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Species associated with cytospora canker on *Populus tremuloides*

Jeff B. Kepley[1], F. Brent Reeves[1],
William R. Jacobi[2*], & Gerard C. Adams[3]

[1] *Department of Biology, Colorado State University, Ft. Collins, CO 80523 USA*

[2] *Dept. of Bioagricultural Sciences & Pest Management, Colorado State University, Ft. Collins, CO 80523 USA*

[3] *Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583-0722 USA*

* Correspondence to: *William.jacobi@colostate.edu*

Abstract — A new species that is often associated with *Cytospora chrysosperma* was found commonly on stems of *Populus tremuloides* in Colorado. This fungus is illustrated and described as *Cytospora notastroma* sp. nov., and morphological and molecular data demonstrate that the new species is distinct from *C. chrysosperma, C. nivea, C. translucens,* and other species found on *Populus.* Diagnostic features are superficially visible, darkly pigmented conceptacles circumscribing the ascostromata and conidiomata and surrounding the white to grayish-white discs; some isolates produce a *Phialocephala*-like synanamorph in vitro in addition to the *Cytospora* anamorph.

Key words — aspen, coelomycete, pathogen, systematics, *Valsa*

Introduction

Cytospora canker attacks branches, stems, and roots of many woody species worldwide. Estimates of the number of woody host species found with the disease vary, but 85 or more have been cited by some authors (Sinclair et al. 1987, Farr et al. 1989, Adams et al. 2006). *Cytospora* Ehrenb. is recommended as a holomorphic genus that includes the former genera *Leucocytospora* (Höhn.) Höhn., *Leucostoma* (Nitschke) Höhn., *Valsa* Fr., *Valsella* Fuckel, and *Valseutypella* Höhn. (Rossman et al. 2015).

In Colorado, Hinds (1964) found cytospora canker in 97% of the native aspen (*Populus tremuloides*) stands that he sampled. The mortality and dieback of aspen have been reported across western North America and Rocky Mountains from Canada to Arizona (Frey et al. 2004, Hogg et al. 2005, 2008; Fairweather et al. 2008, Worrall et al. 2008, 2010; Zegler et al. 2012).

In Colorado, results of aerial surveys in 2006 indicated that as many as 140,000 acres were impacted, with as much as 10% of the aspen affected in some areas (Bartos & Shepperd 2006). Mortality in some areas may also affect lateral roots, which would hinder vegetative regeneration via root suckering. Death of mature trees can be rapid (a year or two) and is believed often to begin at epicenters followed by radial spread throughout an aspen stand. Studies by Worrall et al. (2008) in southwestern Colorado concluded that agents such as *Encoelia pruinosa* (Ellis & Everh.) Tork. & Eckblad and *Ganoderma applanatum* (Pers.) Pat., which typically kill mature trees in aspen stands, are unimportant in the present mortality. Rather, a group of secondary agents is involved, among which are *Cytospora* species causing cytospora canker. This canker on aspen is usually caused by *Cytospora chrysosperma* (Pers.) Fr. [= *Valsa sordida* Nitschke], which in the current epidemic is often present and believed to play a major role in mortality (Marchetti et al. 2011). Sinclair et al. (2005) stated that *C. chrysosperma* has been inconsequential in natural forests but can cause devastating losses in nursery seedbeds, storage, newly established forest plantations, and landscape or shelterbelt plantings of *Populus* spp. A study by Jacobi et al. (1998) concluded that aspen sprout mortality following harvesting in Colorado results from drought and/or drought coupled with root flooding-induced stress followed by infection by the canker fungi *C. chrysosperma* and *Dothiora polyspora* Shear & R.W. Davidson. It is apparent that despite the predisposing and inciting factors associated with aspen mortality, *Cytospora* species are often involved in aspen disease in natural or commercial forests in the western United States, and in many instances *C. chrysosperma* is believed to be the specific agent (Hinds 1964, Krebill 1972, Hinds & Krebill 1975, Ross 1976, Juzwik et al. 1978, Walters et al. 1982, Jacobi et al. 1998). Additionally, aspen growing in urban forests in the Rocky Mountain region are often found with the disease (Kepley & Jacobi 2000).

Problems associated with species identification of *Cytospora*

A thorough understanding is lacking of the *Cytospora* species occurring on aspen and other *Populus* spp. in North America in Colorado, the southern Rocky Mountains, and the Great Plains regions as well as elsewhere in the United States. Spielman (1983, 1985) lists several species and the national host index (http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm) records five on aspen (*Cytospora chrysosperma, C. leucosperma* (Pers.) Fr., *C. leucostoma* (Pers.) Sacc., *C. nivea* Fuckel, *C. translucens* Sacc.). The causal organism responsible for cytospora canker on aspen in Colorado is typically reported to be *C. chrysosperma,* while several investigative studies have employed *C. chrysosperma* isolated from aspen in Colorado (Guyon et al. 1996, McIntyre et al. 1996, Kepley & Jacobi 2000). Fungal identification has mostly been based on morphological characteristics and host association.

Use of morphological characters (e.g., stromatic tissues, locular size, shape, and arrangement, conidiogenesis, and spore characteristics of the conidioma) for accurate identification of *Cytospora* spp. is quite problematic (Adams et al. 2005), given the plasticity known to occur with diagnostic features (Adams et al. 2002). Spielman (1983) believed that for taxa within *Valsa*, most morphological characters could be modified to some degree. Such morphological variation combined with a poor understanding of host ranges has hindered species delimitation (Spielman 1985). Furthermore, because the current identification system relies on key morphological characteristics forming on bark tissues of woody plants in nature, no workable system exists for identifying *Cytospora* species in vitro or on inoculated host tissues (Adams et al. 2005).

Species causing cytospora canker on *Populus tremuloides*

During examinations of cankers on aspen stems, we found that another morphologically distinct *Cytospora* species is frequently present with *C. chrysosperma*. This species forms a conidioma of rosette-like to labyrinthine locules and leucocytosporoid form (e.g., having entostromatic tissue separated from host tissues by a dark line of delimiting conceptacle; Adams et al. 2005). It typically co-occurs with and superficially resembles *C. chrysosperma,* a species that forms large labyrinthine locules and cytosporoid form (e.g., without delimiting conceptacle). In addition to morphology, isoenzyme analyses and vegetative compatibility studies readily separate isolates of the unknown *Cytospora* species from those of *C. chrysosperma* (Kepley 2009). Almost certainly the two taxa have been confused in past reports, and cytospora canker on aspen in Colorado is most likely caused by more than one species of *Cytospora,* contradicting what is typically reported in the literature. Three *Cytospora* species have been reported on aspen in the southern Rocky Mountains and adjacent regions: *C. nivea* (Ellis & Everhart 1892, Gilbertson et al. 1979, Shaw 1973), *C. translucens* (Eslyn 1960), and *C. chrysosperma*. *Cytospora nivea* from North America was segregated as "*C. pseudonivea*" [≡ *Leucostoma pseudoniveum* Lar. N. Vassiljeva] based on the smaller ascospore size compared with European specimens including the type of *C. nivea* (Vasilyeva et al. 2008). In the eastern states, *C. leucosperma* and *C. leucostoma* have also been reported on aspen. The leucocytosporoid form of *Cytospora* is shared by the unknown species from Colorado and *C. nivea, C. translucens,* and *C. leucostoma*. Additionally, the unknown species shares with *C. translucens* a superficially visible ring of the conceptacle surrounding the disc at the bark surface. This characteristic is well illustrated and described by Hubbes (1960) for his specimens of *C. translucens*. As the presence of *C. nivea* and *C. translucens* is so rarely reported on aspen in the southern Rocky Mountains that the records may be misidentifications, we have endeavored to verify the reports during this study.

Molecular methods for inferring fungal phylogeny and fungal identification

Molecular phylogenetic analysis has been beneficial in identifying *Cytospora* species because many species have overlapping morphology and the morphology can vary with bark thickness, environment, and host factors (Adams et al. 2005). Approximately 110 *Cytospora* species have been described (Kirk et al. 2008). Many were described based on host range, and host range is no longer considered a reliable characteristic for species delineation (Adams et al. 2005). Anamorph and teleomorph morphological data and other types of phenotypic character data (e.g., culture morphology, growth rates, substrate utilization, pigment production, isozyme analysis, pathogenicity) when correlated with molecular phylogenetic studies should provide insight with respect to taxonomically informative characters. Sorting out species complexes and properly identifying pathogens are critical in designing control strategies, plant disease quarantine regulation, breeding disease resistance, understanding disease biology and epidemiology, and facilitating communication among plant pathologists, mycologists, and quarantine specialists (O'Donnell et al. 2004). Cytospora canker on aspen in the Rocky Mountains is currently thought to be caused by a complex of fungi; in the absence of genetic or morphological analyses of these fungi, our primary goal was to use sequence, cultural, and morphological analyses to clarify species relationships among *Cytospora* spp. on aspen in Colorado.

Materials & methods

Isolates and specimens studied

Information on isolates is listed in Table 1 and previous publications (Adams et al. 2005, 2006). Isolates are usually linked to a herbarium specimen except those from international culture collections (Adams et al. 2005, 2006). Whenever possible, isolates believed to represent a common species were chosen from different continents, differing ecoregions, and different hosts to support international species concepts. The examined herbarium specimens represented species concepts of *C. nivea* [≡*Valsa nivea*] and *C. translucens* [*Valsa translucens*], particularly those from Colorado, the southern Rocky Mountains, and adjacent regions: **NYBG Herbarium J.B. Ellis Collection:** #68 & #1543 *V. nivea* on *P. tremuloides*, Short Creek, Custer Co., Colorado, coll. D.E. Cockerell; #204 *V. nivea* on *P. tremuloides,* Ten Mile Creek, Clarke [sic, see Lewis & Clark] Co., Montana, coll. F.W. Anderson; **ISC Herbarium L.H. Tiffany Collection:** #320903 *V. translucens* on *P.* ×*canadensis*, Holst St. For., Iowa; #326295 *V. nivea* on *P. tremuloides*, Iowa City, Iowa; **MICH Herbarium:** #71305–71315 *V. translucens* on *Salix* spp. from: Little Laramie, Wyoming, USA; South Dakota, USA; London, Ontario, Canada; Vienna, Austria; Munich and Westphalia, Germany; and Riga, Latvia.

DNA extractions

Mycelia for genomic DNA extractions were obtained from cultures grown for one week in potato-dextrose broth under ambient light and temperature conditions.

Approximately 1 cm^2 of mycelium was ground, extracted, and purified using the MasterPure™ Yeast DNA Purification Kit (EPICENTRE® Biotechnologies, Madison, WI) following the manufacturer's instructions. DNA yields were calculated on the basis of UV absorbance and dilution, and purity estimated by the ratio of UV absorbance at A_{260}/A_{280}.

PCR amplification, gel electrophoresis, and sequencing

For PCR studies, template DNA was diluted with sterile, double-distilled water as needed to provide a final concentration of ca. 200 ng/µl. The primer pairs for amplification of the ITS1+5.8S+ITS2 region were ITS1f F (forward primer) and ITS4 R (reverse primer) (Gardes & Bruns 1993, White et al. 1990). EF1 F and EF2 R (Geiser et al. 2004), EF1-526 F and EF1-1567 R (Rehner 2001), and EF1-728 F and EF1-986 R (Carbone & Kohn 1999) were used for amplification of the EF-1α gene. Protocols of the FailSafe™ PCR System with PreMix choice (Epicentre®) were used for amplification reactions. PCR cycle conditions were those of Touchdown PCR (Korbie & Mattick 2008) that started with an annealing temperature of 60°C and was reduced by 1°C for each cycle until 50°C was reached.

Amplified samples (10 µl ea.) were fractionated by electrophoresis on 1% agarose gels buffered in sodium boric acid (Brody & Kern 2004). Electrophoresis was conducted until major bands in staggered sets of samples were well separated as suggested by Rehner (2001) for isolating desired PCR products. Upon completion of electrophoresis, gels were placed in the refrigerator at 4°C to firm up the gel texture followed by rapid band excision with a sterile scalpel. PCR products in gel slices were purified prior to DNA sequencing using the QIAquick Gel Extraction Kit (Qiagen Sciences, Inc., Germantown, MD) according to the manufacturer's instructions when a microcentrifuge was used for gel extraction. Both purified (exposed to UV light) and unpurified PCR products were submitted to Macrogen USA, Inc., (Rockville, MD) for sequencing. PCR products were sequenced in both directions using the same primer pairs that were used in the amplification reactions.

Phylogenetic analyses

Automatic alignment of the individual ITS1-5.8S-ITS2 rDNA (ITS) and partial EF-1α sequential data sets of sequences was performed in MUSCLE 3.8.31 (Edgar 2004) followed by refinement via direct examination and editing in MEGA version 5.0 (Tamura et al. 2011). Insertions/deletions (indels) and gaps introduced for alignment purposes were handled as pairwise-deletions, a process that removed the gaps from the analysis if the gaps had a higher percentage of ambiguous sites than the site coverage cutoff parameter, which we specified at 95% in MEGA (100% cutoff is no ambiguous sites) (Tamura et al. 2011).

To infer phylogenetic relationships, 87 taxa datasets were constructed separately from the ITS and EF-1α sequences. The partition-homogeneity test (ILD test, Farris et al. 1994) was performed in PAUP 4b10 (Swofford 2003) to assess the validity of combining the two molecular datasets. Concordance of the ITS and EF-1α datasets was evaluated using 1000 bootstrap replications (Felsenstein 1985) and 1000 random additions of taxa replicates per partition replicate with tree bisection-reconnection (TBR) branch swapping and MulTrees active.

Table 1. *Cytospora* and *Diaporthe* taxa used for phylogenetic studies.

Species	Isolate	Origin	Host	NCBI Accession no. [ITS/EF-1α sequence]
C. abyssinica	CBS116819	Ethiopia	*Eucalyptus globulus*	AY347352/JX439558
	CBS117004	Ethiopia	*Eucalyptus globulus*	AY347354/JX438559
C. acaciae	CBS468.69	Spain	*Ceratonia siliqua*	DQ243804/JX438560
C. annulata	CBS118089	NY, USA	*Acer rubrum*	AY347345/JX438576
C. berkeleyi	CBS116824	CA, USA	*Eucalyptus globulus*	AY34734/JX438561
	CBS116825	CA, USA	*Eucalyptus globulus*	AY347351/JX438562
C. ceratosperma	AR98007	MA, USA	*Vaccinium* sp.	AY188992/AY188991
	CBS116.21	Netherlands	*Fagus sylvatica*	AY347335/JX438577
	CO_C14	CO, USA	*Populus tremuloides*	JX438635/JX438548
C. chrysosperma [allele 1]	NE_TFR3w	MT, USA	*Populus tremuloides*	JX438641/JX438549
[allele 2]	NE_TFR3w	MT, USA	*Populus tremuloides*	JX438641/JX438550
C. cincta	NE_A48	MI, USA	*Malus ×domestica*	AF191170/JX438579
C. coenobitica	CBS283.74	Netherlands	*Betula verrucosa*	JX438610/JX438578
C. curreyi {a}	CBS148.42	Switzerland	*Larix* sp.	AF191172/JX438580
C. diatrypelloidea	CBS116826	Australia	*Eucalyptus globulus*	AY347368/JX438563
	CBS120062	Australia	*Eucalyptus globulus*	AY347368/JX438563
C. diatrypoides	NE_Healy1-2	AK, USA	*Alnus tenuifolia*	JX438612/JX438584
	NE_JacLeuco	CO, USA	*Alnus tenuifolia*	JX438611/JX438583
	NE_ESPAlnus2	CO, USA	*Alnus tenuifolia*	JX438613/JX438584
	NE_StanMoist	AK, USA	*Alnus tenuifolia*	JX438614/JX475137
C. eriobotryae	CBS116846	India	*Eriobotrya japonica*	AY347327/JX438564
C. eucalypticola	CBS116853	South Africa	*Eucalyptus saligna*	AF260265/JX438590
	CBS116851	South Africa	*Eucalyptus dunnii*	AY347360/JX438591
C. eugeniae	IMI062499	Malaysia	*Eugenia aquea*	AY347348/JX438587
	CBS116837	Indonesia	*Eugenia* sp.	AY347344/JX438586
	IMI057979	Tanzania	*Anacardium occidentale*	AY347347/JX438589
C. friesii	CBS113.81	Germany	*Abies alba*	AY347318/JX438592
C. germanica {a}	CBS196.42	Switzerland	Unknown	AY347325/JX438593
C. kunzei/pini	ATCC20502	Canada	*Pinus contorta*	JX438615/JX438594
	NE_Waterloo	MI, USA	*Pinus strobus*	JX438616/JX438598
	NE_BogueScots	MI, USA	*Pinus sylvestris*	JX438617/JX438597
	CBS118094	MI, USA	*Picea pungens*	AY347320/JX438595
	CBS118093	MI, USA	*Picea glauca*	AY347320/JX438596
C. kunzei/pini				
[allele 1] {a}	CBS197.42	Switzerland	*Pinus sylvestris*	AY347332/JX438546
[allele 2] {a}	CBS197.42	Switzerland	*Pinus sylvestris*	AY347332/JX438547
C. leucosperma	CBS191.42	Switzerland	*Taxus baccata*	AY347330/JX438576
	CBS116809	NJ, USA	*Acer rubrum*	AY347339/JX438576
C. leucostoma	NE_RCommon	FL, USA	Unknown twig	JX463524/JX438599
	NE_HigLake4	MI, USA	*Alnus rugosa*	JX475137/JX438600
	NE_Lp8	MI, USA	*Prunus serotina*	AF191177/JX438601
	ATCC74091	WV, USA	*Betula alleghaniensis*	JX438618/JX438602
C. magnoliae	IMI259790	LA, USA	*Magnolia* sp.	JX438623/JX438565
C. mali	ATCC56632	Japan	*Malus ×domestica*	AF192326/JX438571
C. melanodiscus	NE_Worrall4	CO, USA	*Alnus tenuifolia*	JX438619/JX438608
	NE_JimsLand2	AK, USA	*Alnus tenuifolia*	JX438605/JX438621
	NE_Worrall2b	CO, USA	*Alnus tenuifolia*	JX438606/JX438620

C. mougeotii	CBS198.50	Norway	*Picea abies*	AY347329/JX438566
C. nitschkei	CBS116854	Ethiopia	*Eucalyptus globulus*	AY347356/JX438567
C. nivea	CBS118562	South Africa	*Malus ×domestica*	DQ243796/JX438607
	CBS259.34	Switzerland	*Populus nigra*	AF191174/JX438532
	CBS109489	Russia	*Populus* sp.	JX438624/DQ862035
C. nivea/translucens	NE_OSUAlnus	OR, USA	*Alnus tenuifolia*	JX438625/JX438534
C. notastroma	NE_Cottonwd16	MI, USA	*Populus deltoides*	**JX438626/JX438535**
	NE_HigginLake5	MI, USA	*Alnus rugosa*	**JX438627/JX438536**
	NE_NiveaPR	MI, USA	*Populus ×canadensis* 'Robusta'	**JX438537/JX438628**
	CO_K3	CO, USA	*Populus tremuloides*	**JX438631/JX438539**
	CO_L1	CO, USA	*Populus tremuloides*	**JX438634/JX438538**
	CO_K16	CO, USA	*Populus tremuloides*	**JX438630/JX438540**
	CO_K20	CO, USA	*Populus tremuloides*	**JX438629/JX438541**
	NE_TFR8	MT, USA	*Populus tremuloides*	**JX438542/JX438633**
	NE_TFR5	MT, USA	*Populus tremuloides*	**JX438543/JX438632**
"C. parapersoonii"	NE_LCN	MI, USA	*Prunus persica*	AF191181/JX438544
	NE_T28.1	CA, USA	*Prunus simonii*	AF191176/JX438545
C. pinastri {a}	CBS185.42	Switzerland	*Abies alba*	AY347336/JX438572
C. pinastri–western NA	CBS118567	BC, Canada	*Pseudotsuga menziesii*	AF192551/JX438573
	CBS118092	BC, Canada	*Chamaecyparis* sp.	AF192550/JX438574
	CBS196.50	Italy	*Thuja* sp.	AF192311/JX438575
C. pruinosa	CBS118555	South Africa	*Olea europaea* subsp. *africana*	DQ243790/JX463522
	PPRI6334	South Africa	*Olea europaea*	DQ243789/JX438581
C. pruinosa {a}	CBS201.42	Switzerland	*Syringa vulgaris*	DQ243801/JX438582
C. punicae	CBS199.50	Turkey	*Punica granatum*	JX438622/JX438568
C. rhizophorae	ATCC38475	LA, USA	*Rhizophora mangle*	DQ996040/JX438609
	ATCC66924	HI, USA	*Haliclona caerulea*	DQ092502/JX438609
C. sacchari	CBS160.33	India	*Saccharum officinarum*	DQ243811/JX438569
C. schulzeri	CBS118559	South Africa	*Malus ×domestica*	DQ243792/JX438603
	CBS118570	MI, USA	*Malus ×domestica*	DQ243802/JX438604
C. sp. undetermined	NE_W7b	WY, USA	*Alnus tenuifolia*	JX438554/JX438638
	NE_FlorWP	WI, USA	*Pinus strobus*	JX438639/JX438555
	NE_Pisqua	NC, USA	*Tsuga canadensis*	JX438640/JX438556
	CBS118566	South Africa	*Acacia nilotica*	DQ243800/JX438557
C. translucens {a}	CBS152.42	Switzerland	*Salix* sp.	AF191182/JX438552
	NE_JacTissue5	CO, USA	*Salix* sp.	JX438637/JX438553
C. valsoidea	CBS117003	Indonesia	*Eucalyptus urophylla*	AF192312/JX438570
Cytospora sp. {b}	CBS116814	CA, USA	*Sequoia sempervirens*	AY347340/JX438585
	CBS116816	CA, USA	*Eucalyptus paniculata*	AY347365/JX438585
D. ampelina	—	South Africa	*Vitis vinifera*	JQ038888/AY745084
D. vaccinii	CBS160.32	MA, USA	*Oxycoccus macrocarpos*	GQ250326

{a} = Reference cultures deposited by Défago as standards for her 1935 European species concepts.

{b} = *Valsa eucalypti* Cooke & Harkn.

Collection acronyms: **ATCC** = American Type Culture Collection, Manassas VA, USA; **AR** = Amy Rossman collections, USDA-ARS, Beltsville MD, USA; **CBS** = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **CO** = William R. Jacobi collections, Colorado State University, Ft. Collins CO, USA; **IMI** = International Mycological Institute, CABI Bioscience, Egham, Surrey, UK; **NE** = Gerard Adams collections, University of Nebraska, Lincoln NE, USA; **PPRI** = South African NCF, Plant Protection Research Institute, Pretoria, South Africa.

The arbitrarily numbered alleles indicated under *C. chrysosperma* and *C. kunzei/pini* refer to two EF-1α sequences present in the hyphae.

Evolutionary analyses were conducted using MEGA. Stochastic models for estimating evolutionary distance between sequences with maximum likelihood (ML) (Felsenstein 1981) were calculated for the combined data sets of DNA nucleotides of the ITS and EF-1α regions. The optimal models for the pattern of nucleotide substitution, the evolutionary rate differences among sites, and indel evolution were selected for phylogenetic reconstructions (Nei & Kumar 2000). The EF-1α dataset was treated as non-coding rather than protein coding nucleotides because only a small percentage of the sequence included an exon region. A phylogeny tree was inferred using the ML heuristic method and the Close-Neighbor-Interchange search method with initial random addition of 100 trees in MEGA. Strains of *Diaporthe* species including *D. vaccinii* GQ250326 strain CBS160.32 and *D. ampelina* JQ038888 strain STEU 7005, and AY745084 strain OH-9 served as outgroup for ITS and EF-1α data sets. Nucleotide sequences of species of *Valsa* and *Cytospora* deposited in NCBI by other researchers were not included in this study because of problems in verifying the identification. Final results were summarized as the ML tree with the best negative log likelihood values, branch support values of clade credibility, and bootstrap confidence limits calculated with 1000 bootstrap replications.

Morphological studies

For examination of fruiting bodies on bark (natural state), conidiomata and ascostromata were excised from aspen tissues by cutting deep enough into the bark tissues to remove entire fruiting bodies. For examination of fruiting bodies from cultural specimens, mature pycnidia-like conidiomata (those oozing spore masses) were cut out with a portion of the modified Leonian's agar medium (20 ml per 9 cm Petri dish; Leonian 1923). These cultures were seven weeks old and grown at 25°C in the dark. Protocols for fixing, embedding, and sectioning were modified from Adams et al. (2005). Measurements of characteristic structures from fruiting bodies on bark were derived from 20 observations when possible. Distilled water and several stains: phloxine-KOH, lactophenol-cotton blue, and Melzer's solution, were used as mounting media for observing general morphological characteristics, e.g., stromatal tissues, perithecial wall tissues, conidiogenous cells, asci, and spores. Sections were stained with 0.001% aqueous toluidine blue.

Cultural characteristics were determined in triplicate from isolates (C1, C14, K3, K16, K20, and L1) grown in 9 cm Petri plates (wrapped with wax-film) containing 20 ml modified Leonian's agar under 12 hours continuous light and 12 hours continuous darkness at 25°C for 28 days. Colors were determined according to Rayner (1970). Measurements of conidiomata, conidia, and hyphae were based on 20 observations each per isolate. Distilled water, and the aqueous stains listed prior were again used as mounting media for microscopic examinations.

Results

Molecular phylogenetic characterization

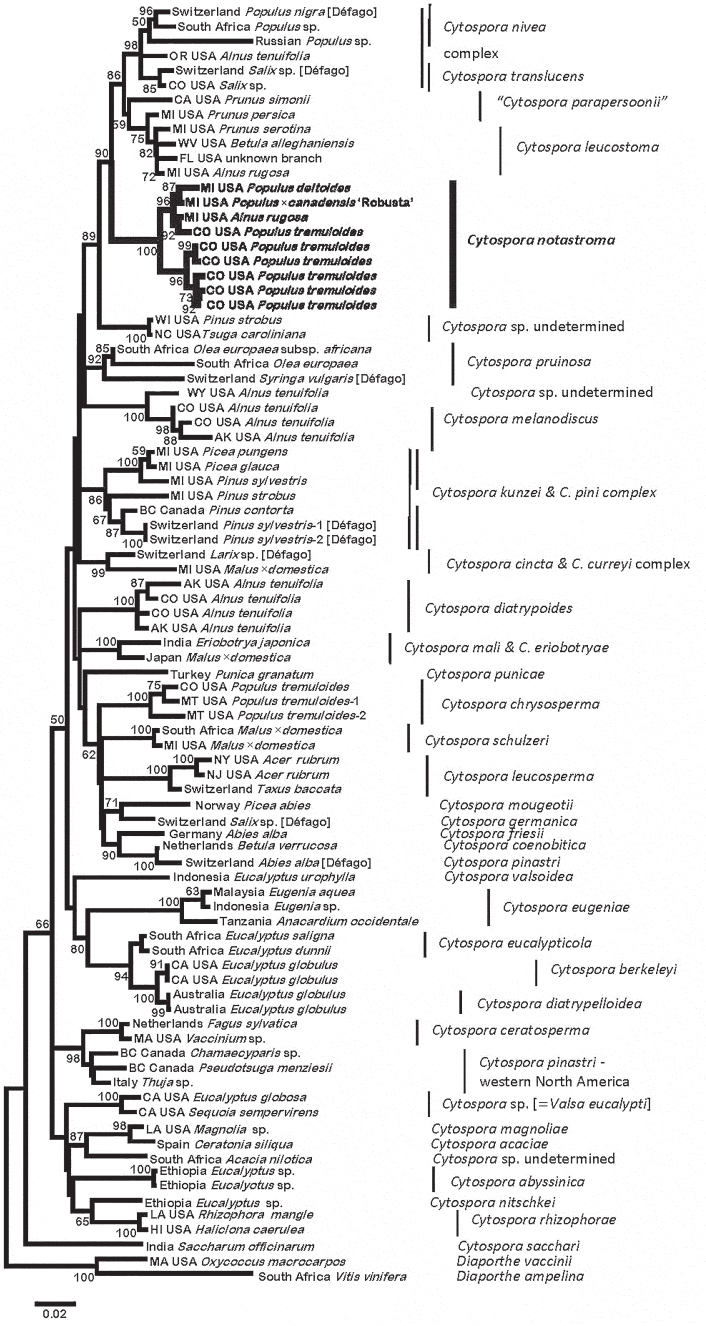
PCR products of the ITS were approximately 540 base pairs (bp) and those of the EF-1α were approximately 300 bp, but the length of the intron varied among isolates. As the partition homogeneity test indicated phylogenetic

congruence between the two data sets (ρ = 0.003), the data sets were combined. Alignment of the combined ITS and EF-1α sequence data set included 87 taxa and 633 sites (excluding gaps introduced for alignment) with 490 parsimony-informative characters out of 1121 total sites (ITS = 626 sites with gaps, EF-1α = 495 sites with gaps). Maximum parsimony analysis yielded 12 equally parsimonious trees with tree length of 1370 steps and scores of CI (consistency index) = 0.391971, RI (retention index) = 0.719150, and RCI (rescaled consistency index) = 0.281886 (for all sites) (Farris 1989). The final tree was drawn in MEGA and Microsoft PowerPoint version 2010 (Microsoft, USA).

Evaluation of models of nucleotide substitution in MEGA yielded the General-Time-Reversible (GTR) model (Tavaré 1986) as optimal. Substitution pattern and rates of evolution for DNA nucleotide sequences were estimated under the GTR model with a discrete Gamma distribution (GTR+G) used to model evolutionary rate differences among sites (5 categories, 4 nucleotides, and indel gaps as partial-deletions at 95%). The 50% majority rule bootstrap values are displayed on the strict consensus tree (Plate 1) with tree-length (as sum of branch lengths, SBL) of 1.97608505, Ln likelihood of –7741.06, and a transition/transversion ratio of 1.5418. Terminal isolates on the phylogenetic tree were designated with names in regular fonts that include location and in italic fonts for host of origin followed by a number. The number represents a unique DNA sequence that corresponds to one or more isolates given in Table 1 or other publications. Names to the right of the vertical bars represent described species and species complexes.

Phylogenetic analysis (Plate 1) strongly supports (100% bootstrap confidence) a cluster of isolates from aspen within a holophyletic (monophyletic) clade. We describe the unique morphology of the fungi in this clade as a new species, *C. notastroma.* It forms a sister group to the clade containing *C. nivea, C. translucens, C. leucostoma,* and "*C. parapersoonii*" [≡ *Leucostoma parapersoonii* G.C. Adams et al.]. Two subgroups are inferred and supported by 94–98% bootstrap confidence within /notastroma clade. Colorado isolates from aspen exhibiting dieback or mortality cluster in both *C. notastroma* subgroups and also in the clade of *C. chrysosperma* with 100% bootstrap support. *Cytospora nivea* and *C. translucens* are not readily distinguished from one another by the combined ITS and EF-1α sequence and intermediate isolates appear to exist (e.g., Oregon_*Alnus*1). "*Cytospora pseudonivea*" represented by isolate Russia_*Populus*1 (AR 3413 = CBS 109489; Vasilyeva et al. 2008), is conspecific with Switzerland_*Populus*1, an isolate that G. Défago (1935) designated as typical for the species concept of *C. nivea* in Europe.

A well-supported clade of mixed nomenclatural species is treated as representing a single species complex that is named after the oldest or most


Switzerland Populus nigra [Défago]
South Africa Populus sp.
Russian Populus sp.
OR USA Alnus tenuifolia
Switzerland Salix sp. [Défago]
CO USA Salix sp.
CA USA Prunus simonii
MI USA Prunus persica
MI USA Prunus serotina
WV USA Betula alleghaniensis
FL USA unknown branch
MI USA Alnus rugosa
MI USA Populus deltoides
MI USA Populus × canadensis 'Robusta'
MI USA Alnus rugosa
CO USA Populus tremuloides
CO USA Populus tremuloides
CO USA Populus tremuloides
CO USA Populus tremuloides
CO USA Populus tremuloides
CO USA Populus tremuloides
WI USA Pinus strobus
NC USA Tsuga caroliniana
South Africa Olea europaea subsp. africana
South Africa Olea europaea
Switzerland Syringa vulgaris [Défago]
WY USA Alnus tenuifolia
CO USA Alnus tenuifolia
CO USA Alnus tenuifolia
AK USA Alnus tenuifolia
MI USA Picea pungens
MI USA Picea glauca
MI USA Pinus sylvestris
MI USA Pinus strobus
BC Canada Pinus contorta
Switzerland Pinus sylvestris-1 [Défago]
Switzerland Pinus sylvestris-2 [Défago]
Switzerland Larix sp. [Défago]
MI USA Malus × domestica
AK USA Alnus tenuifolia
CO USA Alnus tenuifolia
CO USA Alnus tenuifolia
AK USA Alnus tenuifolia
India Eriobotrya japonica
Japan Malus × domestica
Turkey Punica granatum
CO USA Populus tremuloides
MT USA Populus tremuloides-1
MT USA Populus tremuloides-2
South Africa Malus × domestica
MI USA Malus × domestica
NY USA Acer rubrum
NJ USA Acer rubrum
Switzerland Taxus baccata
Norway Picea abies
Switzerland Salix sp. [Défago]
Germany Abies alba
Netherlands Betula verrucosa
Switzerland Abies alba [Défago]
Indonesia Eucalyptus urophylla
Malaysia Eugenia aquea
Indonesia Eugenia sp.
Tanzania Anacardium occidentale
South Africa Eucalyptus saligna
South Africa Eucalyptus dunnii
CA USA Eucalyptus globulus
CA USA Eucalyptus globulus
Australia Eucalyptus globulus
Australia Eucalyptus globulus
Netherlands Fagus sylvatica
MA USA Vaccinium sp.
BC Canada Chamaecyparis sp.
BC Canada Pseudotsuga menziesii
Italy Thuja sp.
CA USA Eucalyptus globosa
CA USA Sequoia sempervirens
LA USA Magnolia sp.
Spain Ceratonia siliqua
South Africa Acacia nilotica
Ethiopia Eucalyptus sp.
Ethiopia Eucalyptus sp.
Ethiopia Eucalyptus sp.
LA USA Rhizophora mangle
HI USA Halicilona caerulea
India Saccharum officinarum
MA USA Oxycoccus macrocarpos
South Africa Vitis vinifera
0.02
Cytospora nivea complex
Cytospora translucens
"Cytospora parapersoonii"
Cytospora leucostoma
Cytospora notastroma
Cytospora sp. undetermined
Cytospora pruinosa
Cytospora sp. undetermined
Cytospora melanodiscus
Cytospora kunzei & C. pini complex
Cytospora cincta & C. curreyi complex
Cytospora diatrypoides
Cytospora mali & C. eriobotryae
Cytospora punicae
Cytospora chrysosperma
Cytospora schulzeri
Cytospora leucosperma
Cytospora mougeotii
Cytospora germanica
Cytospora friesii
Cytospora coenobitica
Cytospora pinastri
Cytospora valsoidea
Cytospora eugeniae
Cytospora eucalypticola
Cytospora berkeleyi
Cytospora diatrypelloidea
Cytospora ceratosperma
Cytospora pinastri - western North America
Cytospora sp. [=Valsa eucalypti]
Cytospora magnoliae
Cytospora acaciae
Cytospora sp. undetermined
Cytospora abyssinica
Cytospora nitschkei
Cytospora rhizophorae
Cytospora sacchari
Diaporthe vaccinii
Diaporthe ampelina

common species until further study reveals otherwise (Adams et al. 2005, 2006). For example, the clade referred to as the *C. chrysosperma* species complex in earlier studies (Adams et al. 2006) was shown to include specimens identified as *C. eutypelloides* Sacc., *C. hariotii* Briard, *C. minuta* Thüm., and *C. tritici* Punith.

Polytomy has been lessened in the combined ITS and EF-1α tree (Plate 1) compared to single gene trees (see Fig. 1 in Adams et al. 2005); however evolutionary relationships still cannot be fully resolved among several clades.

Taxonomy

Cytospora notastroma Kepley & F.B. Reeves, **sp. nov.** Plates. 2, 4, 6C,D, 7

MycoBank MB 801154

Differs from *Cytospora chrysosperma* by its prominent dark conceptacles visible from the bark surface delimiting the stroma and disc of both ascostromata and conidiomata.

Type: USA, Colorado: Upper Poudre Canyon east of Cameron Pass on dead bole of *Populus tremuloides* in grove, 20 Aug 2004, collectors J. Kepley & F.B. Reeves (**Holotype,** NEB318541).

Etymology — *notastroma*, a shortened form of the Latin word *notabilistromatica*, referring to the notable encircling zone of conceptacle tissue superficially visible around the disc of the ascostroma and the disc of the conidioma.

Ascostromata immersed in bark, erumpent, ovoid to circular 2.0–3.0 × 1.2–1.8 mm, leucostomoid circinateous, 6–15 perithecia arranged circinately in well developed orangish, cinnamon, olive-gray to creamish-white entostroma composed of cells forming textura angularis and intricata, conceptacles prominent, olive-black to black, apparent on the surface of the bark. Discs prominent, snowy-white to grayish-white, nearly flat, circular to ovoid 0.4–0.55(–0.65) mm diam, furfuraceous, composed of cells forming a textura angularis and intricata, 2–10 laterally to vertically inserted ostioles, surrounded by an ovoid to circular zone of olive-black to black conceptacle tissue apparent on the surface of the bark. Ostioles olive-black to black (45–)60–100(–120) µm diam, nearly level to slightly above disc surfaces. Perithecia olive-black to black, globose (0.25–)0.3–0.40(–0.50) mm diam, inclined, walls of textura epidermoidea. Asci free, clavate to obclavate (33–)37–43 × 8–11 µm, apical apparatus non-amyloid, 8-spored. Ascospores biseriate, allantoid, thin-walled, hyaline (salmon-colored in mass), and

Plate. 1. Reconstructed phylogeny of *Cytospora* species based on maximum likelihood analysis of non-coding ITS and EF-1α DNA sequences. The tree is a bootstrap consensus tree of 50% majority rule with –Ln likelihood score = 7741.06, SBL tree length = 1.97608505 steps). Branch lengths correspond to inferred genetic distances with the scale bar representing a 2% nucleotide divergence. The numbers at the nodes represent bootstrap support values based on 1000 resamplings (values ≥50% are shown).

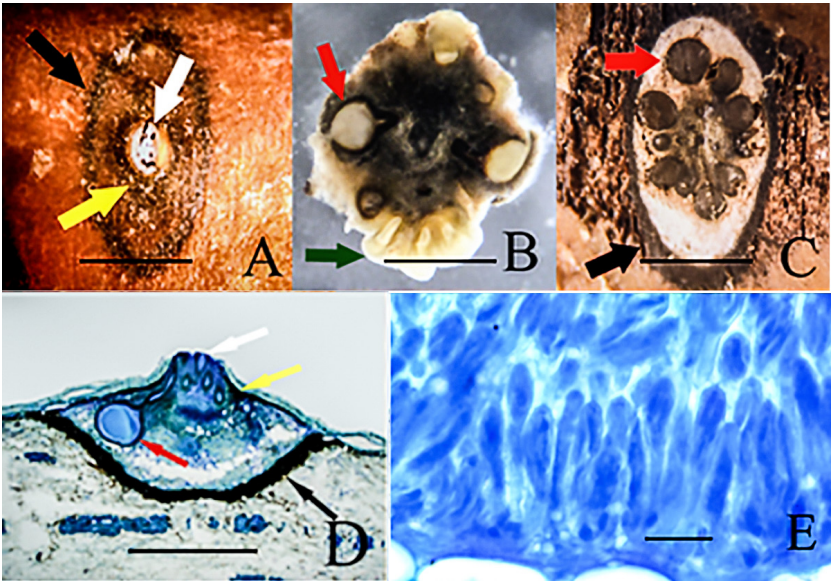


Plate 2. *Cytospora notastroma* ascostromata. A. Erumpent ascostroma with prominent ovoid snowy-white disc and emerging black ostioles (white arrow), obscure black conceptacle tissue (yellow arrow), and black conceptacle (black arrow). B. Excised ascostroma showing teleomorph and anamorph in same stroma; perithecium (red arrow) and locular chambers of anamorph (green arrow). C. Horizontal cross section showing circinate perithecia surrounded by well developed cinnamon to creamish-white entostroma, globoid perithecium (red arrow), and conceptacle (black arrow). D. Vertical section with ostiole emerging through disc (white arrow), black conceptacle tissue surrounding the disc (yellow arrow), laterally inclined perithecium surrounded by entostroma (red arrow), and conceptacle (black arrow). E. Clavate asci (with ascospores) floating freely in perithecial centrum. Scale bars: A–D = 1.0 mm, E = 15 um.

aseptate (7.5–)8.0–9.5 × 1.5–2.0 µm. Anamorph conidiomata usually interspersed amongst teleomorphs but sometimes present in the same stromata as the teleomorphs. Conidiomatal stromata immersed in bark, erumpent, labyrinthine to rosette-like leucocytosporoid, ovoid to circular 1.5–2.5 × 1.0–1.5 mm, conceptacles prominent, olive-black to black. Discs prominent, white to grayish-white, nearly flat, circular to ovoid 0.25–0.40 mm diam, furfuraceous, composed of amorphous material and cells forming a textura angularis, 1–3 ostioles, surrounded by an ovoid to circular zone of olive-black to black conceptacle tissue. Ostioles olive-gray, olive-black to black, 75–150(–170) µm diam, nearly level to slightly above disc surfaces. Locules multi-chambered, subdivided by invaginations into regular to irregular radially arranged chambers sharing common walls, 100 × 300 µm diam, surrounded

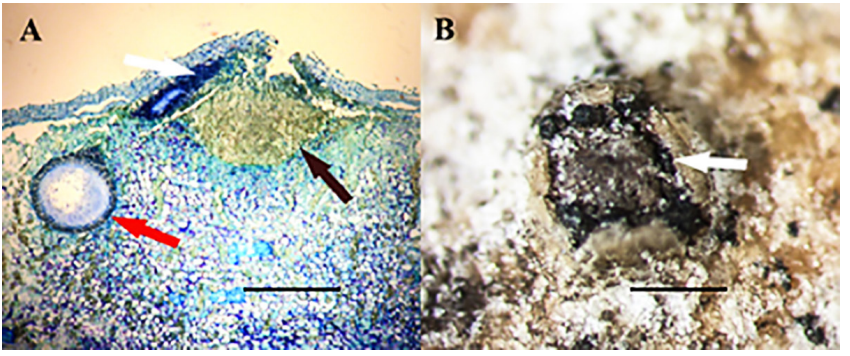


Plate 3. *Cytospora chrysosperma* ascostromata. A. Vertical section with ostiole at disc margin (white arrow); stromatic tissue below disc (black arrow); globose perithecium (red arrow) lacking well developed entostroma (note lack of conceptacle delimiting stroma). B. Erumpent ascostroma with emerging ostioles (white arrow) at margin of prominent tan to gray circular disc. Scale bars: A-B = 0.5mm

with well developed cinnamon, olive-gray to creamish-white stromata of textura angularis and textura intricata. Conidioma conidiophores hyaline and branched 6.0–10.0 × 1.0–1.5 µm, inclusive of phialides, arise from basal cells (2.0–)3.0–4.5 × 1.5–3.0 µm, embedded in a continuous gelatinous matrix. Conidioma conidiogenous cells enteroblastic phialidic, cylindrical, tapering to the apices, minute collarettes. Conidioma conidia hyaline (salmon-colored in mass), eguttulate, allantoid, aseptate 3.0–5.0 × 1.0 µm. *Phialocephala*-like hyphomycetous anamorph formed in culture on modified Leonian's agar (20 ml/9 cm Petri dish) at 25°C with 12 hours continuous light and 12 hours continuous darkness; synanamorphs located in older regions of young cultures (7–10 d old) where pigmentation is forming and hyphae are aggregated into ball-like clusters. Hyphomycetous conidiophores arising from main hyphae, mononematous, darkly pigmented, variable in length (short to quite long) and numbers of septa (three or more), often subtended by basal cells. Branching variable (dichotomous to three or more), occurring in series, initiated at or near septa. Hyphomycetous conidiogenous cells phialidic, cylindrical (5.0–12.0 × 2.0–3.0 µm), taper to apices. Hyphomycetous conidia hyaline, allantoid, aseptate.

Culture characteristics — Colony growth olivaceous-black (top and reverse) on modified Leonian's agar (20 ml/9 cm Petri dish) after 28 d at 25°C with 12 hours continuous light and 12 hours continuous darkness. Hyphae generally appressed and growing down into the agar. Pycnidium-like conidiomata greenish-black, often covered with white, smoke-grey to olivaceous-grey hyphae, and typically forming vertically oriented beaks/necks. Exuded cirrhi milky-white.

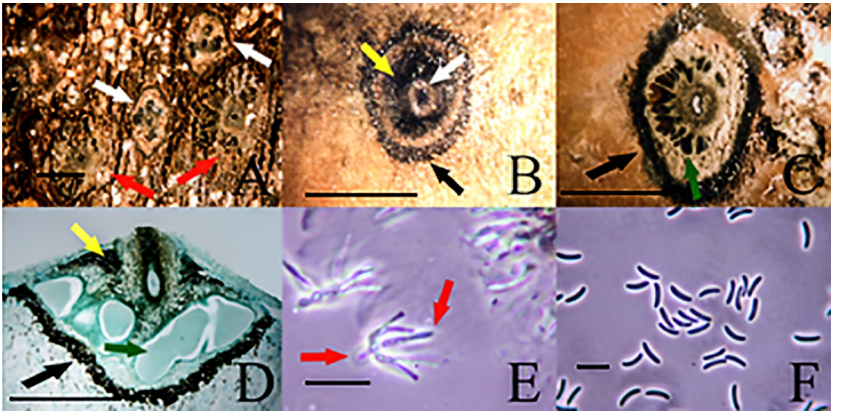


Plate 4. *Cytospora notastroma* conidiomata. A. Interspersed conidiomata of *C. notastroma* (white arrows) and *C. chrysosperma* (red arrows). B. Erumpent conidioma; well-developed black conceptacle tissue (yellow arrow); prominent circular grayish-white disc with two emerging black ostioles (white arrow); black conceptacle (black arrow). C. Horizontal cross-section; rosette-like multi-chambered locules surrounded by well developed creamish-white to olive-gray stromatic tissues (green arrow); conceptacle (black arrow). D. Vertical section; conceptacle tissue surrounding the disc (yellow arrow); rosette-like multi-chambered locules sharing common walls surrounded by well developed stromatic tissues (green arrow); conceptacle delimiting the stroma (black arrow). E. Conidiogenous cells and spores; basal cell subtending branching phialidic conidiophores (left-most arrow); spore at apex of phialide (right-most arrow). F. Hyaline, eguttulate, allantoid, and aseptate conidia. Scale bars: A–D = 1.0 mm, E = 8 um, F = 2 um

Additional material examined: **USA**, **Colorado**: Pingree Park, on dead bole of *Populus tremuloides*, 25 May 2004, collectors J.B. Kepley & F.B. Reeves (NEB318542, NEB318543).

Hosts — *Populus tremuloides* Michx., *P. deltoides* W. Bartram ex Marshall, *P.* ×*canadensis* Moench 'Robusta' [*P. nigra* × *P. deltoides*], *Alnus incana* subsp. *rugosa* (Du Roi) R.T. Clausen

Distribution — USA (Alaska, Colorado, Montana, Michigan)

Notes — Conidiomata of *C. notastroma* are more common on aspen than the teleomorphs, although they occasionally occur in the same stroma. Conidiomata often co-occur interspersed amongst *C. chrysosperma* conidiomata (Plate 4a) and are of similar size and external shape. However, the conidiomata of the two species are readily distinguished, as those of *C. notastroma* have prominent olive-black to black conceptacles visible on the bark surface that delimit the stroma including the white to grayish-white disc (Plate 4b). Entostromatic tissue surrounding the locules is cinnamon, olive-gray to creamy-white in color, and can be variable in terms of development. Well-developed conceptacle tissue causes conidiomata to take on a distinctive target-like appearance (Plate 4).

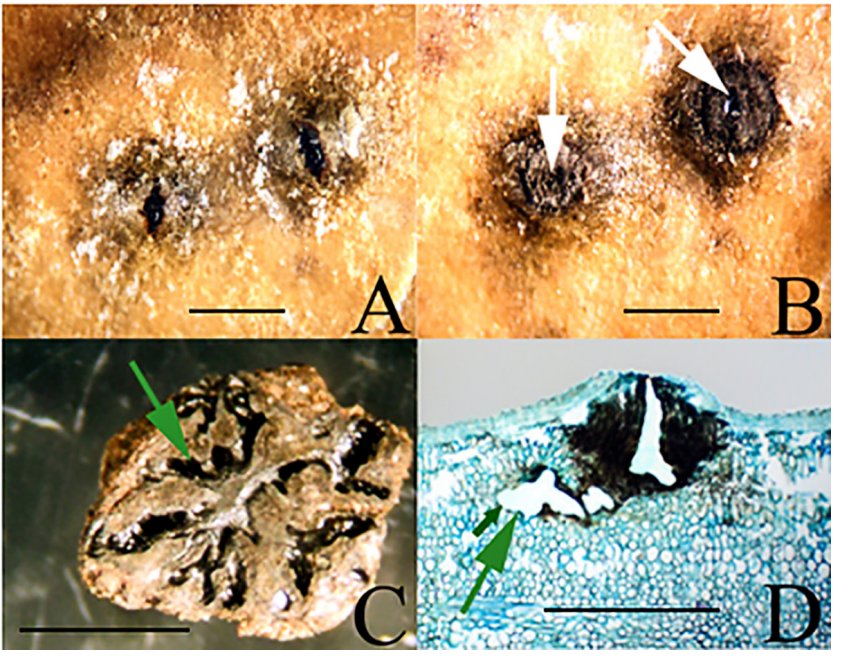


Plate 5. *Cytospora chrysosperma* conidiomata. A. Erumpent circular conidiomata (note lack of conceptacles and conceptacle tissues). B. Conidiomata with prominent gray, olive-gray to olive-black circular discs, each with single black ostiole (white arrows). C. Conidioma with labyrinthine multi-chambered locules surrounded by gray to olive-green stromatic tissues (green arrow). D. Vertical cross section with labyrinthine multi-chambered locules sharing common walls surrounded by dark stromatic tissues (green arrow); stroma of the conidioma is better developed than entostroma of the ascostroma); note lack of conceptacle and conceptacle tissue. Scale bars: A-D = 1.0 mm

In contrast, *C. chrysosperma* (Plate 5) lacks conceptacles delimiting the conidiomata and conceptacle tissues surrounding the discs; the discs are gray, olive-gray to olive-black.

Ascostromata of *C. notastroma* are similar in size and shape to those of *C. chrysosperma* but have a prominent dark conceptacle visible from the bark surface delimiting the stroma and disc (Plate 2). Such zone lines become less prominent with increasing depth and can vary among specimens or between ascostromata on the same specimen, ranging from well developed to somewhat obscure. When well developed, the conceptacle tissue gives the ascostroma a distinct target-like appearance. The zone line tends to extend less deeply into the fruit body in comparison to similar tissues found in conidiomata.

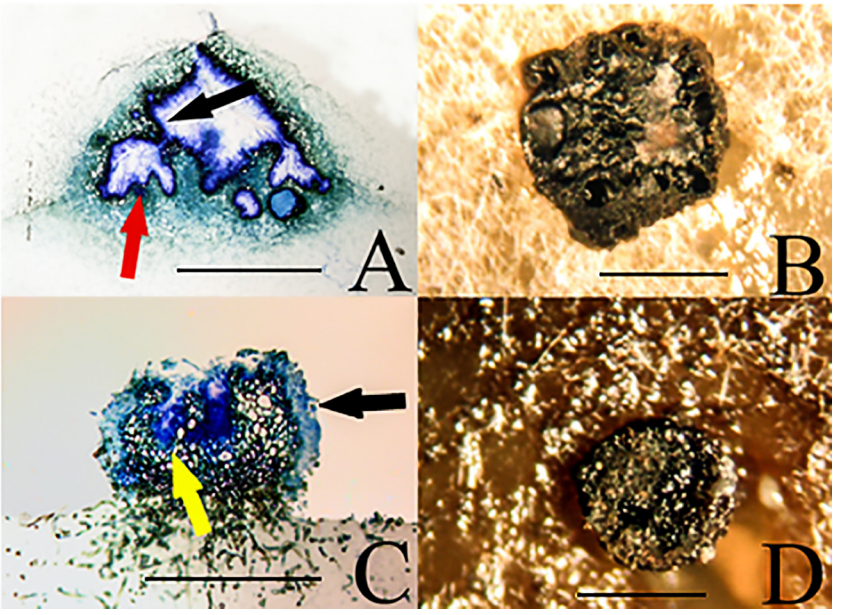


Plate 6. Conidiomata in vitro. ***Cytospora chrysosperma***. A. Vertical section of conidioma of isolate C 14 with multi-lobed locular chambers (red arrow) and layer of conidiophores (black arrow) interspersed with long gelatinous hyphal cells lining the walls of the locular structure. B. Horizontal cross section through conidioma of isolate C 14 showing the complex labyrinthine locular structure. ***Cytospora notastroma***. C. Vertical section of conidioma of isolate 3 with invaginations lined with conidiophores (yellow arrow) and conidiophores lining the surface of the pycnidium (black arrows). D. Horizontal cross section through conidioma of isolate K3 showing the simple structure without an enclosed multi-lobed arrangement of locules. Scale bars: A–D = 2.0 mm

Ascostromatal discs are snowy-white to grayish-white and powdery or flaky. The ostioles emerge through the disc and may be scattered, in rows, or in a circular arrangement on the disc surface; as few as two or as many as 10 ostioles may be visible. Perithecia are surrounded by well-developed orangish, cinnamon, olive-gray to creamish-white entostroma (Plate 2c).

Ascostromata and conidiomata of *C. translucens* and *C. nivea* (including the smaller spore form, *"C. pseudonivea"*) share with *C. notastroma* the leucostomoid form with perithecia embedded in entostroma and with delimiting conceptacle forming the dark zone lines. *Cytospora translucens,* but not *C. nivea*, shares the dark zone line ring of conceptacle surrounding the disc that is visible on the plant surface through a thin epidermis. Many other characters of *C. translucens* and the small form of *C. nivea* overlap those of *C. notastroma*. On average, *C. translucens* forms much smaller ascostromata with fewer perithecia and

larger ascospores. The smaller form of *C. nivea* also generally forms smaller ascostromata. The conidiomata of *C. translucens* are smaller with less complex labyrinthine chambers than *C. notastroma,* whereas those of *C. nivea* have more complex labyrinthine chambers than *C. notastroma*.

Cytospora chrysosperma ascostromata lack the delimiting conceptacle and discs are not snowy-white (Plate 3) as compared with *C. notastroma, C. nivea,* and *C. translucens*. Ascostromatal discs of *C. chrysosperma* are dark and sometimes obscured by perithecial beaks. Ostioles are circinately arranged around the margin of the disc, and beaks are often swollen and may be fused with adjacent beaks.

In vitro, conidiomata produced by *C. notastroma* are simple with invaginations (Plate 6). In contrast, conidiomata produced by isolates of *C. chrysosperma* have a complex structure comprised of multi-lobed locular chambers enclosed within the conidioma (Plate 6). Cultures of *C. notastroma* (isolates K3, K16, K20, L1) differ distinctly from those of *C. chrysosperma* (isolates C1 and C14; Plate 7) on common media like potato dextrose or malt extract agars. On Leonian's medium *C. notastroma* cultures tend to be dark with hyphae generally appressed and typically growing within the agar, and conidiomata are often covered with white, smoke-grey to olivaceous-grey hyphae. In contrast, *C. chrysosperma* cultures are light in color and zonate, with aerial hyphae dense and quite tall; numerous pycnidia-like conidiomata, some covered with white, buff to honey-colored hyphae, form primarily in the darker zones. Cultures of *C. notastroma* do not show the scalloped concentric rings of growth at irregular distances within the colony as described for *"C. pseudonivea"* (Vasilyeva et al. 2008, as *Leucostoma pseudoniveum*).

Hyphal tips collected from five-day-old cultures and mounted in water showed that isolates of *C. notastroma* had wide hyphae (4.0–5.5 µm diam.) that were bead-like and wavy in appearance. Additionally, a bursting of hyphal tips was observed in young (ca. seven days old) cultures. Hyphae produced by *C. chrysosperma* isolates are considerably narrower (1.5–2.5 µm diam.) and uniformly straight and no lysing was observed. Young (i.e., ca. 7–10 days old) cultures of *C. notastroma* produced a *Phialocephala*-like anamorph (Plate 7). These synanamorphs were located in older regions of cultures just beginning to form pigmentation where hyphae aggregated into ball-like clusters.

Discussion

A main objective of the present study was to use molecular techniques to sort out the "species complex" associated with cytospora canker of aspen in the Rocky Mountains and adjacent regions of the Great Plains. Based upon evolutionary analyses of ITS and EF-1α nucleotide sequence data sets,

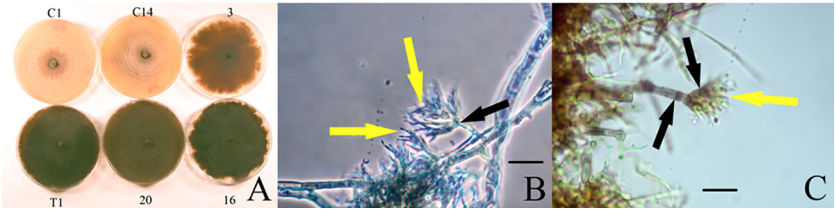


Plate 7. Cultural characteristics. A. Cultures of *Cytospora chrysosperma* isolates (C1 and C14) are light in color and zonate (conidiomata forming in the darker zones) unlike isolates of *C. notastroma* (K3, K16, K20, T1). ***Cytospora notastroma***. B. *Phialocephala*-like synanamorph produced by isolates K16 and K20. Dichotomous branching of conidiophores (black arrow); clusters of phialidic conidiogenous cells (yellow arrows). C. *Phialocephala*-like synanamorph produced by isolates K16 and K20. Septa (black arrows); phialidic conidiogenous cells of conidiogenous apparatus (yellow arrows). Scale bars: B–C = 5.0 um.

isolates of *C. notastroma* were strongly supported as a holophyletic clade with eastern isolates from *Populus deltoides* (eastern cottonwood), *P.* ×*canadensis* 'Robusta' (Carolina poplar), and *Alnus incana* subsp. *rugosa* (speckled alder) in Michigan. This implies that the Colorado and Michigan isolates are members of one widespread (phylogenetic) species occurring on at least two host genera. The other species isolated from aspen was identified as *C. chrysosperma* based on morphology and homologous sequences. *Cytospora chrysosperma* is more prevalent on aspen than *C. notastroma* and the pathogenicity of the latter has not yet been documented.

Study of historical herbarium specimens collected in the Americas and the opportunity to collect similar specimens from similar locations and compare morphology and DNA homology of the ITS and EF-1α sequences when possible have led us to conclude that *C. notastroma* has been confused with *C. nivea* in eastern and western North America. For example, our study of herbarium specimens indicates that several determined as *C. nivea* by J.B. Ellis (Ellis & Everhart 1892) and H. Kern (Kern 1957) represent *C. notastroma*. Ellis & Everhart (1892) and Kern (1957) had noted that the dimensions of stromata, perithecia, asci, ascospores, and conidia of the material from the western hemisphere were consistently smaller than those of European material and the type specimen of *C. nivea* (Vasilyeva et al. 2008).

Cytospora notastroma also shares with *C. translucens* the encircling conceptacle superficially visible under the thin epidermis and bark of tree stems and branches, and the leucostomoid character of the stroma. The fact that the visible encircling conceptacle may be absent in collections on thicker bark may have led to misidentification as *C. nivea*. Furthermore, comparison of ITS sequence of specimens identified as *C. nivea* and *C. translucens* in

NCBI GenBank accessions from Iran and China supports our conclusions that *C. nivea* and *C. translucens* are difficult to differentiate genetically. The collection of an isolate from *Salix* sp. in Colorado that is conspecific with European isolates identified as *C. translucens* by Défago (agreeing in morphology and DNA sequence homology) may be fortuitous but is also due to our concerted efforts toward discovering and verifying the species presence. As *C. translucens* may not be present on aspen and is rarely reported on *Salix* spp. in the Rocky Mountains, it may not be endemic.

We believe the smaller forms of *C. nivea* described by Ellis & Everhart (1892), Gilman et al. (1957), and Kern (1957) from Colorado, Montana, Iowa, and Michigan most likely represent *C. notastroma.* We assume many similar collections will be homologous in DNA sequence to *C. notastroma*, but we cannot exclude the possibility that some may have sequence homology to *C. nivea*. Resolving interrelationships among a well-supported clade and neighboring clades, unfortunately, is somewhat problematic in *Cytospora* because of polytomies occurring in the phylogram may result from either much homoplasy across the genus (Sanderson & Donoghue 1989, Farr et al. 2002) or "very real polytomies in the tree" (Hall 2008). Farr et al. (2002) reported problems with homoplasy across the related diaporthalean genus, *Diaporthe*.

Cytospora notastroma is somewhat unusual in that the anamorph occasionally forms within the same stromatic tissues as the teleomorph; nonetheless, this does occur in a few species such as *C. cincta* Sacc. and *C. massariana* Sacc. (Adams et al. 2005). In both nature and culture, *C. notastroma* specimens are readily distinguished from those of *C. chrysosperma,* which increases the usefulness of our research for forest and plant pathologists. Cultural variation among *C. notastroma* isolates is relatively negligible, although isolate K3 produced more lobate growth, was less darkly pigmented, and did not produce pycnidia-like conidiomata with beaks. Additionally, isoenzyme studies place isolate K3 in a different subgroup from isolates K16, K20, and L1 with a genetic similarity (based on Jaccard's coefficient) of 47% (Kepley 2009). Our phylogeny clusters Colorado isolate K3 more closely with the eastern isolates from speckled alder, eastern cottonwood, and *P.* ×*canadensis* 'Robusta' of Michigan. However, as isolate K3 was collected from an urban landscape, the geographic origin of the host is unknown. In this sexually reproducing species, isoenzyme profiles would be expected to differ between populations east of the Mississippi and those of the southern Rocky Mountain region. Obviously, further genetic, molecular, and morphological studies should be conducted for this phylogenetic cluster of taxa in order to resolve possible sympatric species.

Although the occurrence of two synanamorphs (albeit in culture) was quite unexpected, two other studies have reported similar observations: Helton &

Konicek (1961) described "naked conidiophores" arising from dichotomous branching of hyphal tips in isolates from stone fruit trees; Hildebrand (1947) reported a similar occurrence with isolates of *C. leucostoma* from peach trees (our phylogenetic analyses indicate the *C. notastroma* isolates as distinct but closely related to *C. leucostoma*). The naked conidiophores described in the latter studies apparently were not *Phialocephala*-like in morphology, and whether naked conidiophores or a *Phialocephala*-like anamorph occur in the field is not known.

With time, methods for identifying fungi and assessing their phylogeny will continue to improve, and databases will continue to enlarge. Datasets of 28S rDNA, β-tubulin, and histone 3A sequences are nearly complete for 100 *Cytospora* taxa and will, it is to be hoped, remove the polytomies in the current phylogeny. It is important to note that phylogenetic analyses allow only formulation of inferences and hypotheses. Hall (2008) summarizes this quite succinctly, stating that "the right tree doesn't exist" and "all methods implicitly acknowledge that the trees produced are only a subset of the possible trees that are consistent with the data." Recognizing new *Cytospora* species is important when they are encountered in association with major plant epidemics. Given their endophytic and pathogenic nature and broad host range, future studies are needed to determine the biology, ecology, and physiology of these organisms.

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Literature cited

Adams GC, Surve-Iyer RS, Iezzoni AF. 2002. Ribosomal DNA sequence divergence and group I introns within the *Leucostoma* species *L. cinctum, L. persoonii,* and *L. parapersoonii* sp. nov., ascomycetes that cause cytospora canker of fruit trees. Mycologia 94: 947–967. http://dx.doi.org/10.2307/3761863

Adams GC, Wingfield MJ, Common R, Roux J. 2005. Phylogenetic relationships and morphology of *Cytospora* species and related teleomorphs (*Ascomycota, Diaporthales, Valsaceae)* from *Eucalyptus*. Studies in Mycology 52: 1–144.

Adams GC, Roux J, Wingfield MJ. 2006. *Cytospora* species (*Ascomycota*, *Diaporthales*, *Valsaceae*): introduced and native pathogens of trees in South Africa. Australasian Plant Pathology 35: 521–548. http://dx.doi.org/10.1071/AP06058

Bartos DL, Shepperd WD. 2006. Draft of notes from the aspen "die-off summit." 19 p. Accessed online February 11, 2013: http://www.aspensite.org/pdf/Die-off/Aspen-Summit-Summary.pdf

Brody JR, Kern SE. 2004. Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. BioTechniques 36: 214–216.

Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553–556. http://dx.doi.org/10.2307/3761358

Défago G. 1935. De quelques Valsées von Höhnel parasites des arbres à noyau dépérissants. Beiträge zur Kryptogamenflora der Schweiz 8(3). 111 p.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5): 1792–1797. http://dx.doi.org/10.1093/nar/gkh340

Ellis JB, Everhart BM. 1892. The North American *Pyrenomycetes*. A contribution to mycologic botany. Ellis & Everhart, Newfield, NJ.

Eslyn WE. 1960. New records of forest fungi in the Southwest. Mycologia 52: 381–387. http://dx.doi.org/10.2307/3755953

Fairweather ML, Geils BW, Manthei M. 2008. Aspen decline on the Coconino National Forest. 53–62, in: M McWilliams (ed.). Proceedings of the 55th Western International Forest Disease Work Conference; 2007, October 15–19, 2007; Sedona, AZ. College of Natural Resources, Utah State University, Logan, Utah.

Farr DF, Bills GF, Chamuris GP, Rossman AY. 1989. Fungi on plants and plant products in the United States. APS Press, St. Paul, MN. 1252 p.

Farr DF, Castlebury LA, Rossman AY. 2002. Morphological and molecular characterization of *Phomopsis vaccinii* and additional isolates of *Phomopsis* from blueberry and cranberry in the eastern United States. Mycologia 94: 494–504. http://dx.doi.org/10.2307/3761783

Farris JS. 1989. The retention index and the rescaled consistency index. Cladistics 5: 417–419. http://dx.doi.org/10.1111/j.1096-0031.1989.tb00573.x

Farris JS, Kallersjo M, Kluge AG, Bult C. 1994. Testing significance of incongruence. Cladistics 10: 315–319. http://dx.doi.org/10.1111/j.1096-0031.1994.tb00181.x

Felsenstein J. 1981. Evolutionary trees from DNA sequences: a Maximum Likelihood approach. Journal of Molecular Evolution 17: 368–376. http://dx.doi.org/10.1007/BF01734359

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791. http://dx.doi.org/10.2307/2408678

Frey BR, Lieffers VJ, Hogg EH, Landhausser SM. 2004. Predicting landscape patterns of aspen dieback: mechanism and knowledge gaps. Canadian Journal of Forest Research 34: 1327–1390. http://dx.doi.org/10.1139/x04-062

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113–118. http://dx.doi.org/10.1111/j.1365-294X.1993.tb00005.x

Geiser DM, Jiménez-Gasco MdM, Kang S, Makalowska I, et al. 2004. FUSARIUM-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. European Journal of Plant Pathology 110(5–6): 473–479. http://dx.doi.org/10.1023/B:EJPP.0000032386.75915.a0

Gilbertson RL, Cummins GB, Darnall ED. 1979. Indexes to W.G. Solheim's Mycoflora Saximontanensis Exsiccata. Mycotaxon 10: 49–92.

Gilman JC, Tiffany LH, Lewis RM. 1957. Iowa ascomycetes II. *Diaporthaceae: Valseae*. Iowa State College Journal of Science 31(4): 623–647.

Guyon JC, Jacobi WR, McIntyre GA. 1996. Effects of environmental stress on the development of cytospora canker of aspen. Plant Disease 80: 1320–1326. http://dx.doi.org/10.1094/PD-80-1320

Hall BG. 2008. Phylogenetic trees made easy: A How-To Manual. 3rd edition. Sinauer Associates, Inc. Sunderland, MA. 233 p.

Hayova VP, Minter DW. 1998. *Leucostoma niveum*. I.M.I. Descriptions of Fungi and Bacteria 1362. 3p.

Helton AW, Konicek DE. 1961. Effects of selected. *Cytospora* isolates from stone fruits on certain stone fruit varieties. Phytopathology 51: 152–157.

Hildebrand EM. 1947. Perennial peach canker and the canker complex in New York, with methods of control. Cornell University, Agricultural Experiment Station, Memoir 276. 61 p.

Hinds TE. 1964. Distribution of aspen cankers in Colorado. Plant Disease Reporter 48: 610–614.

Hinds TE, Krebill RG. 1975. Wounds and canker diseases on western aspen. U.S.D.A. Forest Service, Forest Pest Leaflet 152. 9 p.

Hogg EH, Brandt JP, Kochtubajda B. 2005. Factors affecting interannual variation in growth of western Canadian aspen forests during 1951–2000. Canadian Journal of Forest Research 35: 610–622. http://dx.doi.org/10.1139/x04-211

Hogg EH, Brandt JP, Michaellian M. 2008. Impacts of a regional drought on the productivity, dieback, and biomass of western Canadian aspen forests. Canadian Journal of Forest Research 38: 1373–1384. http://dx.doi.org/10.1139/X08-001

Hubbes M. 1960. Systematische und physiologische Untersuchungen an Valsaceen auf Weiden. Phytopathologische Zeitschrift 39(1): 65–93.

Jacobi WR, Kelly EF, Troendle CA, Anqwin PA, Wettstein CA. 1998. Environmental conditions and aspen regeneration failure. U.S.D.A. Forest Service, Rocky Mountain Region, Renewable Resources, Forest Health Management, Technical Report R2-60 (Golden, CO). 24 p.

Juzwik J, Nishijima T, Hinds TE. 1978. Survey of aspen cankers in Colorado. Plant Disease Reporter 62: 906–910.

Kepley JB. 2009. Colorado *Cytospora* complex on *Populus tremuloides* Michx. PhD dissertation, Department of Biology, Colorado State University, Fort Collins

Kepley JB, Jacobi WR. 2000. Pathogenicity of *Cytospora* species on six hardwood species. Journal of Arboriculture 26: 326–332.

Kern H. 1957. Untersuchungen über die Umgrenzung der Arten in der Ascomycetengattung *Leucostoma.* Phytopathologische Zeitschrift 30: 149–180.

Kirk, P.; Cannon, P.; Minter, D.; Stalpers, J.; 2008. Ainsworth and Bisby's dictionary of the fungi (ed.10). Wallingford: CABI. 771 p.

Knight DH. 2001. Summary: aspen decline in the West? U.S.D.A. Forest Service, Proceedings, Rocky Mountain Research Station P–18.

Korbie DJ, Mattick JS. 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nature Protocols 3(9): 1452–1456. http://dx.doi.org/10.1038/nprot.2008.133

Krebill RG. 1972. Mortality of aspen on the Gros Ventre elk winter range. U.S.D.A. Forest Service, Intermontane Forest and Range Experiment Station, Research Paper INT-129 (Ogden, UT). 16 p.

Leonian LH. 1923. The physiology of perithecial and pycnidial formation in *Valsa leucostoma.* Phytopathology 13: 257–272.

Marchetti SB, Worrall JJ, Eager T. 2011 Secondary insects and diseases contribute to sudden aspen decline in southwestern Colorado, USA. Canadian Journal of Forest Research 41: 2315–2325. http://dx.doi.org/10.1139/x11-106

McIntyre GA, Jacobi WR, Ramaley AW. 1996. Factors affecting cytospora canker occurrence on aspen. Journal of Arboriculture 22: 229–233.

Nei M, Kumar S. 2000. Molecular evolution and phylogenetics. Oxford University Press, Oxford.

O'Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T. 2004. Genealogical concordance between mating type locus and seven other nuclear genes supports formal recognition of nine

phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41: 600–623. http://dx.doi.org/10.1016/j.fgb.2004.03.003

Rayner RW. 1970. A mycological colour chart. Commonwealth Mycological Institute, Kew, UK. 34 p.

Rehner SA. 2001. Primers for Elongation Factor 1-α (EF1-α). http://www.aftol.org/pdfs/EF1primer.pdf (Accessed online February 01, 2013)

Ross WD. 1976. Fungi associated with root diseases of aspen in Wyoming. Canadian Journal of Botany 54: 734–744. http://dx.doi.org/10.1139/b76-079

Rossman AY, Adams GC, Cannon PF, Castlebury LA, Crous PW, Gryzenhout M, Jaklitsch WM, Mejia LC, Stoykov D, Udayanga D, Voglmayr H, Walker DM. 2015. Recommendations of generic names in *Diaporthales* competing for protection or use. IMA Fungus 16(1) 145–154. http://dx.doi.org/10.5598/imafungus.2015.06.01.09

Sanderson MJ, Donoghue MJ. 1989. Patterns of variation in levels of homoplasy. Evolution 43: 1781–1795. http://dx.doi.org/10.2307/2409392

Shaw CG. 1973. Host fungus index for the Pacific Northwest – I. Hosts. Washington State University Agricultural Experiment Station Bulletin 765. 121 p.

Sinclair WA, Johnson WT. 2005. Diseases of trees and shrubs. Comstock Publishing Associates, Cornell University Press, Ithaca, NY. 660 p.

Spielman LJ. 1983. Taxonomy and biology of *Valsa* species on hardwoods in North America, with special reference to species on maples. Ph.D. dissertation. Cornell University. Ithaca, NY. 175 p.

Spielman LJ. 1985. A monograph of *Valsa* on hardwoods in North America. Canadian Journal of Botany 63: 1355–1378. http://dx.doi.org/10.1139/b85-190

Swofford DL. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Inc. Sunderland, MA

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731–2739. http://dx.doi.org/10.1093/molbev/msr121

Tavaré S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Lectures on Mathematics in the Life Sciences (American Mathematical Society) 17: 57–86.

Vasilyeva LN, Rossman AY, Farr DF. 2008 [”2007”]. New species of *Diaporthales* from eastern Asia and eastern North America. Mycologia 99(6): 916–923. http://dx.doi.org/10.3852/mycologia.99.6.916

Walters JW, Hinds TE, Johnson DW, Beatty J. 1982. Effects of partial cutting on diseases, mortality, and regeneration of Rocky Mountain aspen stands. U.S.D.A. Forest Service, Rocky Mountain Forest and Range Experiment Station, Research Paper RM-240 (Ft. Collins, CO). 12 p.

White TJ, Bruns T, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322 in: MA Innis et al. (eds), PCR Protocols: a Guide to Methods and Applications. San Diego, Academic Press.

Worrall JJ, Egeland L, Eager T, Mask RA, Johnson EW, Kemp PA, Shepperd WD. 2008. Rapid mortality of *Populus tremuloides* in southwestern Colorado, USA. Forest Ecology and Management 255: 686–696. http://dx.doi.org/10.1016/j.foreco.2007.09.071

Worrall JJ, Marchetti SB, Egeland L, Mask RA, Eager T, Howell B. 2010. Effects and etiology of sudden aspen decline in southwestern Colorado, USA. Forest Ecology and Management 260: 638–648. http://dx.doi.org/10.1016/j.foreco.2010.05.020

Zegler TJ, Moore MM, Fairweather ML, Ireland KB, Fulé PZ. 2012. Aspen mortality near the southwestern edge of its range. Forest Ecology and Management 282: 196–207. http://dx.doi.org/10.1016/j.foreco.2012.07.004