Phialophora verrucosa* Med. (Conant 1937). Reclassification of phialophora-like anamorphs (Gams 2000) and subsequent ribosomal DNA analyses (Harrington & McNew 2003) have since shown that members of the genus *Cadophora* are anamorphs of the Helotiales (Leotiomycetes), and are distinct from the genus *Phialophora*, which resides in the Chaetothryiales (Eurotiomycetes).

Most *Cadophora* species are primarily isolated from soil and plants, either interacting as plant pathogens, root colonizers, or saprobes. For example, the vascular pathogen *Cadophora gregata* (Allington & D.W. Chamb) T.C. Harr & McNew causes Brown stem rot of soybean (Allington & Chamberlain 1948), a disease which has a significant economic impact on the soybean industry in the United States. Isolates resembling *Cadophora malorum* (Kidd & Beaumont) W. Gams cause wood-decay of kiwifruit (Di Marco *et al.* 2004). *Cadophora melinii* Nannf. and *Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew have also been isolated from the wood of kiwifruit trees suffering from trunk hypertrophy and longitudinal bark cracks, and *C. melinii* is considered the pioneer wood colonizer in this disease complex (Prodi *et al.* 2008). *Cadophora* species have also been isolated from soil (Arenz *et al.* 2006) and decayed wood (Blanchette *et al.* 2004) in 'extreme' environments, with *C. malorum*, *C. luteo-olivacea*, and *C. fastigiata* causing soft rot of wood huts in Antarctica.

Recently, the role of *Cadophora* in the decline of grapevine (*Vitis vinifera* L.) has been questioned, based on species reports from California, South Africa, Spain, Uruguay, and Canada. In particular, *C. luteo-olivacea* has been isolated from both asymptomatic (Halleen *et al.* 2007; Casieri *et al.* 2009) and symptomatic grapevine wood, in nursery (Navarrete *et al.* 2011) and field plants (Rooney-Latham 2005; Úrbez-Torres *et al.* 2014) showing black streaking of xylem vessels, the typical internal symptoms of Esca and Petri disease (Mugnai *et al.* 1999), or from decayed and discolored wood observed at the graft union of declining *V. vinifera* 'Syrah' plants and rootstocks (Gramaje *et al.* 2010). When inoculated to potted grapevines in pathogenicity assays, this species has been shown to cause wood lesions and black streaking in longitudinal stem sections (Rooney-Latham 2005; Halleen *et al.* 2007; Gramaje *et al.* 2011; Úrbez-Torres *et al.* 2014). The isolation of *C. luteo-olivacea* from grapevines co-infected with the wood-infecting fungi (i.e. trunk pathogens) *Togninia minima* (Tul. & Tul.) Berl. and *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams suggest that *C. luteo-olivacea* may be associated with the trunk diseases Esca and, in young vineyards, Petri disease (Gramaje *et al.* 2011; Úrbez-Torres *et al.* 2014). *Cadophora luteo-olivacea* is also recovered from nursery stock and vineyard soils, suggesting that the fungus can spread to vineyards via contaminated nursery stock or from soil-borne inoculum (Halleen *et al.* 2007; Gramaje *et al.* 2011; Agusti-Brisach *et al.* 2013). Another less common *Cadophora* species, identified as *C. melinii* based on ITS phylogenies, albeit with low statistical support, has been isolated from grapevine in Spain, although the isolate used in controlled inoculations was not found to be pathogenic (Gramaje *et al.* 2011). As grapevine trunk diseases have a serious economic impact on grape production worldwide, development of effective management strategies depends on a clear identification of the pathogens involved.

In North America, *C. luteo-olivacea* has been recovered from declining grapevines in California (Rooney-Latham 2005), Pennsylvania (Overton *et al.* 2005), and British Columbia, Canada (Úrbez-Torres *et al.* 2014). Aside from these few reports, little is known regarding the diversity of *Cadophora* species involved in grapevine decline, their geographic distribution in North America, and their ability to degrade grapevine wood. Accordingly, we characterized different *Cadophora* species recovered from the decayed wood of grapevines sampled from eastern and western North America, which provided a good comparison of isolates from a diverse range of vineyard environments. Approximately 85 % of grapes in North America are grown in the US state of California (Anonymous 2014), which has a Mediterranean climate ideal for *V. vinifera* cultivars for production of wine grapes (e.g. 'Cabernet Sauvignon'), table grapes (e.g. 'Thompson seedless'), and raisin grapes (e.g. 'Fiesta'). The continental climate of eastern North America does not accommodate widespread plantings of *V. vinifera*. Instead, the most widely-planted grape is the eastern North American species *Vitis labruscana* 'Concord', for production of juice and preserves. Also planted in eastern North America are cold-tolerant hybrids of *V. vinifera*, *V. labruscana*, and other North American *Vitis* species, such as *Vitis rupestris* (e.g. 'Aurore', 'Cayuga White'). The eastern North American region with the highest grape acreage is the US state of New York (Anonymous 2014). For areas with a similarly cold climate to New York, sites were also chosen in the nearby mid-Atlantic state of Ohio, the New England states of Connecticut, Massachusetts, New Hampshire, Rhode Island, and Vermont, and the Canadian Provinces of Ontario and Québec. Sites were also chosen in the Atlantic seaboard states of Maryland and Virginia, in part because of their relatively warmer climate.

Materials and methods

Grapevine sampling and fungal isolation

Vineyards were surveyed for general symptoms of trunk diseases, including low vigor, stunted canopy, and dead spurs, and also for retrained cordons or trunks, which is a common management practice to limit the spread of the pathogens from infected tissues to healthy tissues, further limiting inoculum availability for vine-to-vine disease spread. A total of 1260 samples (860 in eastern North America, 400 in California) were collected from wood cankers or discolored wood, revealed by cutting through symptomatic spurs, cordons, and trunks, according to Petit *et al.* (2011). Microbiological

isolations of fungi from the wood samples were performed according to Baumgartner *et al.* (2013). Briefly, 4 × 4 × 4-mm wood pieces cut from the margins of diseased wood were sterilized in 0.6 % sodium hypochlorite and plated onto potato dextrose agar (PDA, Difco, Detroit, MI) amended with tetracycline (1 mg L^{-1}). Plates were incubated at 25 °C in darkness for up to 21 d. From eastern North America and California vineyards, 79 and 18 isolates with morphological characteristics of *Cadophora* species were recovered, representing an incidence of 9 % and 4.5 % respectively, from the total number of wood samples examined. Of those, we selected 25 isolates representative of distinct morphological groups (Table 1), mainly based on colony morphology; they were subsequently single-spore purified to PDA plates, to perform phylogenetic and morphological analyses, and pathogenicity tests.

Phylogenetic analyses

Genomic DNA was isolated from liquid cultures grown at 25 °C in 2 ml of potato dextrose broth (PDB) at 150 RPM after 14 d (DNeasy Plant kit; Qiagen), following the manufacturer's instructions. Amplification of portions of the 18S and 28S ribosomal DNA (rDNA) including the intervening internal transcribed spacer regions and 5.8S rDNA (ITS1–5.8S–ITS2) were performed using the primer set ITS1 and ITS4 following the protocol of White *et al.* (1990). Amplification of translation elongation factor 1-α (TEF1-α) utilized the primer set EF1-688F and EF1-1251R (Carbone & Kohn 1999). Amplification of portions of the beta-tubulin (BT) locus using primers T1 and T2 (O'Donnell & Cigelnik 1997) and Bt2a and Bt2b (Glass & Donaldson 1995) produced multiple PCR products for some isolates, therefore a novel BT primer set, BTCadF 5′ MATGCGTGAAATYGTAAGT3′ and BTCadR 5′ TCAGCACCCT CAGTGTAATG3′ (PCR program: 94 °C initial denaturation for 4 min followed by 35 cycles of 94 °C denaturation for 1 min, 56 °C annealing for 30 s, 72 °C extension for 1 min, and a final extension at 72 °C for 10 min) was designed based on the following available BT sequences of Helotiales fungi: *Meliniomyces variabilis* Hambl. & Sigler, *Meliniomyces bicolor* Hambl. & Sigler, *Ascocoryne sarcoides* (Jacq.) J.W. Groves & D.E. Wilson, and *Sclerotinia sclerotiorum* (Lib.) de Bary (http://genome.jgi-psf.org/programs/fungi/index.jsf). PCR products were visualized on a 1.5 % agarose gel (120 V for 25 min) to validate presence and size of amplicons followed by purification via Exonuclease I and recombinant Shrimp Alkaline Phosphatase (Affymetrix) and sequenced in both directions on an ABI 3730 Capillary Electrophoresis Genetic Analyzer at the College of Biological Sciences Sequencing Facility at the University of California, Davis.

Nucleotide sequences were proofread and edited in Sequencher v. 5.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and deposited in GenBank (Table 1). BLASTn searches of GenBank identified homologous ITS sequences with high sequence identity from type isolates and were included for phylogenetic reference. In addition, we included ITS sequences of *Cadophora* isolates representative of taxa previously recovered from grapevine (Table 1). Multiple sequence alignments were assembled in MEGA v. 6 (Tamura *et al.* 2013) and manually improved where necessary in Mesquite v. 2.74 (Maddison & Maddison 2010). Alignments were submitted to

TreeBASE under accession number S16293. Each dataset (ITS, TEF1-α, BT, and combined) was analyzed using maximum likelihood (ML) and maximum parsimony (MP). For ML analyses, the Akaike Information Criterion (AIC) implemented in MEGA v. 6 (Tamura *et al.* 2013) was used to identify the best-fit model of nucleotide evolution for each dataset. The ML analyses were conducted in MEGA v. 6 (Tamura *et al.* 2013) utilizing the Nearest-Neighbor-Interchange heuristic method and topological support was assessed by 1000 bootstrap replicates. For MP analyses heuristic searches consisted of 1000 random sequence additions with the tree-bisection-reconnection branch swapping algorithm in PAUP* v. 4.0b10 (Swofford 2002), gaps were treated as missing data. Branch stability was estimated by 1000 bootstrap replications for each dataset. Sequences of *Cadophora finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew served as the outgroup taxon in all analyses. Concordance among datasets ($P \geq 0.01$) was evaluated with the partition homogeneity test (PHT) in PAUP* v. 4.0b10 (Swofford 2002). Concordant datasets were combined and analyzed as described above.

Morphology

Morphological characterization of isolates U5, U17, ONC1, NYC1, NYC14, NYC12, NHC1 CBS 101539, and CBS 111743 was assessed on malt extract agar (MEA) and/or 1 % PDA after eight to 21 d at 25 °C in the dark, to determine cultural and microscopic characteristics of conidia, conidiophores, phialides, and collarettes. Colony colors were determined using taxonomic description color charts (Rayner 1970). Slides were produced by mounting aerial hyphae in water and 30 measurements were made of each microscopic morphological structure at 1000× magnification.

For micrographs acquisition, suspensions of aerial mycelium and spores were stained with the fluorochrome Calcofluor White Stain M2R (CFW; Sigma–Aldrich®, Switzerland). CFW selectively binds to cellulose, and chitin and was used here for sensitive, direct microscopic examination of fungal cultures. Staining was achieved by placing a 1.5 μl drop of 0.1 % CFW onto 6.5 μl fungal suspension on glass slides, covering it with a coverslip, followed by immediate microscopic examination. The chitin walls stained with CFW were observed under a compound microscope (Leica DM500B model) equipped with ×100 objective lens, a mercury short-arc lamp (Osram HXP model), and a set of Leica optic filters. The Leica filter cube N3 green (excitation filter BP 564/12, suppression filter BP 600/40) was used for acquiring all micrographs. The microscope was equipped with a Leica color video camera (model DFC 310 FX).

Optimal growth temperature was tested by culturing each isolate in triplicate on MEA in the dark at temperatures ranging from 5 °C to 35 °C at 5 °C increments. Mycelial plugs (5 mm diam.) were taken from the margin of an actively-growing culture and transferred to the center of 90-mm diam. petri dishes. Radial growth was measured after 13 d. Two measurements perpendicular to each other were taken of the colony diameter. Analyses of variance (ANOVAs) were used to test the null hypotheses that conidial dimensions and colony diameters were equal among isolates. Homogeneity of variance was evaluated prior to ANOVA. ANOVA was performed using

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Table 1 — *Cadophora* isolates collected from vineyards in North America, and type specimens used for phylogenetic comparisons.

Species	Isolate[a]	Location	Year collected	Collector	Host	Substrate	GenBank Accession[d]		
							ITS	Beta-tubulin	TEF1-α
Cadophora fastigiata	A168; CBS 307.49[T]	Sweden	1949	S.O. Pehrson	Not recorded	Not recorded	AY249073	KM497131	KM497087
C. finlandica	CBS 444.86[T]	Finland	1975	C.J.K. Wang	Not recorded	Not recorded	AF486119	KM497130	KM497086
C. luteo-olivacea	A175; CBS 141.41[T]	Sweden	1939	M. Sulfatfabrik	Not recorded	Not recorded	AY249066	KM497133	KM497089
C. luteo-olivacea	A19	California, USA	1998	H. Scheck	*Vitis vinifera*	Vascular discoloration	KM497038	KM497119	KM497075
C. luteo-olivacea	A41	California, USA	1998	H. Scheck	*V. vinifera* 'Chardonnay'	Vascular discoloration	KM497039	KM497120	KM497076
C. luteo-olivacea	A42	California, USA	1998	H. Scheck	*V. vinifera* 'Chardonnay'	Vascular discoloration	KM497040	KM497121	KM497077
C. luteo-olivacea	U5*	California, USA	1999	S. Rooney-Latham	*V. vinifera* 'Sangiovese'	Vascular discoloration	KM497041	KM497122	KM497078
C. luteo-olivacea	U7	California, USA	1999	S. Rooney-Latham	*Olea europa*	Vascular discoloration	KM497044	KM497125	KM497081
C. luteo-olivacea	U8	California, USA	1998	H. Scheck	*V. vinifera* 'Semillon'	Vascular discoloration	KM497042	KM497123	KM497079
C. luteo-olivacea	U17*	California, USA	1998	H. Scheck	*V. vinifera* 'Chardonnay'	Vascular discoloration	KM497043	KM497124	KM497080
C. luteo-olivacea	U21	California, USA	1998	H. Scheck	*V. vinifera*	Vascular discoloration	KM497045	KM497126	KM497082
C. luteo-olivacea	U22	California, USA	1998	H. Scheck	*V. vinifera* 'Chardonnay'	Vascular discoloration	KM497046	KM497127	KM497083
C. luteo-olivacea	U53	California, USA	2004	S. Rooney-Latham	*V. vinifera* 'Chardonnay'	Vascular discoloration	KM497047	KM497128	KM497084
C. luteo-olivacea	U56	California, USA	2004	S. Rooney-Latham	*V. vinifera* 'Syrah'	Vascular discoloration	KM497048	KM497129	KM497085
C. luteo-olivacea	FI2131	Uruguay	Unknown	Unknown	*V. vinifera*	Petri disease symptoms	HQ586008		
C. luteo-olivacea	Clo-28	Spain	2007	D. Gramaje	*Vitis berlandieri×* *V. rupestris* 1103P	Vascular streaking	HQ661091		
C. luteo-olivacea	CBS 109924	South Africa	2000	F. Halleen	*V. vinifera* 'Sultana'	Asymptomatic *V. vinifera*	EF042108		
C. luteo-olivacea	PARC314	British Columbia, Canada	2008—2012	J.R. Úrbez-Torres	*V. vinifera* 'Chardonnay'	Vascular streaking	KF764524		
C. malorum	CBS 165.42[T]	Netherlands	1942	S.A. de Lange	*Amblystoma mexicanum*	Not recorded	AY249059	KM497134	KM497090
C. melinii	A164; CBS 268.33[T]	Sweden	1934	E. Melin	Not recorded	Not recorded	AY249072	KM497132	KM497088
C. melini	ONC1*	Ontario, Canada	2008	P.E. Rolshausen	*V. vinifera* 'Cabernet Franc'	Wood canker	KM497033	KM497114	KM497070
C. melini	U11	California, USA	1997	H. Scheck	*V. vinifera* 'Sangiovese'	Vascular discoloration	KM497032	KM497113	KM497069
C. novi-eboraci	NYC1*	New York, USA	2008	P.E. Rolshausen	*V. vinifera* 'Cabernet Sauvignon'	Wood canker	KM497035	KM497116	KM497072
C. novi-eboraci	NYC2	New York, USA	2008	P.E. Rolshausen	*V. labruscana* 'Concord'	Wood canker	KM497034	KM497115	KM497071
C. novi-eboraci	NYC13	New York, USA	2008	P.E. Rolshausen	*Vitis vulpina*	Wood canker	KM497036	KM497117	KM497073
C. novi-eboraci	NYC14*T	New York, USA	2008	P.E. Rolshausen	*V. labruscana* 'Concord'	Wood canker	KM497037	KM497118	KM497074
C. novi-eboraci[b]	CBS 101359	Italy	1998	F. Calzarano	*Actinidia chinensis*	Stained wood	DQ404350	KM497135	KM497092
C. orientoamericana	CTC1	Connecticut, USA	2008	P.E. Rolshausen	*V. vinifera* 'Chardonnay'	Wood canker	KM497012	KM497093	KM497049
C. orientoamericana	CTC2	Connecticut, USA	2008	P.E. Rolshausen	*Vitis* hybrid 'Marechal Foch'	Wood canker	KM497013	KM497094	KM497050
C. orientoamericana	CTC4	Connecticut, USA	2008	P.E. Rolshausen	*V. vinifera* 'Cabernet Franc'	Wood canker	KM497014	KM497095	KM497051
C. orientoamericana	CTC5	Connecticut, USA	2008	P.E. Rolshausen	*Vitis* hybrid 'Cayuga white'	Wood canker	KM497015	KM497096	KM497052
C. orientoamericana	MAC2	Massachusetts, USA	2008	P.E. Rolshausen	*Vitis* hybrid 'Aurore'	Wood canker	KM497016	KM497097	KM497053
C. orientoamericana	MDC1	Maryland, USA	2008	P.E. Rolshausen	*V. vinifera* 'Cabernet Sauvignon'	Wood canker	KM497017	KM497098	KM497054
C. orientoamericana	NHC1*T	New Hampshire, USA	2008	P.E. Rolshausen	*Vitis* hybrid 'Niagara'	Wood canker	KM497018	KM497099	KM497055
C. orientoamericana	NHC2	New Hampshire, USA	2008	P.E. Rolshausen	*Vitis* hybrid 'Chancellor'	Wood canker	KM497019	KM497100	KM497056
C. orientoamericana	NHC3	New Hampshire, USA	2008	P.E. Rolshausen	*Vitis* hybrid 'Marechal Foch'	Wood canker	KM497020	KM497101	KM497057

C. orientoamericana	NYC3	2008	New York, USA	P.E. Rolshausen	*V. labruscana* 'Concord'	Wood canker	KM497021	KM497102	KM497058
C. orientoamericana	NYC6	2008	New York, USA	P.E. Rolshausen	*V. riparia*	Wood canker	KM497022	KM497103	KM497059
C. orientoamericana	NYC9	2008	New York, USA	P.E. Rolshausen	*V. labruscana* 'Concord'	Wood canker	KM497023	KM497104	KM497060
C. orientoamericana	NYC11	2008	New York, USA	P.E. Rolshausen	*V. vinifera* 'Chardonnay'	Wood canker	KM497024	KM497105	KM497061
C. orientoamericana	NYC12*	2008	New York, USA	P.E. Rolshausen	*V. vinifera* 'Chardonnay'	Wood canker	KM497025	KM497106	KM497062
C. orientoamericana	OHC1	2008	Ohio, USA	P.E. Rolshausen	*V. labruscana* 'Concord'	Wood canker	KM497028	KM497109	KM497065
C. orientoamericana	VAC1	2008	Virginia, USA	P.E. Rolshausen	*V. vinifera* 'Sauvignon blanc'	Wood canker	KM497026	KM497107	KM497063
C. orientoamericana	VTC1	2008	Vermont, USA	P.E. Rolshausen	Vitis hybrid 'Vignoles'	Wood canker	KM497027	KM497108	KM497064
Cadophora sp.	Cme-1	2007	Spain	D. Gramaje	*V. vinifera* 'Syrah'	Vascular streaking	HQ661096		
C. spadicis	QCC1	2008	Québec, Canada	P.E. Rolshausen	*V. vinifera* 'Gamay'	Wood canker	KM497031	KM497112	KM497068
C. spadicis	RIC1	2008	Rhode Island, USA	P.E. Rolshausen	*V. vinifera* 'Cabernet Sauvignon'	Wood canker	KM497029	KM497110	KM497066
C. spadicis	RIC3	2008	Rhode Island, USA	P.E. Rolshausen	Vitis hybrid 'Vidal'	Wood canker	KM497030	KM497111	KM497067
C. spadicis[c,T]	CBS 111743	2002	Italy	N. Paola	*A. chinensis*	Vascular discoloration	DQ404351	KM497136	KM497091

a Type, ex-type, and neotype isolates are denoted by 'T'. Isolates used for morphological characterization and in the pathogenicity assay are denoted by an asterisk.
b Previously identified as *C. malorum*.
c Previously identified as *C. melinii*.
d Sequences in bold were produced in this study.

the MIXED procedure in SAS v. 9.2 (SAS Institute, Cary, NC, USA), and the main effect of isolate was treated as a fixed effect. For significant effects ($P < 0.05$), means were compared by Tukey's tests. Reverse-transformed means and 95 % confidence limits are presented for data that were transformed prior to ANOVA.

Pathogenicity tests

Seven isolates, representative of four of the *Cadophora* species identified by molecular analyses (Table 1), were selected for inoculation to the woody stems of potted *V. vinifera* 'Chardonnay' in the greenhouse. For each isolate, inoculum consisted of a conidial suspension obtained after 21 d incubation in the dark on PDA. Conidia were harvested by flooding each PDA plate with 2 ml of sterile water and gently scrapping the agar surface with a sterile glass rod. The suspension was filtered through two layers of sterile cheesecloth to remove aerial mycelium. Conidia concentrations were estimated with a hemocytometer and adjusted with sterile water to 1×10^6 conidia ml^{-1}.

Plants were propagated from dormant cuttings according to Travadon *et al.* (2013). Briefly, starting in March 2012, cuttings were callused at 30 °C and 100 % humidity in a mixture of perlite and vermiculite (1:1, vol/vol) for 20 d. Once shoot and root initials emerged from the callused 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 (American National Can) to prevent inoculum desiccation. Non-inoculated controls were mock-inoculated with sterile water. 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, Marysville, OH, USA). Plants were grown in the greenhouse at the University of California Experimental Station in Davis from April 2012 to April 2014 [natural sunlight photoperiod, 25 ± 1 °C (day), 18 ± 3 °C (night)]. Each year, in early November, plants were pruned to 2 to 3-bud canes and the temperature was dropped [10 ± 2 °C (day), 4 ± 2 °C (night)] until the end of January. Plants were watered twice per week for 15 min using a drip-irrigation system (0.5 l h^{-1}). Two replicate experiments were performed one week apart on two sets of plants propagated in two separate greenhouses. In each experiment, 12 plants were inoculated with each isolate and arranged in a completely randomized design.

Pathogenicity was evaluated approximately 24 m after inoculation by measuring the extent of wood discoloration above and below the inoculation site. First, the green shoots, roots, and bark of each plant were removed and the woody stems were surface sterilized in 1 % sodium hypochlorite for 2 min and rinsed with deionized water. The extent of wood discoloration above and below the inoculation site was measured with a digital caliper. To confirm that the pathogen was responsible for wood discoloration, pathogen recovery was attempted by cutting ten pieces (2 × 5 mm) of wood from the distal margin of the lesions followed by surface sterilization in 0.6 % sodium hypochlorite (pH 7.2) for 30 s and

rinsed twice for 30 s in sterile deionized water, and plated on 1 % PDA amended with tetracycline (5 mg ml^{-1}) and ampicillin (250 mg ml^{-1}), and 2 % MEA amended with streptomycin (100 mg ml^{-1}) and ampicillin (250 mg ml^{-1}) in the dark at approximately 22 °C for 14–21 d.

The length of wood discoloration (LWD) was used as a measure of pathogenicity. Normality and homogeneity of variances were evaluated using normal probability plots and Levene's test, respectively. Log-transformation was applied to LWD to meet parametric assumptions. ANOVAs were used to determine the effect of the isolate on LWD. ANOVAs were performed using the MIXED procedure in SAS, with experiment considered as random effects. Means were calculated using the LSMEANS procedure. Pairwise mean differences with control level (non-inoculated control) were analyzed using Dunnett's test ($P < 0.05$). Recovery from inoculated plants was a second measure of pathogenicity. Recovery rate was calculated as the percentage of plants from which the pathogen was recovered, out of the total number of inoculated plants. To assess the main effect of isolate on recovery rate, generalized linear mixed models were performed using the GLIMMIX procedure in SAS, which utilizes the *logit* link function to accommodate binomial data. The factor experiment was considered as random effect. Recovery rates of the non-inoculated controls (all of which were zero) were excluded from the analyses.

Results

Phylogenetic analyses

For ML analyses, the best-fit model of nucleotide evolution was deduced based on the AIC (K2+G for ITS and TEF1-α and T92+I for BT). Analyses of individual loci (ITS, TEF1-α, and BT) yielded similar trees, which differed primarily in intraspecies relationships and the order of divergence among *Cadophora* species. Alignment of ITS resulted in a 528-character dataset (400 characters were constant, 44 characters were parsimony-uninformative, and 84 characters were parsimony informative). MP analyses produced 72 equally-most parsimonious trees of 180 steps and a consistency index (CI) and retention index (RI) of 0.878 and 0.977, respectively. ML and MP analyses revealed that the novel *Cadophora* isolates were divided into five well-supported clades (≥83 %/≥88 % ML and MP bootstrap values, respectively), which included *Cadophora malorum* CBS 165.42 nested within the *Cadophora luteo-olivacea* clade, which also included isolates of *C. luteo-olivacea* from other grape-growing regions (Fig S1). Two isolates (CBS 111743 and Cme-1) previously characterized as *Cadophora melinii* clustered distantly from one another and from the type isolate.

Alignment of TEF1-α resulted in a 460-character dataset (317 characters were constant, 42 characters were parsimony-uninformative, and 101 characters were parsimony informative). MP analyses produced 24 equally-most parsimonious trees of 209 steps, and CI and RI of 0.847 and 0.968, respectively. Analysis of TEF1-α and ITS produced topologically-similar trees, with five well-supported clades (≥98 %/≥99 %). However, *C. malorum* CBS 165.42 was strongly

supported (100 %/100 %) as the sister group to *C. luteo-olivacea* (Fig S2).

Alignment of BT resulted in a 538-character dataset (250 characters were constant, 43 characters were parsimony-uninformative, and 245 characters were parsimony informative). MP analyses produced 238 trees of 470 steps, and CI and RI of 0.821 and 0.968, respectively. ML and MP analyses revealed that the recovered *Cadophora* isolates were divided into four well-supported (≥96 %/≥98 %) clades, similar to analyses of ITS and TEF1-α. However, *Cadophora fastigiata* CBS 307.49 was moderately to highly supported (85 %/92 %) as sister to the clade that contained isolates collected from Rhode Island and Québec, Canada, including *C. melinii* CBS 111743 (isolated from kiwi in Italy). Additionally, *C. malorum* CBS 165.42 and *C. luteo-olivacea* isolates represented a strongly-supported monophyletic lineage, although *C. luteo-olivacea* was poorly to highly (≤70 %/96 %) supported as a monophyletic group (Fig S3).

Test for concordance between datasets using the PHT revealed that these data (ITS, TEF1-α, and BT) were not significantly inconcordant (P = 0.01), and were combined and analyzed as above. Alignment of the combined dataset consisted of 1526 characters (967 characters were constant, 129 characters were parsimony-uninformative, and 430 characters were parsimony informative). Thirteen isolates clustered with strong support (86 %/100 %) with the ex-type specimen *C. luteo-olivacea* CBS 141.41 (Fig 1). Two isolates, U11 and ONC1, formed a monophyletic group with the type specimen *C. melinii* CBS 268.33, which was strongly supported as sister to the type species for the genus, *C. fastigiata* CBS 307.49. Four isolates from New York (NYC1, NYC2, NYC13, and NYC14) clustered with CBS 101359, which is currently misidentified as *C. malorum*, and so we propose to name this clade *Cadophora novi-eboraci*. Sequences from two isolates from Rhode Island and one from Québec grouped with strong support (100 %/100 %) with an isolate (CBS 111743) currently misidentified as *C. melinii*, which was originally isolated from kiwi. We propose to name this clade *Cadophora spadicis*. Seventeen isolates collected from eight states in the eastern United States clustered as a strongly-supported group (99 %/100 %) with no known species name, and so we propose to name this group *Cadophora orientoamericana*.

Morphology

After 13 d on MEA, isolates U17 and U5 produced morphologically-similar colonies with smooth margins. Isolate U5 produced an olivaceous black felty center (Fig 2A), whereas isolate U17 produced flat, olivaceous black centers harboring fissures (Fig 2B). Isolate ONC1 produced a felty colony, with white to hazel hyphae, with a smooth margin (Fig 2C). Isolates NYC1 and NYC14 were virtually identical on MEA with the production of a white flat colony with smooth margins and aerial mycelial tufts in the center (Fig 2D and E). Isolate CBS 101359 grew very slowly with an irregular margin and a white colony (Fig 2F). Isolate NYC12 produced a felty colony that was white with a smooth margin (Fig 2G). NHC1 produced a white colony with a smooth, regular, and flat margin with some aerial hyphae toward the center (Fig 2H). Isolate CBS 111743 produced a vinaceous buff to fawn colony with

Fig 1 — Single most likely tree (ln likelihood-6479.4859) resulting from the analysis of three-gene combined dataset (ITS, TEF1-α, and BT). Numbers in front and after the slash represent maximum likelihood and maximum parsimony bootstrap values, respectively. Values represented by an asterisk were less than 70 %. Scale bar represents the number of substitutions per site.

aerial hyphae located in the center, whereas the margin was smooth and regular (Fig 2I).

After 13 d growth on MEA at 25 °C in the dark, average colony radial growth differed significantly among the seven isolates examined (P [$F_{(8,18)} > 321.48$] < 0.0001), ranging from 9.5 mm for NYC1 to 42.5 mm for ONC1 (Table 2). Growth at incremental temperatures of 5 °C from 5 °C to 35 °C on MEA for 13 d in the dark revealed that optimal growth temperature

was 20 °C for all isolates except U17, which reached slightly larger colony diameter at 25 °C (Fig 3). The minimum temperature for growth for all isolates was 5 °C. None of the isolates grew at 35 °C. Two of the isolates, NYC14 and NYC1, had little to no growth at 30 °C and had overall slower growth rates. At 25 °C after 13 d in the dark, growth rates were 0.78, 0.92, and 1.12 mm per day for *C. novi-eboraci* isolates NYC1, NYC14, and CBS 101359, respectively, 2.11 and 2.33 mm per day for
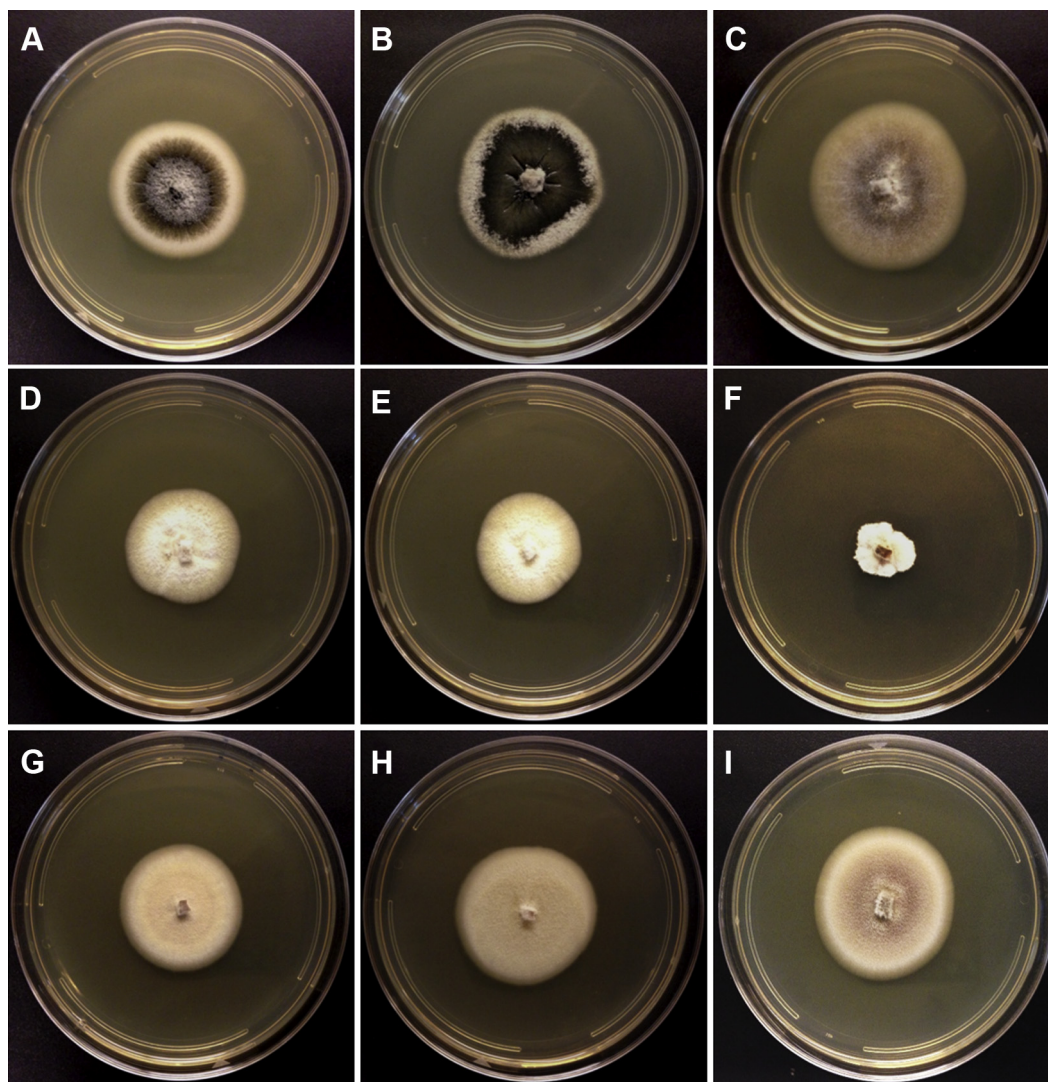
Fig 2 − Cultures of (A) *C. luteo-olivacea* U17, (B) *C. luteo-olivacea* U5, (C) *C. melinii* ONC1, (D) *C. novi-eboraci* NYC1, (E) *C. novi-eboraci* NYC14, (F) *C. novi-eboraci* CBS 101359, (G) *C. orientoamericana* NHC1, (H) *C. orientoamericana* NYC12, (I) *C. spadicis* CBS 111743, cultured on malt extract agar at 25 °C in the dark after 15 d.

Table 2 − Colony radial growth and conidial morphology of seven isolates, representing five *Cadophora* species sampled from vineyards in North America.

Species	Isolate	Colony diameter[a] (mm)	Mean conidia length[b] (µm)	Mean conidia width[b] (µm)
Cadophora luteo-olivacea	U5	29.7 [28.2−31.2] D	5.22 [5−5.4] C	2.17 [2−2.3] B
C. luteo-olivacea	U17	41.3 [39.8−42.8] EF	4.56 [4.4−4.7] A	1.94 [1.8−2.1] A
C. melinii	ONC1	42.5 [41−44] F	4.66 [4.5−4.8] AB	2.13 [2−2.3] B
C. novi-eboraci	NYC1	9.5 [8−11] A	5.77 [5.6−6] D	2.28 [2.1−2.4] B
C. novi-eboraci	NYC14	11.2 [9.7−12.7]AB	4.9 [4.7−5.1] AB	2.33 [2.2−2.5] B
C. novi-eboraci	CBS 101359	14.6 [13.1−16.1] B	5.25 [5.1−5.4] C	2.73 [2.6−2.9] CD
C. orientoamericana	NHC1	25.2 [23.7−26.7] C	6.53 [6.3−6.7] E	3.02 [2.9−3.2] D
C. orientoamericana	NYC12	23.8 [22.3−25.3] C	5.48 [5.3−5.7] CD	3.56 [3.4−3.7] E
C. spadicis	CBS 111743	38.7 [37.3−40.3] E	5.03 [4.8−5.2] BC	1.92 [1.8−2.1] A

a Colony diameter was measured after 13 d growth at 25 °C on malt extract agar. Each value is the mean of three cultures. Numbers in brackets indicate 95 % confidence limits. Means followed by the same letter are not significantly different (P < 0.05; Tukey's test).
b Each value is the mean of thirty conidia measurements. Numbers in brackets indicate 95 % confidence limits. Means followed by different letters are significantly different (P < 0.05; Tukey's test).
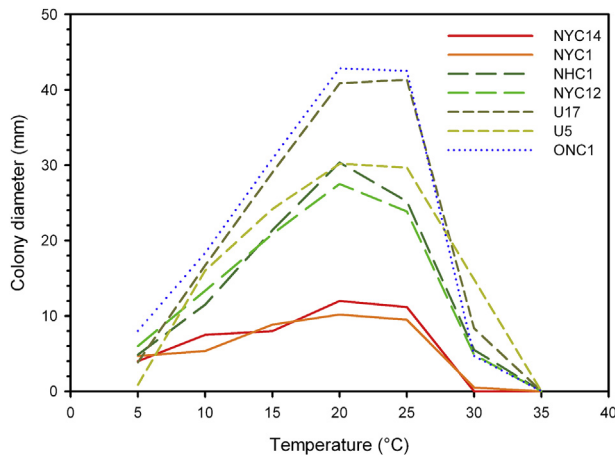
Fig 3 — Average colony diameter of *C. luteo-olivacea* isolates U17 and U5, *C. novi-eboraci* NYC1 and NYC14, *C. melinii* ONC1, and *C. orientoamericana* NYC12 and NHC1, assessed on malt extract agar (MEA) after 13 d growth in the dark at temperatures ranging from 0 to 35 °C, in 5 °C increments. Three MEA plates per isolate were used.

Cadophora orientoamericana isolates NYC12 and NHC1, respectively, 3.14 and 2.32 mm per day for *C. luteo-olivacea* isolates U17 and U5, respectively, 3.29 mm per day for *C. melinii* isolate ONC1, and 2.97 mm per day for *C. spadicis* isolate CBS 111743.

Cadophora luteo-olivacea isolates U5 and U17 produced pale hazel, septate hyphae 2.82 μm and 2.41 μm wide, respectively, conidiophores were simple, mostly straight, septate, pale hazel, for U5 10.67−(25.7)−61.11 × 1.94−(2.27)−2.91 μm and U17 9.7−(21.27)−34.92 × 1.94−(2.29)−2.91 μm, conidiogenous cells located laterally or terminally, monophialidic, hyaline, mostly cylindrical and tapering toward apex, for U5 8.73−(12.23)−21.34 × 2.42−(3.07)−3.88 μm and U17 8.73−(13.06)−25.22 × 1.94−(2.26)−2.91 μm, conidia hyaline, mostly biguttulate, oblong elliptical or ovoid, some with slight medial constriction, single, aseptate, for U5 4.85−(5.22)−6.3 × 1.94−(2.17)−2.42 μm and U17 3.88−(4.56)−5.33 × 1.45−(1.94)−2.42 μm. *Cadophora melinii* isolate ONC1 produced medium brown to dark brown septate hyphae 2.52 μm wide, conidiophores were simple, straight, septate, medium brown, 6.79−(18.8)−62.08 × 2.42−(2.52)−2.91 μm, conidiogenous cells located laterally or terminally, monophialidic, medium brown, straight or curved, 7.76−(10.52)−17.46 × 1.94−(2.79)−3.88 μm with cup-shaped flaring medium brown collarettes, conidia light brown, mostly biguttulate, cylindrical to oblong ellipsoidal, single, aseptate, 3.88−(4.66)−5.33 × 1.94−(2.13)−2.91 μm. *Cadophora orientoamericana* isolates NYC12 and NHC1 produced hyaline, septate hyphae 3.13 and 2 μm wide, respectively, conidiophores were simple or branched, medium, septate, hyaline, for NYC12 9.7−(30.58)−52.38 × 2.42−(3.05)−3.88 and NHC1 11.64−(25.8)−51.41 × 1.94−(2.63)−3.39 μm, conidiogenous cells terminal, single, hyaline, for NYC12 6.79−(12.44)−21.34 × 2.42−(3.02)−3.88 and for NHC1 7.76−(9.02)−13.58 × 2.42−(2.84)−3.39 μm, conidia hyaline, ovoid, cylindrical to elliptical, biguttulate, single, aseptate, for NYC12 4.85−(5.48)−6.79 × 2.42−(3.56)−4.36 for NHC1 5.34−(6.53)−7.76 × 2.42−(3.02)−3.4 μm. *Cadophora novi-eboraci* isolates NYC1, NYC14, and CBS 101359 produced hyaline, septate

hyphae 2.42, 2.47, and 2.07 μm wide, respectively, conidiophores simple or branched, septate, hyaline, for NYC1 11.64−(31.1)−54.32 × 1.45−(1.97)−2.42 μm, for NYC14 9.7−(31.13)−59.17 × 1.94−(1.87)−2.42 μm, for CBS 101359 7.76−(29.55)−59.17 × 1.45−(1.82)−1.94 μm, conidiogenous cells terminal or lateral, obclavate, mostly monophialidic some multiphialidic, hyaline, for NYC1 5.82−(9.53)−12.61 × 1.45−(2.1)−2.91 μm, for NYC14 6.79−(10.63)−17.46 × 1.94−(2.18)−2.91 μm, for CBS 101359 5.82−(8.02)−10.67 × 1.94−(2.42)−2.91 μm, conidia hyaline, ovoid to oblong elliptical, single, aseptate, some biguttulate, for NYC1 4.85−(5.77)−6.79 × 1.94−(2.28)−2.91 μm, for NYC14 3.88−(4.9)−5.82 × 1.94−(2.33)−2.91 μm, and for CBS 101359 4.85−(5.25)−6.79 × 2.42−(2.73)−2.91 μm. *Cadophora spadicis* isolate CBS 111743 produced simple or branched, septate, pale brown hyphae 3.12 μm wide, conidiophores simple to branched, septate, pale brown, 9.7−(22.63)−53.35 × 1.45−(1.95)−2.42 μm, conidiogenous cells lateral or terminal, long, mostly monophialidic some multiphialidic, brown to light brown, 7.76−(11.44)−17.46 × 1.94−(2.42)−2.91 μm, conidia light brown, oblong cylindrical, aseptate, single, some biguttulate, 3.88−(5.03)−6.3 × 1.45−(1.92)−2.42 μm. There were significant differences in conidia length (P [$F_{(8,261)}$ > 41.72] < 0.0001) and in conidia width (P [$F_{(8,261)}$ > 63.96] < 0.0001) among the nine isolates examined. Isolates NYC12 and NHC1 had particularly wide conidia, relative to other isolates (Table 2).

Taxonomy

Based on phylogenetic analyses (ML and MP) of three nuclear loci, three distinct and strongly-supported clades (*Cadophora novi-eboraci*, *Cadophora orientoamericana*, and *Cadophora spadicis*), for which no applied species names exist, were identified. Additionally, *C. melinii* CBS 111743 and *C. malorum* CBS 101359 are currently misidentified, and thus we propose the following new species to properly circumscribe these taxa:

Cadophora novi-eboraci Travadon, Lawrence, Rooney-Latham, Gubler, Wilcox, Rolshausen & K. Baumgartner, **sp. nov.**

MycoBank No.: MB 810073; Fig 4A and B.

Etymology: The name refers to the geographic location from which isolates were collected, New York State.

No teleomorph observed.

Colony diameter 11.16 mm in 13 d at 25 °C on MEA, slow growing, white flat colony with smooth margins and aerial mycelial tufts in the center. Hyphae hyaline, smooth, straight, branched, septate, 1.94−(2.47)−3.39 μm wide. Conidiophores arising from agar surface or aerial hyphae, simple or branched, septate, hyaline, 9.7−(31.13)−59.14 × 1.94−(1.87)−2.42 μm. Conidiogenous cells terminal or lateral, monophialidic, obclavate, flexous, hyaline, 6.79−(10.63)−17.46 × 1.94−(2.18)−2.91 μm. Conidia hyaline, ovoid, cylindrical to oblong elliptical, biguttulate, single, aseptate, 3.88−(4.9)−5.82 × 1.94−(2.32)−2.91 μm. Collarettes short, flaring, cup-shaped, 0.97−(1.23)−1.45 × 0.97−(1.26)−1.45 μm.
Holotype: ATCC NYC14, isolated from wood canker of *Vitis labruscana* cv. 'Concord', collected by P.E. Rolshausen in New York, USA in 2008.

Cadophora orientoamericana Travadon, Lawrence, Rooney-Latham, Gubler, Wilcox, Rolshausen & K. Baumgartner, **sp. nov.**
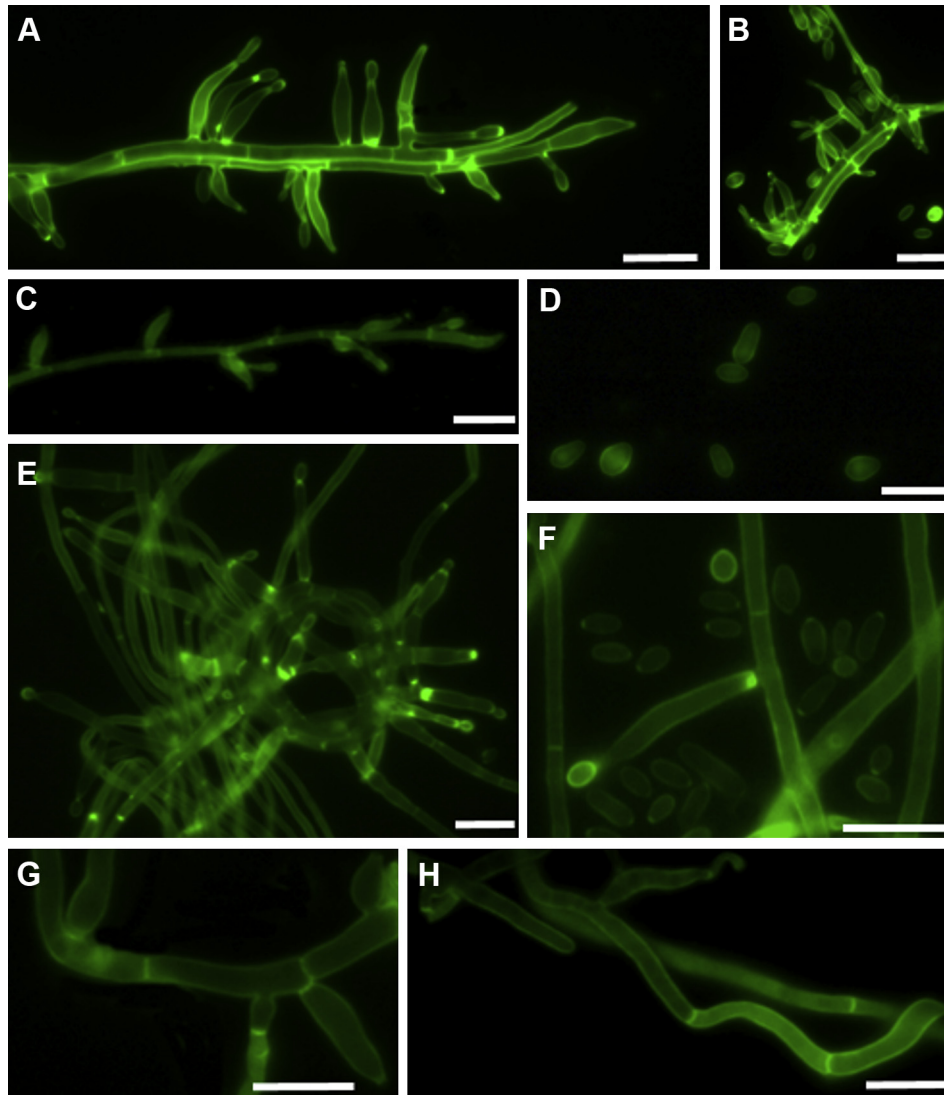
Fig 4 − Micrographs depicting the morphology of conidiogenous cells and conidia of *C. novi-eboraci* isolates NYC14 (A and B) and CBS 101359 (C and D), *C. spadicis* isolate CBS 111743 (E and F), and *C. orientoamericana* isolate NHC1 (G and H). Scale bar = 10 μm.

MycoBank No.: MB 810074; Fig 4G and H.

Etym.: The name refers to eastern North America, the geographic origin of the isolates for this species.

No teleomorph observed.

Colony diameter 25.16 mm in 13 d at 25 °C on MEA, white, smooth regular margin with some aerial hyphae toward the center. Hyphae hyaline, smooth, straight, branched, septate, 1.94−(2)−2.43 μm wide. Conidiophores arising from agar surface or aerial hyphae, simple or branched, medium, septate, hyaline, 11.64−(25.8)−51.41 × 1.94−(2.63)−3.39 μm. Conidiogenous cells terminal or lateral, single, hyaline, 7.76−(9.02)−13.58 × 2.42−(2.84)−3.39 μm. Conidia hyaline, ovoid, cylindrical to elliptical, biguttulate, single, aseptate, 5.34−(6.53)−7.76 × 2.42−(3.02)−3.4 μm. Collarettes short, flaring, cupshaped, 0.97−(1.53)−1.94 × 0.97−(1.47)−1.94 μm.
Holotype: ATCC NHC1, isolated from wood canker of *Vitis* hybrid cv. 'Niagara', collected by P.E. Rolshausen in New Hampshire, USA in 2008.

Cadophora spadicis CBS 111743 (Prodi, Sandalo, Tonti, Nipoti & A. Pisi) Travadon, Lawrence, Rooney-Latham, Gubler, Wilcox, Rolshausen & K. Baumgartner, **sp. nov.**

MycoBank No.: MB 810075; Fig 4E and F.

Etym.: The name refers to the distinctive vinaceous buff to fawn color of the colony.

No teleomorph observed.

Basionym: *Cadophora melinii* A. Prodi, Sandalo, Tonti, Nipoti & A. Pisi, Journal of Plant Pathology **90**: 3 (2008).

Colony diameter 38 mm in 13 d at 25 °C on MEA, vinaceous buff to fawn, white smooth regular margin with some aerial tufts of hyphae toward the center. Hyphae fawn to hazel, smooth, straight, branched, septate, 2.42−(3.12)−3.88 μm wide. Conidiophores arising from agar surface or aerial hyphae, simple or branched, medium, septate, fawn, 9.7−(22.63)−53.35 × 1.45−(1.95)−2.42 μm. Conidiogenous cells terminal or lateral, single, vinaceous buff to fawn, 7.76−(11.44)−17.46 × 1.94−(2.42)−2.91 μm. Conidia light hazel,

oblong cylindrical, aseptate, single, some biguttulate, 3.88−(5.03)−6.3 × 1.45−(1.92)−2.42 μm. Collarettes short, flaring, cup-shaped, 0.97−(1.66)−1.94 × 1.45−(1.86)−1.94 μm. Neotype: CBS 111743, isolated from kiwi tree, collected by N. Paola in Italy in 2002.

Pathogenicity tests

All isolates of *Cadophora luteo-olivacea*, *Cadophora melinii*, *Cadophora novi-eboraci*, and *Cadophora orientoamericana* caused wood lesions and black, vascular discolorations spreading above and below the inoculation site in the woody stems of potted 'Chardonnay' plants in the greenhouse, as observed 24 m post-inoculation (Fig S4). Wood discolorations caused by all seven *Cadophora* isolates were significantly longer than those of non-inoculated plants ($P < 0.05$; Dunnett's test; Table 3), indicating that each isolate was pathogenic. *Cadophora novi-eboraci* isolate NYC14 (25.7 mm) and *C. orientoamericana* NHC1 (23.8 mm) caused the largest discolorations (Table 3). The wood immediately surrounding the inoculation sites of non-inoculated control plants was discolored, but the discoloration was restricted (7.8 mm; N = 24 plants, averaged across experiments) and no pathogenic fungi were isolated from these lesions. From lesions of the inoculated plants, we recovered fungal colonies that matched morphologically the isolates inoculated to the plants. Recovery rate of the seven isolates ranged from 36 % for *C. melinii* ONC1 to 79 % for *C. orientoamericana* NHC1 and did not differ significantly among isolates (Table 3; $P > 0.05$; Tukey's test). Both the extent of wood discolorations and re-isolation success rates suggest that all seven *Cadophora* isolates are wood pathogens. Inoculated plants did not show apparent foliar symptoms that are typically associated with a trunk disease in the vineyard, such as tiger-stripe pattern, stunting or dieback of the green shoots.

Discussion

This study constitutes the first comprehensive characterization of *Cadophora* species recovered from the wood of grapevines exhibiting dieback and wood cankers, typical symptoms of trunk diseases, in North America. Five *Cadophora* species were identified, including three newly-described species, *Cadophora novi-eboraci*, *Cadophora orientoamericana*, and *Cadophora spadicis*, for which type specimens are described and deposited in publicly-available collections. Species delimitation was mainly based on comparisons of three nuclear loci, an unprecedented effort for this genus. Such sequence data will assist greatly in the identification of these fungi for mycologists and plant pathologists, as the number of species previously identified from grapevine, for example, was likely underestimated due to the lack of available sequence and morphological data for type specimens. Previous to this study, only *Cadophora luteo-olivacea* was reported from grapevine in North America (Rooney-Latham 2005) and the largest diversity reported from grapevine worldwide was limited to two species, including *C. luteo-olivacea* (Gramaje *et al.* 2011). We further characterized the pathogenicity of four *Cadophora* species *in planta* by means of controlled inoculations and demonstrated the role of *C. luteo-olivacea*, *Cadophora melinii*, *C. novi-eboraci*, and *C. orientoamericana* as grapevine trunk pathogens.

Prior to this study, the identification of *Cadophora* species has primarily been based on morphology and comparisons of ITS sequences. The genus *Cadophora*, related (Helotiales; *Phialocephala* W.B. Kendr.), and non-related (Chaetothyriales; *Phialophora*) phialophora-like species have suffered from taxonomic flux since their inception because of morphological overlap that exist among these taxa. Most notably, morphological similarities between these taxa occur in the production of phialides with flaring, hyaline collarettes. The genera *Cadophora* and *Phialocephala* are generally distinguished by phialide complexity and conidial length, with the former producing phialides singly or in groups of two or three and conidia longer than 4 μm, whereas the latter often produces densely packed heads of phialides and conidia shorter than 4 μm (Day *et al.* 2012). Morphological examination of phialides and conidial dimensions supported the results observed by Day *et al.* (2012), where *Cadophora* species predominately produced solitary phialides or small clusters of phialides in groups of two or three and conidial lengths that were greater than 4.5 μm. Analysis of conidial dimensions of isolates in this study revealed significant overlap among and within species,

Table 3 − Mean lesion lengths and recovery rates of seven *Cadophora* isolates from the woody stems of *V. vinifera* 'Chardonnay' at 24 m post-inoculation. Each value is the mean of 12 observations per experiment and two replicate experiments.

Species	Isolate	Mean length of wood discoloration (mm)[a]	Recovery rate[b]
Cadophora luteo-olivacea	U5	20.7 [14.3−30] B	0.43 A
C. luteo-olivacea	U17	16.4 [11.3−23.8] B	0.48 A
C. melinii	ONC1	21.7 [14.9−31.5] B	0.36 A
C. novi-eboraci	NYC1	14.8 [10.2−21.5] B	0.52 A
C. novi-eboraci	NYC14	25.7 [17.7−37.4] B	0.37 A
C. orientoamericana	NHC1	23.8 [16.5−34.5] B	0.79 A
C. orientoamericana	NYC12	21.9 [15−31.8] B	0.65 A
Non-inoculated control	−	7.8 [5.4−11.4] A	0

a Numbers in brackets indicate 95 % confidence limits. Means that are significantly greater than that of the non-inoculated control are followed by 'B' ($P < 0.05$; Dunnett's tests).
b Means followed by the same letter are not significantly different ($P < 0.05$; Tukey's test).

suggesting that conidial sizes should not be used for diagnostic purposes. Nonetheless, conidia of the two *C. orientoamericana* isolates examined were, on average, wider than 3 μm and significantly wider than all isolates, except *C. novi-eboraci* CBS 101359 (average width of 2.73 μm), suggesting that wide conidia are characteristic of *C. orientoamericana*. Colony growth rates also varied among species with three distinct categories: slow growth exhibited by isolates of *C. novi-eboraci*, medium growth by isolates of *C. orientoamericana*, and fast growth by *C. luteo-olivacea*, *C. melinii*, and *C. spadicis*; colony growth rates may be informative for identification of these species.

The geographic distribution of *Cadophora* species in North America may reflect their adaptation to contrasting environments. All *Cadophora* isolates had optimal growth rate at 20 °C, except *C. luteo-olivacea* U17 which optimal growth occurred at 25 °C, suggesting that this latter species may be better adapted to the warm, Mediterranean climate found in California, relative to *C. novi-eboraci* and *C. orientoamericana*, which were only recovered from the cooler, continental climate of eastern North America. This hypothesis is further supported by the higher growth rates of eastern North American *C. orientoamericana* isolates at 5 °C, as compared to California isolates *C. luteo-olivacea*. Nonetheless, more intense sampling would be necessary to draw any firm conclusions on *Cadophora* species distribution and its relation to environmental conditions, as our sampling scheme was focused on grapevine plants suffering from decline in targeted vineyards. The hypothesis of climate-driven distribution is supported by Blanchette *et al.* (2010), who recovered multiple *Cadophora malorum* isolates from degraded wood huts in Antarctica, whereas only one *C. luteo-olivacea* isolate was recovered from this extremely cold environment. Our findings suggest that *C. luteo-olivacea* is predominantly affecting vineyards in Mediterranean climates, as previously reported (Rooney-Latham 2005; Halleen *et al.* 2007; Gramaje *et al.* 2011; Navarrete *et al.* 2011), whereas distinct *Cadophora* species cause a trunk disease of vineyards in the cooler climate of eastern North America.

Due to the few morphological differences that could distinguish *Cadophora* species, DNA sequences were critical for species delimitation. Previous work has shown that the biology and morphology of *C. malorum* and *C. luteo-olivacea* are highly similar; however, ITS sequences are distinct and did not support them as independent monophyletic groups (Harrington & McNew 2003). In our study, the analysis of ITS sequences placed *C. malorum* CBS 165.42 within the *C. luteo-olivacea* group. Previous reports indicated that *C. malorum* should be sister to *C. luteo-olivacea* based on analysis of ITS (Harrington & McNew 2003; Gramaje *et al.* 2011). The analysis of an additional nuclear locus, TEF1-α, was able to strongly discriminate between these two taxa, confirming them as independent lineages and not synonyms. The inclusion of TEF1-α hence supported the taxonomy of Gams (2000) and was most informative in distinguishing these two species.

The genus *Cadophora* has received little interest in phylogenetic studies and this questions the accuracy of species identification in publicly-available culture and molecular databases. For example, isolate CBS 101359 is currently identified as *Phaeomoniella chlamydospora* in the CBS database,

whereas other authors classified this reference isolate as *C. malorum* (Di Marco *et al.* 2004; Prodi *et al.* 2008). Our findings based on three nuclear loci revealed that isolate CBS 101359 represents a new species, *C. novi-eboraci*, and was previously misidentified as *C. malorum* causing kiwi tree trunk hypertrophy (Di Marco *et al.* 2004). Similarly, isolate CBS 111743 is currently misidentified as *C. melinii* in public databases and other studies on *Cadophora* (Prodi *et al.* 2008; Gramaje *et al.* 2011). Our results based on the use of type-specimen sequences indicated that isolate CBS 111743 should be considered a new species, *C. spadicis*, and this isolate represents the neotype specimen for this newly-described species.

Before this study, only two *Cadophora* species were reported from grapevine wood, including *C. luteo-olivacea* and a second species likely misidentified as *C. melinii* based on ITS phylogeny because of low statistical support (Gramaje *et al.* 2011). By sequencing three loci from type specimens in addition to new species, the *Cadophora* complex occurring on grapevine now includes five species. Our results revealed the presence of *C. melinii* on grapevine, with one isolate from California and another from Ontario, Canada clustering with strong support with the type specimen for this species in our multigene phylogeny. These findings suggest that the three isolates that are part of a second *Cadophora* species from grapevine in Spain were misidentified as *C. melinii* (Gramaje *et al.* 2011) and that they represent, instead, a sixth species occurring on grapevine for which no type specimens are currently available. Further molecular characterization of the previously-mentioned isolates using additional loci and integrating the sequences produced in the current study should allow a taxonomic reevaluation of the *Cadophora* species complex occurring on grapevine in Spain.

The involvement of *C. luteo-olivacea* in the complex of fungi causing Petri disease and Esca of grapevines was first suggested by Halleen *et al.* (2007), who showed this species can colonize grapevine pruning wounds and wounded trunks in South Africa, causing vascular discoloration up to 438 mm in trunks of *V. vinifera* 'Periquita' after 14 m incubation under field conditions. In this above-mentioned study, however, inoculations of young grapevine cuttings propagated from dormant wood with the same *C. luteo-olivacea* isolate did not result in wood discolorations larger than control plants after 3 m incubation in the greenhouse (Halleen *et al.* 2007). In other studies, *C. luteo-olivacea* has been either classified as nonpathogenic or weakly pathogenic, based on relatively short-term inoculations of grapevine cultivars or rootstocks [*e.g.*, 3 m (Navarrete *et al.* 2011), 31 d and 21 weeks (Úrbez-Torres *et al.* 2014)]. More surprisingly, after only a 14-week incubation period under greenhouse conditions, inoculations of rootstock cuttings with *C. luteo-olivacea* produced lesions up to 92 mm (Gramaje *et al.* 2011). In the present study, we inoculated young plants with conidial suspensions of four *Cadophora* species (*C. luteo-olivacea*, *C. melinii*, *C. novi-eboraci*, and *C. orientoamericana*) and obtained wood discolorations ranging from 15 to 26 mm after a long 24-m incubation period, supporting the necessity to conduct long-term experiments to allow sufficient wood degradation by *Cadophora* species (Halleen *et al.* 2007; Navarrete *et al.* 2011). Our findings suggest that all isolates representing four *Cadophora* species included in our pathogenicity assays can be considered slow wood colonizing

fungi. Despite their apparent slow colonization process, the perennial status of grapevines makes them at risk of slowly declining over decades due to *Cadophora* infections.

Considering the presence of *Cadophora* isolates in asymptomatic, nursery planting material (Halleen *et al.* 2007), in vineyard nursery soils (Agusti-Brisach *et al.* 2013), and in the nursery at different stages of grapevine propagation (Gramaje *et al.* 2011), the possible infection courts of vines planted in the field remain hypothetical until further epidemiological studies are conducted. Halleen *et al.* (2007) demonstrated the ability of *Cadophora* conidia to artificially infect pruning wounds in the field. In order to reveal the role of pruning wounds as natural infection courts, a further step would require detecting aerial *Cadophora* conidia in the vicinity of vines in the field. The use of detection tools based on specific molecular markers, which can now be designed for each new species described in this study, should prove helpful for such investigations, as well as for the specific detection of these fungi *in planta*. Prevention of pruning wound infections should then rely on strategies developed for other trunk pathogens of grape (Weber *et al.* 2007; Rolshausen *et al.* 2010; Sosnowski *et al.* 2013), provided timing of spore release are known in different viticulture regions. Alternatively, if vineyard soils constitute the main source of inoculum for grapevine infections, disease management practices based on soil disinfestation and amendments, plant-based resistance to infection, and prophylactic cultural practices should be investigated.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2014.11.002.

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