



Pathogenicity and distribution of two species of *Cytospora* on *Populus tremuloides* in portions of the Rocky Mountains and midwest in the United States



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ABSTRACT

Historically, *Cytospora* canker of quaking aspen was thought to be caused primarily by *Cytospora chrysosperma*. However, a new and widely distributed *Cytospora* species on quaking aspen was recently described (*Cytospora notastroma* Kepley & F.B. Reeves). Here, we show the relative pathogenicity, abundance, and frequency of both species on quaking aspen in portions of the Rocky Mountain region, and constructed species-level phylogenies to examine possible hybridization among species. We inoculated small-diameter aspen trees with one or two isolates each of *C. chrysosperma* and *C. notastroma* in a greenhouse and in environmental growth chambers. Results indicate that both *Cytospora* species are pathogenic to drought-stressed aspen, and that *C. chrysosperma* is more aggressive (i.e., caused larger cankers) than *C. notastroma*, particularly at cool temperatures. Neither species cause significant canker growth on trees that were not drought-stressed. Both *C. chrysosperma* and *C. notastroma* are common on quaking aspen, in addition to a third, previously described species, *Cytospora nivea*. Multiple *Cytospora* species often co-occur on the same host tree, and evidence of hybridization among species exists.

1. Introduction

Quaking aspen (*Populus tremuloides* Michx.) is one of the few native hardwood species found in the Rocky Mountain region of western North America (Little, 1971). Although quaking aspen is widely distributed across the continent, in the western half of the United States, it thrives in montane environments, where summer temperatures are cool, and moisture is plentiful (Little, 1971). When aspen grow on marginal sites, such as the dry Front Range of the Rocky Mountains in Colorado, they are prone to damage by environmental stress and subsequent infestation by secondary insects and diseases (Hinds, 1985). Foremost among these is *Cytospora* canker, caused by at least two *Cytospora* species.

Cytospora has been described as a wound parasite, a stress-related pathogen, and an opportunistic saprobe (Sinclair and Lyon, 2005; Hinds, 1985). It is not considered to be an aggressive pathogen of most host trees unless drought or another stressor prevents the host from successfully limiting fungal colonization (Hubert, 1920). *Cytospora* species invade host tissue through wounds or other openings in the bark (Long, 1918; Hinds, 1985). At least two *Cytospora* species have been isolated from asymptomatic host tissues, including on aspen and alder (Garner, 1967; Worrall et al., 2010). Chapela (1989) observed that some infected aspen displayed cankers only after a period of drying.

Conversely, McIntyre et al. (1996) examined healthy quaking aspen for presence of *Cytospora* species but did not recover any isolates from asymptomatic phloem tissues. The same study also showed, however, that *Cytospora* could be consistently isolated from surface bark of aspen trees from June–November (McIntyre et al., 1996). Host wound response intensities, with and without *Cytospora* infections, have been investigated in various host tree species, including quaking aspen and a poplar hybrid (Bloomberg and Farris, 1963; Banko and Helton, 1974; Bertrand and English, 1976; Biggs et al., 1983; Wisniewski et al., 1984; Biggs, 1984; Biggs, 1986; Guyon et al., 1996; McIntyre et al., 1996). Drought-stressed quaking aspen trees have been shown to remain susceptible to infection by *Cytospora* species for more than a week (McIntyre et al., 1996).

The identity of the *Cytospora* species that occur on hardwoods, including quaking aspen, has historically been complicated and confusing. Spielman (1985) clarified much of the confusion in a monograph of the *Cytospora* (*Valsa*) genus, in which she noted that nearly every morphological characteristic was highly variable. Thus, many isolates previously described as individual species were likely variants of half a dozen species (Spielman, 1985). Adams et al. (2005) noted that many additional *Cytospora* species were described based on the host on which they were found, although it is now known that a single

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Cytospora species can infect multiple hosts (Adams et al., 2005). The same study described the worldwide distribution of *Cytospora*, and its species complexes based on the characteristics of anamorphic and teleomorphic fruiting body structure (Adams et al., 2005). Based on these and observations by Spielman (1985) and others, both Adams et al. (2005) and Kepley and Jacobi (2000) stated that the four teleomorphic genera historically associated with *Cytospora* canker (*Leucostoma*, *Valsella*, *Valseutypella*, and *Valsa*) should be condensed into one genus. As Spielman (1985), Kepley and Jacobi (2000) and Adams et al. (2005, 2006) observed, the wide range of variability in morphological characteristics is likely due to environmental and host conditions, in addition to various phenotypic differences.

Past inoculation studies, ostensibly using *C. chrysosperma*, examined various environmental stressors on host trees and the extent to which these stressors impacted canker development (Burks, 1994; McIntyre et al., 1996; Guyon et al., 1996). Deficiencies of certain macronutrients, including plant available nitrogen and iron, resulted in larger cankers (Burks, 1994). Guyon et al. (1996) examined the effect of drought, flooding, and defoliation on the expansion of *Cytospora* canker on aspen and cottonwood. Drought and severe (75–100%) defoliation had the greatest influence on canker growth; cankers on severely defoliated trees were much larger than non-defoliated control trees or trees with 50% defoliation (Guyon et al., 1996).

Kepley et al. (2015) recently described a new species of *Cytospora* in quaking aspen based on analyses of morphological, isozyme, and genetic marker sequences. This new species, named *C. notastroma*, often forms pycnidia and perithecia over layers of dark stromatal tissue, resulting in a target-shaped ring surrounding the ostiole (Kepley and Jacobi, 2000; Kepley et al., 2015). The pathogenicity of this species on quaking aspen has not been examined. The central purpose of this study was to build on the work of Kepley et al., (2015) by comparing *C. notastroma* with *C. chrysosperma* on host trees in a greenhouse and in the wild.

The relative pathogenicity and genetic relationship of *C. chrysosperma* and *C. notastroma* on quaking aspen are unknown. Thus, the objectives of this study were to: (1) perform Koch's postulates, and prove the pathogenicity of *Cytospora notastroma* on quaking aspen (relative to the known pathogen *C. chrysosperma*), (2) determine whether the two species behave similarly under different temperature ranges on quaking aspen, and (3) estimate the relative abundance of *C. chrysosperma* and *C. notastroma*, the two most commonly occurring species on quaking aspen in Colorado and select locations throughout the western and midwestern US. To address these objectives, we conducted a series of inoculation trials with aspen under various environmental conditions, collected and cultured *Cytospora* species from infected bark tissue, and classified the isolates as belonging to either *C. notastroma* or *C. chrysosperma*.

2. Materials and methods

2.1. Sample collection

Symptomatic quaking aspen from across a large area (Fig. 1) were sampled for *Cytospora* canker. For the inoculation portion of the study, two *C. chrysosperma* isolates ('BDSR 1.2', 'DG11A') were cultured (and purified by single-sporing) from aspen bark collected in the Red Feather Lakes area (N40.752976, W105.498122), and from Poudre Canyon (N40.682286, W105.642762) Colorado, during the spring and summer of 2012 (Fig. 1). The holotype of *C. notastroma* is isolate RCKEP3A which was originally collected by J. Kepley from quaking aspen bark from the Roaring Creek campground (N40.714227, W105.734967) in Poudre Canyon in October of 2002. *Cytospora notastroma* isolate SW8C was collected from a quaking aspen in the Denver suburb of Aurora, Colorado (N39.728981, W104.813863) in the spring of 2013 (Fig. 1). For the phylogenetic portion of the study, we sampled infected quaking aspen trees from three distinct categories (1) four urban areas in north-

central Colorado (Fort Collins, Denver, Glenwood Springs, and Meeker); (2) from forests outside of Colorado and southern Wyoming, including Utah (the Ashley and Dixie National Forests), northwestern Montana (adjacent to highway 89, near Babb, MT), and east-central Minnesota (Chisago County); (3) four national forests in Colorado and southern Wyoming (Medicine Bow, Arapaho-Roosevelt, White River, and Pike-San Isabel National Forests) (Fig. 1). In a previous study, we established 97 aspen health monitoring plots on five national forests in Colorado and southern Wyoming in 2009–2010 to assess the impact of various damage agents on aspen health in stands designated as healthy or damaged (Dudley et al., 2015). In this study, we revisited a subset of the healthy and damaged plots to collect aspen stems infected with *Cytospora* canker. In Colorado, the forests sampled are mainly east of the Continental Divide (the Pike-San Isabel, Arapaho-Roosevelt, and Medicine Bow National Forests), with one sampled Forest west of the Divide (the White River N.F.). At each sample location, we navigated to the center of each selected plot using a GPS unit (Garmin® eTrex Legend; Garmin Ltd., Olathe, KS). At the center of the plot, we randomly selected a bearing, and established a 10 × 1 m transect along the bearing. The first ten aspen trees with symptomatic *Cytospora* canker along each transect were sampled, and infected tissues (bark or stems, or both) were placed in paper bags, labeled, and stored in a laboratory. We isolated from a minimum of three trees per plot and isolated three cultures per tree. In total, we collected infected aspen tissue from urban areas in Colorado, from two national forests in Utah, as well as individual trees in Montana and Minnesota, for a total of 410 samples from 110 trees. Of these, 200 isolates from 63 trees were confirmed to be *Cytospora* species (Table 1; Fig. 1)

2.2. Culture of *Cytospora* isolates

All isolates were grown on ½ -, or ¼-strength potato dextrose agar (PDA), and a subset were grown on Leonian's modified medium (Leonian, 1921). Plates were stored at room temperature (25 °C) in sterile plastic bins. Spores were extracted from fruiting bodies using a modified procedure developed by Kepley (2009): bark surface was first sprayed with a 75% ethanol/ water solution and allowed to dry; locule chambers were exposed by aseptically slicing off thin layers of the fruiting body to expose chamber entrance, and a droplet of sterile water was pipetted onto the exposed locule chamber, from which a spore mass emerged (Kepley, 2009). A metal streaking loop was used to streak the spore mass onto a plate of media. In the case that fruiting bodies were not present on the surface of bark tissue, small (2–3 mm diameter) pieces of bark were excised from canker margins, surface sterilized, and partially submerged in the media. Plates were sealed with Parafilm® (Bemis Company, Inc., Neenah, WI) and checked daily. Once mycelial growth resembling *Cytospora* was evident, the colony was hyphal tipped and transferred to a fresh plate and further incubated for 7–10 days. After cultures had attained a size of at least 3 cm diameter, approximately a dozen pieces of the culture margin were cut (1–2 mm²), transferred to a liquid medium (potato dextrose broth), and placed in a rotating growth chamber for up to 6 days at 25 °C. Additional pieces of the culture were placed in glycerol and stored at –80 °C, as well as ½-strength potato dextrose agar sealed glass slants and stored at 4 °C. The tissue samples in liquid medium were grown to 1–2 cm in diameter, and after 5–6 days were extracted using a centrifuge or vacuum extractor. Samples were then placed in 2 mm plastic vials and stored in a –20 °C freezer prior to DNA extraction.

2.3. DNA extraction and sequence amplification

Extraction was performed using the Invitrogen Plant DNeasy™ Kit (Invitrogen™ Corp., Carlsbad, CA). Following extraction, nucleotide concentration of the samples was assessed using a NanoDrop™ 2000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific, Wilmington, DE). Samples that contained at least 15 ng/μl of purified

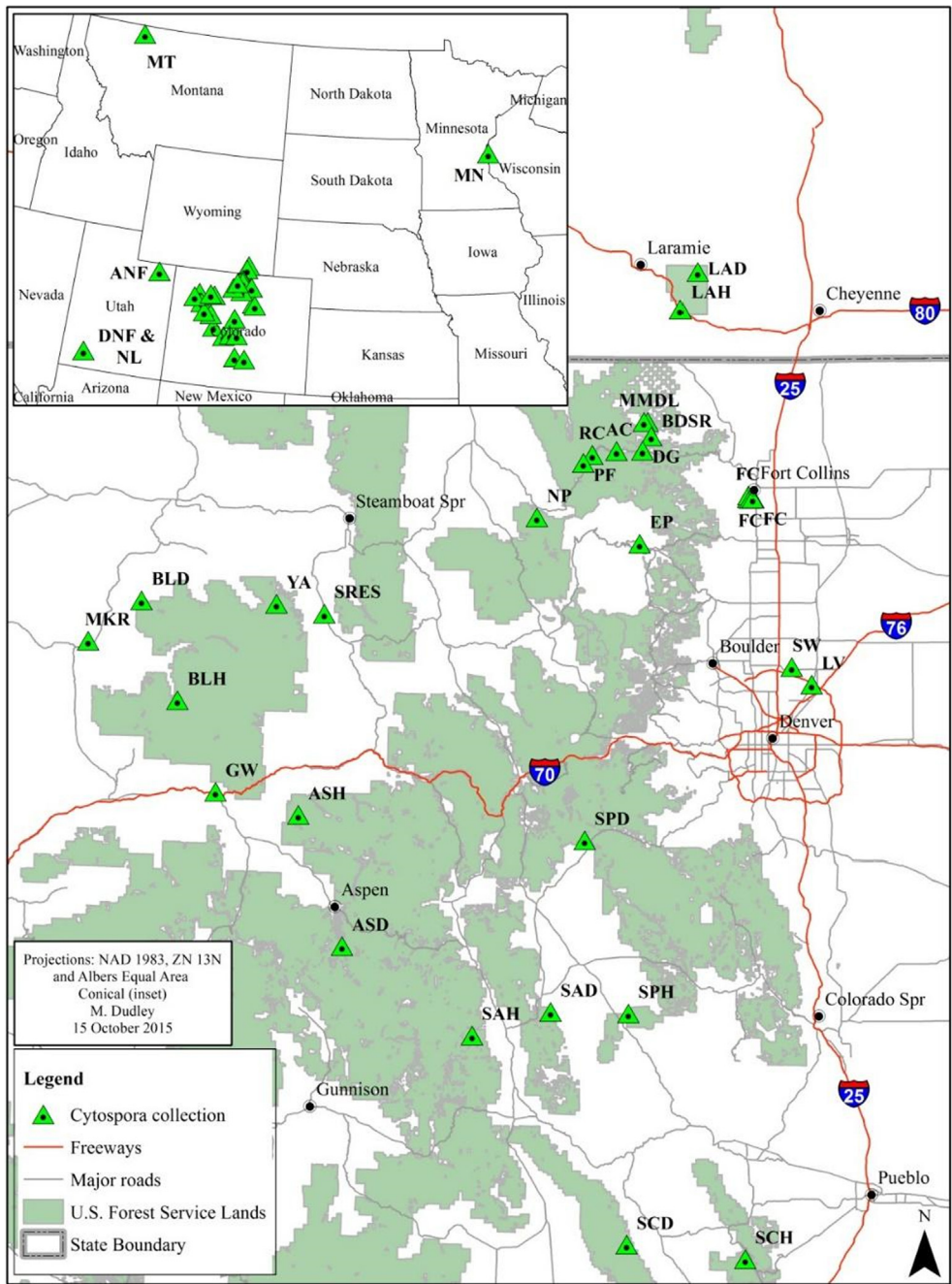


Fig. 1. Map of sample locations used in this study. Materials were collected June 2012–Sept 2014.

Table 1
Numbers of fungal isolates cultured from cankers on quaking aspen included in this study, by geographic region and *Cytospora* species.

Region	No. Trees	No. <i>Cytospora</i> Isolates	No. CH* isolates	No. NO isolates	No. NI isolates	No. other <i>Cytospora</i> †
Eastern CO (urban)	10	36	21	15	0	0
Northern CO	6	15	10	5	0	0
Northwestern CO	10	45	17	12	11	5
Poudre Canyon	5	12	3	9	0	0
South-central CO	16	39	17	4	6	12
Southern WY	8	23	14	7	0	2
Central UT	6	17	5	9	2	1
Eastern MN	1	9	0	9	0	0
Northern MT	1	4	0	4	0	0
Total	63	200	87	74	19	20
Percent of total			43.5%	37.0%	8.5%	10.0%

* CH: *Cytospora chrysosperma*; NO: *Cytospora notastroma*; NI: *Cytospora nivea*.
† Isolates identified to *Cytospora* genus level, but marker sequences did not match any of the three species listed.

DNA were then used for polymerase chain reaction (PCR) amplification, using a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Markers used in this amplification included the internal transcribed spacers and 5.8S subunit of the nuclear rDNA (ITS) using primers ITS1 and ITS4 (White et al., 1990) and a portion of the β -tubulin (*BT*) gene (using primers Bt2a and Bt2b) (Carbone and Kohn, 2001).

2.3.1. *Cytospora* species confirmation: Fragment sequencing and alignment

Approximately half of the samples were sent for sequencing to the Colorado State University Proteomics and Metabolomics Facility, and half to the University of Arizona Genetics Core facility (Tucson, AZ). The CSU Proteomics and Metabolomics Facility uses an Applied Biosystems® 3130xl Genetic Analyzer (Life Technologies, Grand Island, NY). The Arizona facility uses multiple Applied Biosystems® 3730 Genetic Analyzers (Life Technologies, Grand Island, NY). Chromatogram files were examined using software program Sequence Scanner v 1.0 (Applied Biosystems®, Foster City, CA). Base pairs with quality scores of less than 20 were examined for errors, and the sequences' overall quality scores, start and end points, and location and nature of ambiguous base pairs were recorded and annotated in Excel® (Microsoft Corp., Seattle, WA). ITS and *BT* sequences were aligned and trimmed using MUSCLE (multiple sequence comparison by log-expectation) (Edgar, 2004), implemented in the software program Geneious Prime® (v 2019.2.3). Locus sequences were concatenated and isolates with identical concatenated sequences were placed into haplogroups by species, and for each locus separately.

2.3.2. *Cytospora* species confirmation: nucleotide diversity analyses

We used DNAsp5 (Rozas et al., 2003) to estimate genetic diversity parameters for each locus (ITS and *BT*) and concatenated ITS-*BT* dataset. This was done for each species group (*Cytospora notastroma* (NO), *C. nivea* (NI) and *C. chrysosperma* (CH)) as well as the total population. Haplotype diversity (h_D), nucleotide diversity (π), selection (Tajima's D , Fu and Li's F), and recombination were estimated for each locus. Linkage disequilibrium (i.e., the non-random assortment of alleles for a given locus) was also tested for the concatenated ITS-*BT* dataset.

We conducted phylogenetic analyses of *Cytospora* sequences using maximum likelihood (ML) and Bayesian analyses (BA) on each locus independently and on the concatenated dataset. Reference and out-group sequences included *Cytospora nivea* [NCBI accession numbers KF294008.1 (ITS) and EU219135.1 (*BT*)], *C. chrysosperma* [JX438635 (ITS) and KT590414 (*BT*)], *C. notastroma* [JX438627 (ITS)], *C. cypri* [DQ243801 (ITS) and KM034893 (*BT*)] and a *Cryptosphaeria pullmanensis* isolate cultured previously from quaking aspen. DT-ModSel (Minin, et al., 2003) was used to identify the best-suited evolutionary model for each locus. Bayesian analyses tree selection was conducted using Markov-Chain Monte Carlo (MCMC) analysis in software program BEAST, with 1,000,000 independent runs (i.e. chains) and sampling every 1000 runs (Drummond et al., 2012). Data files were formatted using BEAUTi (Drummond et al., 2012). Again, substitution models were previously identified by DT-ModSel. Tracer (v1.2) (Drummond et al., 2012) was run on the parsimony trees produced by BEAST to assess the quality of each BA run. If all Effective Sample Size (ESS) values were confirmed to be greater than 200, then enough MCMC runs were presumed. Tree Annotator v1.8.2 (Drummond et al., 2012) was used to summarize trees compiled by BEAST in a single tree. The evolutionary models selected by DT-ModSel were TIMe + I + G for the ITS-based tree, and TrNe + G for the β -tubulin-based tree. A partition homogeneity test was implemented in PAUP*4a146 to test for congruence between the two loci (Swofford, 2002). PhyML (Guidon and Gascuel, 2003) was used to estimate the ML phylogenies of each locus and the combined dataset with 500 bootstrap replicates and TN93 substitution model.

2.3.3. *Cytospora* species confirmation: genetic distance & principal components analysis

Trimmed, concatenated ITS-*BT* sequences for all haplogroups of each *Cytospora* species (16 total) were uploaded to ClustalOmega (Sievers et al., 2011; www.ebi.ac.uk) to generate an alignment file, including annotation of SNP locations. The Excel add-in program GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) was used to calculate genetic distance and conduct a principle components analysis. Genetic distance and PCA calculations were performed for all *Cytospora* species (*C. chrysosperma*, *C. notastroma*, and *C. nivea*) together and by species independently.

2.3.4. Inoculation study: aspen nursery stock

Two size classes of quaking aspen were used in this study. Sapling-sized quaking aspen (i.e., < 1.0 m tall) that were approximately three years old were used for inoculation trials conducted in a growth chamber and were obtained from the Fort Collins Wholesale Nursery in Fort Collins, CO in 2012. Aspen were grown in standard potting mix and planted in number 1 plastic nursery containers. Pole-sized trees (2–2.5 m tall), 5–7 years old, were used in inoculations trials conducted in a greenhouse setting. These were obtained from the Little Valley tree nursery in Brighton, CO in the spring of 2013. Trees were grown in standard potting mix (of sphagnum moss, perlite, and soil) and planted in number 2 plastic nursery containers. All nursery stock was stored in a shadehouse on the CSU campus for a period of two to three months prior to the inoculation study, and under favorable growth conditions.

2.3.5. Inoculation study: drought stress measurements

Upon commencement of inoculation study, drought stress was induced and measured by determining predawn water potential levels of fully formed leaves. Water potential was measured on trees in growth chambers after at least six hours on the 'dark' cycle. Measurements were taken using a Scholander-type pressure chamber (PMS Instruments, Albany, OR) daily until levels reached drought stress levels, approximately -1.5 MPa. Once drought stress was induced, water potential was measured twice weekly, and small amounts of water (25–250 ml) were added periodically to maintain stressed conditions and to keep the trees alive for the duration of the experiment.

2.3.6. Inoculation study: growth chamber inoculation trials

Four trials, consisting of eight aspen saplings each, were placed in a diurnal growth chamber under warm or cool conditions. Temperatures were set to fluctuate in a cool environment from 15 °C during the light cycle and 12 °C during the dark cycle, and a warm environment from 32 °C during the light cycle to 25 °C during the dark cycle. We used two different growth chambers during this experiment; a Caron® (model 6340-1) (Marietta, OH) growth chamber was used for the cool temperature trials, and a Percival® (model E-54U) (Perry, IA) was used in the warm temperature trials. Both growth chambers used fluorescent lights. Measurements of the light intensity of the two chambers were taken using an Apogee Instruments® Quantum meter (model MQ-100) (Logan, UT) placed roughly 30 cm below the light source. The light intensity of the Caron® growth chamber averaged $75 \mu\text{mol}^{-2} \text{m}^{-2} \text{s}^{-1}$ and the Percival® chamber averaged $28 \mu\text{mol}^{-2} \text{m}^{-2} \text{s}^{-1}$. Two inoculation trials of eight drought-stressed trees each were conducted for each of the two temperature ranges, plus two watered control trees for a total of 34 aspen saplings. Three wound sites per tree were placed at 10 cm intervals along the stem, and surface-sterilized prior to wounding by applying 70% ethanol to the bark surface. Stem diameters at wound sites ranged from 7 to 20 mm. Wounds were produced along the tree stem using a 5- or 7-mm cork borer to remove the bark. A plug of half-strength potato dextrose agar ($\frac{1}{2}$ PDA) of the same diameter, or a $\frac{1}{2}$ PDA plug colonized with a single isolate of either *C. chrysosperma* or *C. notastroma* was inserted into the wound. Each tree was inoculated with one isolate of each *Cytospora* species. All inoculation sites were sealed by wrapping the stem with a 2 cm-wide strip of Parafilm®. *Cytospora*

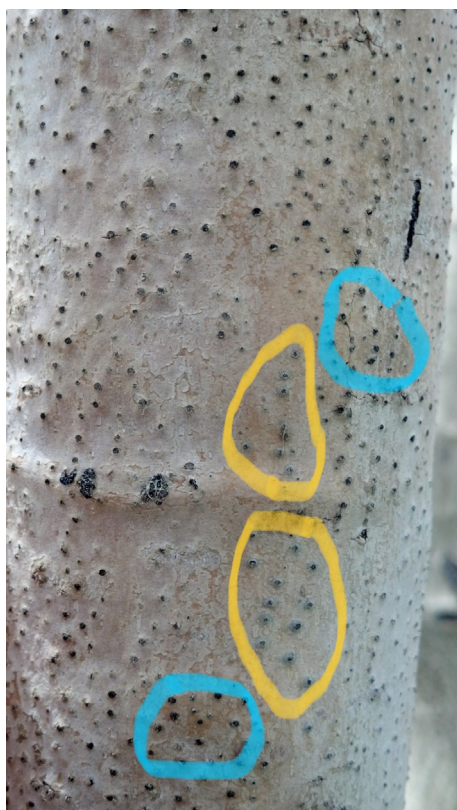


Fig. 2. An aspen trunk infected with *Cytospora* canker. Note the variable morphology of pycnidia formed on the same stem: some fruiting bodies (yellow circles) feature dark stromatal tissue visibly surrounding the ostiole; other pycnidia (blue circles) lack this feature. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nivea was not included in the inoculation trials, as isolates were not obtained and identified until after the conclusion of this portion of the study.

2.3.7. Inoculation study: greenhouse trial

Thirty-two pole-sized aspen trees (2–2 ½ m tall) in standard potting mix and five-gallon plastic pots were placed on benches in mid-July 2013 in a CSU greenhouse. Three-quarters of the trees were drought-stressed until water potentials reached approximately -1.5 MPa, and the remaining one quarter were watered daily. Greenhouse lights remained off for the duration of the experiment. Drought-stressed trees were given 100–250 ml water whenever pre-dawn water potentials exceeded -2.0 MPa, to keep the trees alive for the duration of the experiment. After trees had been stressed for one week, each was inoculated as previously described with two isolates each of *C. chrysosperma* and *C. notastroma*, plus a control plug of sterile media (1/2 PDA) for a total of five inoculated wound sites per tree. Temperature data were recorded hourly using a Watchdog® (Spectrum Technologies, Inc., Plainfield, IL) temperature recorder.

2.3.8. Inoculation study: canker measurements & fungal re-isolation

The protective wraps were removed from wound sites one week after the date of inoculation and examined for evidence of canker growth. Cankers were examined and measured again every 2–3 days until the conclusion of the study. Canker boundaries were defined as the edge of the discolored bark tissue and were re-measured on all trees for all trials (i.e. growth chamber and greenhouse). The number of measurements of canker size varied by trial, with a maximum of seven measurements of cankers on trees in the greenhouse and two

measurements on trees in the growth chamber throughout the trials, which was dependent upon tree health. At the conclusion of each inoculation trial, a total of twelve cankers or wounds (five *C. chrysosperma*, five *C. notastroma*, two control) were sampled, and fungi (if present) were re-isolated to confirm pathogen identity.

2.3.9. Inoculation study: statistical analysis

Analysis of the growth chamber trials data included the log-transformed sum of canker area, calculated as the area of an ellipse, where ellipse area = ((canker length/2) * (canker width/2) * π). The area of the inoculation wound (with a diameter of 5- or 7-mm) was subtracted from the canker area. Statistical analyses were performed using SAS® 9.4 software (SAS Institute, Cary, NC) and SPSS v. 25 (IBM Corp., Armonk, NY). All four temperature trials (i.e. two high, two low) were combined and analyzed together. Growth chamber data were analyzed as a mixed linear model and included temperature, treatment, isolate, measurement time, as well as the temperature, isolate, and treatment interaction within measurement date. The random variables included tree within temperature and wound position (i.e. placement of the inoculum along the stem) by isolate interaction within temperature. Water potential data were analyzed (as the average of two water potential readings per tree) as repeated measures mixed linear models. Average water potential was analyzed as treatment type by measurement date interaction. We dropped drought treatment as a variable from the final analysis once we had confirmed that cankers did not form on watered trees.

The greenhouse inoculation trial data were analyzed separately from the four growth chamber inoculation trials. Because some of the cankers coalesced after two weeks, we used canker size at twelve days post-inoculation in our analyses. Canker area was analyzed as a mixed linear model with treatment (i.e. drought-stressed or watered), isolate, inoculation position (five per tree), and average water potential. Random effects were trees within treatment, as well as the isolate by tree interaction within treatment type. Least-squares means were obtained for all main effects and interactions of interest. Means were significantly different at the $P \leq 0.05$ level. Position was initially included in the analysis but was later removed due to non-significance.

3. Results

3.1. *Cytospora* species confirmation: frequency and distribution of *Cytospora* species

Of the 110 diseased quaking aspen trees we sampled, all had symptoms of *Cytospora* canker, with or without fruiting bodies present. The presence of a *Cytospora* species was confirmed, via culturing, on 71 of the sampled trees. We initially attempted to differentiate *C. notastroma* from *C. chrysosperma* and other possible *Cytospora* species by the presence of a prominent olive-black to black conceptacle that delimited the stroma as described by Kepley et al. (2015). However, this characteristic was variable and sometimes absent in the stroma of *C. notastroma*, depending on bark thickness and the color of the bark (i.e. periderm) (Fig. 2). This was further complicated, however, when a third *Cytospora* species, *C. nivea* was cultured from pycnidia lacking a conceptacle, making species identification based on fruiting body appearance impossible. Therefore, isolates were cultured from pycnidia as previously described. We tentatively identified 366 of the 410 cultured isolates as *Cytospora* species based on production and morphology of pycnidia and conidia in culture, or the growth rate and color of isolates when grown on ¼ or ½-strength potato dextrose agar or Leonian's modified medium. This number was later reduced to 200 *Cytospora* cultures once molecular analyses were conducted. When cultured on Leonian's modified medium, *Cytospora notastroma* had a dark colony appearance with appressed hyphae growing into the agar, whereas *C. chrysosperma* had a light tan or buff-colored colony appearance, as previously described (Kepley et al, 2015; Kepley, 2009). *Cytospora*

notastroma and *C. chrysosperma* were readily distinguishable from each other after 7–10 days growth on Leonian's modified medium. Identification based on culture color was further complicated as we observed *Cytospora nivea* to be variable in color when grown on Leonian's modified medium; ranging from light tan (like *C. chrysosperma*) to dark brown (although not as dark in color as *C. notastroma*).

Of the 200 isolates identified as *Cytospora* species, 44% were *C. chrysosperma* and 37% were *C. notastroma* (Table 1). We detected both *C. chrysosperma* and *C. notastroma* in each of the three main regions sampled (Table 1). *Cytospora nivea* was detected only in northwestern and south-central Colorado and central Utah (Table 1) and only *C. notastroma* was found in eastern Minnesota and northern Montana (Table 1). An additional 20 isolates cultured from trees in northwestern and south-central Colorado, southern Wyoming, and central Utah were identified to the *Cytospora* genus level, but could not be conclusively identified as *Cytospora chrysosperma*, *notastroma*, or *nivea* and were likely an undescribed species (Table 1). We isolated more than one *Cytospora* species from a quarter of the trees sampled (18 of 71) from each of the three sampling regions of Colorado, Wyoming, and Utah that were tested most intensively. Six trees featured *C. chrysosperma* and *C. notastroma*, and four trees had *C. chrysosperma* and *C. nivea*. The remaining eight trees were infected with one of the three identified species, plus an unidentified species of *Cytospora*.

3.1.1. *Cytospora* species confirmation based on phylogenetics

To validate our morphological identifications, the ITS and *BT* sequences of 67 and 45 isolates, respectively, were compared with each other and with NCBI sequences for each of the three species included in this study (Figs. 3 and 4). There was strong bootstrap (BS) (100%) support for the differentiation among *C. chrysosperma*, *C. notastroma* and *C. nivea* isolates based on ITS sequences (Fig. 3). Analysis of the *BT* also supported separation among the three species (Fig. 4). This sequence data concurred with our species confirmations based on morphological features, with the exception that most of the isolates previously thought to be either hybrids or an undescribed *Cytospora* species were in fact *C. nivea*.

Bayesian (BI) and maximum likelihood (ML) analyses of 67 of the combined ITS-*BT* sequences indicated strong (0.99–1.0 posterior probability, PP) and 90–91% bootstrap (BS) support for the differentiation among each of the three *Cytospora* species and was suggestive of intra-specific clades within each species (Fig. 5). The ML-based ITS phylogeny supported only two broad groups of isolates; the first representing a portion (38) of the *C. chrysosperma* isolates, and the second representing all *C. notastroma*, *C. nivea*, and the remaining portion (45) of the *C. chrysosperma* isolates. A BI phylogeny based on the 45 *BT* sequences also supported three distinct species groups for *C. notastroma*, *C. nivea* and *C. chrysosperma* (Fig. 4). Bootstrap values of a ML phylogeny did not support any distinction among the three species (data not shown). The BI phylogeny of the concatenated sequences of 20 haplogroups found three well-supported clades within *C. notastroma*, two within *C. chrysosperma*, and two within *C. nivea* (Fig. 5). However, PP values were not sufficiently high (51%) to parse *C. chrysosperma* from *C. cyperi* (NCBI).

3.1.2. *Cytospora* species confirmation: nucleotide diversity analyses

A partition homogeneity test implemented in PAUP*4a146 to test for congruence between the two loci confirmed incongruence, and therefore phylogenies were generated for each locus separately, as well as for the combined dataset. Tests of haplotype diversity indicated high levels of diversity for each of the species tested (Table 2). Nucleotide diversity was greatest for the *BT*, especially within the *C. notastroma* isolates. Neutrality tests indicated that the loci are under neutral selection and are therefore appropriate for phylogenetic analyses (Table 2). Linkage disequilibrium tests were not significant (Table 3), and recombination occurred at both loci, but was greater for *BT* (Table 3).

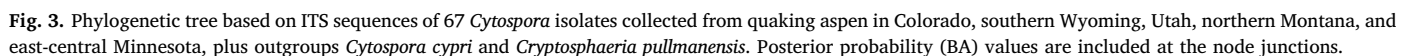
3.1.3. Inoculation study: growth chamber inoculation trials

Drought-stressed trees averaged -1.0 and -0.9 MPa in the warm and cool temperature growth chambers respectively (data not shown). Watered trees averaged -0.7 and -0.45 MPa in the warm and cool growth chambers (data not shown). Least-squares means were obtained and were significantly different at $P \leq 0.05$. We obtained 95% confidence intervals for canker size for each isolate-temperature combination, and intervals for each combination were considered significant at $P \leq 0.05$. Both *Cytospora* species caused canker formation on trees in the warm temperature trial, relative to the control wound (Table 4). Among the drought-stressed saplings inoculated with a *Cytospora* isolate, cankers resulting from *C. chrysosperma* were larger in comparison with the control and the *C. notastroma* inoculated trees (Table 4). Wound sites amended with $\frac{1}{2}$ PDA developed small areas of bark discoloration but did not develop cankers. Such discoloration was distinguishable from cankers based on tissue color and the shape of the margin of the discolored area and was due to drying of the bark. However, not all wound sites inoculated with the two *Cytospora* species developed cankers; the inclusion of the non-cankered wounds in the analysis strongly influenced mean canker size as illustrated in Table 5. For example, of the 16 drought-stressed trees (and 48 wound sites) inoculated and placed in the cool temperature growth chamber, 21 sites inoculated with a *C. chrysosperma* isolate ('DG11A') formed cankers, compared with 16 sites inoculated with a *C. notastroma* isolate ('RCKEP3A') (Table 4). Mean canker size formed by *C. chrysosperma* among all drought-stressed trees used in the growth chamber trials was 64 mm^2 and was significantly larger than cankers formed by the *C. notastroma* isolate (54 mm^2) (Table 4). Regardless of experimental setting, both isolates of *C. chrysosperma* caused canker formation more often than either *C. notastroma* isolate (Table 5). Isolates of both *Cytospora* species were recovered from their respective inoculum sites. Watered trees inoculated with one isolate each of *C. chrysosperma* and *C. notastroma* species did not develop cankers in the low temperature treatment (Table 4).

3.1.4. Inoculation study: greenhouse inoculation trials

Water potential data indicated that drought-stressed trees in the greenhouse averaged -1.11 MPa (data not shown). Watered trees in the greenhouse averaged -0.7 MPa. Over time, trees reached a maximum water potential deficit of -1.55 MPa twelve days into the experiment (data not shown).

The largest cankers formed on drought-stressed trees at sites inoculated with *C. chrysosperma* isolate DG11A (Table 4). However, there was no difference in canker area between the two *C. chrysosperma* isolates (Table 4). When considering only sites where cankers were observed, both *C. chrysosperma* isolates and *C. notastroma* SW8C formed larger cankers than areas of discoloration observed at wound sites in the non-inoculated sites (Table 4). The range in canker sizes among infected aspen (i.e. those trees on which cankers formed) in the greenhouse trial was $38\text{--}6927 \text{ mm}^2$ for the *C. chrysosperma* isolates, and $38\text{--}6024 \text{ mm}^2$ for the *C. notastroma* isolates. Four of the drought-stressed aspen trees did not develop any cankers at all. Wound sites that were not inoculated with a *Cytospora* isolate developed small areas of bark discoloration approximately two weeks after inoculation, but did not develop cankers, except for sites on two trees, which we determined were previously infected with *C. chrysosperma*. Areas of discoloration due to drying of the bark were distinguishable from discolored areas caused by canker formation based on bark tissue color and the shape of the discolored area. None of the inoculation sites on watered trees formed cankers larger than the control, and even sites inoculated with *C. notastroma* isolate RCKEP3A on drought-stressed trees did not produce cankers larger than the watered controls (Table 4).



Adams et al., 2006; Hubbes, 1960) have observed, the morphological features of *Cytospora* are highly variable, and this is true of fruiting body formation on a host, as well as cultured isolates (Spielman, 1985). Spielman (1985) noted that because of this degree of variability, additional species have been described (incorrectly) based on specific isolates over the years, further complicating the species identification of this genus. We also observed considerable variability in the morphology of fruiting bodies we sampled in this study (Fig. 2). Specifically, the dark stromatal tissue layer attributed to *C. notastroma* was not always clearly visible. Kepley et al. (2015) note that *C. translucens* also sometimes produces a dark conceptacle-like ring surrounding the pycnidium. It seems possible that some of the variation in fruiting body morphology may be due to phenotypic differences among individual host trees. Finally, some differences in pycnidia formation observed in this study may be attributed to growth phase of the fungus; if the dark stromatal layer of tissue that is characteristic of *C. notastroma* only forms at

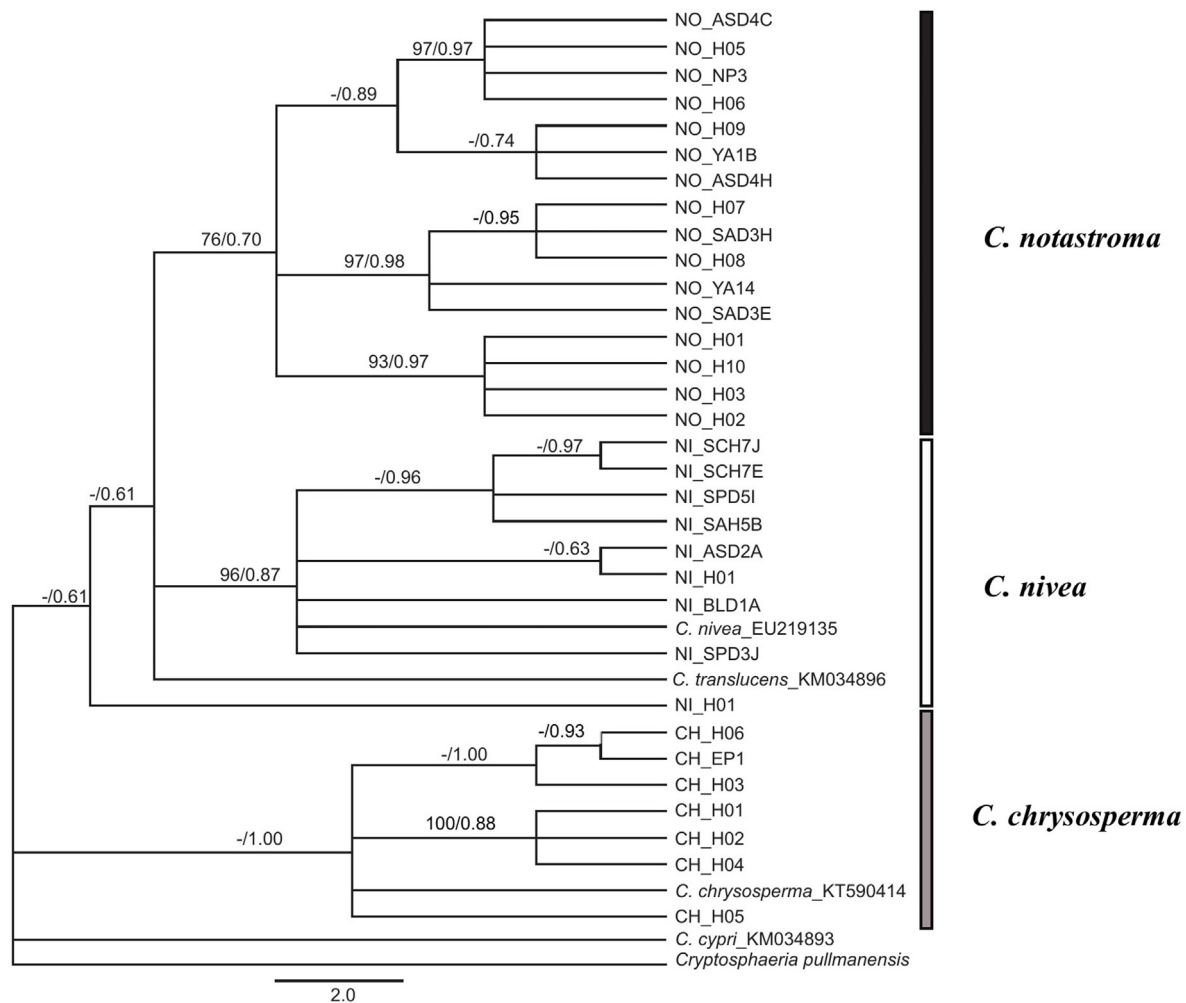


Fig. 4. Phylogenetic tree based on *BT* sequences of 45 *Cytospora* isolates collected from quaking aspen in Colorado, southern Wyoming, Utah, northern Montana, and east-central Minnesota, plus outgroups *Cytospora cypri* and *Cryptosphaeria pullmanensis*. Posterior probability (BA) values are included at the node junctions.

maturation, then perhaps some of the pycnidia later attributed to *C. notastroma* but lacking the dark basal tissue disk simply had not yet matured.

Despite *C. notastroma* being a weaker pathogen, we observed similar frequencies of *C. notastroma* and *C. chrysosperma* (Table 1). We did not discriminate among tree size classes when we sampled infected aspen for *Cytospora* canker, and so we cannot make any inferences as to whether one species is found more frequently on a specific tree size than another. However, further study could investigate whether the two species fill different ecological niches in aspen stands; does one species occur more frequently on shade-weakened understory trees than the other, and are lethal cankers more likely to have been caused by one species over another?

We did not detect differences in haplogroup composition by geographic region. This was somewhat surprising but could be explained by one or two factors. First, only two loci were used in this study, and thus we cannot make statements about intra-species populations. Additional data would certainly help to clarify relationships within species groups. Perhaps the selection of less conservative loci (i.e. greater variability, more SNPs) than the two used herein would allow for the identification of geographic differences. A second (and less likely) possibility explaining why we did not detect geographic differences in haplogroup composition is that there could be more movement (and therefore admixture) of *Cytospora* inoculum over greater distances than previously thought. Kaczynski et al. (2014) examined sapsuckers (*Sphyrapicus nuchalis* Baird) as a possible vector of *Cytospora*

chrysosperma, which was implicated in years-long decline of riparian willow (*Salix* spp.) in Rocky Mountain National Park, Colorado. Roughly one-third of the birds sampled carried *Cytospora* inoculum on either their beaks or feet, or both, and thus sapsuckers were confirmed as a means of pathogen spread from one host to another (Kaczynski et al. 2014). Based on these findings, it may be possible that birds, flying insects, or other vectors are a potential means of pathogen spread.

We observed evidence that could indicate hybridization among the three *Cytospora* species examined in this study. Our results show that recombination readily occurs at both ITS and *BT* loci for all three species. At least ten isolates recovered from infected aspen could not be identified to the species level. In a previous phylogenetic (BA) analysis, three of these ten isolates were alternately grouped with *C. nivea*, *C. notastroma*, or *C. chrysosperma*, depending on the locus. Kepley et al. (2015) observed that ‘intermediate isolates’ existed which could not be distinguished, based upon ITS and Elongation factor 1- α (*EF1 α*) sequences, between *C. nivea* and *C. translucens* (which has also been reported on quaking aspen). Fully 25% of the trees we sampled were infected with more than one *Cytospora* species, and this number could in fact be much higher. Thus, it is possible that observed cankers could be a patchwork of different causal species.

4.2. Inoculation study

The results of the inoculation trials conducted in this study strongly

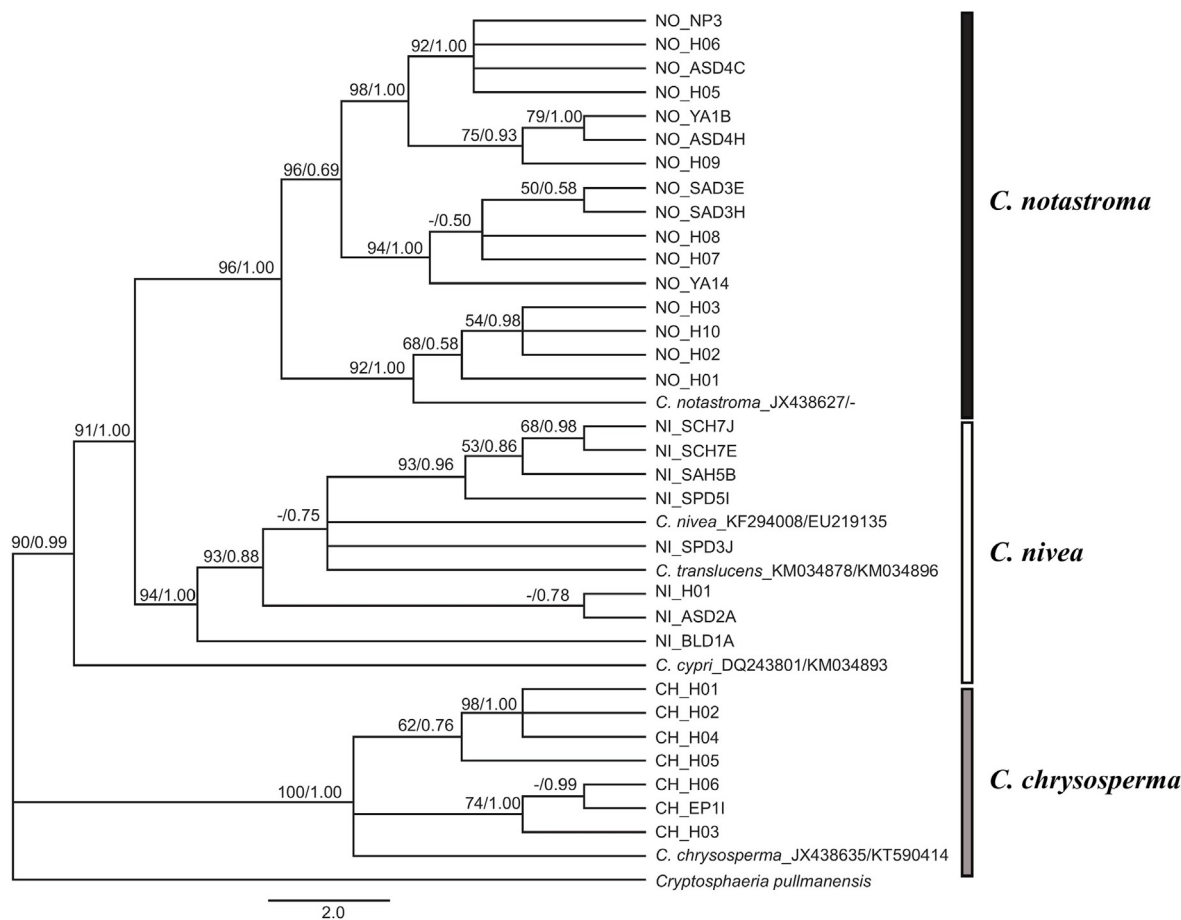


Fig. 5. Phylogenetic tree assembled using posterior probability analysis, representing the concatenated ITS-BT sequences of 34 *Cytospora* isolates collected from quaking aspen in Colorado, southern Wyoming, Utah, northern Montana, and east-central Minnesota, plus an outgroup (*Cryptosphaeria pullmanensis*).

indicate that although *Cytospora notastroma* is pathogenic on quaking aspen, *C. chrysosperma* is the more aggressive species, particularly among trees experiencing some degree of drought stress. Cankers caused by *C. chrysosperma* developed at a faster rate and were larger overall than either isolate of *C. notastroma*. We observed (as have [Kepley et al. 2015](#)) that the two species are often found infecting the same host, sometimes directly adjacent to each other. We speculate that *C. chrysosperma* may further weaken stressed host trees, perhaps making colonization by *C. notastroma* easier. Further, in the greenhouse portion of the study, we observed that *C. notastroma* cankers did not dramatically expand until the conclusion of the trial, when host trees were near death, and *C. chrysosperma* cankers had already thoroughly

colonized the bark tissue.

[Guyon et al. \(1996\)](#) noted that the peak susceptibility of aspen and cottonwood trees to canker formation by *Cytospora* was at -1.6 MPa. The water potential values in this study were largely lower than this amount, and therefore it is possible that greater drought stress could have resulted in greater canker growth of either or both species. We note that *C. chrysosperma* isolates formed cankers that grew substantially, even when water potential values were less severe (e.g., -0.9 MPa) (data not shown).

When we compared the effects of temperature on canker development, we observed significantly less canker growth of *C. chrysosperma* at high temperatures (compared to low temperatures), based on

Table 2
Haplotype and nucleotide diversity measures, with tests for neutrality of individual and combined species of *Cytospora* using two genetic markers and the concatenated sequence.

Marker	Species	Concatenated				ITS				B-tubulin			
		NO*	CH*	NI*	All	NO	CH	NI	All	NO	CH	NI	All
Diversity [†]	h _D	0.967	1.000	0.964	0.991	0.920	0.783	0.917	0.925	0.932	0.879	0.944	0.963
	nucl	0.019	0.019	0.015	0.088	0.006	0.008	0.009	0.041	0.043	0.031	0.023	0.117
Fu_Li [†]	F*	1.21	0.91	-0.03	-2.69	1.45	1.81	-0.24	2.01	-0.67	1.33	-0.58	-0.52
	F	1.25	1.42	0.05	-3.11	1.43	1.67	0.23	2.64	-0.30	2.31	-0.22	0.31
Tajima [†]	D	1.18	1.19	0.19	-1.64	0.87	1.88	0.09	1.57	-0.10	1.68	-0.34	0.26

* NO: *Cytospora notastroma*.
* CH: *Cytospora chrysosperma*.
* NI: *Cytospora nivea*.
† Not significant at P = 0.10.

Table 3

Test results for evidence of recombination events, gene flow, and linkage disequilibrium based on ITS, beta-tubulin or the combined (concatenated) ITS-BT sequences, by *Cytospora* species, or averaged over all isolates.

	Marker	Concatenated				ITS				BT			
		NO*	CH*	NI*	All	NO	CH	NI	All	NO	CH	NI	All
Gene Flow (Nei) [†]	Gst				0.0				0.1				0.1
	Nm				18.5				4.1				10.4
Recomb., by sp. [†]	Sk ²	78.9	104.0	48.9		3.8	7.9	5.0		81.2	47.1	21.8	
	Avg dist.	779.4	782.1	770.4		437.0	439.0	432.7		341.6	342.0	337.0	
	Rm	3	1	2		3	0	1		4	2	0	
Recomb., across sp. [†]	Sk ²				2564				118				437
	Avg dist.				778				437				340
	Rm				31				12				21
Linkage Disequil. [†]	ZnS				0.38								
	Za				0.64								
	ZZ				0.26								
	Wall's B				0.59								
	Wall's Q				0.60								

* NO: *Cytospora notastroma*.

* CH: *Cytospora chrysosperma*.

* NI: *Cytospora nivea*.

† Not significant at P = 0.10.

Table 4

Mean canker area for those sites where cankers formed 12–14 days following inoculation with either *C. chrysosperma* or *C. notastroma* in a cool or warm temperature growth chamber, a greenhouse, and an outdoor setting.

Species	Isolate	Treatment	Canker area (mm ²)		
			Growth chamber, Cool Temp ^{1,2} (n = 48)	Growth chamber, Warm Temp ^{1,2} (n = 48)	Greenhouse Trial ^{2,3} (n = 160)
<i>C. chrysosperma</i>	DG11A	Drought	27 (15, 10) ^{cd}	27 (18, 11) ^{bd}	333 (94, 73) ^c
		Watered	0 [‡]	7.4 [‡]	70 (38, 24) ^{ab}
	BDSR12	Drought	– [†]	–	223 (63, 49) ^c
		Watered	–	–	76 (41, 26) ^{ab}
<i>C. notastroma</i>	RCKEP3A	Drought	12 (7, 4) ^{abc}	56 (6, 7) ^b	79 (22, 17) ^a
		Watered	25 [‡]	– [*]	49 (26, 17) ^a
	SW8C	Drought	–	–	126 (35, 28) ^b
		Watered	–	–	58 (31, 20) ^a
Control	Agar	Drought	11 (6, 4) ^b	7 (5, 3) ^a	77 (22, 17) ^a
		Watered	44 [‡]	0 [‡]	53 (28, 18) ^a

¹ Letters indicate significant difference at the P = 0.05 level; means with the same letter are not significantly different from each other; comparisons are among all isolates in both warm and cool trials.

² Numbers in parentheses are upper and lower confidence interval values, from the mean.

³ 'n' is the total number of inoculation sites for each portion of the study.

* Watered trees in the growth chamber trials do not include a confidence interval because there was only one watered tree per temperature trial.

† Only one isolate per species was used in the growth chamber portion of the study.

* Inoculation site was contaminated with another fungal species, and thus not included.

Table 5

A summary of canker formation caused by two isolates each of *Cytospora chrysosperma* and *C. notastroma* two weeks post-inoculation. Sites were considered to have cankers if the discolored area around the inoculation site of each isolate was greater than the discolored area surrounding the site amended with ½ PDA on each tree.

Species	Isolate	Percent of inoculation sites developing cankers*	n	Mean and range () of canker area(mm ²) [‡]
<i>C. chrysosperma</i>	DG11-A [†]	61%	98	64 (50–77); 173 (38–6927)
	BDSR1-2 [‡]	50%	64	135 (38–5342)
<i>C. notastroma</i>	RCKEP3A [†]	41%	98	54 (50–56); 80 (38–377)
	SW8C [‡]	41%	64	294 (38–6024)

* Three (growth chamber trials) or five (greenhouse trial) inoculation sites per tree, depending on trial environment and tree size.

† Isolates DG11-A and RCKEP3A were used in all trials. Means and ranges of isolates DG11A and RCKEP3A are listed as the combined mean of four growth chamber trials (first group) and the combined mean of the greenhouse and outdoor trials (second group).

‡ Isolates BDSR1-2 and SW8C were used only in the greenhouse inoculation trial.

* Canker means for the greenhouse and outdoor trials are based on measurements taken 14 days after inoculation. 'n' is the number of inoculation sites.

infected aspen tissue (i.e. those trees on which cankers formed). Interestingly, we observed no effect of temperature on the growth of cankers produced by *C. notastroma*. This was contrary to our initial hypothesis, in which we predicted that drought stress combined with high temperatures would result in larger and more rapid canker expansion. Helton and Konicek (1962) observed that temperatures for optimal culture growth ranged from 25 to 35 °C for two purported *Cytospora* species, *C. cincta* and *C. leucostoma*. Additionally, studies of other phytopathogenic fungal species, including *Geosmithia morbida* and the bluestain fungus *Ophiostoma clavigera*, have demonstrated that these organisms cause more extensive disease development under cooler (i.e. 25 °C and below) conditions (Freeland et al., 2011; Solheim and Krokene, 1998). At least two *Cytospora* species occurring on stone fruit trees have been shown to have differential responses to temperature (Hildebrand, 1947; Wensley, 1964; Bertrand and English, 1976). Studies by Hildebrand (1947), Wensley (1964), and Bertrand and English (1976) demonstrated that *C. leucostoma* was more pathogenic (i.e. caused larger cankers) on orchard trees under warm temperatures than *C. cincta*; *C. cincta* was more pathogenic than *C. leucostoma* on trees under lower temperatures.

Our results indicate that *Cytospora chrysosperma* can induce canker formation on drought-stressed host trees under either warm or cool conditions (although it was less aggressive under warm conditions). This tolerance of a rather broad temperature range may be one reason why *C. chrysosperma* is such an effective pathogen of stressed host trees. Earlier studies of temperature and *Cytospora* canker growth on other host species were based on seasonal observations, with *C. leucostoma* isolates producing large, or rapidly-expanding cankers during the warmest months of the year, whereas *C. cincta* produced cankers only during the late-autumn or spring (Hildebrand, 1947; Wensley, 1964; Bertrand and English, 1976). Thus, *C. chrysosperma* may be somewhat unique in that it can cause disease on aspen throughout the growing season and perhaps during the dormant season as well.

We observed, especially in the greenhouse portion of this study, that some of the aspens did not develop cankers at all, despite leaf water potential measurements that indicated drought stress. It has been established that significant phenotypic differences exist among aspen clones in resistance to herbivory (Stevens et al., 2007; Lindroth and St Clair, 2013) as well as disease resistance (Copony and Barnes, 1974; Holeski et al., 2009). Quaking aspen genets often display stark phenotypic differences in morphology; clonal variation in drought and disease resistance have been and continue to be intensively studied (e.g. Griffin et al., 1991; DeWoody et al., 2009; St. Clair et al., 2010; Long and Mock, 2012; Callahan et al., 2013). Also noted in many previous studies of aspen and other *Populus* species (St. Clair et al., 2010; Kanaga et al., 2008; Marron and Ceulemans, 2006), phenotypic traits are often influenced by environmental conditions: for example, Kanaga et al. (2008) and Marron and Ceulemans (2006) describe the interaction between environment and phenotype as a cause of significant phenotypic plasticity. Based on this evidence, it is likely that at least some of the variability in canker development we observed over the course of these experiments may be phenotypic disease resistance among aspen clones. We would like to emphasize that this study examined only the variation in disease development from the perspective of the pathogen; an examination of disease resistance on the part of the host will further clarify the precise cause of variation in disease development from one tree to another, and one clone to another.

5. Conclusions

We have shown that *Cytospora notastroma* is pathogenic on drought-stressed aspen trees, although it appears to be a weaker pathogen (at least under the conditions we tested) than *C. chrysosperma*. These species routinely co-occur on a single tree, and we observed that cankers caused by *C. notastroma* expanded rapidly only on trees already colonized by *C. chrysosperma*. As Kepley (2009) and Kepley et al. (2015)

have previously described, *Cytospora* cankers are often caused by multiple *Cytospora* species. We detected multiple *Cytospora* species on a single host in 25% of the trees we sampled. Whether *Cytospora chrysosperma*, *C. notastroma*, and others hybridize with each other seems likely, but cannot be conclusively determined here. Given the ubiquitous nature of the pathogen, and the ongoing droughts throughout the western US, *Cytospora* canker-caused dieback is likely to continue among drought-stressed aspen stands.

CRedit authorship contribution statement

M.M. Dudley: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **N.A. Tisserat:** Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration. **W.R. Jacobi:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision. **J. Negrón:** Validation, Resources, Writing - review & editing. **J.E. Stewart:** Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing - review & editing, Visualization.

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Appendix A. Supplementary material

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