DISSERTATION

FACTORS INFLUENCING THE HEALTH OF QUAKING ASPEN (*POPULUS TREMULOIDES* MICHX.)

Submitted by

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ABSTRACT

FACTORS INFLUENCING THE HEALTH OF QUAKING ASPEN (*POPULUS TREMULOIDES* MICHX.)

In **Chapter 1** of this dissertation, we analyzed a series of increment cores collected from 260 adult dominant or co-dominant quaking aspen (*Populus tremuloides* Michx.) trees from national forests across Colorado and southern Wyoming in 2009 and 2010. Half of the cores were collected from trees in stands with a high amount of crown dieback, and half from lightly damaged stands. We define the level of stand damage based on stand survey data, in which lightly damaged stands had average crown dieback of 16%, and heavily damaged stands averaged 41%. Upon analysis, two-thirds of the cores collected did not exhibit radial growth correlated with region-wide patterns (e.g. climate) and were classified as having a low cohesive response (LCR). The site variable most predictive of whether a stand exhibited high cohesive response (HCR) or low cohesive response was site elevation, followed by aspect, slope, and canopy closure. Sites with HCR stands were more likely to have aspen bark beetle damage, white rot, and Cryptosphaeria canker. We did not detect relationships between tree growth and summer precipitation from 1900-2008, but there was a relationship between growth and annual precipitation. A growth model included maximum May and July temperatures, as well as the current and previous year's annual precipitation.

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 has recently been described (tentatively named *Cytospora notastroma*). In **Chapter 2** of

this dissertation, we show the relative pathogenicity of both species. Small-diameter aspen trees were inoculated with one or two isolates each of *C. chrysosperma* and *C. notastroma* in a greenhouse, outdoor setting, and in environmental growth chambers. Results indicate that both species are pathogenic to drought-stressed trees and that *C. chrysosperma* was more aggressive than *C. notastroma* at both warm and cool temperatures. Neither species caused significant canker growth on trees that were not drought stressed.

In **Chapter 3**, we investigated the abundance and frequency of *C. notastroma*, relative to *C. chrysosperma*. We wished to estimate the relative abundance of known *Cytospora* species on quaking aspen throughout portions of the Rocky Mountain region, and to construct species-level phylogenies based upon isolates obtained from infected aspen. We report that both *C. chrysosperma* and *C. notastroma* are quite common on quaking aspen, although we recovered C. chrysosperma slightly more often (48% of sequenced cultures) than C. notastroma (42 % of sequenced cultures). We also recovered a third, previously-described species, *C. nivea* in 9% of sequenced cultures. We also found that *Cytospora* species often co-occur on the same host tree (25% of trees sampled), and that evidence of recombination or possible hybridization between the species exists.

The aspen bark beetle, *Trypophloeus populi*, is known as a stress-related damage agent on quaking aspen. In a previous study, we often found *T. populi* attacking host trees also infected with Cytospora canker. In **Chapter 4** of this dissertation, we wished to determine whether *T. populi* is a potential vector of Cytospora canker, and whether *Cytospora* inoculum could be recovered from adult beetles or gallery tissues. We did not recover any *Cytospora* isolates from 161 adult *T. populi* beetles cultured, and only two *Cytospora* isolates from 42 beetle galleries and

seven adult aspen. We suspect that these isolates, cultured from two trees, were a result of a previous infection, as both host trees had extensive cankers as well as *Cytospora* fruiting bodies.

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CHAPTER 1

INFLUENCE OF CLIMATE ON THE GROWTH OF QUAKING ASPEN (*POPULUS TREMULOIDES*) IN COLORADO AND SOUTHERN WYOMING

SUMMARY

We analyzed a series of increment cores collected from 260 adult dominant or co-dominant quaking aspen (*Populus tremuloides* Michx.) trees from national forests across Colorado and southern Wyoming in 2009 and 2010. Half of the cores were collected from trees in stands with a high amount of crown dieback, and half from lightly damaged stands. We define the level of stand damage based on stand survey data, in which lightly damaged stands had average crown dieback of 16%, and heavily damaged stands averaged 41%. Upon analysis, two-thirds of the cores collected did not exhibit radial growth correlated with region-wide patterns (e.g. climate) and were classified as having a low cohesive response (LCR). The site variable most predictive of whether a stand exhibited high cohesive response (HCR) or low cohesive response was site elevation, followed by aspect, slope, and canopy closure. Sites with HCR stands were more likely to have aspen bark beetle damage, white rot, and Cryptosphaeria canker. We did not detect relationships between tree growth and summer precipitation from 1900-2008, but there was a relationship between growth and annual precipitation.

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INTRODUCTION

Quaking aspen (*Populus tremuloides*) is the primary pioneer tree species and one of a few hardwood tree species found in forests throughout the southern Rocky Mountain region (Mueggler 1985). Following a disturbance event, such as a stand-replacing fire, aspen colonize the area, either by seed or through sprouting from existing roots (Mueggler 1985; Barnes 1966). The success of this early-seral species is favored by widespread disturbance events, which often serve to reduce competing populations of late-seral conifer species (e.g. Kulakowski et al. 2013; Lankia et al. 2012; Krasnow and Stephens, 2015). Aspen produces high numbers of suckers following stand-replacing fire or other disturbances (Scheier, and Campbell, 1978; Perala, 1995; Romme et al. 1995), with sucker densities greatest following a complete removal of the overstory. However, regeneration still occurs (though at lower densities) as gaps in the canopy are produced (Shepperd, 1993; Shepperd & Smith, 1993; Shepperd et al. 2001). Studies indicate that, like most other tree species, growth rates of aspen are a function of climatic factors combined with various site and soil characteristics (Hogg, et al, 2008, 2013). The impacts of drought on *P. tremuloides* includes a decrease in leaf size, leaf area index (LAI), and alteration of root water flow properties (Greitner et al. 1994; Siemens and Zwiazek 2003; Krishnan et al. 2006). Severely-stressed individuals display an inhibition of root hydraulic conductivity, as a result of an increase in the ratio of apoplastic to cell-to-cell water transport (Siemens and Zwiazek, 2003). Anderegg et al. (2013) showed that aspen that have undergone drought stress and air embolism are more prone to cavitation during subsequent drought episodes.

The traditional view of aspen regeneration is that seeding events are rare, and that therefore, the majority of saplings were assumed to be vegetative ‘suckers’. This was mainly due to the physiology of aspen seed, and the specific conditions required for successful germination

and survival of young seedlings. McDonough (1979) documented the survival of aspen seedlings grown under a variety of temperature and moisture conditions. He concluded that in addition to requiring exposed mineral soil, aspen seedlings needed ample moisture (i.e. soil water potentials greater than -2.3 atm) for the first few weeks of growth in order for even a small percentage (i.e. < 10%) of the seedlings to survive (McDonough, 1979). Long and Mock (2012) note that there are numerous of examples of aspen regenerating by seed following a major disturbance event, such as the widespread and severe fires in Yellowstone National Park (documented by Kay, 1993), and by genotypic evidence. It is also becoming apparent that the successional niche aspen has been placed in, as a pioneer species which cannot persist among conifers, may be inaccurate. Small stands of aspen may behave in a 'gap-phase' manner, and persist within conifer-dominated stands (Long and Mock, 2012, with unpublished data by Shaw, 2009). Climate change presents a challenge to forest managers, and actions should be taken which aid a forest's resilience to environmental change (Millar, et al, 2007).

Forest health researchers have documented stand mortality in southern Utah and Idaho (Stam et al. 2008; Guyon and Hoffman 2011), Arizona (Fairweather et al. 2008, Zegler et al. 2012), the Pacific Northwest (Flowers and Kohler 2011), the Carson National Forest in New Mexico (J. Jacobs, personal communication, 2015) and the boreal aspen forests of Alberta and Saskatchewan (Brandt et al. 2003; Hogg et al. 2002 & 2008). Worrall et al. (2008) first observed rapid overstory dieback in Colorado during 2005, and coined the term 'sudden aspen decline' (SAD), based on the observation that stands with dying overstory lacked a significant regeneration response (Worrall et al. 2008). Based upon aspen health data from an extensive Colorado and southern Wyoming study (Dudley et al. 2015), we concluded that acute drought was most likely the inciting factor which caused a marked increase in select secondary damage

agents and overstory mortality. In that study, we found that heavily damaged stands with over 38% over story mortality were consistently warmer and drier during the period directly preceding and during the episode of dieback, 2000-2006, than lightly damaged stands (Dudley et al. 2015). Trees growing on sites with sub-optimal growing conditions, such as those with shallow soils and drier sites, tend to be more responsive to climatic events than trees growing on more favorable sites (Fritts, 1976; Stokes and Smiley 1968). Chronologies from tree species closely related to quaking aspen (e.g. plains cottonwood) and from species with similar wood structure (e.g. *Betula* species) have demonstrated that these species respond to climatic events with increased or decreased radial growth (Edmondson et al. 2014; Levanic and Eggertsson 2008). Further, previous studies on the impact of various environmental, site, and biotic agents on aspen growth indicate that drought, frost, defoliation (by forest tent caterpillar, *Malacosoma disstria* Hübner) and poor site conditions all have a negative influence on annual growth (Hogg et al. 2002 & 2008; Strain, 1966; Cooke and Roland, 2008; Ireland et al. 2014; Leonelli et al. 2008).

To date, there are no chronologies available for *Populus tremuloides* from the International Tree Ring Data Bank (NOAA, ITRDB), but several studies have utilized aspen increment cores and cross-sections to age stands (Elliott and Baker, 2004) and to produce regional chronologies (Hogg and Schwarz, 1999; Hogg et al. 2005). Quaking aspen are often difficult to crossdate, due in part to the wood structure (diffuse-porous), which can make ring boundaries difficult to determine, as well as the formation of false rings, or complete lack of ring formation during some years (Speer 2010; P.M. Brown, personal communication, 2011). In this study, we examined a set of tree cores collected from 97 aspen stands throughout the mountainous regions of Colorado and southern Wyoming.

The main questions we wished to answer included: (1) Are some aspen stands predisposed by site, stand, or geographic conditions to produce a highly cohesive radial growth pattern in response to changes in precipitation or temperature? (2) Are there differences in drought impacts on radial growth by region? (3) Do any variables have a larger impact on increment growth than others? (4) Can inferences be made about current and future aspen health (i.e. presence of various diseases and insects) from radial growth and site characteristics?

MATERIALS AND METHODS

Study Area

In 2009 and 2010, we established 97 aspen health survey plots on five national forests to assess the impact of current and future disturbances by diseases, insects and climate (Figure 1.1; Table 1.S2). Approximately half of our survey plots were established in aspen stands classified as heavily damaged and half in lightly damaged stands (though not as a paired-plot design), based upon 2008-2009 U.S.F.S. aerial survey data. Aerial surveyors annually map four categories of aspen stand damage or dieback. These include: (1) aspen stands currently undergoing an apparent defoliation or foliage discoloration event; (2) stands with thinning crowns on at least 25% of adult aspen; (3) stands with moderate (< 50% of stems) levels of overstory mortality; or (4) stands with high (> 50% of stems) levels of overstory mortality (Krist, 2005). We combined damage categories one and two into a 'lightly damaged' group, and categories three and four into a single category for placement of plots in heavily damaged stands. We later verified that heavily damaged stands had much higher rates of overstory dieback than lightly damaged stands (38% and 14%, respectively) (Dudley et al. 2015).

Plot selection

Ranger districts were sampling units within each national forest, with one to four districts sampled per Forest. Districts were selected for sampling if they contained large areas of aspen-dominated forests, as determined by examination of forest type data (based upon a remotely-sensed vegetation data layer, http://ndis.nrel.colostate.edu/coveg/). All spatial data processing and extraction was performed using ArcGIS® 10.0 desktop software (ESRI, Redlands, CA USA). Potential survey points in lightly and heavily damaged stands were generated using the 'Create Random Points' tool in ArcToolbox. Point locations greater than 1 km from a road were eliminated from consideration. Further, final plot locations were chosen from the remaining points to represent a wide range of aspen stands and elevations. Survey points were uploaded to handheld GPS units (Garmin *eTrex® Legend*, Garmin International, Ltd., Olathe, KS, USA). Potential survey stands were to consist of at least 50% aspen stems, and be at least 120 x 20 m in size, or another potential site was located. Each plot consisted of a 100 meter-long transect, which was established starting at the randomly-generated GPS point, and oriented in such a way roughly bisected the stand of interest. Three circular, fixed-area (201 m^2) subplots with an 8 m radius were established along the 100 m transect by selecting three numeric locations from a list of randomly-generated numbers. One adult dominant or co-dominant tree was cored in each subplot, for a maximum of 3 increment cores per transect.

Site and stand data

Stand-level data recorded during core collection included: aspect, percent slope, elevation, stand structure, and percent live stems. In each subplot, the first ten adult aspen (≥ 12 cm DBH) were assessed for percent crown dieback and disease and damage agents. We used morphological characteristics, such as the type of fruiting body and pattern of cankers on the stem to identify specific canker diseases, and morphology and placement of conks to identify

decay fungi. Likewise, we examined trees for general signs of wood borer attack (*Saperda calcarata* or *Agrilus liragus*), which included tunneling, exit holes, and brown stained bark. However, damage was not attributed to either species specifically. Presence of aspen bark beetles (*Trypophloeus populi* or *Procryphalus mucronatus*) was determined based on the presence of small (< 1 mm) exit holes on the bark, cracked bark over galleries, or both.

Increment core sampling and preparation

One adult dominant or co-dominant tree (≥ 12.0 cm) was cored using a 5-mm increment borer at breast height (1.4 m from the base of the tree) in each sub-plot for a total of 260 increment cores. Trees selected for sampling were living, and either healthy or with mild to moderate evidence of disease or damage, but did not show signs or symptoms of white rot fungus (*Phellinus tremulae*), as cores from rotten trees would be unreadable. Some sub-plots (31) did not contain any adult aspen, and thus cores were not collected from these locations. Increment cores collected within the same national forest (and later grouped together as a chronology) were no more than 100 miles apart, and most were within 40 miles of each other. Although cored trees occurred on sites with varying elevation, aspect, and slope steepness, a previous analysis of aspen health, size, and mortality did not reveal major differences among these classifications, and thus we did not stratify our samples by elevation or aspect (Dudley et al. 2015). Cores were air-dried for one week before they were glued to wooden mounting blocks with the vascular tissue vertical. Cores were sanded progressively with 150 or 220 and then 400 and 600-grit sandpaper to produce a smooth surface with cell and fiber structures clearly visible (Speer, 2010). A few cores were broken, discolored, or otherwise unreadable and were omitted from measurement and analysis.

Skeleton plot construction

Cross-dating using the skeleton plot technique (Stokes and Smiley, 1968; Swetnam, 1985), a graphical technique for comparing ring-width, was used to establish an accurate tree age. A master skeleton plot (tree ring chronology) representative of all cores within a sampling area (ranger district) was then constructed, which contained a series of reference years. This allowed the identification of missing rings and elimination of false rings (Stokes and Smiley, 1968).

Annual ring measurement

Annual rings widths were measured using an increnometer (Velmex, Inc, Bloomfield, NY, USA), a digital counter, and associated software program (MEASURE J2X©, VoorTech Consulting, Holderness, NH, USA). The raw data was then checked and statistically crossdated using the software program COFECHA (Holmes, 1983; Grissino-Mayer, 2001), to first determine the correlation between three cores (series) collected within the same transect. These triplicates were then compared to others collected within the same ranger district. All raw core data were standardized using a segment length of 30-years, with 15 years overlap between increments. Core series with correlations of less than 0.42 were examined and corrected (using the software program EDRM (Grissino-Mayer, 2001)), or were placed in the low cohesive group (LCR) and were excluded from further climate-growth analysis if their correlations remained < 0.42, or if they were responding to some growth driver (e.g. possible insect outbreaks or successional processes) other than climate. We are using the terms 'low cohesive response' and 'high cohesive response' to describe groups of trees which respond together in a similar manner to climatic influences. We also examined each series' mean sensitivity rating (ms_x) (Speer, 2010), though this measure was not used in this study to divide series into high cohesive groups (HCR) (i.e. those trees responding to climate effects), and LCR groups (Table 1.A2). For each

national forest, the master chronology (i.e. a dataset which contains series with the highest possible correlations) represented samples within a distinct geographic area. Series collected from the Medicine Bow and Routt National Forests were combined into a single category, due to their relative proximity to each other, and to create a larger sample size.

The final detrended chronologies for the (1) Medicine Bow and Routt, (2) Pike, and (3) San Isabel National Forest were produced using the program ARSTAN (Cook, 1985). A 25-year smoothing spline was used to remove autocorrelative influences on growth (i.e. non-climate related growth trends, such as tree age). This relatively short spline was chosen due to the relatively short lifespan of aspen; the chronology used in this study covers 108 years. A similar approach has previously been used to examine climatic influences on the growth a similarly short-lived species, *Betula pubescens* (Levanič and Eggertsson, 2008). The residual chronology, which contains no autocorrelation, was used in this analysis for each national forest, as it is most appropriate for regression analysis (Speer, 2010). An individual series' inclusion in the final chronology was determined based upon the expressed population signal (EPS), an indication of whether an observed signal (i.e. trend) is stand- or single tree-dominated (Wigley et al. 1984) (Table 1.A2), as well as $\bar{r}$ (running r-bar), a measure of the common signal strength in a series (Table 1.A2) (Speer, 2010). Inclusion of any one series which resulted in an EPS value of less than 0.80 was excluded from the final chronology (Wigley et al. 1984; Youngblut and Luckman, 2013). Chronologies for each national forest (Figure 1.A5-A7) were constructed by importing data into a single spreadsheet using the computer program YUX (Grissino-Mayer, 2001).

Of the 260 cores collected, crossdated and measured, two-thirds were excluded (due to low series correlations in COFECHA) from the climate-growth analyses, due to lack of response to clearly identifiable growth variable (i.e. climate). Only those cores designated as HCR were

used in analyses with climate variables. This included cores from 18 trees on the Medicine Bow and Routt National Forests, 21 trees on the Pike National Forest, and 30 trees on the San Isabel National Forest. Although sample depth for this study is lower than the average used in other dendroclimatological studies, it is comparable to other, recent dendroclimatological studies (Levanič and Eggertsson, 2008; Rayback et al. 2011; Decaulne et al. 2012; Dawadi et al. 2013).

Maximum temperature and precipitation data

All site-specific climate data used in this study were obtained from spatial PRISM (Parameter Regression Independent Slopes Model) datasets (Daly et al. 2002; Daly et al. 2008). Monthly and annual weather data over a time span of 109 years (1900-2008) were downloaded as a series of 800 m resolution grids from the PRISM climate group's website (http://prism.oregonstate.edu). Site-specific weather data were extracted for each plot location using the Sample tool within ArcToolbox. Data for sample locations were selected and averaged together by national forest, in order to match the locations of cores included in the final three chronologies. Precipitation data were compiled and used in three ways: (1) an annual dataset from 1900-2008; (2) a monthly dataset from 1950-2008; (3) a three-month running average from 1950-2008. We chose the time period 1950-2008 because our original analysis included El Nino Southern Ocean (ENSO) surface temperature data (NOAA, Climate Prediction Center) for this same time period. Maximum temperature data were compiled for the months of May, June, July, and August from 1900-2008.

Soil and geologic data

Soil survey data for the four national forests included in the study was downloaded from the digital general soil map of the United States (STATSGO2) as statewide ESRI® shapefiles from the USDA Natural Resources Conservation Service, Geospatial Data Gateway site (USDA, NRCS). This dataset includes soil series associations for each polygon, as well as geologic data.

Soil series and geologic formations of interest were selected by intersecting each layer with core collection plot locations, and then exporting the tabular data of the resulting shapefile. Characteristics of the dominant three soil series for each area were used in analyses, and included parent material, particle size class, mineralogy class, cation exchange capacity (CEC) class, depth of the 'A' horizon(s), and total soil depth.

Statistical Analysis: Site and stand comparisons: HCR or LCR tree presence

All statistical analyses were performed using SAS© 9.4 and Jmp Pro© software packages (The SAS Institute Inc., Cary, NC). Stand and site conditions from HCR and LCR core collection plots were first examined with the categorical regression tree (CRT) function within Jmp Pro© software. Potential variables for use in later logistic regression models were selected based on the LogWorth score (where LogWorth = –log (p-value)). The minimum LogWorth score accepted was 1.0, equal to a P-value of 0.10. Splitting values (nodes) were established to maximize LogWorth scores. A 0.10 P-value was chosen for this and other analyses in this study due to lack of significance of some measures at the P=0.05 level; thus, for the sake of continuity, we chose a cutoff of P=0.10. The likelihood of a stand containing HCR trees was also modeled as a logistic regression with a Spearman correlation coefficient of various site and stand variables of interest with the PROC LOGISTIC program.

Statistical Analysis: Site and stand characteristics: stand structure and disease or insect presence

Site and stand descriptive data, including basal area ha^{-1}, average stand health status score (an index value, where 1= completely healthy tree, 5= long-dead tree), percent dead crown, percent live adult aspen stems (≥ 12.0 cm DBH), and percent conifer encroachment were analyzed at the plot level. Additionally, the presence of several common canker diseases, such as Cytospora (*Cytospora* spp.), sootybark (*Encoelia pruinosa*), black (*Ceratocystis fimbriata*), and

Cryptosphaeria (*Cryptosphaeria lignota*) cankers, as well as white rot (*Phellinus tremulae*) disease were analyzed at the plot level, as well as two types of insect damage (wood borers and aspen bark beetles). All variables were analyzed as mixed linear models in PROC GLIMMIX. Least-squares estimates were calculated for each variable by ranger district within national forest, continental divide position (east or west), stand type (healthy or damaged), and tree response type (HCR or LCR). Means were considered significant if $P \leq 0.10$.

Statistical Analysis: Climatic comparisons: precipitation and maximum temperature

The residual chronology was analyzed as annual incremental growth by maximum monthly temperature (May-August), total annual precipitation, and national forest. An initial examination of the data included simple Spearman correlation coefficients between increment and climate data, calculated in the PROC CORR program.

Ring width indices (RWI) were also modeled with climatic data by year and national forest with the PROC MIXED and PROC REG programs. Maximum temperature was analyzed as monthly values (May-August) over 108 years (1900-2008) and precipitation as annual values for the same time period. In addition to the year-to-year RWI and temperature and precipitation analysis, we also modeled yearly RWI with the previous year's total precipitation (i.e. 'lagged' precipitation). These models utilized categorical temperature and precipitation data. Categories were determined by calculating 1.67 and 2 standard errors from the mean (approximately equivalent to 90 and 95% confidence intervals, and to *P*-values of 0.10 and 0.05) based on precipitation and temperature means by national forest (Table 1.2). Values between the 90-95% CI represented the 'mild' categories (e.g. 'mildly warm' or 'mildly dry'), and values beyond the 95% CI represented the more severe category (e.g., 'very warm or 'very dry'). Values which fell within the 90 % CI limits were considered to be within the normal range. Variables included in

the random statement for all models performed were precipitation (current or lagged) by temperature (month) within year.

Best subset regression analysis of increment data with maximum temperature current or lagged precipitation was performed using the GLMSELECT function in SAS. The model selection method used was stepwise, and selection criteria were based on AICc, an adjusted version of AIC (Akaike Information Criterion).

Finally, we analyzed annual precipitation data by ten-year increments from 1900-2008 for differences between HCR and LCR series, as well as between these series within healthy or heavily damaged stands, and by national forest. All means used were least-squares means, and significant differences were compared at the $P = 0.10$ level.

RESULTS

Site and stand comparisons: HCR versus LCR trees

Examination of ARSTAN output data indicated that the majority of cored trees did not respond uniformly to climatic influences over the 108 year period. After we had verified that there were no cross-dating or measurement errors, we examined which, if any, site factors contributed to this phenomenon. Most of the cores collected from sites on the White River National Forest were either LCR, or the standardized chronologies had expressed population signal (EPS) values of less than 0.80, and were excluded. Mean sensitivity rating was high (0.30-0.37) for nearly all of the series examined, even when a series' correlation coefficient was below the threshold level (0.42). Categorical regression analysis revealed four predictors of whether a site would produce a HCR or LCR tree. Regression tree nodes (splits) were based on (1) site elevation; (2) site percent slope; (3) site aspect; (4) canopy closure (Figure 1.2). Of these, site elevation was the single best predictor of whether a stand would produce a HCR or LCR tree (*P*

= 0.0003). Sites at high elevations (above 2836 meters) tended to have fewer LCR trees overall, and steep, high elevation sites with open canopies were significantly more likely to produce HCR trees, relative to similar sites with closed canopies (Figure 1.2). The second split, on slope category, indicated that HCR trees were not detected on sites with low percent slope (<6 %), and occurred on less than 40% of high elevation sites with steep slopes (P = 0.028) (Figure 1.2). Site aspect was also a significant predictor of response type; sites with LCR trees did not occur on sites with North-East, East, or South-East aspects (P = 0.078) (Figure 1.2). Sites with closed canopies were more likely to produce LCR trees (P = 0.023) (Figure 1.2).

Site and stand characteristics: Stand structure and disease or insect presence

We detected no meaningful differences in site or stand descriptive variables (basal area ha^{-1}, health status score, percent dead crown, percent live stems, adult aspen stems ha^{-1}, or percent conifer encroachment) among sites producing HCR trees and those producing LCR trees (Table 1.A1; Figure 1.A1-A4). We did detect differences in frequency of select damage agents between the two tree response types (Figure 1.3). White trunk rot, Cryptosphaeria canker and aspen bark beetles were more prevalent among sites with HCR trees than those with LCR trees (Figure 1.3).

Climatic comparisons: precipitation and maximum temperature

There were significant relationships between three-month precipitation averages and RWI (based upon Spearman correlation coefficients), and this varied by national forest (Table 1.2). Annual precipitation amounts generally decreased with latitude (i.e. Medicine Bow and Routt receive more precipitation than Pike and San Isabel National Forests) (Figure 1.4A). Correlations between precipitation and growth were positive and strongest for sites on the Medicine Bow and Routt and Pike National Forests for the precipitation three-month averages

for February-April, April-June, and for May-July. On the Pike National Forest, there were also significant, positive correlations between increment growth and the precipitation averages for March-May (Table 1.2). Among series collected from sites on the San Isabel National Forest, growth was negatively correlated with precipitation averages from August through October of the same year (Table 1.2). Negative correlations were detected between growth and maximum May and June temperatures for sites on the Medicine Bow and Routt National Forests, and maximum May temperatures for sites on the Pike National Forest (Table 1.3).

The final model of RWI and climatic influences included both maximum temperature and precipitation ($P = 0.0002$) (Table 1.4). Maximum May and July temperatures, combined with both annual precipitation (of the same calendar year), and the annual precipitation of the previous year, were selected based on the AICc score (Table 1.4). When maximum monthly temperature was removed from both of the precipitation models, differences in RWI were predicted by forest and the current or previous year's annual precipitation (Figures 5 and 6; Figures A5-A7). Trees on the Pike and San Isabel National Forests produced larger annual rings when either the current or previous year's annual precipitation was above average, than during periods of normal or below-average precipitation. This pattern was not observed among cores taken from the Medicine Bow and Routt National Forests; average increment did not vary among the three precipitation classes (Figures 5 & 6; Figures A5-A7). We further noted that the standard error of annual growth for years with normal precipitation and monthly temperature was very small, relative to error values for growth under other conditions (Figures A8-A10). This pattern persisted across all three national forests, and ranged from 0.018-0.019, relative to the standard errors of other temperature and precipitation combinations (0.076-0.174).

Analysis of ten-year averaged annual precipitation by tree sensitivity (HCR or LCR), stand type (healthy or damaged) and national forest indicated pronounced differences (Figures A11-A15). We detected differences between LCR and HCR sites located within heavily damaged stands, but not between LCR and HCR sites located within lightly damaged stands (Figures A11-A12). There were marked differences between HCR and LCR sites on the Medicine Bow and Routt and San Isabel National Forests, but not between HCR and LCR sites on the Pike National Forest (Figures A13 & A14). HCR sites on the Medicine Bow and Routt National Forests consistently received significantly more annual precipitation for every decade since 1900 (Figure 1.A13). This pattern was reversed on the San Isabel National Forest, where HCR sites received less annual precipitation for every decade since 1900, except for 1910-1919 (Figure 1.A15).

DISCUSSION

In our analyses of site and stand characteristics of stands producing HCR or LCR trees, four tested variables stood out as predictors of tree sensitivity. The four main predictors of whether a site produces HCR or LCR trees were: site elevation, site slope, site aspect, and stand structure, all of which suggest the influence of water availability. We have previously described the differences between cores from the White River National Forest and the four other Forests surveyed. The conclusions reached by Hogg et al. (2013), based upon a soil moisture index (SMI) model, accurately predicted lags in tree growth occurring on sites with deeper soils. We suspect that a 'hydrologic lag' (Hogg et al. 2013) may be occurring on at least some of the sites with LCR trees. Sites on the White River National Forest occurred on soils with a mean epipedon depth of 65.8 cm, relative to 13-19 cm on the other four national forests (data not

shown). Increment cores from HCR trees clearly indicate key drought years and years of moisture surplus (e.g. 1924, 1939; 1957 & 1982) (Figure 1.4), but these patterns were largely absent among LCR cores across all national forests.

The dramatic difference in responses of proximal trees to widespread drought events could also represent phenotypic differences in drought tolerance from one clone to another, as has been documented by Griffin et al. (1991). Mounting genetic evidence suggests that aspen stands are often not comprised of a single clone, but of many distinct individuals (DeWoody et al. 2009; Long and Mock, 2012). Based on this, some of the variability in drought response among trees growing near (i.e. within 100 meters) each other may be a reflection of varying drought tolerance among genotypes. This explanation is likewise applicable when examining the range of growth responses under conditions other than average maximum monthly temperature and precipitation. While sampled trees responded similarly to normal moisture and temperature, tree responses to weather conditions outside of average range varied considerably, although it is uncertain whether the apparent variability in drought tolerance present in these populations is sufficient to protect them from an increasingly warmer and drier habitat. Recently, a study of genetic variability of quaking aspen stands throughout North America indicated that in comparison to stands in the northern and eastern portions of its range, aspen stands in the south-western US have lower within-population diversity (Callahan et al. 2013). Such decreased allelic richness, as postulated by Callahan et al. (2013) does not bode well for these populations, perhaps making them more vulnerable to the prolonged episodes of drought predicted for the southwestern United States under climate change.

We note that aspen stands on the White River National Forest, which were sampled and measured, but the increment data were not used in RWI analyses, differed from stands on other

national forests. Of the more than 40 increment cores collected from the WRNF, only about a dozen or so had sufficiently high correlation coefficients (in COFECHA). These were later excluded from the final chronologies due to their low EPS values (from ARSTAN). Key site differences are likely the reason that stands on the WRNF did not respond to climatic influence with the same consistent and stand-wide variations in growth. We suspect that the aspen stands on the WRNF are less likely to experience drought events than those on the other national forests we surveyed. This is likely in part due to the dramatically deeper surface soils which occur in the area, and in part to phenotypic differences in drought tolerance (discussed above). Measured frequency of Cytospora canker is a reasonable proxy for assessing drought stress in a stand, as the causal organism, *Cytospora* spp., successfully colonizes healthy tissue when the host tree is experiencing some environmental stressor, usually drought (Guyon et al. 1996; Christensen, 1940). Surveyed adult aspen on the WRNF had very low levels of Cytospora canker, relative to the other national forests; in 2009-2010, we observed Cytospora canker on about 2% of adult live aspen stems, relative to 13-51% of adult live aspen elsewhere (data not shown) (Dudley et al. 2015).

Our analyses of RWI and annual precipitation, (Figures 5 & 6), which includes the current or previous years' annual precipitation, appears to be more applicable to the southerly locations of the study area. Trees on the Pike and San Isabel National Forests clearly responded favorably to years of above-average precipitation, but those on the Medicine Bow and Routt National Forests did not. It is important to note that the average annual precipitation range for the Medicine Bow and Routt National Forests is over 30% higher than for the Pike National Forest (Table 1.1A; Figure 1.4). This may be one reason that the trees on these northern forests do not respond as strongly to years of low precipitation. A dry year in the northern portion of the study

area could represent twice as much precipitation as is received in a dry year in the southern portion of the study area (Table 1.1A).

The timing of precipitation over the course of a year also differs dramatically between forests in the north (White River and Medicine Bow and Routt National Forests) and the central and southern portions of Colorado (Pike and San Isabel National Forest) (Figure 1.A16). Both forests in the southern portion of the study area receive much of their annual precipitation between April and September, whereas the forests in the north receive most of their precipitation from September to April, and mainly as snowfall (Figure 1.A16). In addition to moisture content, snowpack also reduces environmental stress on aspen because it insulates the roots during freeze events, as was shown by Hogg et al. (2002) in the aspen parklands of Alberta, Canada. A recent study of aspen decline and climate factors indicated that a major driver of aspen mortality is precipitation received between April and September, paired with maximum summer temperatures, a result similar to the model produced from this study (Worrall et al. 2013).

The negative correlation between RWI and the three-month precipitation average from August- October for sites on the San Isabel National Forest could be a reflection of the dependency of these stands upon monsoonal moisture. The monsoon season, which typically begins mid-July in Colorado, is often preceded by periods of hot, dry weather (Doeskin, 2003). Late monsoonal moisture could therefore, account for the negative correlation of August-October precipitation with RWI; the later the arrival of the summer storms, the more pronounced the drought in areas dependent upon these weather patterns.

We note that maximum temperatures during the spring (May) and mid-summer (July) have a significantly negative impact on the growth of trees on the Pike and Medicine Bow and Routt National Forests (Table 1.1B), independent of precipitation. This finding is similar to that

of Hanna and Kulakowski (2012) and Spond et al. (2014), who observed negative relationships between RWI of aspen and seasonal maximum temperatures. It is therefore likely that as the incidence of extreme heat events increase (as predicted by the most recent IPCC report for the coming century) (Stocker et al. 2013), aspen will continue to experience conditions which are not conducive to optimal growth (Rehfeldt et al. 2009). It should also be noted, however, that this relationship between drought and stem mortality isn't always clear; a recent large-scale study of aspen stands throughout the western United States have shown that while aspen mortality is influenced by drought, these impacts can be obscured by stand dynamics and stand age (Bell et al. 2014).

It has been well documented that aspen stands experiencing environmental stress are highly prone to certain insects and diseases, such as wood borers, bark beetles, and (as noted above) Cytospora canker (Hogg et al. 2008; Marchetti et al. 2011; Worrall et al. 2008 & 2010; Guyon et al. 1996; Christensen, 1940). Drought, as well as secondary disease and damage agents and excessive browsing by ungulates, can result in stands with high levels of mortality and low levels of regeneration (Rogers et al. 2013; Hanna and Kulakowski, 2012; Worrall et al. 2008 & 2010).

Maintaining aspen stands on western landscapes, especially under prolonged drought conditions, may require proactive management actions, such as overstory removal (through mechanical or prescribed fire treatments) and ungulate exclusion (Rogers et al. 2013). We observed that stands which responded to drought conditions (i.e. HCR stands) were spatially distinctly located, and not randomly found across the aspen forest types. The series examined in this study also indicate that quaking aspen in Colorado and southern Wyoming have varying degrees of tolerance to drought, and this tolerance is likely the result of a complex of genotype

and site conditions. Based on the variation in spatial pattern and degree of drought tolerance, proactive management will need to be tailored to the stand or district level.

FIGURES AND TABLES

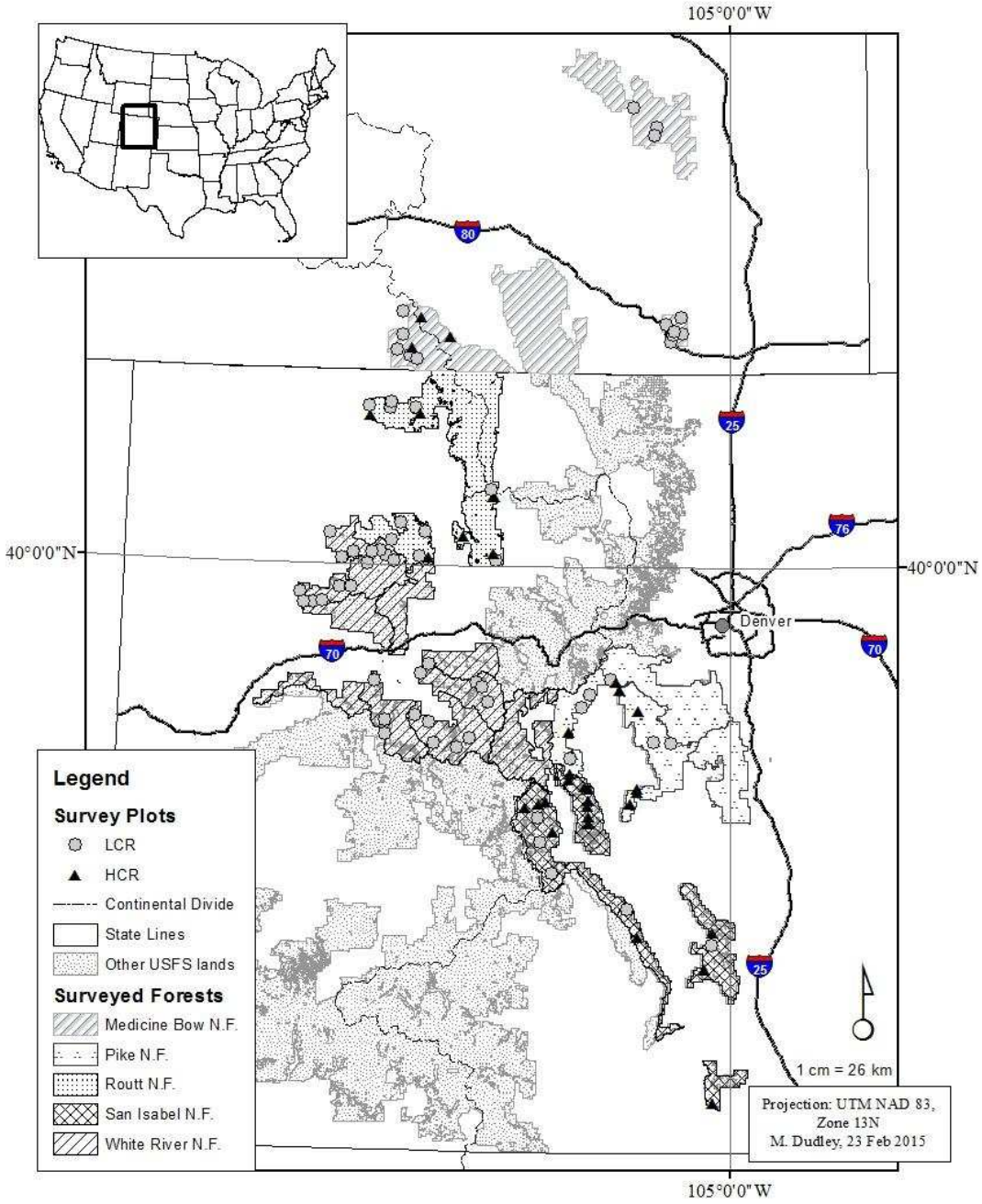

Figure 1.1. Survey area and increment core collection sites in Colorado and southern Wyoming, 2009-2010. A high cohesive response (HCR) plot refers to whether one or more of the trees sampled per transect were responsive to region-wide climate signals. Low cohesive response (LCR) trees were not responsive to region-wide climate signals.

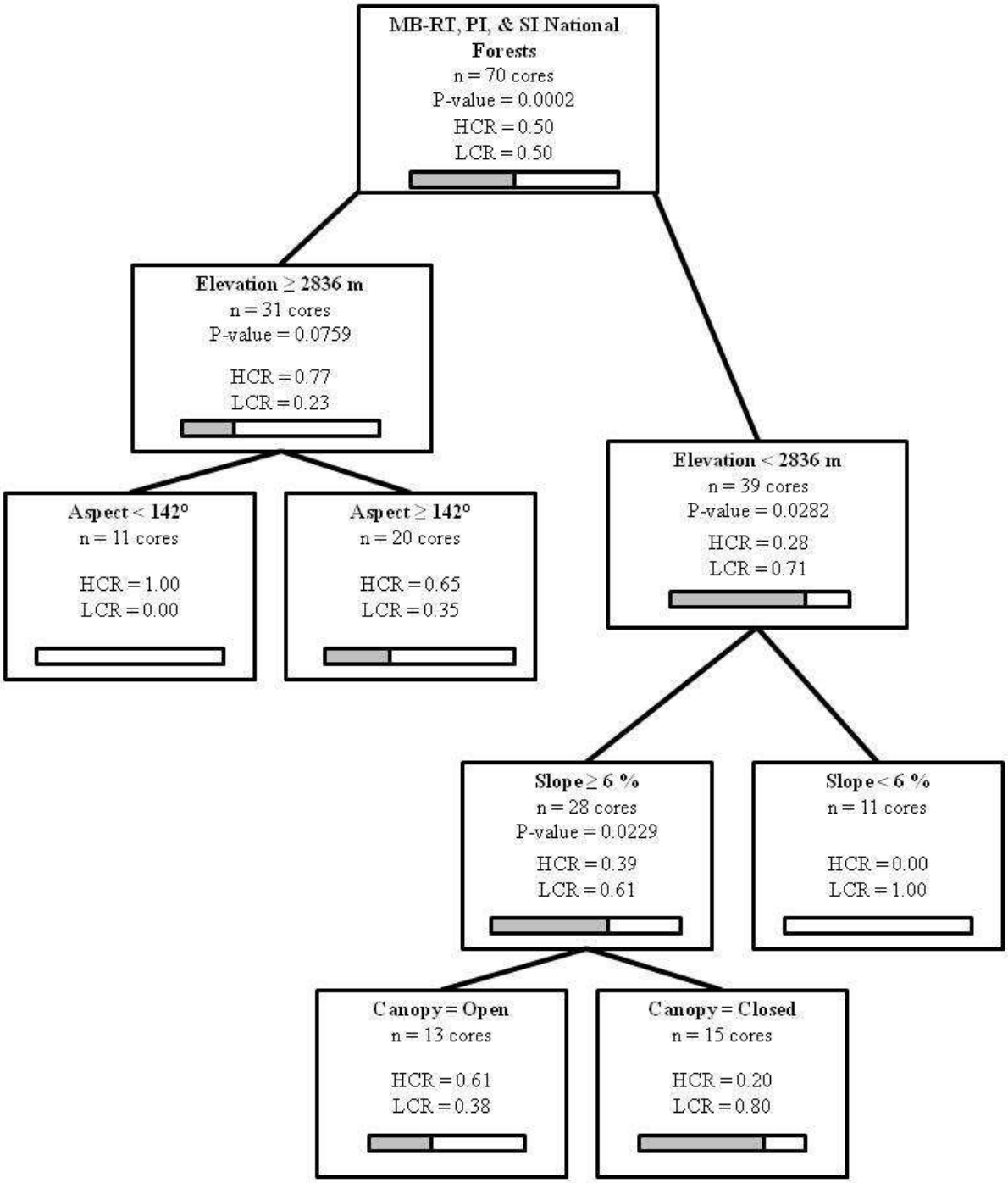

Figure 1.2. Categorical regression tree of plots containing likelihood of high cohesive response (HCR) or low cohesive response (LCR) series (i.e. increment cores) occurring in plots with various site, stand and soil characteristics. Grey and white bars indicate the proportion of transects (with up to three cores per transect) exhibiting LCR or HCR. N = 70, RSquare = 0.381, splits = 4.

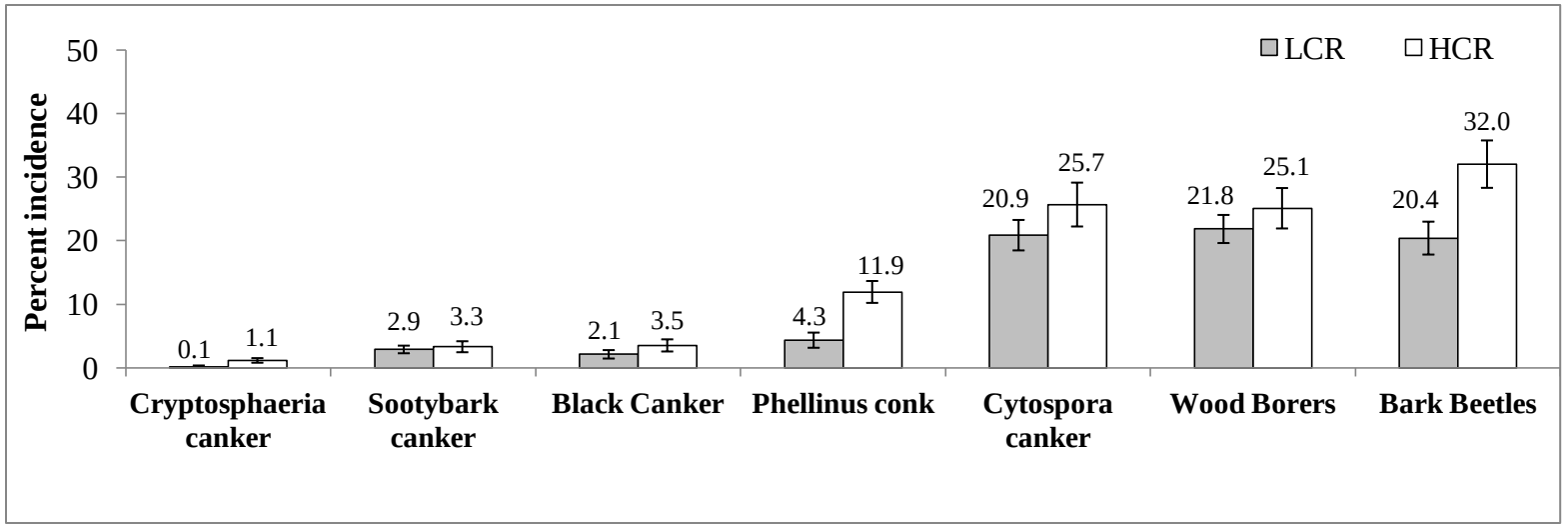

Figure 1.3. Average occurrence of five common fungal diseases and two damaging insect groups on adult aspen, by low cohesive response (LCR) or high cohesive response (HCR) tree type as recorded in a stand health survey 2009-2010. All means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at $p = 0.10$.

Damage agents included in the above categories are as follows: Cryptosphaeria canker- *Cryptosphaeria lignyota*; sootybark canker- *Encoelia pruinosa*; black canker- *Ceratocystis fimbriata*; Phellinus conk- *Phellinus tremulae*; Cytospora canker-*Cytospora sordida* and *V. notastroma*; wood borers- *Agrilus liragus* and *Saperda calcarata*; bark beetles- *Trypophloeus populi* and *Procryphalus mucronatus*.

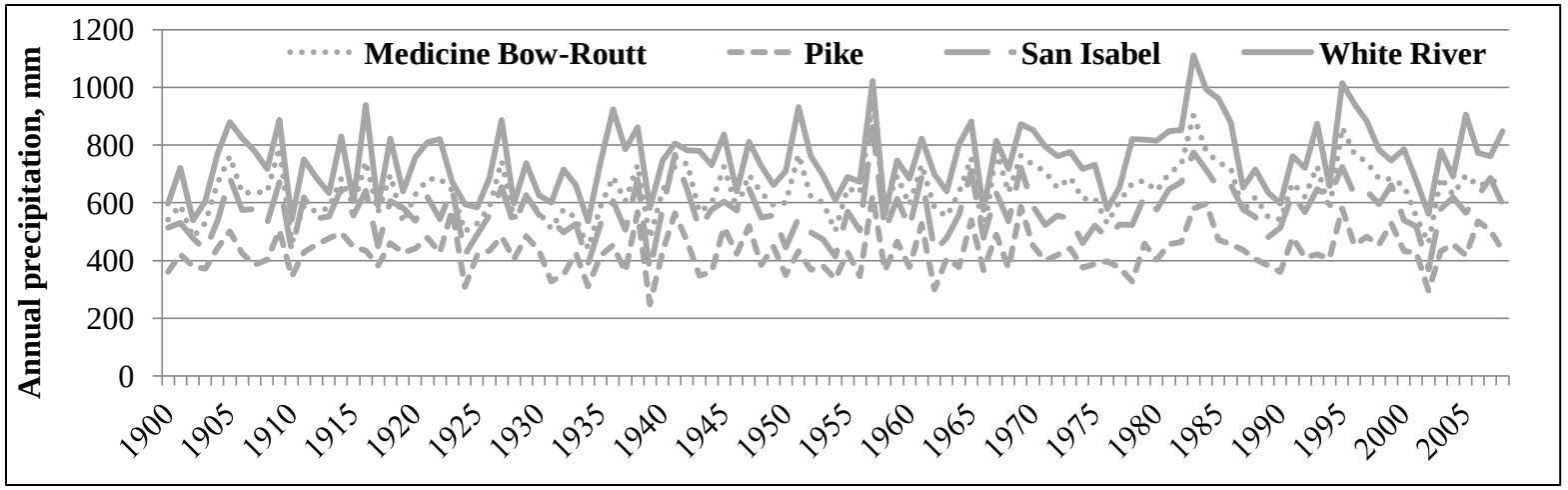

Figure 1.4A. Annual average precipitation (mm) for four National Forests, 1900-2008.

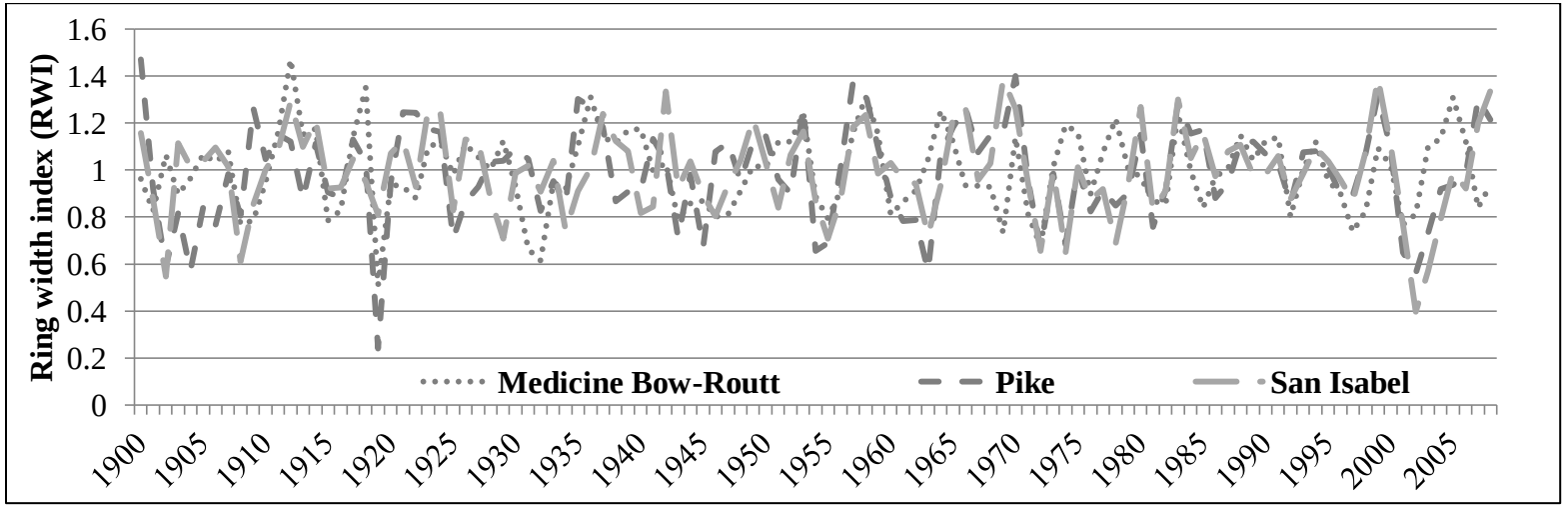

Figure. 1.4B. Average annual RWI of HCR trees from three national forests (in mm), 1900-2008.

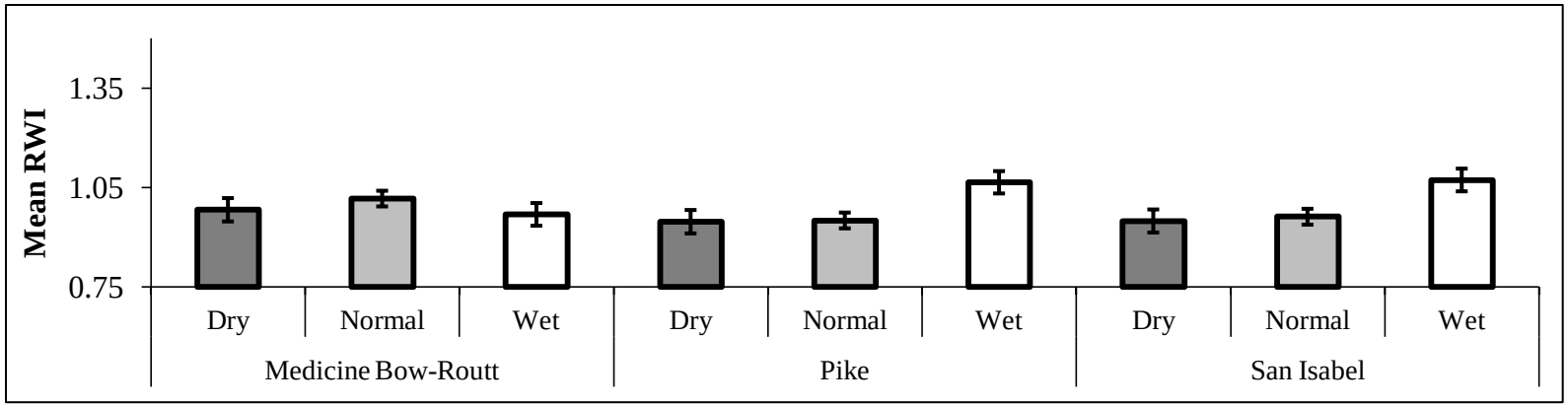

Figure 1.5. Average annual RWI (mm) of adult aspen among three national forests and three moisture classes*, as modeled with the corresponding year's total precipitation (by plot, averaged to National Forest), 1900-2008. All means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at $p = 0.10$.

*Moisture classes are based on a 90% CI of annual precipitation data (1900-2008), where 'normal' includes years with precipitation amount within 90% range, and 'dry' and 'wet' years are those above or below range cutoff. RWI is represented based on 69 tree cores (18 MBRT, 21 Pike, 30 SI).

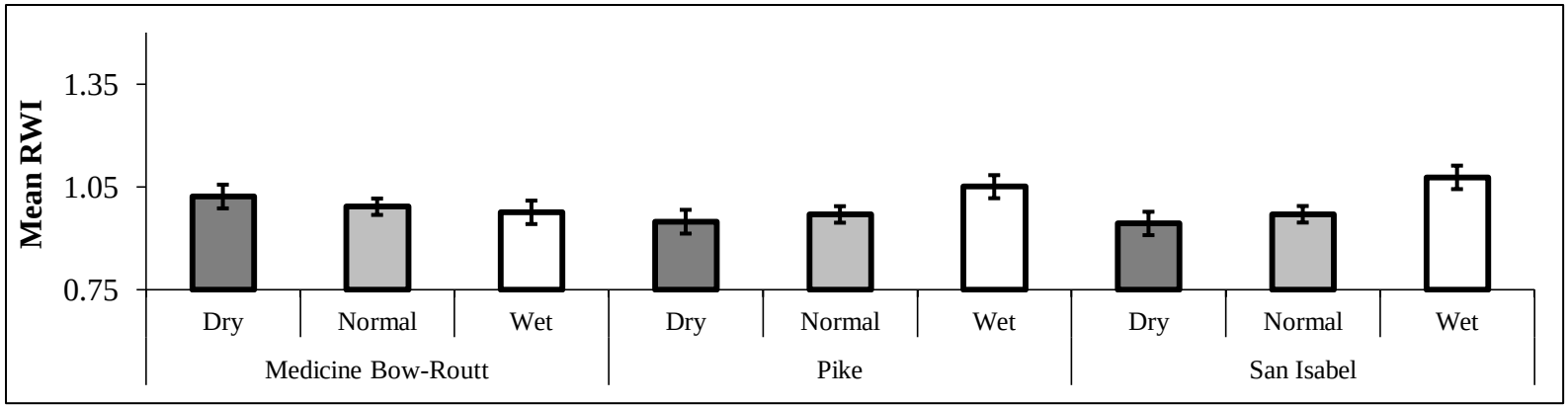

Figure 1.6. Average annual RWI (mm) of adult aspen among three national forests and three moisture classes*, as modeled with one year-lagged annual precipitation (by plot, averaged to National Forest), 1900-2008. All means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at $p = 0.10$.

Table 1.1A. Mild, severe, and normal annual precipitation values for three national forests from 1900-2008. Mild and severe categories represent 1.67 and 2 standard errors from the mean.

	Precipitation (mm)				
	Very wet	Mildly wet	Normal	Mildly dry	Very dry
MB & R*	> 827	796-827	486-796	456-486	< 456
Pike	> 573	549-573	314-549	291-314	< 291
San Isabel	> 746	717-746	424-717	395-424	< 395

*Medicine Bow (MB) and Routt (R) National Forests

Table 1.1B, top & bottom. Mild, severe, and normal maximum monthly temperature values for three National Forests from 1900-2008. Mild and severe categories represent 1.67 and 2 standard errors from the mean.

	May					June				
	Temperature (°C)									
	Very cool	Mildly cool	Normal	Mildly warm	Very warm	Very cool	Mildly cool	Normal	Mildly warm	Very warm
MB & R*	< 9.8	9.8-10.4	10.4-17.0	17.0-17.6	> 17.0	< 16.0	16.0-16.5	16.5-22.1	22.1-22.7	> 22.7
Pike	< 7.8	7.8-8.5	8.5-15.5	15.5-16.2	> 16.2	< 14.5	14.5-15.1	15.1-21.0	21.0-21.6	> 21.6
San Isabel	< 9.8	9.8-10.5	10.5-17.0	17.0-17.6	> 17.6	< 16.3	16.3-16.9	16.9-22.4	22.4-22.9	> 22.9

	July					August				
	Temperature (°C)									
	Very cool	Mildly cool	Normal	Mildly warm	Very warm	Very cool	Mildly cool	Normal	Mildly warm	Very warm
MB & R*	< 20.2	20.2-20.6	20.6-24.9	24.9-25.3	> 25.3	< 19.4	19.4-19.8	19.8-23.4	23.4-23.8	> 23.8
Pike	< 19.0	19.0-19.4	19.4-23.9	23.9-24.3	> 24.3	< 18.4	18.4-18.7	18.7-22.5	22.5-22.9	> 22.9
San Isabel	< 19.9	19.9-20.3	20.3-24.6	24.6-25.0	> 25.0	< 18.7	18.7-19.1	19.1-23.0	23.0-23.4	> 23.4

*Medicine Bow (MB) and Routt (R) National Forests

Table 1.2. Spearman correlation coefficients (top, each cell) and P values (bottom, each cell) for correlations between RWI (of HCR series) and three-month average precipitation values from 1950-2008, by National Forest. Means in bold indicate significance at p = 0.10. n = 59 years.

	Dec-Feb	Jan-Mar	Feb-Apr	Mar-May	Apr-Jun	May-Jul	Jun-Aug	Jul-Sep	Aug-Oct	Sep-Nov	Oct-Dec	Nov-Jan
MB & RT*	0.07	0.16	**0.22**	0.16	**0.24**	0.21	0.15	0.08	0.03	0.05	0.10	0.07
P-value	0.60	0.23	**0.0971**	0.22	**0.0729**	0.11	0.25	0.53	0.85	0.73	0.43	0.59
Pike	0.02	0.12	**0.36**	**0.35**	**0.41**	**0.30**	0.16	0.07	-0.00	-0.04	0.012	-0.06
P-value	0.90	0.36	**0.0052**	**0.0121**	**0.0013**	**0.0196**	0.22	0.62	0.98	0.74	0.93	0.65
San Isabel	0.02	0.14	0.19	0.10	0.09	-0.08	-0.04	-0.15	**-0.23**	-0.09	0.02	0.05
P-value	0.89	0.28	0.14	0.47	0.51	0.55	0.76	0.25	**0.0776**	0.49	0.87	0.71
n	58	59	59	59	59	59	59	59	59	59	59	59

*Medicine Bow (MB) and Routt (R) National Forests

Table 1.3. Spearman correlation coefficients and p-values of associations between RWI and maximum monthly temperatures, 1950-2008. Bold figures indicate significant relationships at p = 0.10. n= 59 years.

	May	June	July	August
MB & RT*	-0.1540	**-0.2142**	-0.1828	0.1054
P value	0.2442	**0.1034**	0.1659	0.4267
Pike	**-0.2795**	-0.2022	-0.1725	0.1284
P value	**0.032**	0.1247	0.1915	0.3325
San Isabel	-0.1047	0.0145	-0.0112	-0.1856
P value	0.4299	0.9132	0.9328	0.1594

*Medicine Bow (MB) and Routt (RT) National Forests

Table 1.4. Model selection of effects for the prediction of annual RWI of quaking aspen. Numbers 2 and 3 are included in the final model, which includes steps 1-4. Climate data are from 1950-2008.

Step	Effect entered	Number	Model R-square	Adjusted R-square	AIC	AICC	F Value	Pr > F
0	**Intercept**	1	0	0	-799.00	-798.97	0	1
1	**Maximum July Temp**	2	0.0322	0.0292	-807.61	-807.54	10.72	0.0012
2	**Annual Precipitation**	3	0.0629	0.057	-816.04	-815.92	10.5	0.0013
3	**Maximum May Temp**	4	0.0699	0.0612	-816.48	-816.29	2.42	0.1207
4	**Lagged Annual Precipitation**	5	0.0767	0.0651*	-816.85*	-816.58*	2.34	0.1272
	Final model	1-5	0.0767	0.0651	816.85	-816.58	6.62	**0.0002**

*Indicates optimal criterion value. No effects were removed in this model selection. Final model P-value was calculated using the F-statistic (6.62), numerator degrees of freedom, k-1 (3), and the denominator degrees of freedom, N-k (320).

Figures 1.A1-A4. Characteristics of adult aspen (≥ 12.0 cm DBH) by National Forest, stand type, and tree response type, as recorded during a 2009-2010 aspen health survey. Data are averaged to the plot level, and designated HCR if at least one of the three cores was HCR. All means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at $p = 0.05$.

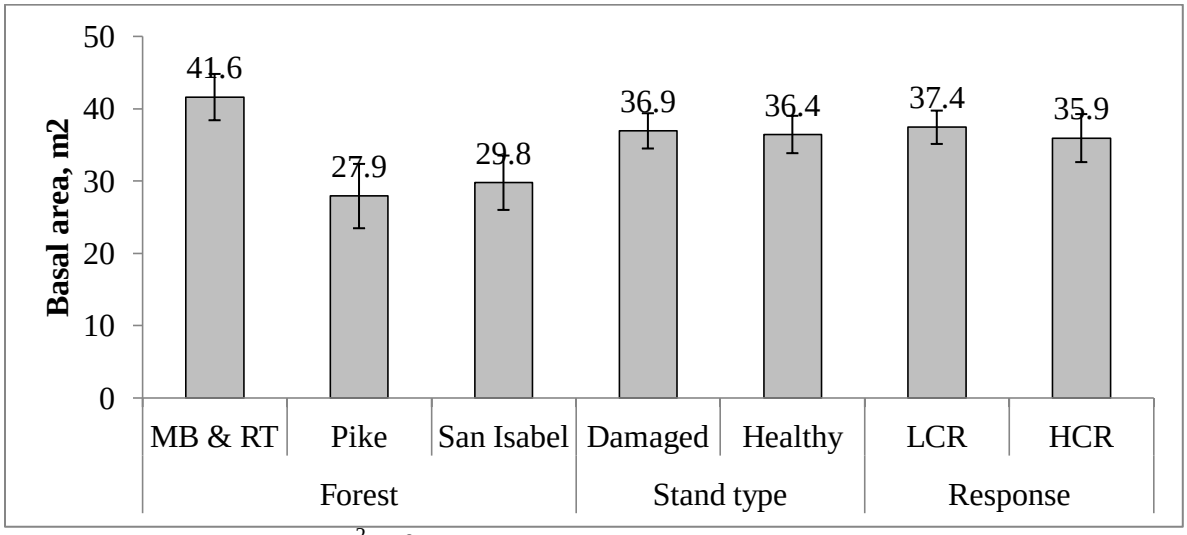

Figure 1.A1. Basal area (m^2) of adult aspen per hectare in Colorado and southern Wyoming surveyed in 2009-2010.

Medicine Bow (MB) and Routt (RT) National Forests

Low (LCR) and high (HCR) response types

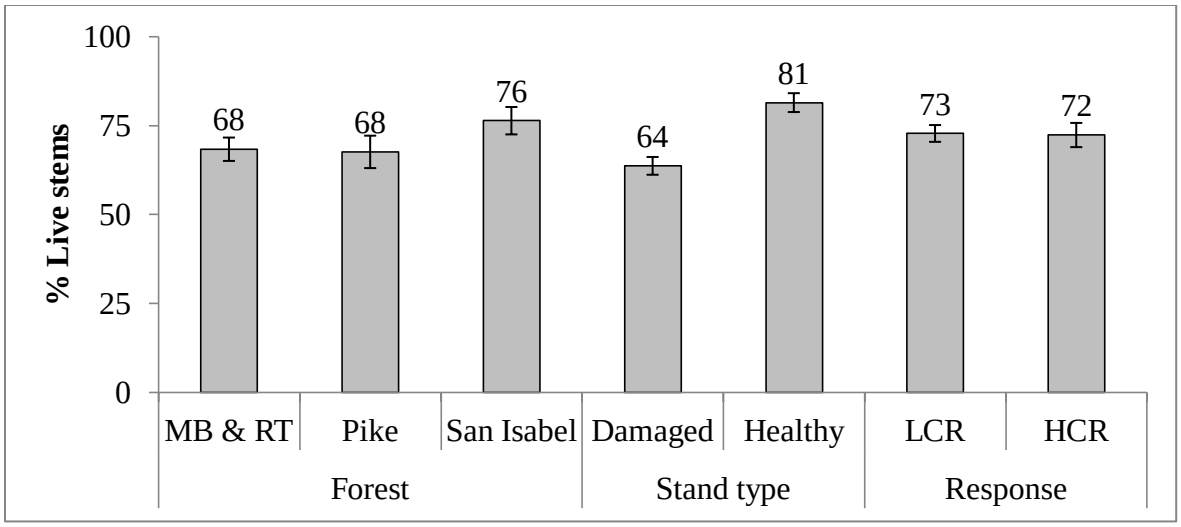

Figure 1.A2. Live adult aspen stems in Colorado and southern Wyoming surveyed in 2009-2010.

Medicine Bow (MB) and Routt (RT) National Forests
Low (LCR) and high (HCR) response types

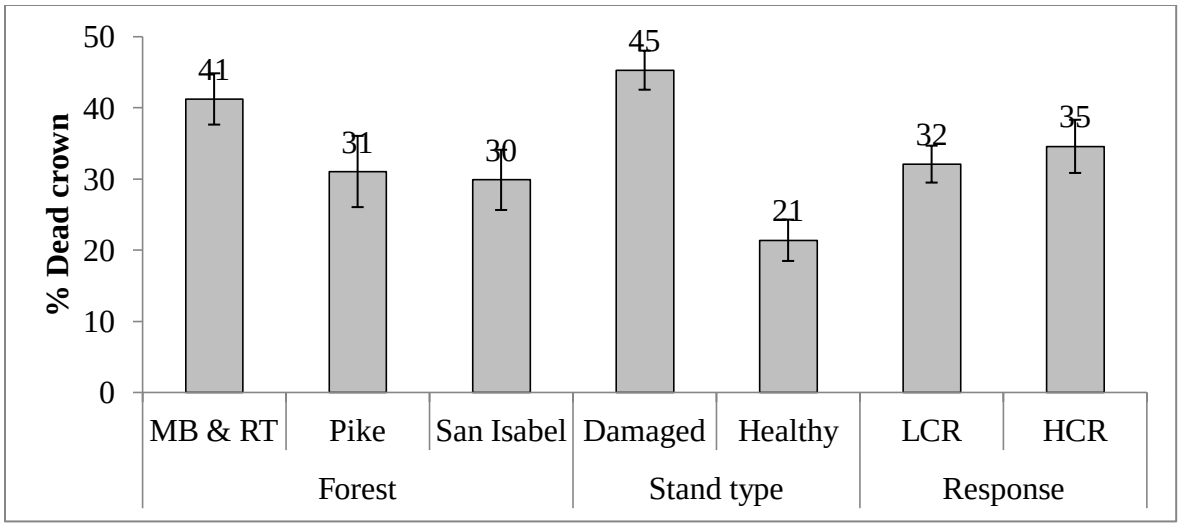

Figure 1.A3. Average dead crown of adult aspen in Colorado and southern Wyoming surveyed in 2009-2010.

Medicine Bow (MB) and Routt (RT) National Forests

Low (LCR) and high (HCR) response types

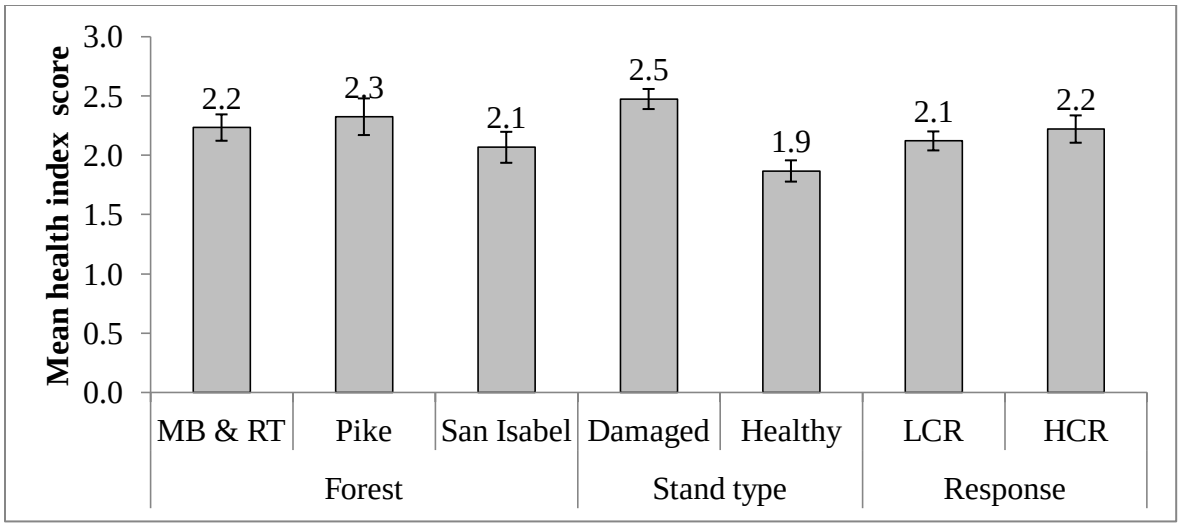

Figure 1.A4. Average health index score (1-3; 1=very healthy, 2= marginal, 3= dying) of adult live aspen in Colorado and southern Wyoming surveyed in 2009-2010.

MB & RT = Medicine Bow and Routt National Forests

Low (LCR) and high (HCR) response types

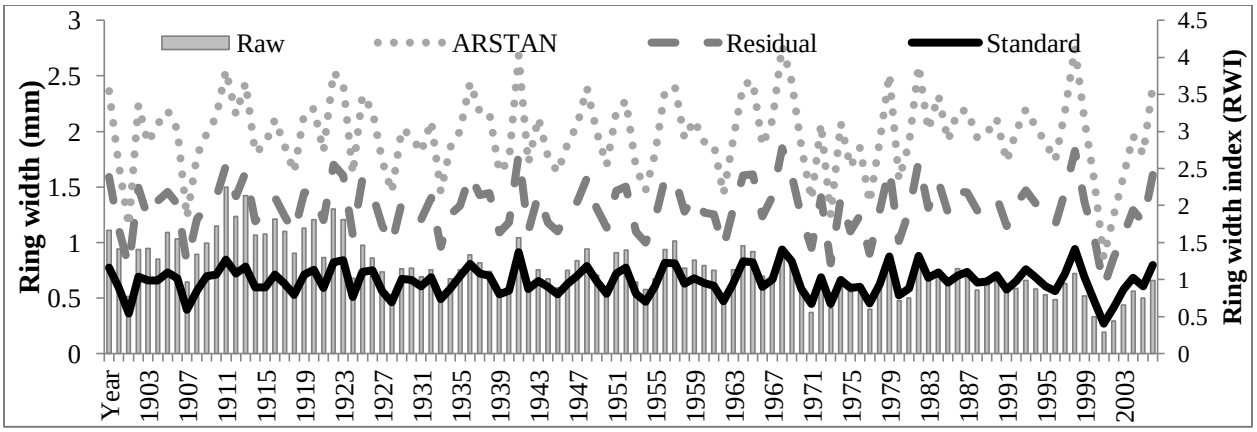

Figure 1.A5. All four (stacked) chronologies of adult aspen from increment cores collected from the Medicine Bow and Routt National Forests, 2009-2010.

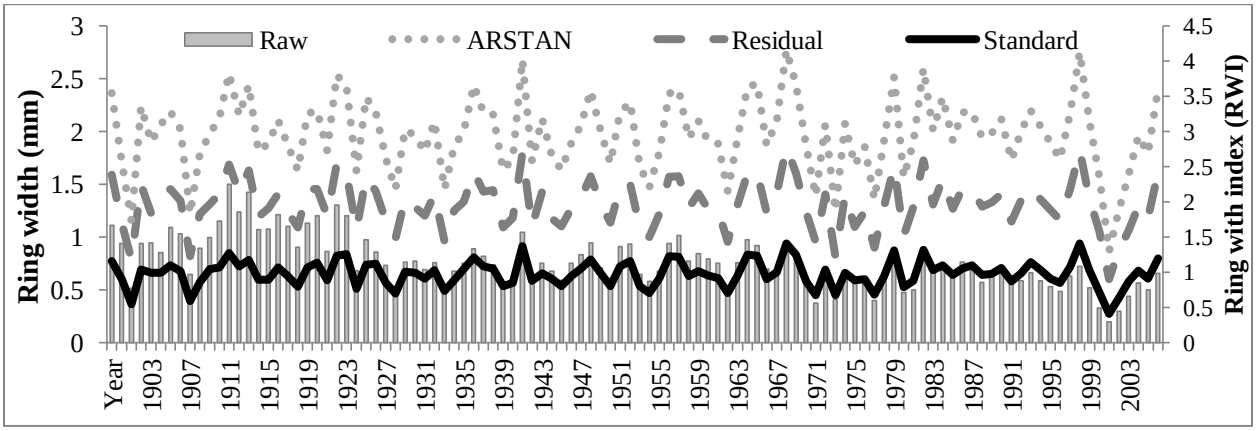

Figure 1.A6. All four chronologies of adult aspen from increment cores collected from the Pike National Forest, 2009-2010.

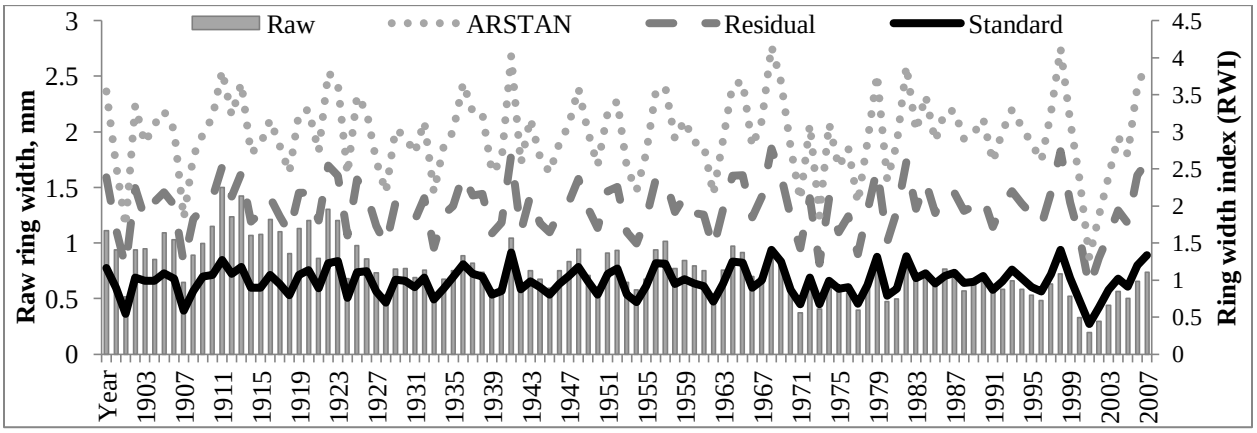

Figure 1.A7. Three chronologies and the raw ring width values of adult aspen from increment cores collected from the San Isabel National Forest, 2009-2010.

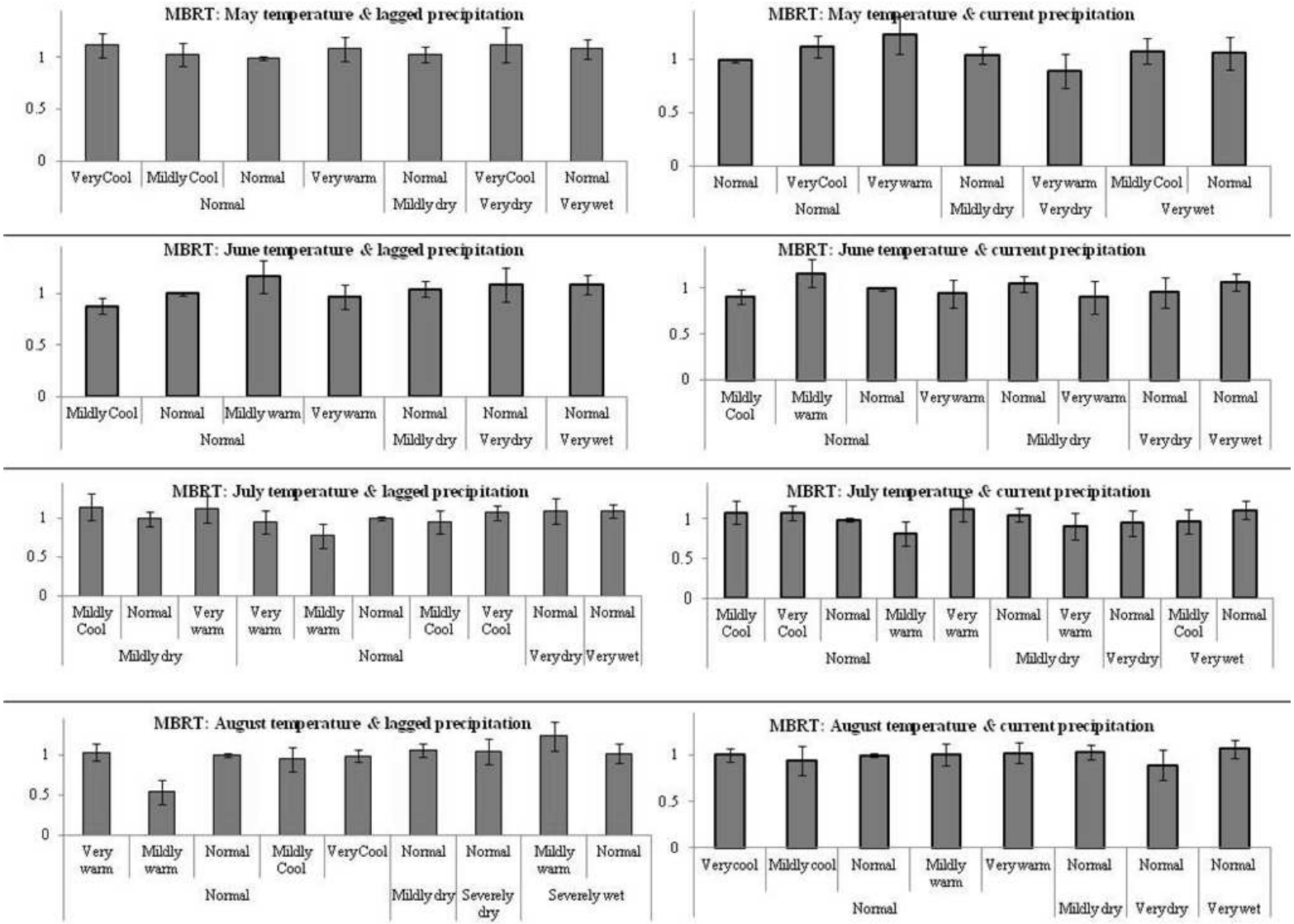

Figure. 1.A8. Modeled RWI among series on the Medicine Bow and Routt National Forests (MBRT), with monthly maximum temperature and either the current or lagged annual precipitation. Precipitation and maximum temperature categories are identical to those listed in Tables 1A & 1B. Error bars represent LSD; bars which do not overlap are significantly different at P=0.10.

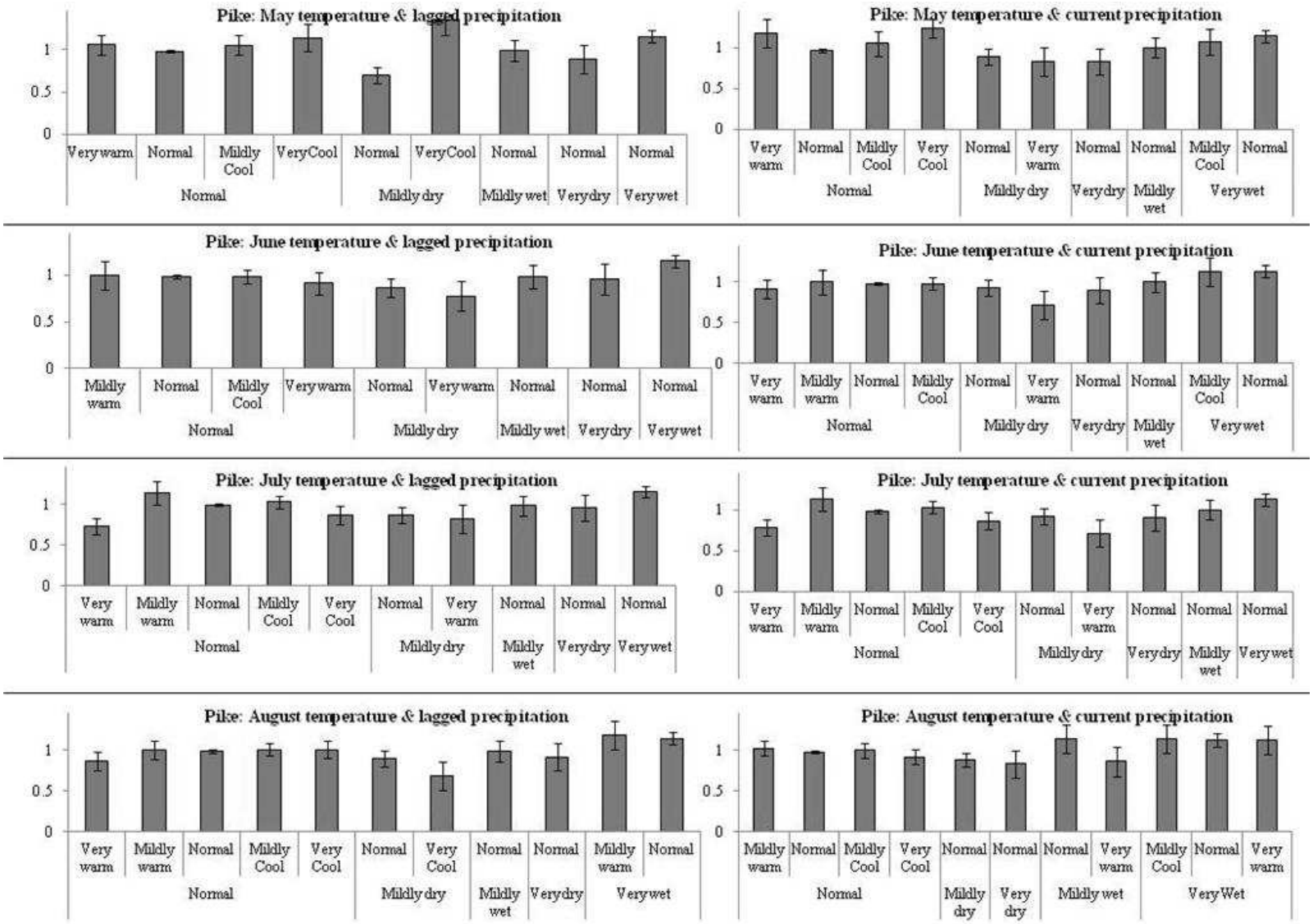

Figure. 1.A9. Modeled RWI among series on the Pike National Forest, with monthly maximum temperature and either the current or lagged annual precipitation. Precipitation and maximum temperature categories are identical to those listed in Tables 1A & 1B.Error bars represent LSD; bars which do not overlap are significantly different at *P*=0.10.

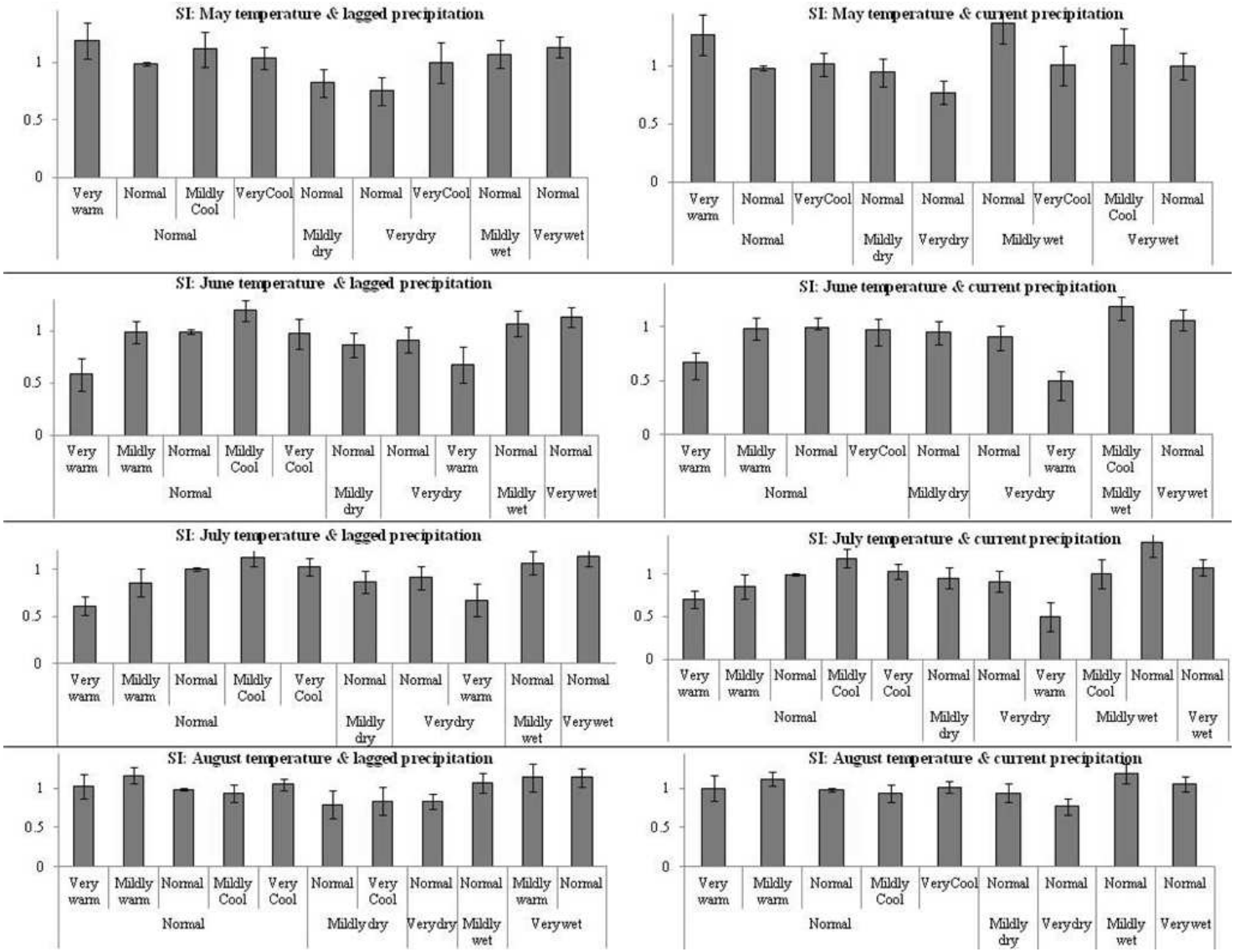

Figure. 1.A10. Modeled RWI among series on the San Isabel National Forest (SI), with monthly maximum temperature and either the current or lagged annual precipitation. Precipitation and maximum temperature categories are identical to those listed in Tables 1A & 1B.Error bars represent LSD; bars which do not overlap are significantly different at P=0.10.

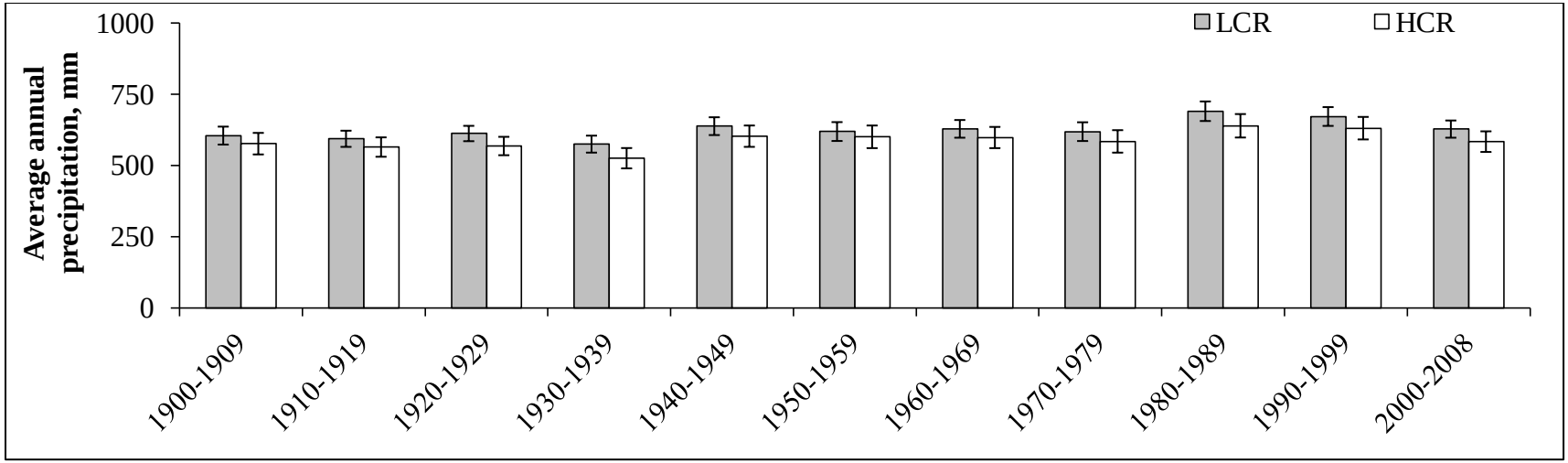

Figure 1.A11. Average annual precipitation (mm) by decade among HCR and LCR sites within lightly damaged stands. Means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at *P*=0.10.

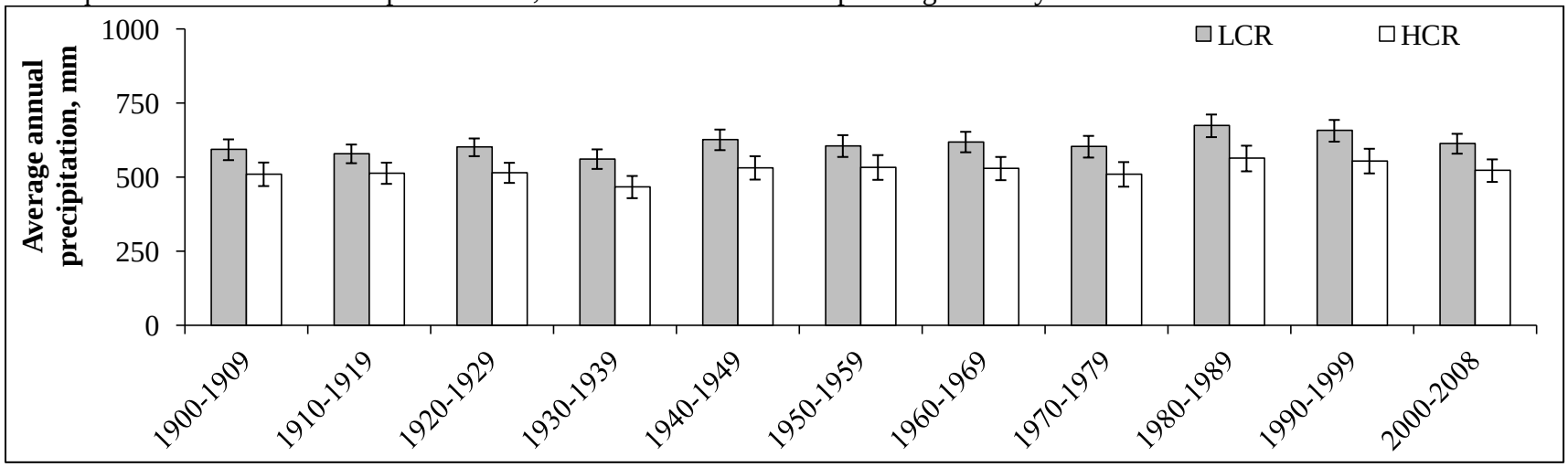

Figure 1.A12. Average annual precipitation (mm) by decade among HCR and LCR sites within heavily damaged stands. Means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at *P*=0.10.

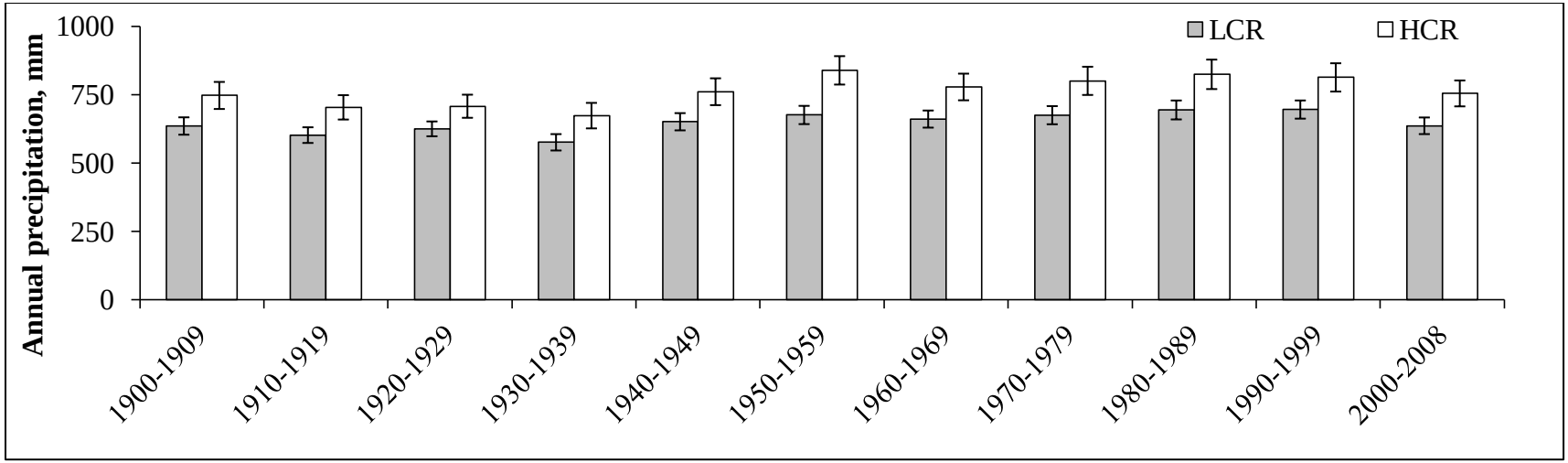

Figure 1.A13. Average annual precipitation (mm) by decade among HCR and LCR sites within the Medicine Bow and Routt National Forests. Means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at *P*=0.10.

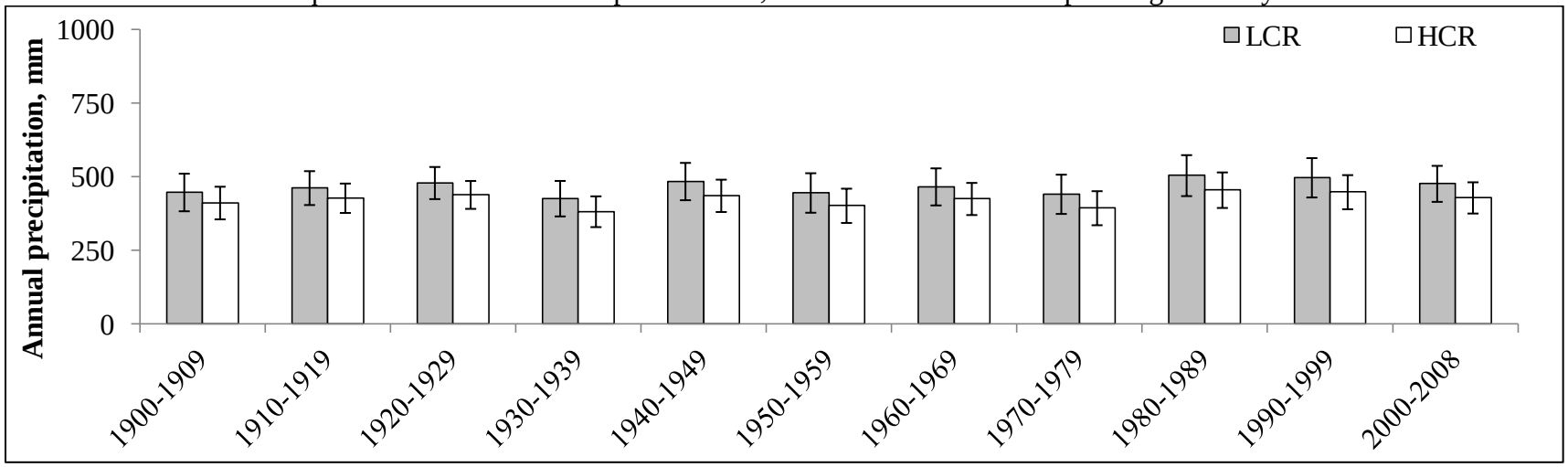

Figure 1.A14. Average annual precipitation (mm) by decade among HCR and LCR sites within the Pike National Forest. Means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at *P*=0.10.

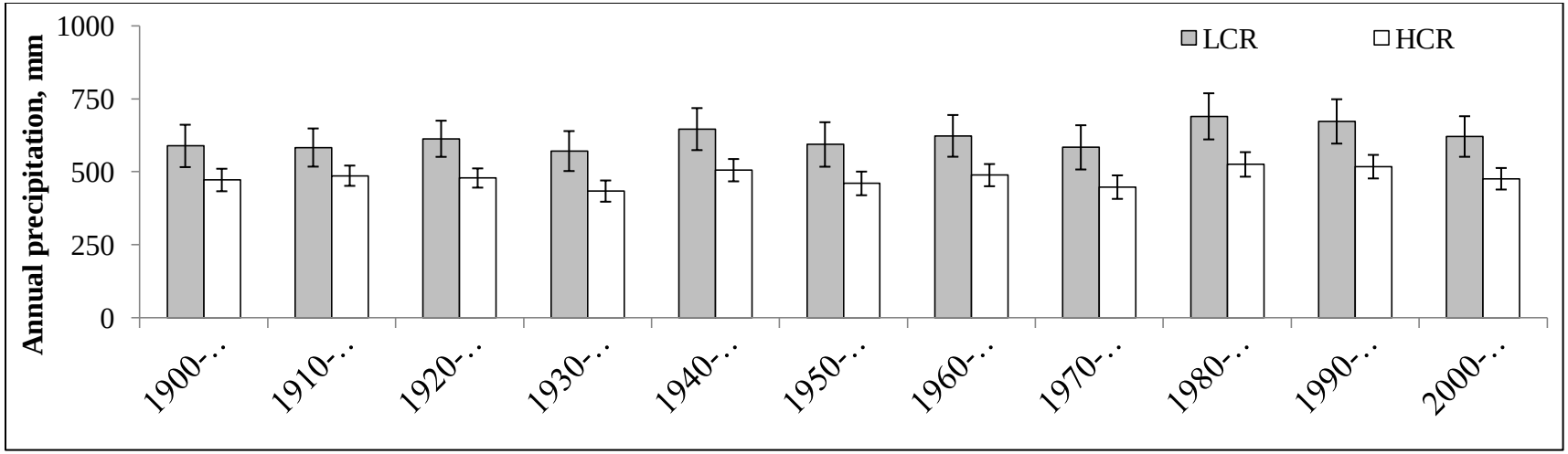

Figure 1.A15. Average annual precipitation (mm) by decade among HCR and LCR sites within the San Isabel National Forest. Means are least-square means. Error bars represent LSD; bars which do not overlap are significantly different at *P*=0.10.

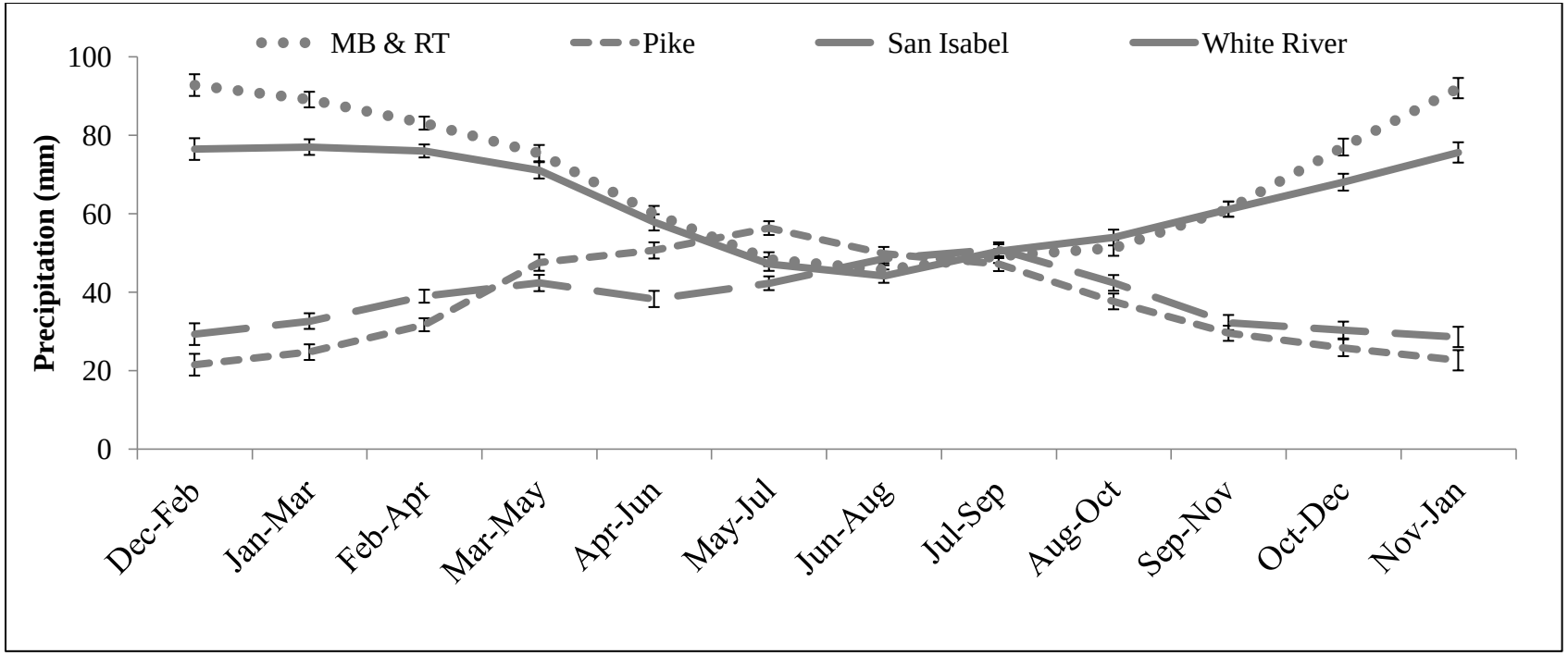

Figure 1.A16. Three-month running averages of precipitation (mm), by National Forest from 1950-2008. Error bars represent LSD; bars which do not overlap are significantly different at *P*=0.10.

*MB & RT = Medicine Bow and Routt National Forests

Table 1.A1. Descriptive statistics of the residual chronologies used in RWI analyses from the output of two software programs (ARSTAN and COFECHA) from tree cores collected from adult quaking aspen on five National Forests in Colorado and southern Wyoming in 2009-2010.

	Residual Chronologies (ARSTAN)							Raw Chronologies-(COFECHA)	
	Running r-bar								
National Forest	Year interval	No. series	rbar	EPS	Years	Total No. series	Mean, mm (SE)	Mean Sensitivity	SIC†
Medicine Bow & Routt	1890-1940	15	0.253	0.832	1883-2008	21	1.00 (0.158)	0.318	0.428
	1915-1960	20	0.208	0.838					
Pike	1860-1910	14	0.307	0.856	1857-2008	18	1.00 (0.205)	0.373	0.45
	1885-1935	17	0.300	0.878					
	1910-1960	18	0.311	0.888					
San Isabel	1860-1910	19	0.390	0.922	1856-2008	30	0.992 (0.177)	0.365	0.467
	1885-1935	25	0.260	0.898					
	1910-1960	29	0.302	0.925					
White River*	1910-1960	7	0.353	0.801	1906-2008	8	0.99 (0.344)	0.326	0.362

*Series not used in RWI analysis
†Series intercorrelation coefficient

Table 1.A2. Site and stand characteristics of healthy and damaged aspen stands on eleven ranger districts and five national forests. Estimates are least-squares means. Superscript letters indicate significance differences between the means at $p \leq 0.10$. Means in the same column with the same letter are not significantly different from each other.

Forest	Stand type	Site type[x]	Elevation (m)	Aspect (degrees)	% Slope	% Conifer Encroachment	Depth 'A' horizon	Stems ha^{-1} (adults)	Stems ha^{-1} (understory)	Basal area ha^{-1} (m^2) (adults)	QMD† (cm) (adults)	% Live stems (adults)	% Live stems (understory)	% Dead Crown (adults)
Medicine Bow & Routt	Healthy	LCR*	2559^{a}	155^{bc}	11.8^{a}	10.5^{b}	16.4^{a}	2446^{b}	10612^{b}	108.2^{c}	23.8^{bc}	85.4^{bc}	93.0^{cd}	22.3^{ab}
		HCR*	2641^{a}	112^{ab}	14.3^{a}	7.8^{ab}	19.9^{a}	2178^{b}	9750^{b}	89.5^{bc}	24.5^{bc}	82.5^{bc}	91.1^{cd}	25.9^{b}
	Damaged	LCR	2580^{a}	162^{c}	12.6^{a}	5.2^{a}	19.8^{a}	2103^{b}	9689^{b}	79.4^{bc}	23.1^{bc}	61.3^{a}	92.9^{cd}	47.7^{c}
		HCR	2712^{b}	204^{c}	23^{bc}	0.5^{a}	21.3^{a}	1792^{ab}	6854^{bc}	81.9^{bc}	24.4^{bc}	63.3^{a}	91.6^{bcd}	48.6^{c}
Pike	Healthy	LCR	2921^{bc}	175^{bc}	6^{a}	5.5^{ab}	14^{a}	1200^{ab}	3854^{a}	19.9^{a}	17.2^{ab}	50.0^{a}	95.0^{bcd}	50.6^{c}
		HCR	3069^{c}	187^{bc}	12.3^{ab}	0.4^{a}	14.5^{a}	1800^{ab}	4625^{abc}	80.2^{b}	21.4^{ab}	79.5^{bc}	64.2^{a}	7.9^{a}
	Damaged	LCR	3077^{c}	185^{bc}	23^{bc}	4.7^{a}	13.6^{a}	1864^{ab}	4083^{ab}	50.0^{ab}	17.0^{a}	63.6^{a}	75.1^{abc}	39.4^{c}
		HCR	3040^{c}	43^{a}	8a	4.7^{a}	13^{a}	2425^{ab}	4035^{ab}	75.1^{abc}	20.1^{ab}	62.8^{ab}	92.9^{bcd}	44.6^{bc}
San Isabel	Healthy	LCR	2678^{ab}	162^{bc}	30.2^{c}	7.0^{ab}	15.3^{a}	2042^{ab}	5166^{ab}	63.6^{ab}	20.7^{ab}	81.7^{bc}	77.1^{abc}	18.6^{a}
		HCR	2941^{c}	181^{c}	18.8^{ab}	7.8^{ab}	13.7^{a}	2025^{ab}	4725^{abc}	61.4^{ab}	20.1^{ab}	82.0^{bc}	73.5^{abc}	23.8^{ab}
	Damaged	LCR	-	-	-	-	-	-	-	-	-	-	-	-
		HCR	2914^{c}	133^{b}	18.1^{ab}	10.6^{b}	15^{a}	2036^{b}	6172^{abc}	52.4^{a}	18.1^{ab}	65.2^{a}	76.3^{abc}	44.0^{c}
White River	Healthy	LCR	2693^{ab}	228^{c}	26.6^{bc}	6.9^{ab}	65.6^{b}	2121^{b}	4350^{ab}	82.8^{b}	22.2^{b}	89.1^{bc}	74.3^{abc}	13.9^{a}
		HCR	-	-	-	-	-	-	-	-	-	-	-	-
	Damaged	LCR	2660^{ab}	229^{c}	31.6^{c}	1.5^{a}	66^{b}	1233^{a}	4883^{ab}	62.8^{ab}	26.0^{c}	67.6^{a}	96.0^{d}	45.7^{c}
		HCR	-	-	-	-	-	-	-	-	-	-	-	-

* Low (LCR) and high (HCR) cohesive response types. †QMD: quadratic mean diameter. QMD=$\sqrt{(\frac{BA*Frequency}{k*TpHa})}$

Table 1.S1A. Specific site data for each core sampling plot within the Medicine Bow and Routt National Forests used in dendroclimatic analyses.

National Forest	Plot number	No. cores	Elevation	Aspect	Slope position	% Slope	Other tree species	Dominant plants
Medicine Bow & Routt N.F.	BCD1	3	2552	173	Backslope	38	*Pinus contorta*	*Carex* sp., *Geranium* sp.
	BCH1	2	2680	170	Backslope	10	*Abies lasiocarpa*	*Carex* sp., *Lupinus* sp.
	BCH4	1	2680	154	Backslope	6	*Abies lasiocarpa, Pinus flexilis*	*Lupinus* sp., *Symphoracarpus* sp.
	HPD2	3	2660	330	Toeslope	10	*P. tremuloides* only	*Lupinus* sp.
	HPH2	3	2400	40	Backslope	25	*Picea engelmanii*	*Monarda* sp.
	YAD1	2	2912	240	Toeslope	36	*P. tremuloides* only	*Pastinaca* sp., *Delphinium* sp.
	YAD3	1	2715	160	Backslope	18	*P. tremuloides* only	*Pastinaca* sp., *Delphinium* sp.
	YAH1	3	2766	100	Backslope	11	*Pinus contorta, Picea engelmanii*	*Pastinaca* sp., *Veratrum* sp.
	YAH4	2	3013	156	Backslope	10	*Picea engelmanii, Abies lasiocarpa*	*Fragaria* sp., *Wyethia* sp.

Table 1.S1B. Specific site data for each core sampling plot within the Pike National Forest used in dendroclimatic analyses.

National Forest	Plot number	No. cores	Elevation	Aspect	Slope position	% Slope	Other tree species	Dominant plants
Pike N.F.	SPD1	1	3183	279	Backslope	20	*Picea engelmanii*	*Fragaria* sp., *Rosa* sp.
	SPD3	2	3123	45	Backslope	6	*P. tremuloides* only	*Arctostaphylos* sp.
	SPD4	2	2957	40	Backslope	10	*P. tremuloides* only	*Rosa* sp., *Thermopsis* sp.
	SPD7	2	3100	170	Backslope	20	*P. tremuloides* only	*Poa* spp.
	SPH2	3	3000	20	Summit	13	*P. tremuloides* only	*Juniperus* sp.
	SPH3	3	3060	159	Backslope	15	*P. tremuloides* only	*Poa* spp.
	SPH4	3	3020	147	Backslope	8	*P. tremuloides* only	*Juniperus* sp.
	SPH6	2	3137	301	Backslope	11	*Picea engelmanii*	*Fragaria* sp., *Lupinus* sp.

Table 1.S1C. Specific site data for each core sampling plot within the San Isabel National Forest used in dendroclimatic analyses.

National Forest	Plot number	No. cores	Elevation	Aspect	Slope position	% Slope	Other tree species	Dominant plants
San Isabel N.F.	SAD1	2	2973	110	Backslope	30	*Pinus ponderosa*	*Juniperus* sp., *Rosa* sp.
	SAD2	1	2776	10	Backslope	10	*Picea engelmanii*	*Arctostaphylos* sp., *Juniperus* sp.
	SAD3	1	2877	204	Backslope	10	*P. tremuloides* only	*Ribes* sp., *Thermopsis* sp.
	SAD4	2	2984	45	Backslope	10	*Abies lasiocarpa*, *Picea engelmanii*	*Thermopsis* sp., *Iris* sp.
	SAD5	3	2982	270	Backslope	11	*Picea engelmanii*	*Artemesia* sp., *Thermopsis* sp.
	SAD6	2	2932	170	Backslope	15	*P. tremuloides* only	*Lupinus* sp., *Rosa* sp.
	SAD7	3	3006	48	Backslope	17	*P. tremuloides* only	*Thermopsis* sp., *Eriogonum* sp.
	SAD8	3	3034	214	Backslope	5	*P. tremuloides* only	*Thermopsis* sp., *Geranium* sp.
	SAH2	1	3118	180	Backslope	35	*P. tremuloides* only	*Poa* spp.
	SAH3	2	2836	345	Toeslope	13	*Abies lasiocarpa*, *Picea engelmanii*	*Acer* sp.
	SAH6	3	3010	48	Backslope	14	*Picea engelmanii*	*Thermopsis* sp., *Lupinus* sp.
	SAH7	3	2907	71	Valley bottom	8	*Picea engelmanii*	*Thermopsis* sp., *Juniperus* sp.
	SCD1	1	2445	116	Backslope	27	*Pinus ponderosa*	*Poa* spp., *Thermopsis* sp.
	SCD2	1	2951	146	Summit	18	*Pinus edulis*, *Pseudotsuga menziesii*	*Artemesia* sp., *Eriogonum* sp.
	SCD3	1	3156	28	Backslope	65	*Picea engelmanii*	*Thermopsis* sp., *Rosa* sp.
	SCH1	1	2980	110	Backslope	17	*Picea engelmanii*, *Pinus flexilis*	*Pastinaca* sp., *Ribes* sp.
	SCH2	1	2695	329	Toeslope	26	*Picea engelmanii*, *Pinus ponderosa*	*Fragaria* sp., *Juniperus* sp.

Table 1.S2. Increment core collection site locations used in this study.

Plot name	UTM, Easting	UTM, Northing
ASD06	302968.9	4333840.9
ASD07	302915.2	4341742.2
ASH06	296896.4	4364379.2
RID01	261907.0	4409443.7
RIH01	267115.2	4410244.0
SCD03	446913.1	4217969.8
SCH01	490202.4	4123126.2
SCH03	489489.9	4212748.6
SCH04	441219.1	4233311.4
YAD01	347853.4	4446339.5
ASD01	323321.4	4368330.3
ASD02	355434.0	4363062.0
ASD03	361506.7	4352074.8
ASD04	320266.1	4344812.4
ASD05	343649.4	4326412.2
ASH01	327775.1	4373695.8
ASH02	358239.3	4360189.0
ASH03	351338.2	4331725.7
ASH04	328034.7	4340693.3
ASH05	330751.1	4328836.1
SCD01	490219.8	4220510.1
SCD02	485636.8	4198899.6
YAD03	365115.1	4469252.6
YAD02	366275.1	4432525.5
YAD04	307019.3	4444652.6
YAH01	364894.9	4436663.5
YAH02	365725.0	4468970.2
YAH03	312373.6	4454103.4
YAH04	328281.5	4434662.2
YAH05	325894.5	4448819.0
BLD02	271669.7	4448902.4
BLH03	278179.8	4434469.6
BLD03	303707.4	4435629.3
BLH04	300199.7	4438553.3
BLH06	295514.8	4438082.0
BLH01	308000.0	4432352.5
BLH05	293184.6	4431318.4
BLD05	284884.1	4437876.8
BLD06	276941.0	4418036.5
BLD01	254956.4	4415416.9
BLH02	256603.1	4409264.6
BLD04	283510.1	4418310.4
HPD02	294672.5	4515795.8
HPD01	294659.6	4521540.7
HPD03	306570.4	4519745.7

HPH03	306758.7	4523487.6
HPH01	320758.6	4520019.1
HPH02	323140.2	4516454.6
HPH04	363956.3	4472872.4
SPD01	447092.5	4300274.0
SPH01	456224.6	4329026.2
SPH02	447437.5	4302032.1
SPD04	410266.9	4322323.6
SPD05	466391.6	4327784.5
SPD06	419834.5	4355886.3
SPD07	415313.3	4348309.1
SPD03	408116.2	4334529.0
SPH06	443070.6	4293541.5
SPD02	431745.2	4364322.1
SPH03	435144.2	4363217.0
SPH04	436941.1	4358524.2
SPH07	447748.7	4346356.3
SPH05	408703.3	4319654.2
SAD02	398955.0	4277701.1
SAD04	409072.7	4310590.3
SAD05	419397.4	4302439.3
SAH07	418499.2	4292680.3
SAD06	419557.8	4295488.5
SAD07	419165.2	4286537.9
SAD08	419148.5	4282656.9
SAH05	398458.0	4254223.5
SAH06	417050.5	4303573.2
SAD03	407883.1	4307614.8
SAH01	390470.5	4286082.3
SAH03	390972.4	4293957.6
SAH02	382800.7	4291891.8
SAH04	391876.6	4272116.1
SAD01	394942.8	4295194.9
YAD05	322520.5	4434960.0
DOD01	458318.0	4679403.0
DOD02	457354.0	4674913.0
DOH01	445366.0	4690422.0
LAD02	472883.0	4561898.0
LAH02	466252.0	4556893.0
LAH01	463409.0	4566839.0
LAD01	472334.0	4570564.0
LAD03	467864.0	4560941.0
LAH03	467987.0	4562645.0
BCH03	317819.0	4549730.0
BCD04	321287.0	4547852.0
BCD01	318951.0	4554172.0
BCH01	324273.0	4571358.0
BCD02	313493.0	4574912.0

BCH02	313781.0	4561531.0
BCD03	310327.0	4552837.0
BCH04	340699.0	4560114.0
SCH02	456259.5	4204016.7

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CHAPTER 2

PATHOGENICITY OF TWO SPECIES OF *CYTOSPORA* TO QUAKING ASPEN (*POPULUS TREMULOIDES* MICHX.)

SUMMARY

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 has recently been described (tentatively named *Cytospora notastroma*). Here, we show the relative pathogenicity of both species. Small-diameter aspen trees were inoculated with one or two isolates each of *C. chrysosperma* and *C. notastroma* in a greenhouse, outdoor setting, and in environmental growth chambers. Results indicate that both species are pathogenic to drought-stressed trees and that *C. chrysosperma* was more aggressive than *C. notastroma* at cool temperatures. Neither species caused significant canker growth on trees that were not drought stressed.

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 best in montane environments, where summer temperatures are cool, and moisture is plentiful (Little, 1971). When grown on marginal sites, such as the dry, eastern front range of the Rocky Mountains in Colorado, aspen is prone to damage by stress and subsequent impact by 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 saprophyte (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). Worrall et al. (2010) showed that *Cytospora umbrina* is present in asymptomatic cambial tissue of alder (*Alnus incana* ssp. *teniufolia*), and could be isolated from host tissues for up to 5 cm from canker margins. Conversely, McIntyre et al. (1996) examined healthy quaking aspen for presence of *Cytospora* species, but not did 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). In some *Cytospora* host species, excessive gum and starch production at the site of infection appear to be a major cause of vessel occlusion and tissue death (Banko and Helton, 1974). 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; 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) showed that aspen exposed to drought conditions remained susceptible to infection up to ten days post-wounding, relative to four days post-wounding in watered trees. Bloomberg and Farris (1963) showed that poplars wounded by scorching of the bark produced tannins and lignin at the wound site, and

when wounds were inoculated with an isolate of *C. chrysosperma*, levels of both defensive compounds were greatly increased. Canker growth varied inversely with the amount of tannin produced, both in terms of numbers of tanniferous cells, and the size of the tanniferous zone (Bloomberg and Farris, 1963).

Biggs et al. (1983) described the histopathology of *C. leucostoma* in peach. Infection begins with large-diameter, wedge-shaped hyphae that grow into the bark components of periderm, cortex, and phloem tissues, killing host cells as they advance (Biggs et al. 1983). This disruption of host tissue is followed by inter- and intra-cellular growth by smaller-diameter hyphae (Biggs et al. 1983). The fungus invades xylem tissues and moves faster longitudinally, via pits in cell walls, or through direct attack with penetration pegs, giving the disease its typical elongated shape (Biggs 1984). Host tissues respond by producing necrophylactic periderm or non-suberized impervious tissue, although the presence of pathogen mycelium inhibits the host's ability to form these defensive structures (Biggs 1984). Biggs (1986) identified a critical period of 10-14 days post-wounding for the host to prevent widespread colonization by the fungus. A thickness of only three cells of phellem tissue in the phloem is sufficient to halt the spread of the pathogen (Biggs 1986).

The phylogeny 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 *Valsa* (*Cytospora*) 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 now it is known that a single *Cytospora* species can infect multiple

hosts. Adams et al. (2006) also described the worldwide distribution of *Cytospora* and its species complexes based on the characteristics of anamorphic and teleomorphic fruiting body structure. 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, Adams, and others have observed, the wide range of variability in morphological characteristics is likely due to environmental and host conditions, in addition to various phenotypic differences (Spielman, 1985; Adams et l. 2005 & 2006; Kepley and Jacobi 2000).

Kepley et al. (2015) have recently described a new species of *Cytospora* in quaking aspen based on analysis 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; Figure 2.. 1). However, the pathogenicity of this species to quaking aspen has not been examined.

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.; 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). Further, canker size was inversely related to tree water potential when it dropped below (i.e. became more negative than) -1.6 MPa (Guyon et al. 1996).

The relative pathogenicity, abundance, and phylogeny of *C. chrysosperma* and *C. notastroma* on quaking aspen are unknown. The objectives of this study are: (1) is *C. notastroma* pathogenic to quaking aspen? (2) Are there differences in pathogenicity between *C. chrysosperma* and *C. notastroma* on quaking aspen? (3) Do the two species behave similarly under different temperature ranges on quaking aspen? To address these questions, we conducted a series of inoculation trials with two phylogenetically distinct isolates each of *C. chrysosperma* and *C. notastroma* on seedling (< 1.4 m tall) and sapling-sized aspen trees (1-2 cm DBH) in the greenhouse, outdoor, and growth chamber settings to simulate various light and temperature combinations

MATERIALS AND METHODS

Fungal cultures

Two *C. chrysosperma* isolates ('BDSR 1.2', 'DG11A') were cultured from quaking aspen bark collected in the Red Feather Lakes, Colorado, area (N40.752976, W-105.498122), and from the Dadd Gulch trailhead (N40.682286, W-105.642762), Poudre Canyon, Colorado, respectively. The *C. notastroma* isolate RCKEP3A was originally collected by J. Kepley, from quaking aspen bark collected from the Roaring Creek campground (N40.714227, W-105.734967) in the Poudre Canyon in October of 2002, and represents the holotype of this species. The *C. notastroma* isolate SW8C was collected from a quaking aspen in the Denver suburb of Aurora, Colorado (39.728981, -104.813863). The single-spore isolates were identified to species level by morphological characteristics of the pycnidia and perithecia in bark, colony

appearance and color on Leonian's modified growth medium (Leonian, 1921), and later by sequence comparisons of their rDNA internal transcribed spacer (ITS) regions.

Aspen nursery stock

Seedling-sized quaking aspen 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. Seedling-sized trees (2-2.5 m tall) were obtained from the Little Valley tree nursery in Brighton, CO, in the spring of 2013. Trees were grown in standard potting mix and planted in number 2 plastic nursery containers.

Leaf water potential measurements

Drought stress was induced and measured by determining pre-dawn 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.

Growth chamber inoculation trials

Eight, 65-90 cm-tall seedling-sized aspen were placed in a diurnal growth chamber, with temperatures set to fluctuate in a cool environment, from 15 °C during the light cycle and 12 °C during the dark cycle, or 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 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 on the tier below the light source. The light intensity of the Caron® growth chamber averaged 75 $\mu mol^{-2}m^{-2}s^{-1}$ and the Percival® chamber averaged 28 $\mu mol^{-2}m^{-2}s^{-1}$. 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 seedling-sized aspen trees. Three wound sites were placed at 10 cm intervals along the stem, and surface-sterilized prior to wounding by rubbing the bark surface with 70% ethanol. Wounds were produced along the tree stem using a 7-mm cork borer to remove the bark. A plug of half-strength potato dextrose agar (1/2 PDA) of the same diameter, or a ½ PDA plug colonized with either a single isolate of *C. chrysosperma* or *C. notastroma* was inserted into the wound. The wound was then sealed by wrapping the stem with a 2 cm-wide strip of Parafilm®.

Greenhouse and outdoor inoculation trials

Thirty-two sapling-sized aspen trees in standard potting mix and five-gallon plastic pots were placed on benches in July 2013 in a Colorado State University greenhouse and 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, in order 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 inoculations per tree. Temperature data was recorded hourly using a Watchdog® (Spectrum Technologies, Inc., Plainfield, IL) temperature recorder.

In mid-September, a second, identical, inoculation trial was initiated, this time outdoors. Rainwater was excluded from the drought treatment group by clear plastic sheeting attached to the tree base with parafilm or electrical tape and covering the pot, and drought was induced to -1.0 to -1.5 MPa. In both trials, canker size was measured one week after inoculation, and again every 2-3 days afterwards until nighttime temperatures dropped low enough to initiate tree dormancy. Hourly weather data for the outdoor trial site was obtained from the Colorado Climate Center's weather station, located on the Colorado State University campus (http://ccc.atmos.colostate.edu/~autowx/fclwx_access.php) (Table 2.S1).

Canker measurements

Canker size was first assessed one week after inoculation. At that time, Parafilm® was removed and initial canker length and width were recorded in millimeters. Canker boundaries were determined as the edge of the discolored bark tissue, and were re-measured on all trees for all trials (i.e. growth chamber, greenhouse, and outdoors) at least once, and were re-measured every other day following the initial measurements for the greenhouse and outdoor trials. The total number of measurements varied by trial, with a maximum of seven measurements of cankers on trees in the greenhouse, three measurements on trees outdoors, and two measurements on trees in the growth chamber setting. The number of measurements taken for each trial was dependent upon tree health, and in the case of the outdoor trial, the onset of autumn.

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). Statistical analyses were performed using SAS© 9.4 software (SAS Institute, Cary,

NC). 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 with temperature, treatment, isolate, measurement time, as well as the temperature, isolate, treatment interaction within measurement date. The random variables included tree within temperature, and position by isolate by position interaction within temperature. We obtained 95% confidence intervals for canker size for each isolate-temperature combination, and intervals for each combination were considered significant at $P \leq 0.05$. Water potential data were analyzed (as the average of two water potential readings per tree) as a repeated measures mixed linear model. Average water potential was analyzed as treatment type by measurement date interaction. Least-squares means were obtained and were considered to be significantly different at the $P \leq 0.05$ level. 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 and outdoor inoculation trial data were analyzed together and separately from the four growth chamber inoculation trials. Because some of the cankers on trees in the greenhouse trial coalesced after two weeks, we used canker size at twelve days post-inoculation for both trials in our analyses. Canker area was analyzed as a mixed linear model with treatment (i.e. drought stressed or watered), isolate, setting (greenhouse or outdoor), 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 considered to be significantly different at the $P \leq 0.05$ level. Position (i.e. placement of the inoculum along the stem) was initially included in the analysis, but was later removed due to non-significance.

RESULTS

Growth chamber inoculation trials

Drought stressed trees averaged -1.0 and -0.9 MPa in the warm and cool temperature growth chambers respectively, whereas watered trees averaged -0.7 and -0.45 MPa in the warm and cool growth chambers (Figure 2.2).

Watered trees inoculated with one isolate each of *C. chrysosperma* and *C. notastroma* species did not develop cankers in either temperature treatment (Table 2.1). Among the seedling-sized aspen inoculated with an isolate of either *C. chrysosperma* or *C. notastroma* or one of each, drought-stressed trees in the cool temperature trial and inoculated with *C. chrysosperma* displayed expanded mean canker growth in comparison with the control and the *C. notastroma* inoculated sites (Table 2.1, Figure 2.3). Both *Cytospora* species caused canker formation on trees in the warm temperature trial, relative to the control wound (Table 2.1, Figure 2.3). Wound sites amended with ½ 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 illustrated in Figure 2.3 and Tables 2.1 and 2.2. For example, of the16 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 2.2). 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 2.2).

Greenhouse inoculation trials

Water potential data indicated that drought-stressed trees in the greenhouse averaged -1.11MPa (Figure 2.4). 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 (Figure 2.5). The effect on mean water potential can be seen in Figure 2.5.

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 cankers 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 2.1; Figure 2.6). The largest cankers were formed on drought-stressed trees at sites inoculated with *C. chrysosperma* isolate DG11A (Table 2.1 Figure 2.6 and 2.9). However, there was no difference in canker area between the two *C. chrysosperma* isolates, and cankers formed by the second *C. chrysosperma* isolate, BDSR12, were not larger than those formed by *C. notastroma* isolate SW8C (Table 2.1; Figures 2.6 and 2.9). When considering only sites where cankers were observed, both *C. chrysosperma* isolates and *C. notastroma* SW8C had larger areas of discoloration than those observed at wounds in the non-inoculated sites (Table 2.2) The combined range in canker sizes among infected aspen (i.e. those trees on which cankers formed) in the greenhouse and outdoor trials was 38-6,927mm^2 for the *C. chrysosperma* isolates, and 38-6024 mm^2 for *the C. notastroma* isolates (Table 2.1). Four of the drought-stressed aspen trees did not develop any cankers at all.

Outdoor inoculation trial

Drought-stressed trees in the outdoor trial averaged -0.9 MPa at the time of inoculation and for the remainder of the experiment, whereas watered trees averaged -0.3 MPa (Figure 2.4). The only significant difference in canker sizes was between drought-stressed trees inoculated with *C. chrysosperma* isolate BDSR12, which formed cankers larger than control sites, but not larger than either of the two *C. notastroma* isolates (P=0.0597), or the second *C. chrysosperma* isolate on drought-stressed or watered trees (Table 2.1; Figures 2.8, 2.11 and 2.12).

DISCUSSION

The results of the inoculation trials conducted in this study strongly 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*. In a concurrent study of the relative distribution and frequency of these species, 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 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, as in the outdoor portion of this study, where values of drought-stressed trees averaged -0.9 MPa throughout the experiment.

When we compared the effects of temperature on canker development we observed significantly less canker growth of *C. chrysosperma* at high temperatures, based on infected aspen (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 assumed that drought stress combined with high temperatures would result in larger and more rapid canker expansion. We note that studies of other phytopathogenic fungal species, including *Geosmithia morbida*, and the bluestain fungus *Ophiostoma clavigera*, have demonstrated that these pathogens 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* is able to cause canker formation on drought-stressed host trees under both warm and cool conditions, although it was less aggressive under warm conditions. It seems possible that this is 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 beyond.

The stark phenotypic differences among quaking aspen clones' morphological characteristics and 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). We observed, especially in the greenhouse portion of this study, that some of the aspen did not develop cankers at all, in spite of showing high vapor pressure deficits, thus were clearly experiencing drought stress. It has been established that significant phenotypic differences 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). Based on this, it seems very likely that at least some of the variability in canker development we observed over the course of these experiments may be a reflection of varying disease resistance among aspen genotypes. Also noted in many previous studies of aspen and other *Populus* species (St. Clair, et al. 2010; Kanaga, et al. 2008; Marron, 2006), phenotypic traits are often influenced by environmental conditions; Kanaga, et al. (2008) and Marron (2006) describe the interaction between environment and phenotype as a cause of significant phenotypic plasticity. In this study, we examined only the variation in disease development from the perspective of the pathogen; we note that 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.

FIGURES AND TABLES

Figure 2.1. *Cytospora chrysosperma* and *C. notastroma* in culture and on inoculated aspen trees. Top: cultures of *C. notastroma* (left) and *C. chrysosperma* (right) on modified Leonian's medium, after two weeks' growth at 25 °C. Bottom row, from left: canker formation with pycnidia and cirri on aspen following inoculations with *C. notastroma* (left two photos), and *C. chrysosperma* (right two photos). Photos: M. Dudley

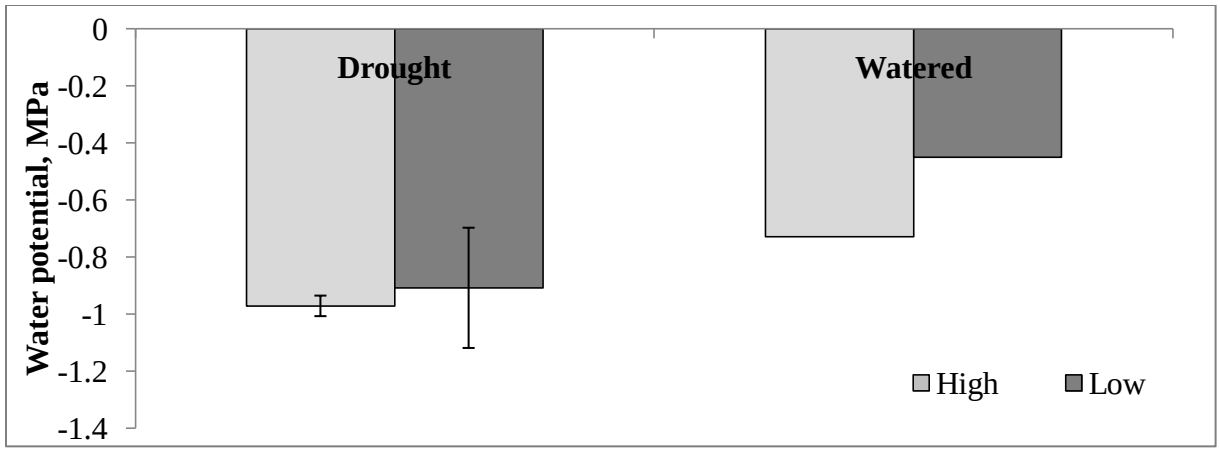

Figure 2.2. Mean pre-dawn water potentials for drought-stressed or watered quaking aspen trees placed in growth chambers at two different day/night (12 hour cycle) temperature regimes, high (32 °C day, 25 °C night), and low (15 °C day, 12 °C night) cycles Error bars represent standard error; bars which do not overlap are significantly different $P \leq 0.05$. Water potential mean for watered trees do not include error bars because only one watered tree per temperature treatment was used in this portion of the experiment.

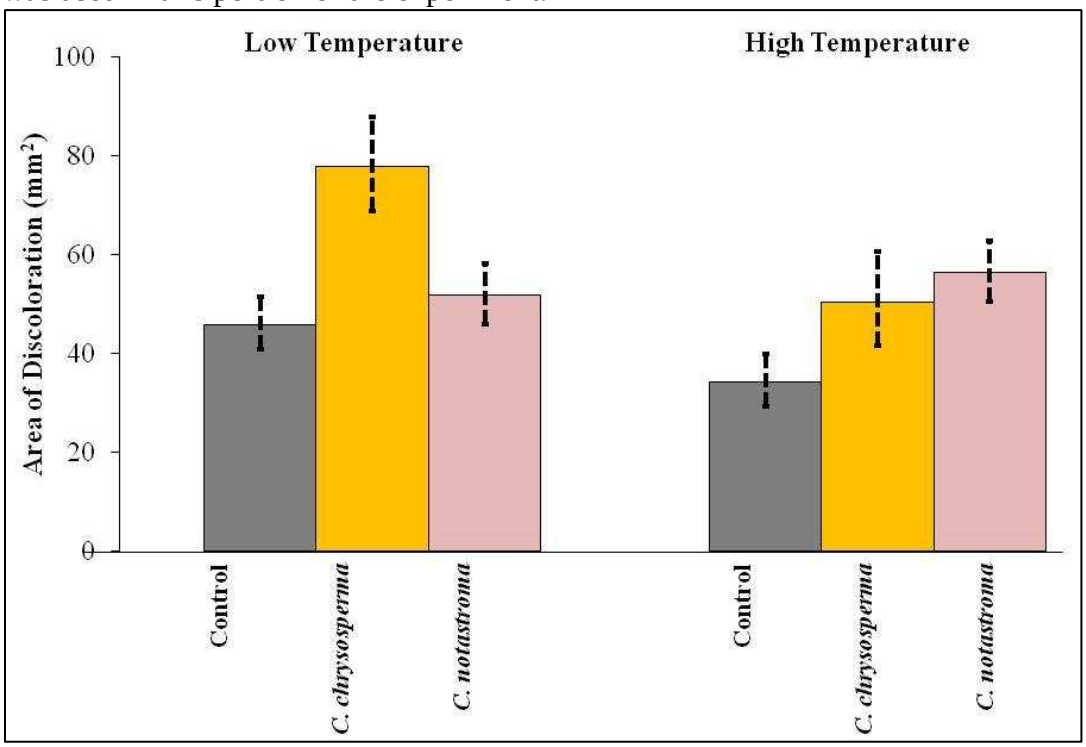

Figure 2.3. Mean canker size on small quaking aspen trees in a growth chamber setting four weeks after inoculation with a *Cytospora chrysosperma* or *C. notastroma* isolate under drought stress. Growth chamber temperature ranges were low (15/12 °C) or high (32/25°C), in 12-hour cycles. Means are least-squares, and dashed lines represent upper and lower limits of a 95 % confidence interval, and those that do not overlap are significant at P=0.05. Data are based upon two trials per temperature range.

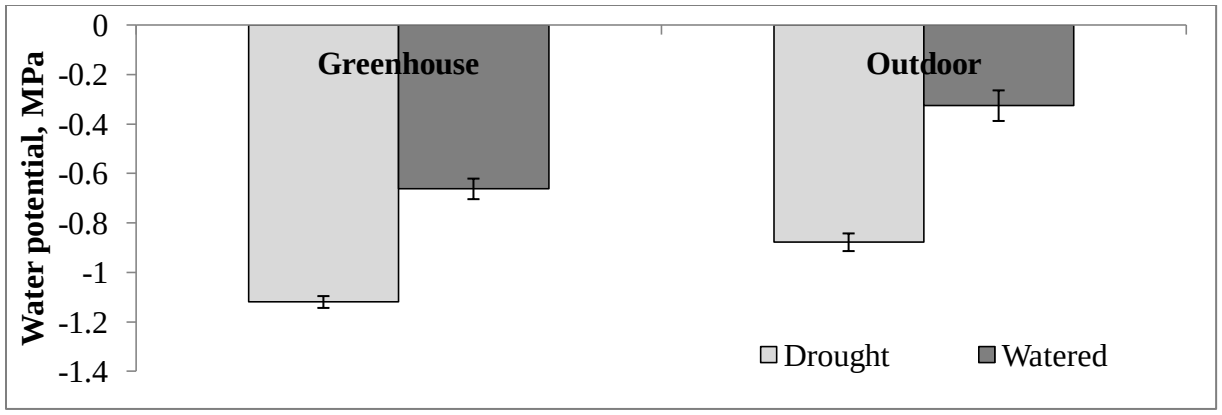

Figure 2.4. Mean pre-dawn water potentials for drought-stressed or well-watered aspen trees placed in a greenhouse or outdoor setting. Error bars represent standard error; bars which do not overlap are significantly different $P \leq 0.05$.

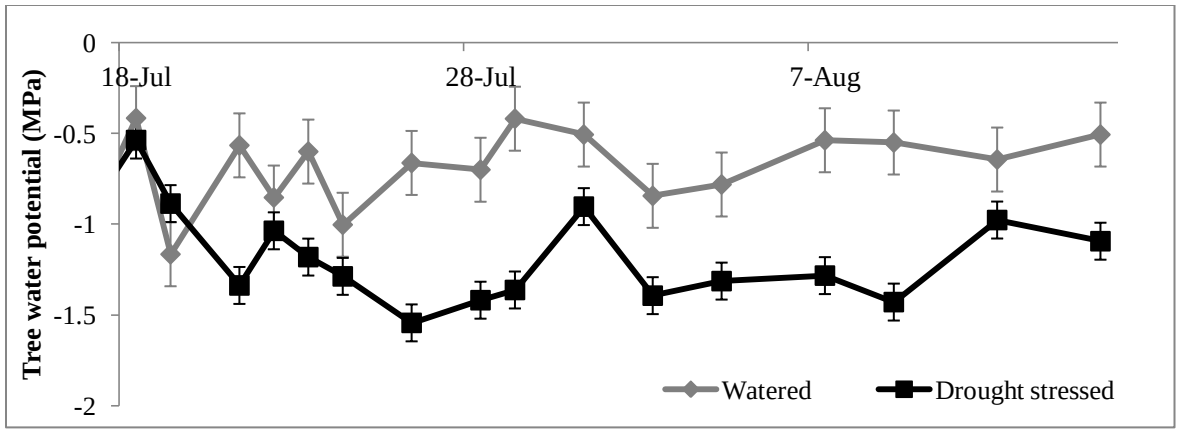

Figure 2.5. Mean pre-dawn tree water potential measurements in a greenhouse on eighteen days from mid-July to mid-August, 2013. Blue line represents pre-dawn water potential of watered trees; red line represents pre-dawn water potential of drought-stressed trees. Trees were inoculated on 23 Jul.

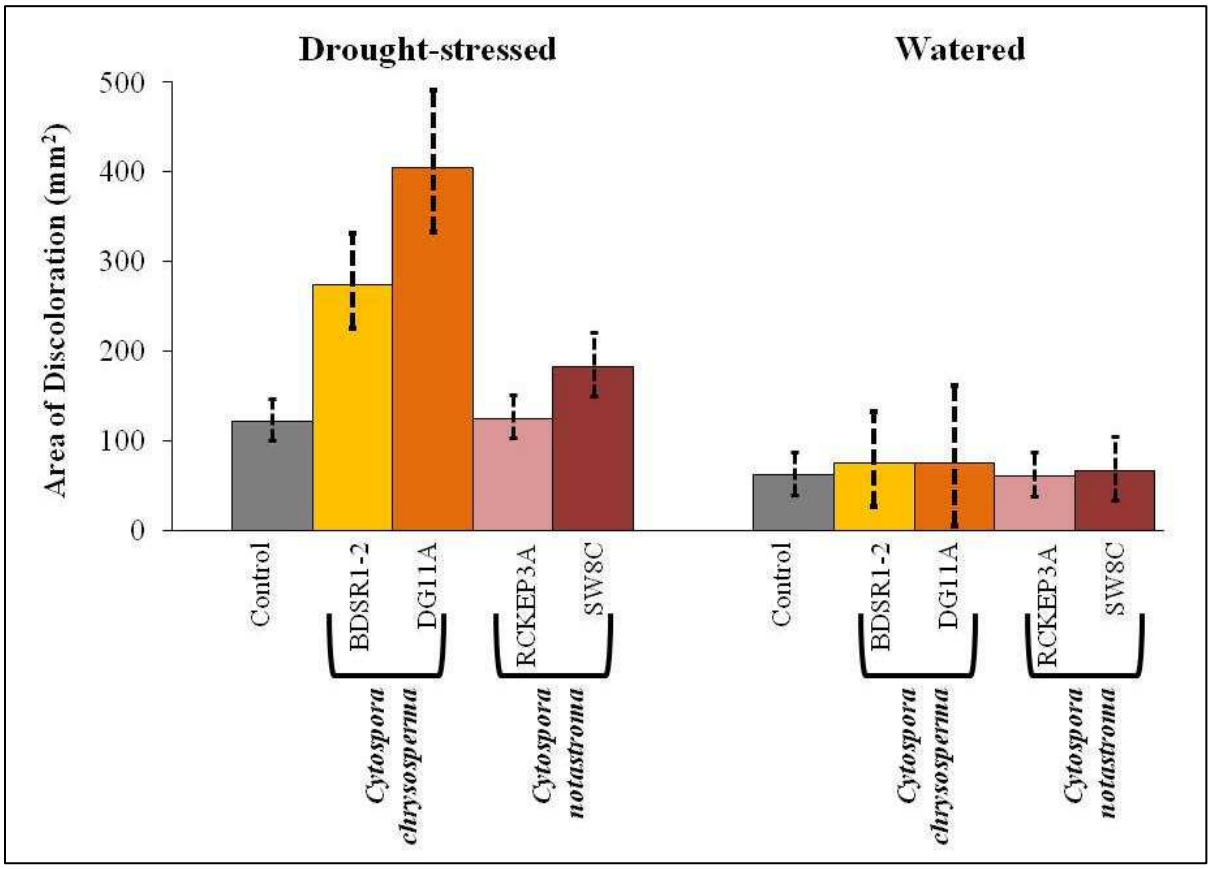

Figure 2.6. Canker size in quaking aspen at 12 days following inoculation with four *Cytospora* isolates under drought-stressed (left) and well-watered (right) conditions in a greenhouse. Wounds in control trees were amended with ½-strength potato dextrose agar. Means are least-squares, dashed lines represent upper and lower limits of a 95 % confidence interval, and those that do not overlap are considered significant at P=0.05.

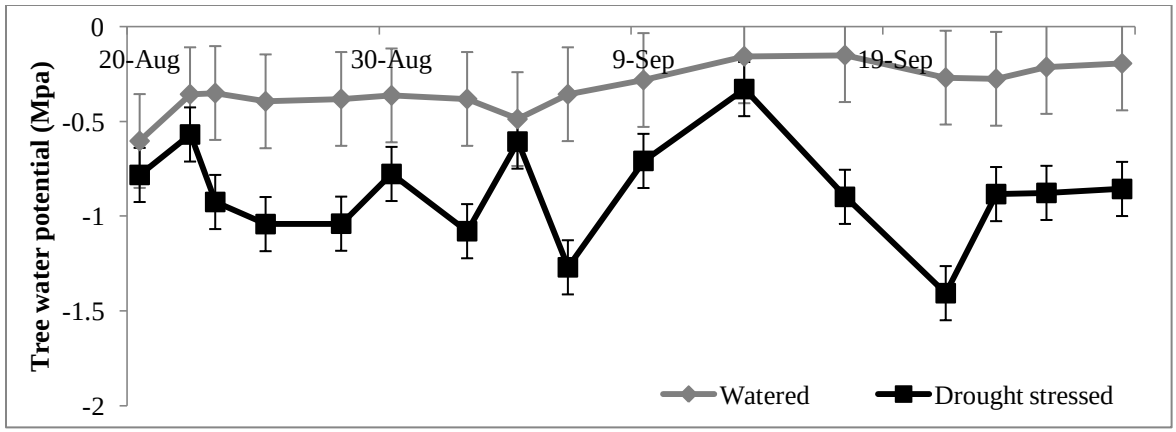

Figure 2.7. Mean pre-dawn tree water potential measurements in an outdoor setting on sixteen days from late August to late September, 2013. Blue line represents well-watered trees, red line represents drought-stressed trees. Trees were inoculated on 19 Sep.

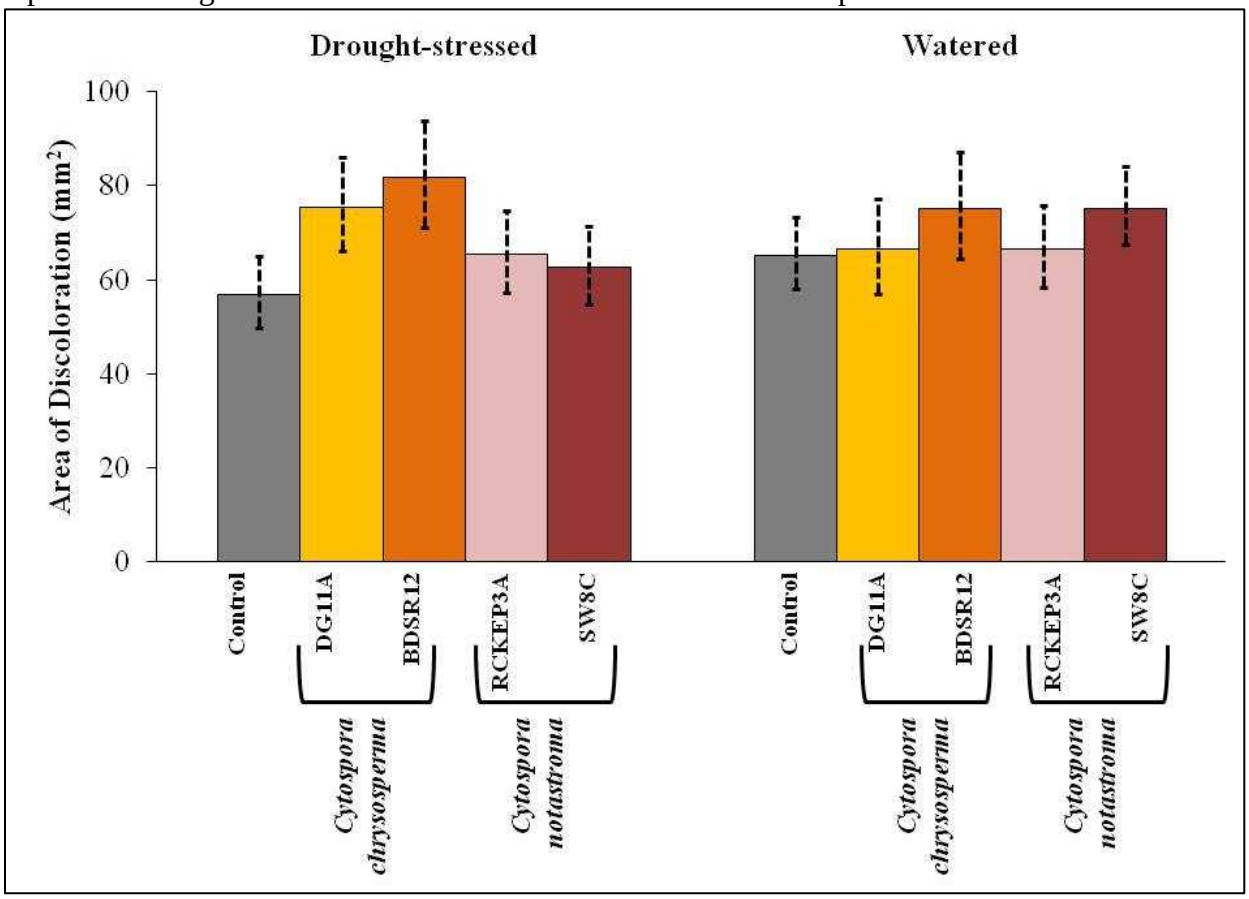

Figure 2.8. Canker size in quaking aspen at 12 days following inoculation with four *Cytospora* isolates under drought-stressed (left) and well-watered (right) conditions in an outdoor setting. Wounds in control trees were amended with a ½-strength potato dextrose agar plug. Means are least-squares, dashed lines represent upper and lower limits of a 95% confidence interval. Lines which do not overlap are significant at P=0.05.

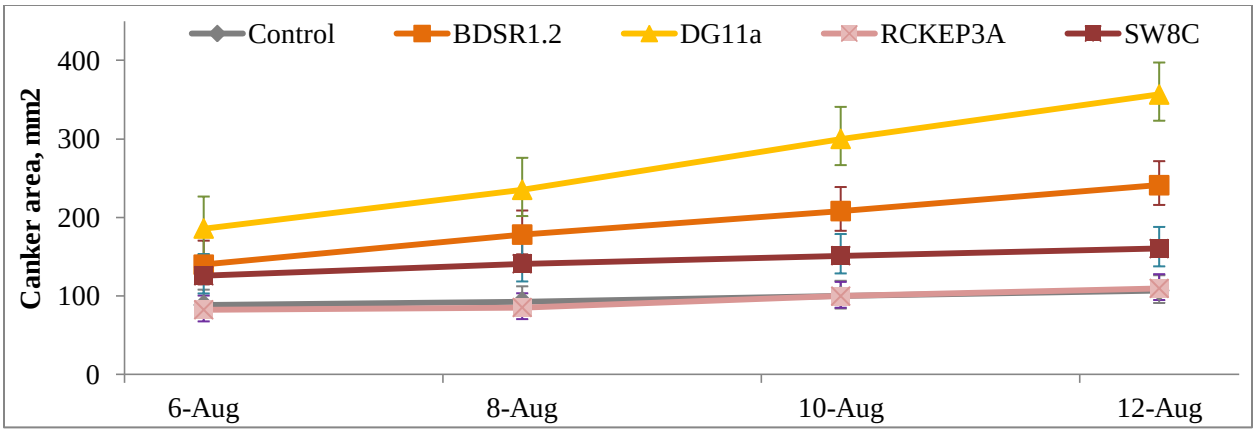

Figure 2.9. Canker development in drought-stressed quaking aspen over 12 days following inoculation (on 7/30/13) with two *Cytospora chrysosperma* (BDSR1.2 and DG11A) and two *C. notastroma* (RCKEP3A and SW8C) isolates in a greenhouse. Wounds in control trees were amended with a ½ strength potato dextrose agar plug. Means are least-squares, lines represent upper and lower limits of a 95% confidence interval. Lines which do not overlap are significant at *P*=0.05.

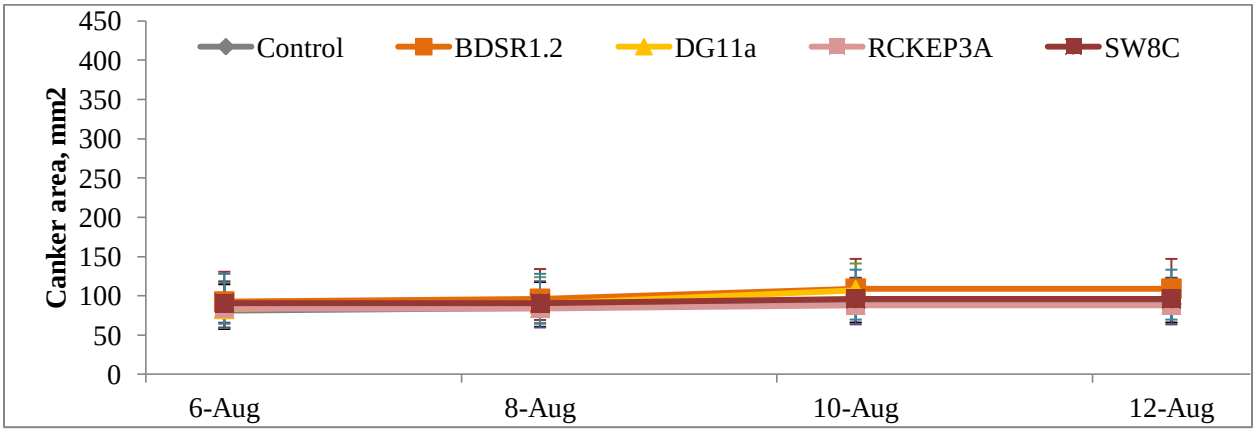

Figure 2.10. Canker development in watered quaking aspen during a two week period following inoculation (on 7/30/13) with two *Cytospora chrysosperma* (BDSR1.2 and DG11A) and two *C. notastroma* (RCKEP3A and SW8C) isolates in a greenhouse. Wounds in control trees were amended with a ½stregth potato dextrose agar plug. Means are least-squares, lines represent upper and lower limits of a 95% confidence interval. Lines which do not overlap are significant at *P*=0.05.

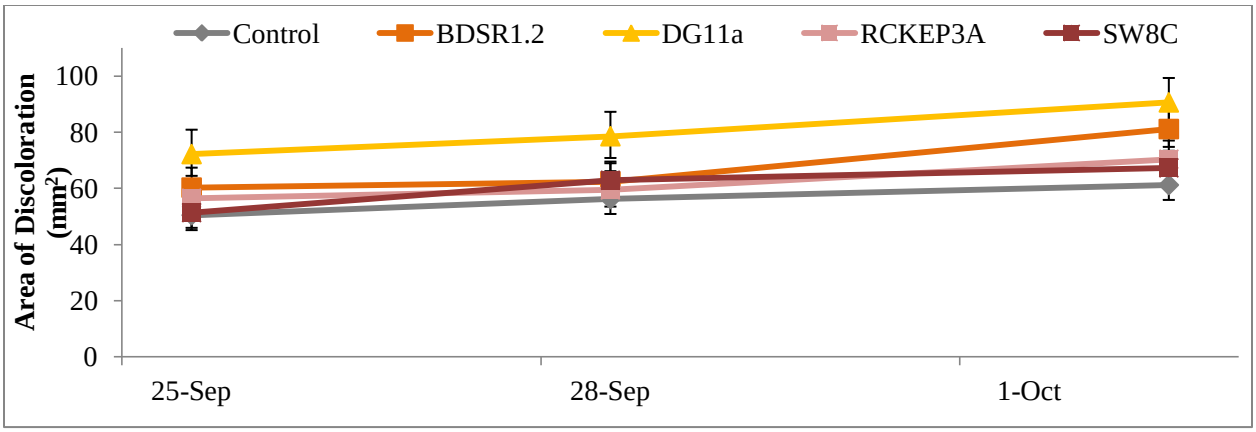

Figure 2.11. Canker development in drought-stressed quaking aspen over 8 days following inoculation (on 9/18/13) with two *Cytospora chrysosperma* (BDSR1.2 and DG11A) and two *C. notastroma* (RCKEP3A and SW8C) isolates in an outdoor setting. Wounds in control trees were amended with a ½ PDA plug. Means are least-squares, lines represent upper and lower limits of a 95% confidence interval. Lines which do not overlap are significant at *P*=0.05.

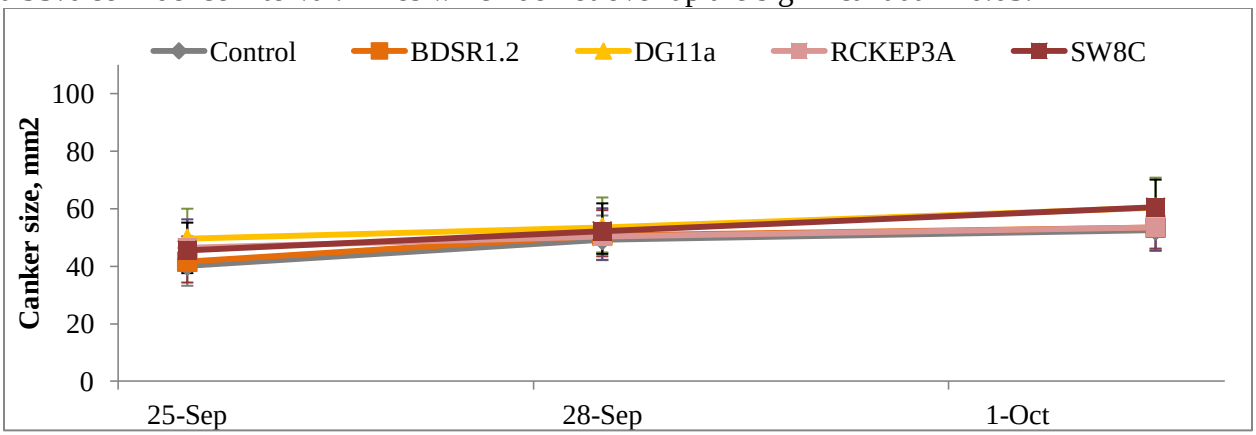

Figure 2.12. Canker development in watered quaking aspen over 8 days following inoculation with (on 9/18/13) two *Cytospora chrysosperma* (BDSR1.2 and DG11A) and two *C. notastroma* (RCKEP3A and SW8C) isolates in an outdoor setting. Wounds in control trees were amended with a ½ PDA plug. Means are least-squares, lines represent upper and lower limits of a 95% confidence interval. Lines which do not overlap are significant at *P*=0.05.

Table 2.1. Mean canker areas for those sites where cankers formed 12-14 days following inoculation with either *Cytospora chrysosperma* or *C. notastroma* in a low or warm temperature growth chamber, a greenhouse, and an outdoor setting.

			Canker area (mm^2)			
Species	**Isolate**	**Treatment**	**Growth chamber, Cool Temp[1, 2, 3] (n=48)**	**Growth chamber, Warm Temp[1, 2, 3] (n=48)**	**Greenhouse Trial[3, 4] (n=160)**	**Outdoor Trial[3, 4] (n=158)**
C. chrysosperma	DG11A	Drought	78 (9, 10)[c]	50 (6, 6) [b]	357 (64, 78)[c]	91 (9, 10)ab
		Watered	49*	25	110 (32, 45)[ab]	60 (11, 13)ab
	BDSR12	Drought	-	-	241 (43, 53[)c]	81 (7, 7)b
		Watered	-	-	109 (32, 45)[ab]	53 (9, 11)ab
C. notastroma	RCKEP3A	Drought	52 (6, 7) [b]	56 (6, 7) [b]	110 (20, 24)[a]	70 (7, 8)ab
		Watered	25	32	88 (26, 36)[a]	54 (9, 11)ab
	SW8C	Drought	-	-	160 (29, 35)[b]	67 (7, 7)ab
		Watered	-	-	96 (28, 39)[a]	61 (11, 13)ab
Control	Agar	Drought	46 (5, 6) [b]	34 (4, 4) [a]	107 (19, 23)[a]	61 (6, 7)a
		Watered	48*	26	90 (26, 37)[a]	52 (9, 11)ab

[1]Watered trees in the growth chamber trials do not include statistics because there was only one watered tree per temperature trial.[2] Comparisons are among all isolates in both warm and cool trials. [3]Numbers in parentheses are upper and lower confidence interval values, from the mean. [4] Greenhouse and outdoor means comparisons are by trial. 'n' is the number of inoculation sites. * A slightly larger-diameter cork-borer was used to inoculate these trees; means thus do not represent cankered area, but a larger wound.

Table 2.2. Canker formation 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)‡**
C. chrysosperma	DG11-A†	61%	98	64 (50-77); 173 (38-6,927)
	BDSR1-2§	50%	64	135 (38-5,342)
C. notastroma	RCKEP3A†	41%	98	54 (50-56); 80 (38-377)
	SW8C§	41%	64	294 (38-6,024)

*Three (growth chamber trials) or five (greenhouse and outdoor trials) 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 and outdoor inoculation trials. ‡Canker means for the greenhouse and outdoor trials are based on measurements taken 14 days after inoculation. 'n' is the number of inoculation sites.

Table 2.3. Mean diurnal temperature ranges and precipitation for the outdoor location used in this study. Greenhouse trial was conducted July-August, 2013; the outdoor trial was conducted September-early October, 2013; growth chamber trials were conducted at various four-week intervals between May 2013 and October 2014.

		Mean daytime temperature (°C)	Mean nighttime temperature (°C)	Mean daily precipitation (mm)*
Greenhouse & outdoor trials	Greenhouse	24.8	20.4	-
	Outdoor	17.3	12.8	5.6
Growth chamber trials	Warm cycle	32.1	26.0	-
	Cool cycle	15 (+-3)	12 (+-3)	-

*Outdoor setting only.

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CHAPTER 3

THE DISTRIBUTION OF TWO *CYTOSPORA* SPECIES ON QUAKING ASPEN (*POPULUS TREMULOIDES* MICHX.) IN SELECTED REGIONS OF THE ROCKY MOUNTAINS AND MINNESOTA IN THE UNITED STATES

SUMMARY

Cytospora canker of quaking aspen was thought to be caused primarily by *Cytospora chrysosperma*. Recently, a new and purportedly widely-distributed *Cytospora* species on quaking aspen has recently been described (*Cytospora notastroma* Kepley & F.B. Reeves,). Although we have previously established that the newly-described species is pathogenic, the abundance and frequency of *C. notastroma*, relative to *C. chrysosperma*, is unknown. Thus, we wished to estimate the relative abundance of known *Cytospora* species on quaking aspen throughout portions of the Rocky Mountain region, and to construct species-level phylogenies based upon isolates obtained from infected aspen. Here, we show: that both *C. chrysosperma* and *C. notastroma* are quite common on quaking aspen, along with a third, previously-described species, *C. nivea*; that *Cytospora* species often co-occur on the same host tree, and that evidence of recombination or possible hybridization between the species exists.

INTRODUCTION

Cytospora canker is a common fungal disease of many woody species throughout temperate regions of the globe (Adams et al. 2005; Sinclair and Lyon, 2005; Spielman, 1983; Hubert, 1918). Host genera known to be susceptible include *Populus*, *Acer*, *Salix*, *Prunus*, *Malus*, *Sambucus*, and *Sorbus*, as well as many conifer species, including those in the *Abies*,

Larix, *Picea*, *Juniperus*, *Cedrus*, *Pseudotsuga*, *Tsuga*, *Thuja*, and *Chamaecyparis* genera (Hubert, 1918; Sinclair and Lyon, 2005). *Cytospora* species are ubiquitous, and most exist as weak saprophytes on bark surface, causing damage only when the host experiences stressful environmental conditions (Sinclair and Lyon, 2005). When such favorable conditions do exist, *Cytospora* rapidly kills cambial tissue, effectively girdling and killing the host tree over months or years (Bloomberg, W.J., 1962a; Bloomberg, W.J., 1962b). Roughly 400 species of *Cytospora* have been described worldwide (Adams et al. 2005), although the disposition of species within the genus has been complicated and confusing (Long, 1918; Hubert, 1918; Christensen, 1940; Guyon, et al. 1996; Adams et al. 2005).

Recently, the teleomorph genus *Valsa* has been eschewed in favor of the more commonly-used genus *Cytospora* (Rossman et al. 2015) Fruiting bodies produced by distantly-related *Cytospora* species may appear identical, although conidial stromata width and branching of conidiophores has been shown to be reliable for the identification of *Cytospora* species occurring on *Eucalyptus* (Adams et al. 2005). Adams et al. (2005) noted that although the morphology of many *Cytospora* species is highly variable, certain species respond to particular cultural conditions (e.g. tolerance of high temperatures, media amended with cyclohexamide).

Historically, the causal organisms of Cytospora canker on *Populus* trees have been described as *Cytospora chrysosperma* (Pers.:Fr.) Fr (formerly *Valsa sordida*) or *C. nivea* (formerly *Valsa nivea*) (Sinclair and Lyon, 2005; Bloomberg 1962a & 1962b) and it has been generally assumed that the main species attacking quaking aspen is *C. chrysosperma* (Sinclair and Lyon, 2005). However, Kepley et al. (2015) have recently described a genetically- and morphologically- distinct *Cytospora* species named *C. notastroma* (Kepley & F.B. Reeves) (Kepley, et al. 2015), from isolates cultured from diseased quaking aspen (*Populus tremuloides*

Michx.) in northern Colorado. The distribution and abundance of this species on quaking aspen has not yet been determined.

The pathogenicity of Cytospora canker to quaking aspen and other host species also experiencing some environmental stress has been well-established (Long, 1918; Hubert, 1918; Christensen, 1940; Guyon et al., 1996; Sinclair and Lyon, 2005). When trees are drought-stressed, Cytospora canker develops very quickly, and may girdle a small diameter trunk or branch (through death and blockage of the vascular tissues) within weeks or months of inoculation (Hubert, 1918; Bloomberg, W.J., 1962a; Bloomberg, W.J., 1962b; Biggs et al. 1983). However, healthy trees grown under non-stressed conditions show little apparent damage from *Cytospora* (Hinds, 1985; Biggs, 1986; Guyon, et al. 1996; Dudley, 2015). *Cytospora nivea* (syn. *Leucostoma niveum, Valsa nivea*) has been documented as a canker-causing fungus, including on quaking aspen in northern British Columbia (Sinclair and Lyon, 2005; Hutchison, 1999). Recently, the pathogenicity of *C. notastroma* has been demonstrated. In a previous study, we showed that although both *C. chrysosperma* and *C. notastroma* are capable of forming cankers on drought-stressed aspen, *C. chrysosperma* is the more pathogenic species under warm or cool conditions, based on rate of canker formation, as well as final canker size (Dudley, 2015).

Throughout western North America, recent episodes of widespread aspen mortality and dieback linked to drought have been well-documented (Hogg, et al. 2002; Worrall, et al. 2008; Worrall, et al. 2010; Anderegg, et al. 2013; Worrall, et al. 2013; Dudley, et al. 2015a & 2015b). Projections for many areas currently suited to quaking aspen indicate drier conditions, and an overall reduction in suitable aspen habitat throughout western North America (Rehfeldt, et al. 2009). Such conditions will be favorable for the development and spread of Cytospora canker (as well as other stress-related damage agents, such as aspen bark beetles and wood-boring insects)

and thus this disease is likely to continue to be prevalent in quaking aspen stands in the future (Marchetti, et al. 2011). Based upon the work of Kepley et al. (2015) and our previous studies, we wished to determine (1) the relative abundance of the two *Cytospora* species most commonly occurring on quaking aspen, *C. chrysosperma* and *C. notastroma*; (2) whether there are other species of *Cytospora* occurring on quaking aspen throughout Colorado and elsewhere.

MATERIALS AND METHODS

Sample areas

We sampled infected quaking aspen trees from three distinct categories (1) four urban areas throughout Colorado (Fort Collins, Denver, Glenwood Springs, and Meeker); (2) from forest settings areas 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 throughout Colorado and southern Wyoming (Medicine Bow, White River, Pike, and San Isabel National Forests). For a previous study, in 2009-2010, we established 97 aspen health monitoring plots on five National Forests in Colorado and southern Wyoming to assess the impact of various damage agents on aspen health in stands designated as healthy or damaged (see Dudley, et al. 2015). In this study, we revisited a subset of the healthy and damaged plots to collect aspen stems with Cytospora canker. The National Forests sampled are mainly east of the Continental Divide (the Pike, San Isabel, and the Medicine Bow N.F.), with one sampled forest west of the Divide (the White River N.F.). These forests contain large and widespread quaking aspen components; the Pike N.F. contains roughly 45,600 ha (487 km^2) of aspen stands, the San Isabel N.F. has about 77,800 ha (779 km^2), the Medicine Bow N.F. has 25,900 ha (389 km^2) and the White River N.F. has

136,500 ha (2264 km^2) of aspen stands, and the Routt National Forest (Yampa and Hahn's Peak Ranger Districts) with 113,100 ha (985 km^2).

Sample collection

Up to two ranger districts were sampled within each national forest. Within each ranger district, one plot in a healthy stand and one in a damaged stand were selected based on whether Cytospora canker was detected on adult or immature aspen during the 2009-2010 survey. We navigated to the center of each selected plot using a GPS unit (Garmin® eTrex Legend). At the center of the plot, we randomly selected a bearing, and established a 10-meter transect along the bearing. The first ten aspen trees with Cytospora canker were sampled, and infected tissue (bark or stems, or both) were placed in paper bags, labeled, and stored in a laboratory. We eventually cultured a minimum of three trees per plot, and three cultures per tree. Sample locations outside of national forests in Colorado and Wyoming were also included. We collected infected aspen tissue from various urban areas throughout 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 (Table 3.1, Figure 3.1; Table 3.A2).

Culture of infected tissues

A minimum of three separate *Cytospora* cultures were obtained from at least three trees per plot. Cultures were selected arbitrarily for further study. All cultures 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 procedure developed by J. Kepley: 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 and partially submerged in the agar. Plates were sealed with Parafilm®, and checked daily. Once single-spore colonies, or mycelial growth resembling *Cytospora* were evident, the colony or hyphal tip was transferred to a fresh plate and further incubated for 7-10 days. After cultures had attained a size of at least 3cm diameter, approximately a dozen pieces of the culture margin were cut (2 mm square), 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, and in 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.

DNA extraction and primer amplification

DNA extraction was performed using the Invitrogen DNA extraction kit. Following extraction, nucleotide concentration of the samples was assessed using a Nanodrop© 2000 sensor (Nanodrop Products, Thermo Scientific, Wilmington, DE). Samples that contained at least 15 ng/µl of purified DNA were then used for polymerase chain reaction (PCR), using a MyCycler ™ Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA). Markers used in this included 5.8S subunit of the rDNA (ITS) (with primers ITS1 and ITS4) and a portion of the β-tubulin (Bt) gene (using primers Bt2a and Bt2b) (Carbone and Kohn, 2001). We also attempted and obtained some level of amplification of the following markers: a portion of the elongation factor 1-α gene

(primers EF728F/EF986R, as well as EF526F /EF1567R) (Carbone and Kohn, 2001; Rehner, 2001); calmodulin (CAL-228F/CAL-737R) (Carbone and Kohn, 2001); methionine aminopeptidase (MAP), as used by Zerillo et al. (2014). We also attempted amplification for single-copy markers MS456 (primers McM7-709 and McM7-1348) (Schmitt, et al. 2009); MS204 (Walker et al. 2012); FG1093 (Walker et al. 2012). In addition, we attempted amplification with a variety of makers developed for *Fusarium solani*: FsGPD; FsACC; FsICL; FsHMG; FsSOD; FsMPD; FsUGP1; FsTOP (Debourgogne et al. 2010). Successful PCR products were purified using a Roche High Pure PCR Product Purification Kit (Roche Diagnostics Corp., Indianapolis, IN), and DNA concentrations were again assessed using the Nanodrop© sensor.

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 an Excel® spreadsheet (Microsoft Corp., Seattle, WA). ITS and β-tubulin sequences were aligned and trimmed using the software program Mega5.2 (Tamura, et al. 2011) with the ClustalW® alignment technique option. Marker sequences were concatenated and isolates with identical concatenated sequences

were placed into haplogroups by species. Haplogroups, as well as individual isolates not matching any haplogroup sequence, were combined and exported to .mas, .fas, .meg, and .nexus files. In addition, separated ITS and β-tubulin sequences for all haplogroups and individual isolates as well as references sequences from NCBI database (www.ncbi.nlm.nih.gov) and outgroup (*Cryptosphaeria pullmanensis*) were exported into fasta and nexus file formats for later use. SNPs occurring in only one sample were checked by examining the trace files, and edits were made to sequences where appropriate.

Phylogenetic analysis

DNAsp5 (Rozas et al. 2003) was used to estimated genetic diversity parameters for each locus and the combined dataset, ITS and Bt for each species group (NO, NI and CH) and the total population. For each locus, for haplotype diversity (h_D), nucleotide diversity (π), selection (Tajima's D, Fu and Li's F), recombination were estimated. Linkage disequilibrium was also tested for the combined ITS and Bt dataset.

We conducted phylogenetic analyses of *Cytospora* sequences using maximum parsimony and Bayesian analyses (BA) on each locus independently and also on the combined dataset of the internal transcribed spacer (ITS) region of the 5.8 subunit of the rDNA, and region 2 of the β-tubulin locus. Reference and outgroup sequences used in this analysis 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 *Crytosphaeria pullmanensis* isolate we cultured previously from quaking aspen. A partition homogeneity test was implemented in using the software package PAUP*4a146 to test for congruence between the two loci (Swofford, 2002). DT-Modsel (Minin, et al. 2003) was used to identify the best suited evolutionary model for each locus. BA

tree selection were conducted using Markov-Chain Monte Carlo (MCMC) analysis in software program BEAST, with 1,000,000 independent runs (i.e. chains) and sampling every 1,000 runs (Drummond et al. 2012). Data files were converted to .xml format 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 TIMef+I+G for the ITS-based tree, and TrNef+G for the β-tubulin-based tree. Partition homogeneity tests confirmed incongruence of the two markers, and so separate trees were assembled for ITS and Bt. A web-based version of the software PhylML (http://www.hiv.lanl.gov/content/sequence/PHYML; Guidon and Gascuel, 2003) was used to assemble maximum parsimony phylogenies of each maker sequence, with 500 bootstrap replicates and TN93 substitution model.

Genetic Distance & Principle Components Analysis

Trimmed, concatenated ITS and beta-tubulin sequences for all haplogroups of each *Cytospora* species (16 total) were uploaded to ClustalOmega (Sievers, et al., 2011; www.ebi.ac.uk), where an alignment file was obtained, including annotation of SNP locations. The alignment was edited and manipulated in Microsoft Excel. The Excel add-in program GenAlEx 6.5 (Peakall and Smouse, 2006 & 2012) was used to calculate genetic distance and conducted a principle components analysis based upon the genetic distance results. Genetic distance and PCA calculations were performed for the three combined *Cytospora* species (*C. chrysosperma, C. notastroma*, and *C. nivea*) and by species.

RESULTS

Frequency and distribution of Cytospora *species*

Of the 110 diseased quaking aspen trees sampled, all had evidence of Cytospora canker, with or without fruiting bodies present. The presence of a particular *Cytospora* species was confirmed 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 bloom (i.e. periderm) (see Figure A3.1). Therefore, isolates were cultured from pycnidia as previously described. We putatively 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 on Leonian's modified media (Table 3. 1; Table 3.A1). When grown on Leonian's media, *Cytospora notastroma* had a dark colony appearance with appressed hyphae growing into the agar whereas *C. chrysoperma* had a light tan or buff-colored colony appearance, as previously described (Figure A3. 2) (Kepley et al, 2015; Kepley, 2009). *Cytospora nivea* was variable in color when grown on Leonian's media, and ranged from light tan to dark brown, although not as dark in color as *C. notastroma* (Figure A3.3).

To validate our morphological identifications, the ITS and β-tubulin sequences of 67 and 45 isolates, respectively, were compared (Figures 3.S1 and 3.S2). There was strong (100%) support for differentiation between *C. chrysosperma, C. notastroma* and *C. nivea* isolates based on ITS sequences (Figure 3.3). Analysis of a β-tubulin-based tree also supported separation

between the three species (Figure 3. 4). This sequence data also corroborated our species identification based on morphological features, except that most of the isolates tentatively identified as an unknown *Cytospora* species were in fact *C. nivea*. In a separate analysis, three unknown *Cytospora* isolates grouped with *C. chrysoperma* based on ITS sequences, and were grouped with *C. notastroma* based on their β-tubulin sequence.

Of the 190 isolates collected, 48 % were *C. chrysosperma*, 42 % were *C. notastroma*, and 9 % were *C. nivea* (Table 3.1; Table 3.A1). We detected both *C. chrysosperma* and *C. notastroma* in each of the three main regions sampled (Table 3. 1). *Cytospora nivea* was only detected in western and south-central Colorado, as well as central Utah (Table 3.1). Only *C. notastroma* was found in eastern Minnesota, northern Montana, and the Poudre Canyon in northern Colorado (Table 3.1). We isolated more than one *Cytospora* species from a quarter of the trees sampled (18/71) from each of the three of the broad regions of Colorado, Wyoming, and Utah that were sampled most intensively. Five of these featured *C. chrysosperma* and *C. notastroma*, as well as three trees with *C. chrysosperma* and *C. nivea*.

Phylogenetic analysis: posterior probability & bootstrap analyses

We constructed a BA-based phylogenetic tree of the concatenated sequences of 16 haplogroups and 13 individual isolates (Figure 3. 4). Partition homogeneity revealed high support for three species groups (CH, NO, NI), and supported—to a limited extent—intraspecific clades (Figure 3. 4). Posterior probability values supported three possible clades within *C. notastroma*, two within *C. chrysosperma*, and two within *C. nivea* (Figure 3.4). Probability values were not sufficiently high (51%) to parse *C. chrysosperma* from *C. cypri* (NCBI). Analysis of 67 ITS sequences based on a posterior probability phylogeny indicated strong (100%) support for differentiation between each of the three *Cytospora* species, and was

suggestive of intra-specific clades within each species (Figure 3. 2). The maximum parsimony-based ITS tree supported only two broad groups among the 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 (Figure 3.A5) A posterior probability phylogeny based on the 45 Bt sequences also supported three distinct species groups, as well strong support for the possibility of intra-species clades within *C. notastroma*, *C. nivea* and *C. chrysosperma* (Figure 3.3). Bootstrap values of a maximum parsimony phylogeny did not support any distinction between the three species of interest (Figure 3.A6).

Principle Components Analysis

A PCA of all concatenated sequences from two genetic markers indicate a distinct grouping of the three described *Cytospora* species, which is consistent with the concatenated BA-based tree (Figure 3.5; Figure 3.2). Analyses of principle components by species group indicated that the *C. chrysosperma* haplogroups recovered in this study form three clusters (Figure 3. 6), as do the *C. notastroma* haplogroups (Figure 3.7). The clustered hapogroups do not share geographic similarities, at least based upon the ITS-Bt sequences used. An additional six *C. nivea* isolates were included in the species-level PCA, as well as haplogroup 1 (Figure 3. 8).

Tests for neutrality, recombination, and linkage disequilibrium

Tests of haplotype diversity indicated high levels of diversity for each of the species tested (Table 3. 2). Nucleotide diversity was greatest for beta tubulin sequences, especially among *C. notastroma* isolates. Neutrality tests indicated that the loci are neutral (Table 3.2). Linkage disequilibrium tests were not significant (Table 3. 2). Recombination occurred at both loci, but was greater for beta tubulin (Table 3.2).

DISCUSSION

The causal agent of Cytospora canker on quaking aspen has been called a species complex (Kepley et al. 2015; Kepley, 2009), and our results support such a description. Both of the ITS and Bt phylogenies indicate strong support for three species groups, and a Principle Components Analysis of the concatenated ITS-Bt sequences supports this. Phylogenetic trees assembled based on posterior probability indicate the existence of intra-specific clades, and this was supported by species-wise PCA.

As Spielman (1985) and others (Kepley et al. 2015; Kepley, 2009; Adams, 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 particular isolates over the years, further complicating the phylogeny of this genus. We also observed considerable variability in the morphology of fruiting bodies we sampled in this study. 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. Aspen stands are now known to be much less clonal than previously thought, and large, continuous stands may in fact be a conglomeration of many genotypes (DeWoody, 2009; Long and Mock, 2012). Phenotypic variations in resistance to herbivory (Stevens, et al. 2007; Lindroth and St Clair, 2013) and disease (Copony and Barnes, 1974; Lindroth and Hwang, 1996; Holeski, et al. 2009) have been extensively documented. Also documented is the coloration and apparent thickness of the ‘bloom’ on aspen trunks (Pearson and

Lawrence 1958). Pearson and Lawrence (1958) reported that differences in bloom properties varied from stand to stand, and were related to site elevation. We observed that the dark stromatal tissue layer surrounding pycnidia was sometimes obscured by particularly opaque layer of periderm (Figures A3. 1 & A3.4). Finally, it seems possible that 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 characterisitic of *C. notastroma* only forms at maturation, then perhaps some of the pycnidia later attributed to *C. notastroma* but lacking the dark basal tissue disk simply had not yet matured.

In a previous study, we confirmed the pathogenicity of *C. notastroma*, and compared it to *C. chrysosperma* (Dudley, et al. 2015). Both species caused cankers to form on drought-stressed aspen trees, but C. chrysosperma consistently formed larger cankers under different ambient temperatures (Dudley, et al. 2015). In spite of being a weaker pathogen, we observed similar frequencies of *C. notastroma* and *C. chrysosperma*. We did not discriminate between 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 particular tree size than another. However, further study could investigate whether the two species fill different niches in aspen stands; does one species occur more frequently on shade-weakened understory trees than the other? 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 markers were used in this study, and thus we cannot make any statements about intra-species populations. Additional data would certainly help to clarify relationships within species groups. Perhaps the selection of a marker whose sequence is less conserved (i.e. greater variability, more SNPs) than

the two used here would allow for 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 an admixture of genetic sequences) of *Cytospora* inoculum or of greater distances than previously thought. Kaczynksi 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 seems possible that *Cytospora* inoculum is spread by birds, flying insects, or other vectors.

We observed evidence that could indicate hybridization between the *Cytospora* species examined in this study (or other *Cytospora* species). Our results show that recombination readily occurs at both ITS and Bt loci for all three identified species. Further, 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 isolates were alternately grouped with *C. nivea*, *C. notastroma,* or *C. chrysosperma,* depending upon which locus the phylogeny was based. Kepley et al. (2015) observed that 'intermediate isolates' existed which could not be distinguished, based upon ITS and Elongation Factor 1-a (EF1a) sequences, between *C. nivea* and *C. translucens* (which has also been reported on quaking aspen). Finally, we observed that fully 25% of the trees sampled were infected with more than one *Cytospora* species. This could in fact be much higher; we sampled material that could be reached from the ground, and rarely sampled cankers

from entire adult aspen trees. Because we did not sample all cankers on all trees, it is possible that observed cankers could be a patchwork of different causal species.

FIGURES AND TABLES

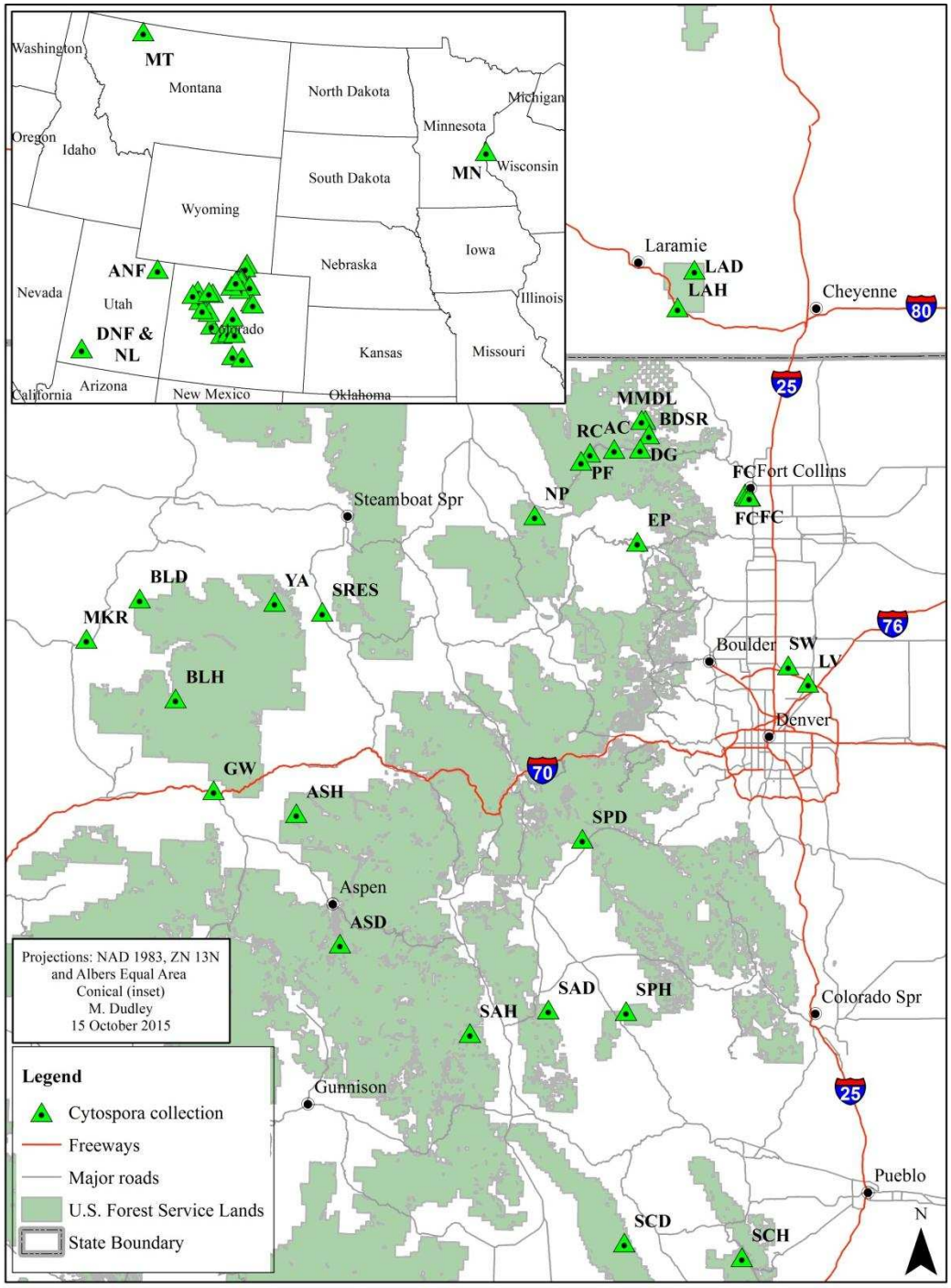

Figure 3.1. Map of sample locations used in this study. Materials were collected June 2012-Sept 2014.

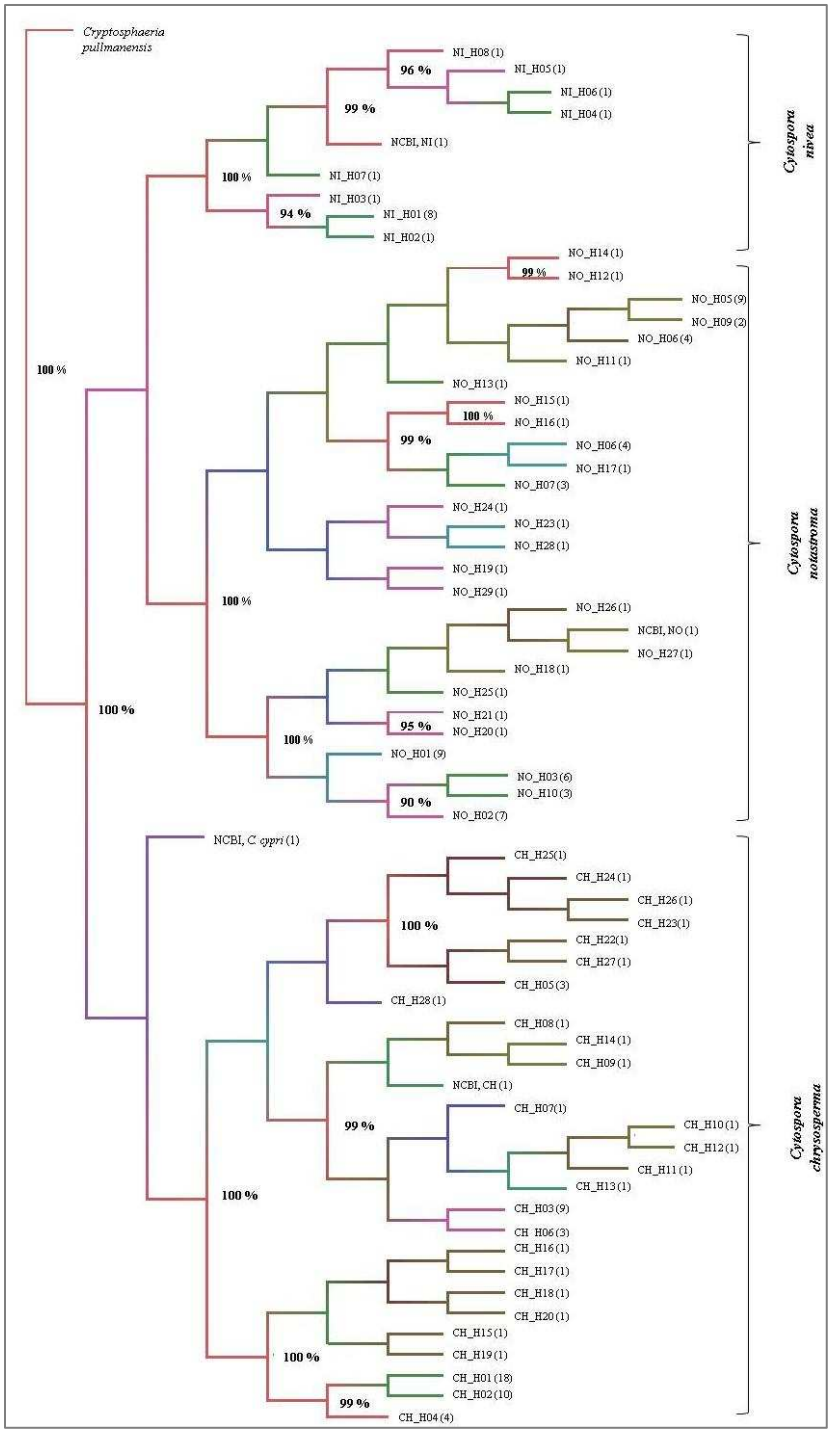

Figure 3.2. Phylogenetic tree based on ITS sequences of 67*Cytospora* isolates collected from quaking aspen throughout Colorado, southern Wyoming, Utah, northern Montana, and east-central Minnesota, plus outgroups *Cytospora cypri* and *Cryptosphaeria pullmanensis*. Posterior probability (BA) values are included at the node junctions.

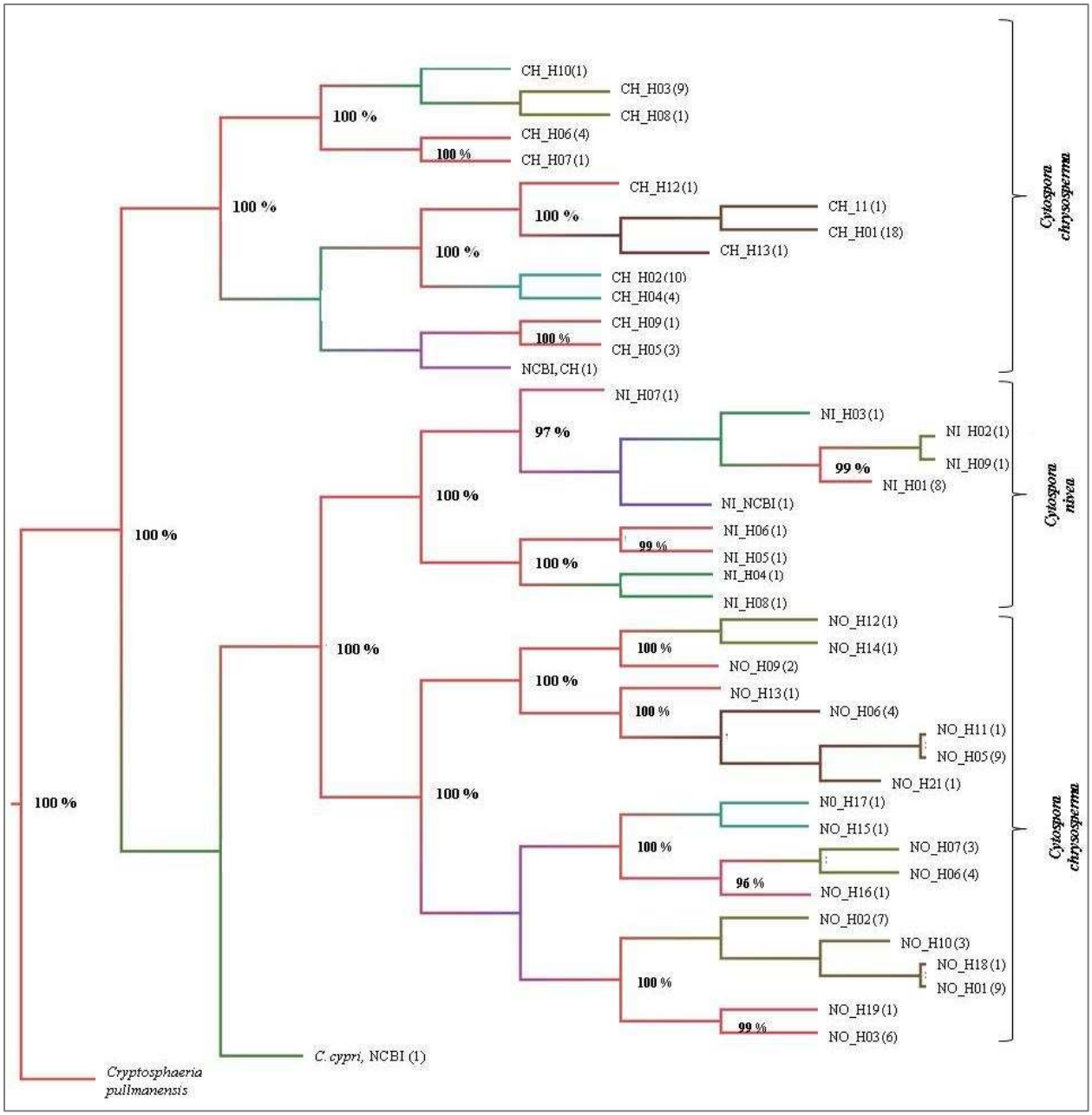

Figure 3.3. Phylogenetic tree based on Bt sequences of 45*Cytopsora* isolates collected from quaking aspen throughout Colorado, southern Wyoming, Utah, northern Montana, and east-central Minnesota, plus outgroups *Cytospora cypri* and *Cryptosphaeria pullmanensis*. Posterior probability (BA) values are included at the node junctions.

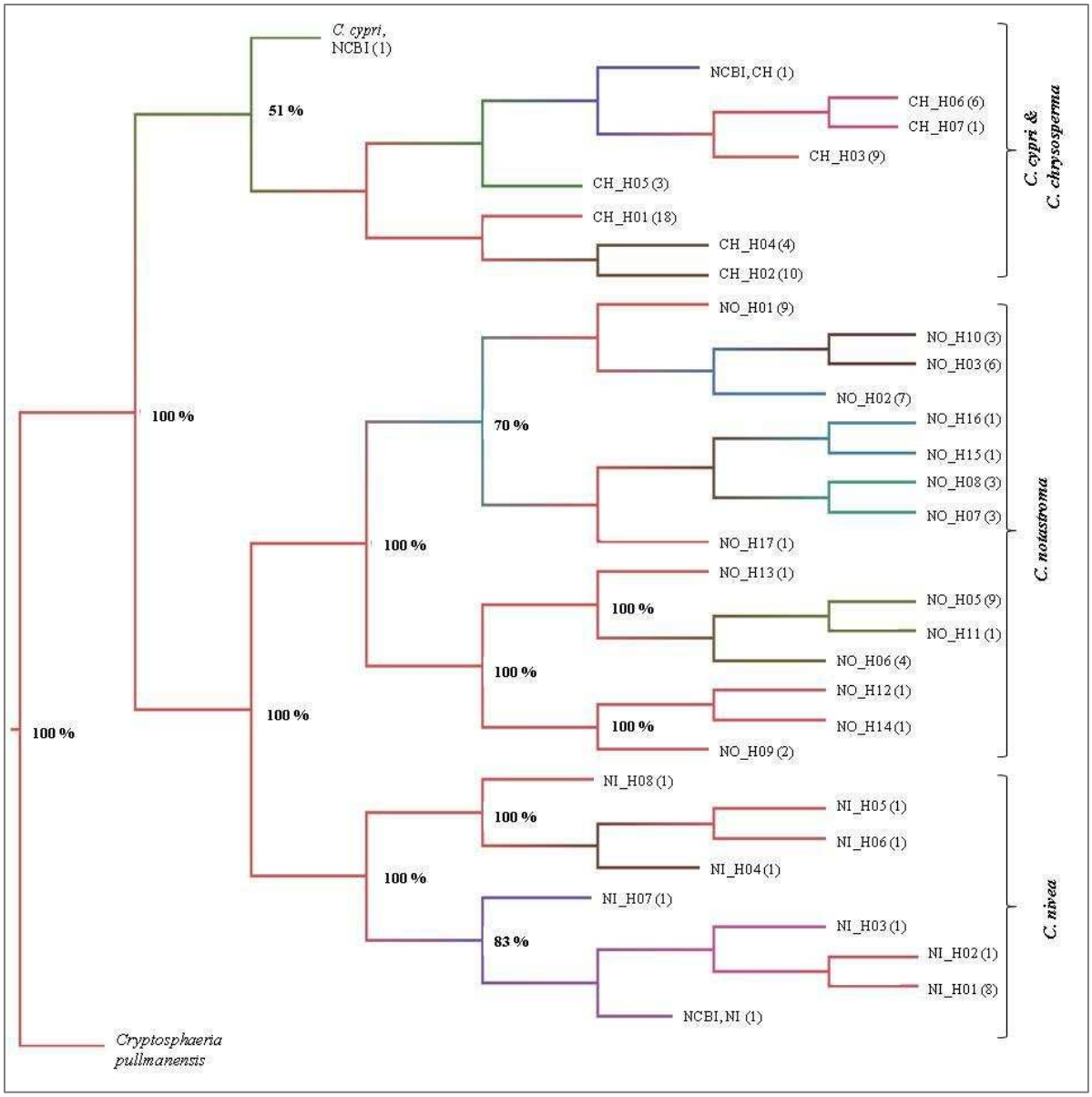

Figure 3.4. Phylogenetic tree assembled using posterior probability analysis, representing the concatenated ITS-Bt sequences of 34 *Cytospora* isolates collected from quaking aspen throughout Colorado, southern Wyoming, Utah, northern Montana, and east-central Minnesota, plus an outgroup (*Cryptosphaeria pullmanensis*).

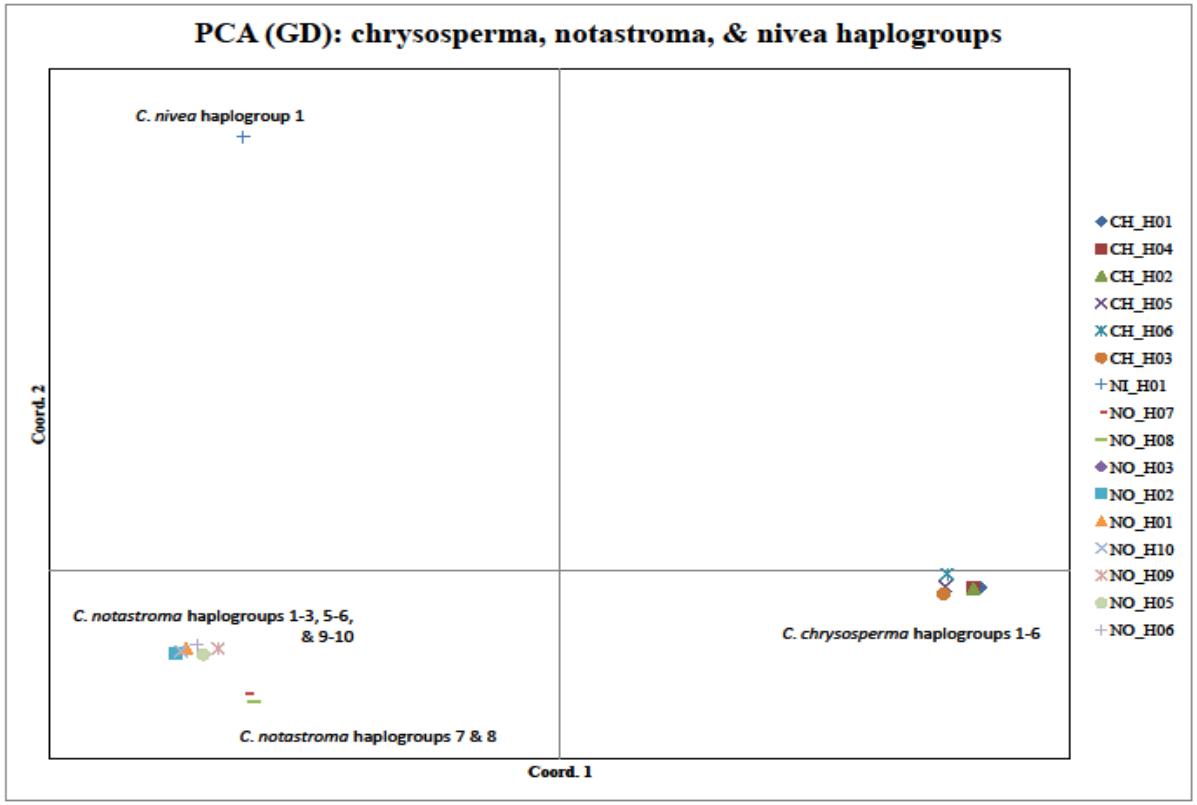

Figure 3.5. Principle Component Analysis (PCoA) of genetic distances representing 101 isolates of *Cytospora chrysosperma*, *C. notastroma*, and *C. nivea* isolated from quaking aspen throughout Colorado, southern Wyoming, Utah, northern Montana, and east-central Minnesota, using concatenated ITS and beta-tubulin regions.

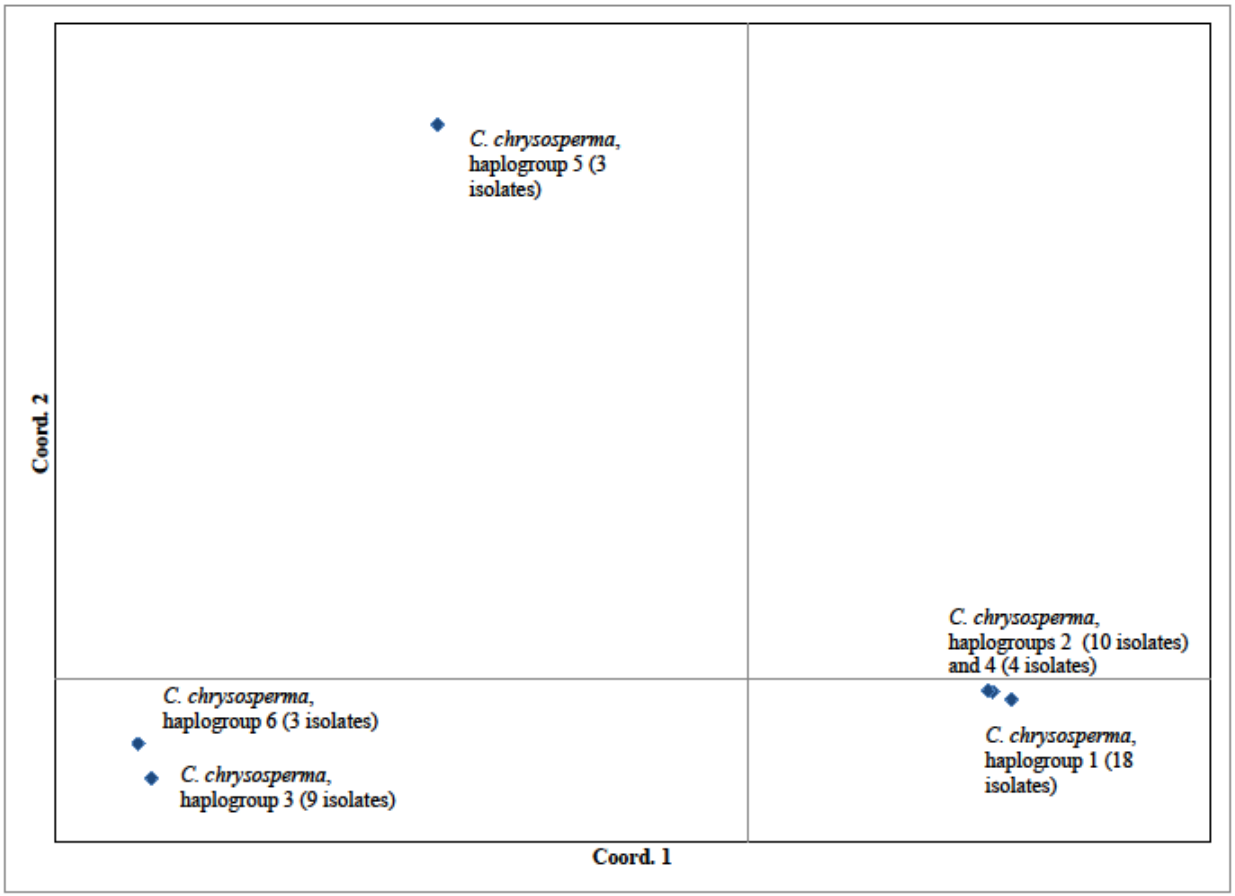

Figure 3.6. Principle Component Analysis (PCoA) of genetic distances representing 47 isolates of *Cytospora chrysosperma*, isolated from quaking aspen using concatenated ITS and beta-tubulin regions.

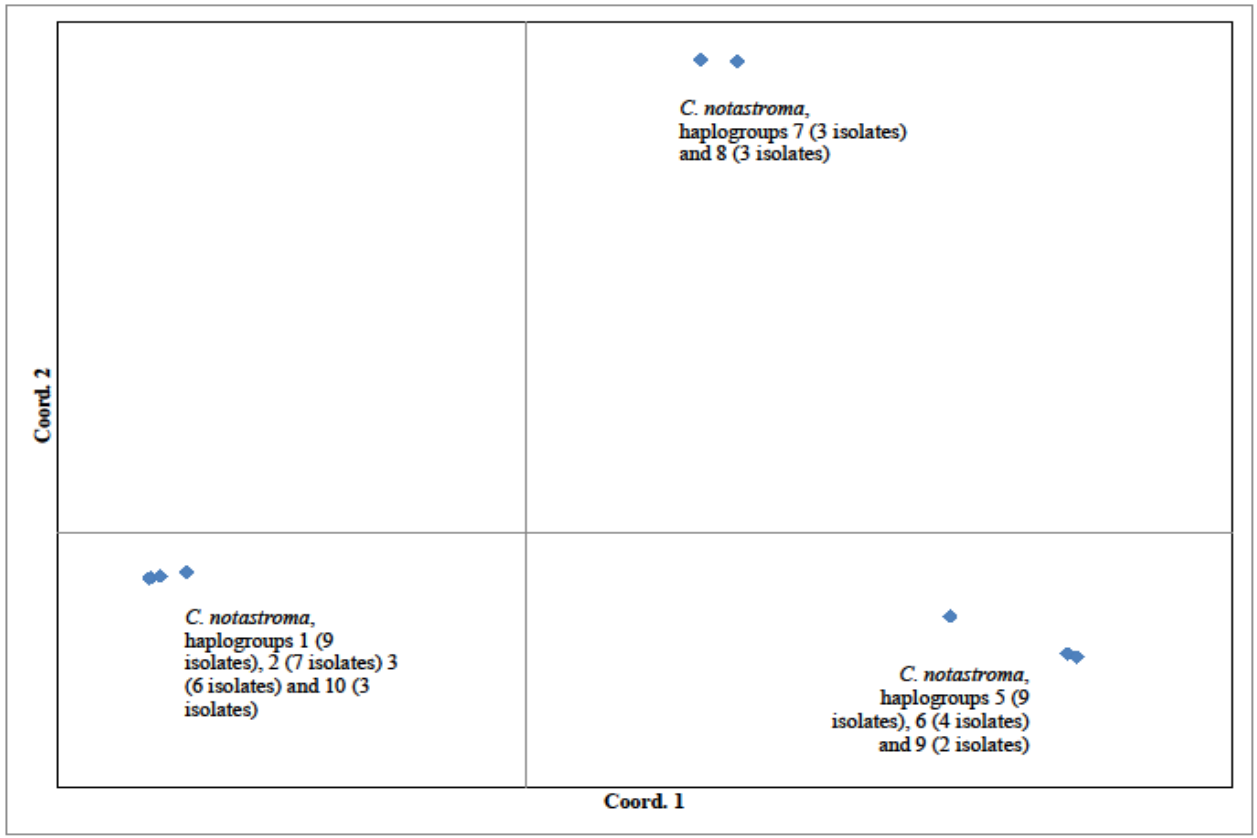

Figure 3.7. Principle Component Analysis (PCoA) of genetic distances representing 47 isolates of *Cytospora notastroma*, isolated from quaking aspen using concatenated ITS and beta-tubulin regions.

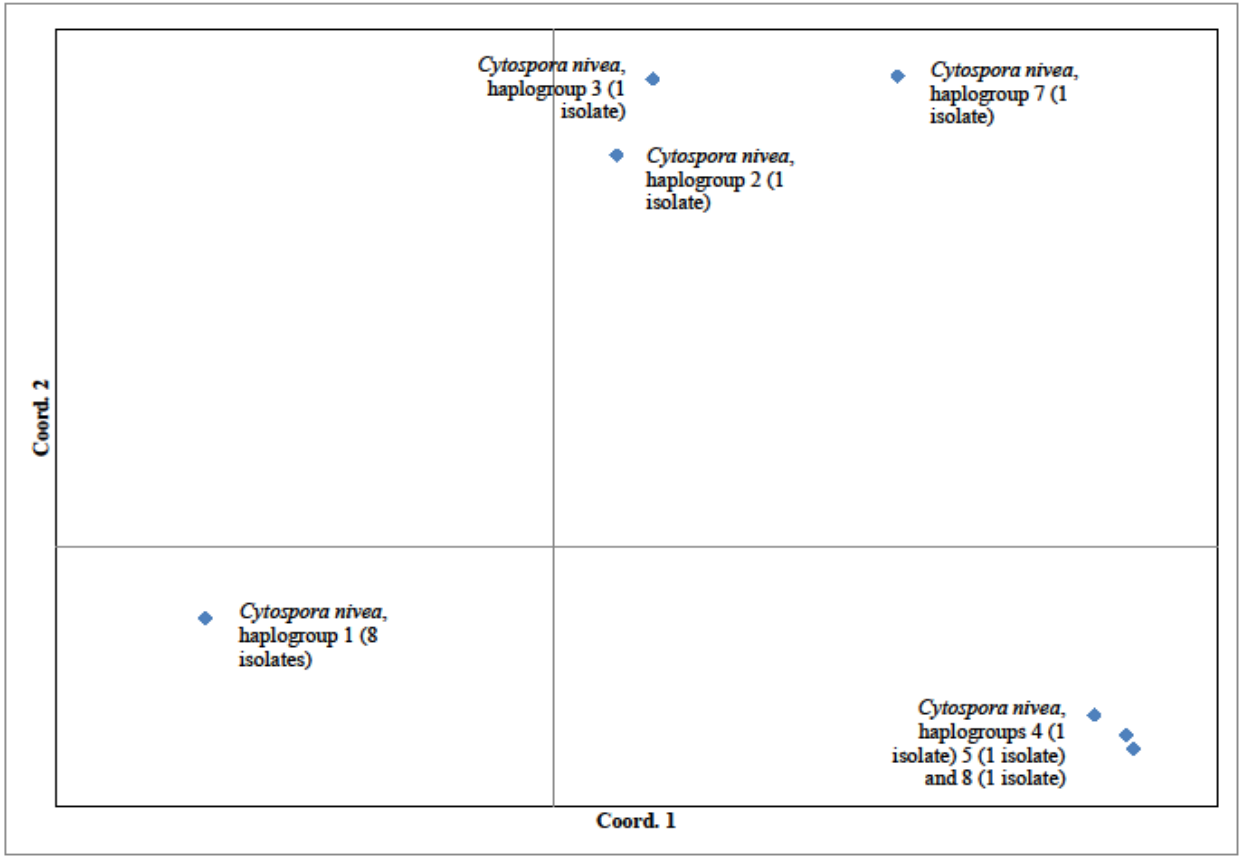

Figure 3.8. Principle Component Analysis (PCoA) of genetic distances representing 47 isolates of *Cytospora nivea*, isolated from quaking aspen using concatenated ITS and beta-tubulin regions.

Table 3.1. Numbers of fungal isolates cultured from cankers on quaking aspen included in this study, by geographic region and *Cytospora* species.

Region	No. Isolates	No. Trees	No. CH isolates*	No. NO isolates	No. NI isolates
Eastern CO (urban)	36	10	21	15	0
Northern CO	16	6	13	3	0
Northwestern CO	20	10	13	6	1
Poudre Canyon	11	5	0	11	0
South-central CO	26	16	12	8	6
Western CO	28	8	11	7	10
Southern WY	23	8	16	7	0
Central UT	17	6	6	9	2
Eastern MN	9	1	0	9	0
Northern MT	4	1	0	4	0
Total	190	71	92	79	19
Sampled, all	410	110			
Total Cytospora	366	106			
Percent of total			**48.4%**	**41.6%**	**8.9%**

*NO: *Cytospora notastroma*; CH: *Cytospora chrysosperma*; NI*: Cytospora nivea*

Table 3.2. 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	Diversity		Fu_Li†		Tajima†
		h_D	nucl	F*	F	D
ITS	NO	0.920	0.00609	1.45	1.43	0.87
	CH	0.783	0.00844	1.81	1.67	1.88
	NI	0.917	0.00912	-0.24	0.23	0.09
	All	0.925	0.04109	2.01	2.64	1.57
B-tubulin	NO	0.932	0.04311	-0.67	-0.30	-0.10
	CH	0.879	0.03111	1.33	2.31	1.68
	NI	0.944	0.02289	-0.58	-0.22	-0.34
	All	0.963	0.11697	-0.52	0.31	0.26
Concatenated	NO	0.967	0.0191	1.21	1.25	1.18
	CH	1.000	0.01934	0.91	1.42	1.19
	NI	0.964	0.01485	-0.03	0.05	0.19
	All	0.991	0.08833	-2.69	-3.11	-1.64

. † None of the measures shown were significant at P=0.10.

Table 3.3. 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	Species	Gene_flow(Nei)		Recombination, by species			Recombination, across *Cytospora* species			Linkage disequilibrium†				
		Gst	Nm	Sk^2	Avg distance	Rm	Sk^2	Avg distance	Rm	ZnS	Za	ZZ	Wall'sB	Wall'sQ
ITS	NO*			3.78	437	3								
	CH‡			7.89	439	0								
	NI‡			4.96	432.67	1								
	All	0.11	4.12				118	437	12					
B-tubulin	NO			81.15	341.55	4								
	CH			47.05	342	2								
	NI			21.79	337	0								
	All	0.05	10.36				437	340.	21					
Concatenated	NO			78.89	779.44	3								
	CH			103.97	782.14	1								
	NI			48.92	770.38	2								
	All	0.03	18.45				2564	778	31	0.38	0.64	0.26	0.59	0.60

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

Table 3.4. All isolates used in analyses in this study, by species and haplogroup.
A. Cytospora chrysosperma.

Species	ITS Haplogrp name	Bt Haplogrp name	No. Isolates/Haplogroup	Isolate ID	Tree No.	Site Name
C. chrysosperma	CH_H01	CH_H01	18	ANF2C	ANF2	ANF
				ASD1F	ASD1	ASD
				BDSR1-2	BDSR1	BDSR
				BLD1G	BLD1	BLD
				BLD4F	BLD4	BLD
				BLD4I	BLD4	BLD
				GW2D1	GW2	GW
				LAD3B	LAD3	LAD
				LAD3D	LAD3	LAD
				LAH1D	LAH1	LAH
				LAH1E	LAH1	LAH
				LAH1H	LAH1	LAH
				LAH2A2	LAH2	LAH
				LAH2A3	LAH2	LAH
				SAH3C	SAH3	SAH
				SAH3D	SAH3	SAH
				SPD4F	SPD4	SPD
				SPD4H	SPD4	SPD
	CH_H02	CH_H02	10	BLD2A	BLD2	BLD
				BLD2E	BLD2	BLD
				BLD4E	BLD4	BLD
				DL1C	DL1	DL
				DL1E	DL1	DL
				DL1H	DL1	DL
				DL1I	DL1	DL
				GW2A	GW2	GW
				GW2B	GW2	GW
				GW2C	GW2	GW
	CH_H03	CH_H03	9	ASH3I	ASH3	ASH
				BLH6C	BLH6	BLH
				DG1-1B	DG1	DG
				DG1-2A	DG1	DG
				FC4A	FC4	FC4
				FC6	FC6	FC6
				FC61B	FC6	FC6
				FC63	FC6	FC6

				MKR22	MKR2	MKR
CH_H04	CH_H04		4	LAH1B	LAH1	LAH
				LAH2B	LAH2	LAH
				SPH2B	SPH2	SPH
				SPH2C	SPH2	SPH
CH_H05	CH_H05		3	LAH6G	LAH6	LAH
				LV1G	LV1	LV
				LV2B	LV2	LV
CH_H06	CH_H06		3	BLD2J	BLD2	BLD
				FC1A	FC1	FC1
				FC2B	FC2	FC2
CH_H07	CH_H07	1		EP1I	EP1	EP
CH_H08	CH_H08	1		ANF2B	ANF2	ANF
CH_H09	-	1		ANF3E	ANF3	ANF
CH_H10	-	1		EP1A	EP1	EP
CH_H11	-	1		EP1F	EP1	EP
CH_H12	-	1		EP1J	EP1	EP
CH_H13	-	1		SW6D	SW1	SW
CH_H14	-	1		MKR2A	MKR2	MKR
CH_H15	-	1		ANF2F	ANF2	ANF
CH_H16	-	1		DL1G	DL1	DL
CH_H17	-	1		DL1F	DL1	DL
CH_H18	-	1		LAD3I	LAD3	LAD
CH_H19	-	1		LAD3J	LAD3	LAD
CH_H20	-	1		SAH6B	SAH6	SAH
CH_H22	-	1		LV1A	LV1	LV
CH_H23	-	1		LV1B	LV1	LV
CH_H24	-	1		LV1C	LV1	LV
CH_H25	-	1		LV1D	LV1	LV
CH_H26	-	1		LV1F	LV1	LV
CH_H27	-	1		LV2A	LV2	LV

B. *Cytospora notastroma*

Species	ITS Haplogrp name	Bt Haplogrp name	No. Isolates/Haplogrp	Isolate ID	Tree No.	Site Name
C. notastroma	NO_H01	NO_H01	9	BDSR2-2	BDSR2	BDSR
				DL3D	DL3	DL
				DL3E	DL3	DL
				FC2A	FC2	FC2
				FC62	FC6	FC6
				MN1A	MN1	MN
				MN1B	MN1	MN
				MN1C	MN1	MN
				MN1E	MN1	MN
	NO_H02	NO_H02	7	NL3B	NL3	NL
				SW5B	SW1	SW
				SW6P3	SW1	SW
				SW6P4	SW1	SW
				SW6P5	SW1	SW
				SW7C	SW1	SW
				SW8P1	SW1	SW
	NO_H03	NO_H03	6	LAD3A	LAD3	LAD
				SW6P2	SW1	SW
				SW7C1	SW1	SW
				SW7D	SW1	SW
				SW8A	SW1	SW
				SW8C	SW1	SW
	NO_H05	NO_H05	9	AC 3-1	AC3	AC
				AC 3-3	AC3	AC
				ASD4B	ASD4	ASD
				BLD3B	BLD3	BLD
				BLD3I	BLD3	BLD
				SRES2	SRES2	SRES
				WLGL1	WLGL1	WLGL
				WLGL2	WLGL1	WLGL
				WLGL3	WLGL1	WLGL
	NO_H06	NO_H06	4	FC3B	FC3	FC3
				RCKEP1A	RCKEP1	RCKEP
				RCKEP2A	RCKEP1	RCKEP
				RCKEP3A	RCKEP1	RCKEP
	NO_H07	NO_H07	3	PF1	PF1	PF

			PF2	PF1	PF
			PF3	PF1	PF
NO_H08	NO_H08	3	LAH6B	LAH6	LAH
			NL4B	NL4	NL
			SCD2F	SCD2	SCD
NO_H09	NO_H09	2	NL4C	NL4	NL
			NP1	NP1	NP
NO_H10	NO_H10	3	ANF1F	ANF1	ANF
			GW2F	GW2	GW
			SRES4C	SRES4	SRES
NO_H11	NO_H11	1	ASD4C	ASD4	ASD
NO_H12	NO_H12	1	ASD4H	ASD4	ASD
NO_H13	NO_H13	1	NP3	NP3	NP
NO_H14	NO_H14	1	YA1B	YA2	YA
NO_H15	NO_H15	1	SAD3E	SAD3	SAD
NO_H16	NO_H16	1	SAD3H	SAH3	SAH
NO_H17	NO_H17	1	YA1-4	YA1	YA
NO_H18	-	1	MNIJ	MN1	MN
NO_H19	-	1	LAD8B	LAD8	LAD
NO_H20	-	1	AC 4-1	AC4	AC
NO_H21	-	1	ANF1E	ANF1	ANF
NO_H23	-	1	LAH6I	LAH6	LAH
NO_H24	-	1	LAH8F	LAH8	LAH
NO_H25	-	1	MN1K	MN1	MN
NO_H26	-	1	MN1F	MN1	MN
NO_H27	-	1	MN1H	MN1	MN

C. *Cytospora nivea*

Species	ITS Haplogrp name	Bt Haplogrp name	No. Isolates/Haplogroup	Isolate ID	Tree No.	Site Name
C. nivea	NI_H01	NI_H01	8	ASD1B	ASD1	ASD
				ASD1H	ASD1	ASD
				ASD2C	ASD2	ASD
				ASH10B	ASH10	ASH
				ASH10E	ASH10	ASH
				DNF1B	DNF1	DNF
				DNF1C	DNF1	DNF
				SPD4D	SPD4	SPD
	NI_H02	NI_H02	1	ASD2A	ASD2	ASD
	NI_H03	NI_H03	1	BLD1A	BLD1	BLD
	NI_H04	NI_H04	1	SAH5B	SAH5	SAH
	NI_H05	NI_H05	1	SCH7E	SCH7	SCH
	NI_H06	NI_H06	1	SCH7J	SCH7	SCH
	NI_H07	NI_H07	1	SPD3J	SPD3	SPD
	NI_H08	NI_H08	1	SPD5I	SPD5	SPD

D. *Cytospora* species

Species	ITS Haplogrp name	Bt Haplogrp name	No. Isolates/ Haplogrp	Isolate ID	Tree No.	Site Name
Cytospora sp.	CS_H01	CS_H01	1	ANF2D	ANF2	ANF
	CS_H03	CS_H03	1	ASH3E	ASH3	ASH
	CS_H26	-	1	ASH3F	ASH3	ASH
	CS_H27	-	1	BLD3D	BLD3	BLD
	CS_H28	-	1	BLD3E	BLD3	BLD
	CS_H24	-	1	LAD7J	LAD7	LAD
	CS_H25	-	1	LAH3E	LAH3	LAH
	CS_H05	-	1	MKR2E	MKR2	MKR
	CS_H20	-	1	SAD1A	SAD1	SAD
	CS_H04	-	1	SAD1E	SAD1	SAD
	CS_H33	-	1	SAD3D	SAD3	SAD
	CS_H06	CS_H06	1	SAH6J	SAH6	SAH
	CS_H21	-	1	SCD2C	SCD2	SCD
	CS_H22	-	1	SCD2D	SCD2	SCD
	CS_H29	-	1	SCH10C	SCH10	SCH
	CS_H23	-	1	SCH8E	SCH8	SCH
	CS_H30	-	1	SCH8H	SCH8	SCH
	CS_H31	-	1	SPD5F	SPD5	SPD
	CS_H32	-	1	SPH3B	SPH3B	SPH
	CS_H02	CS_H02	1	SPH4H	SPH4	SPH

Figure 3.A1. Examples of pycnidia (showing whole and excised) of two *Cytospora* species. Top row: *C. chrysosperma*. Bottom row, left: *C. notastroma*. Bottom row, right: branch with both species present. Here, the bloom thickness and color make distinguishing one species from another difficult.

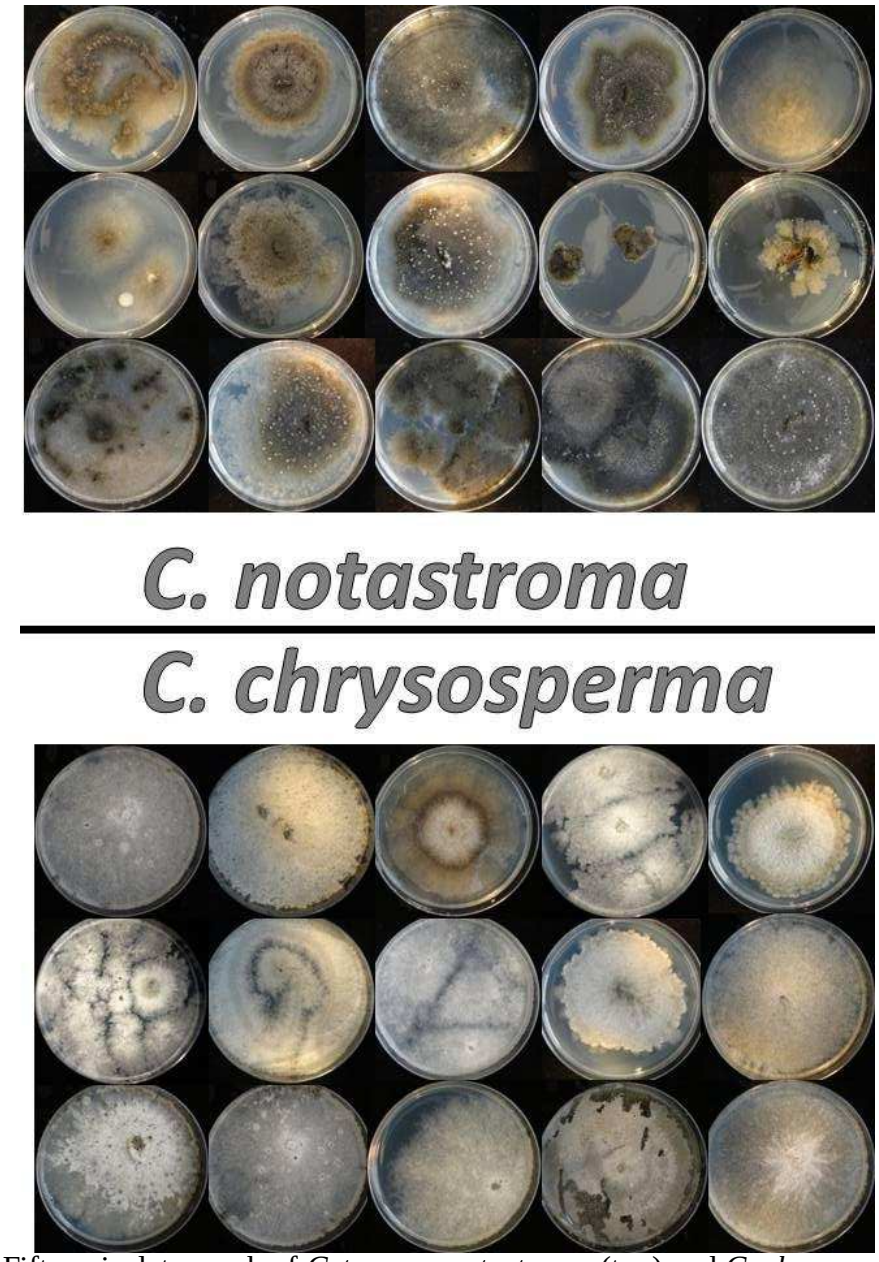

Figure 3.A2. Fifteen isolates each of *Cytospora notastroma* (top) and *C. chrysosperma*, on Leonian's modified medium, approximately two weeks after plating. Photos: Ian Dudley

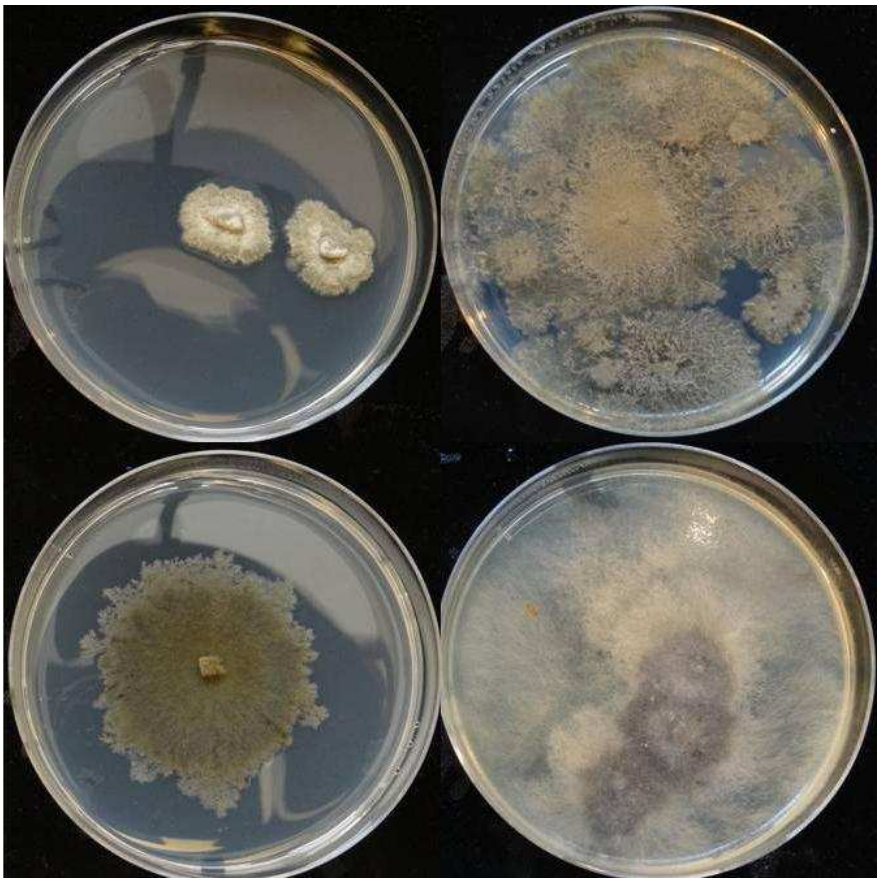

Figure 3.A3. Four isolates of *Cytospora nivea* on Leonian's modified medium, approximately two weeks after plating. Photos: Ian Dudley.

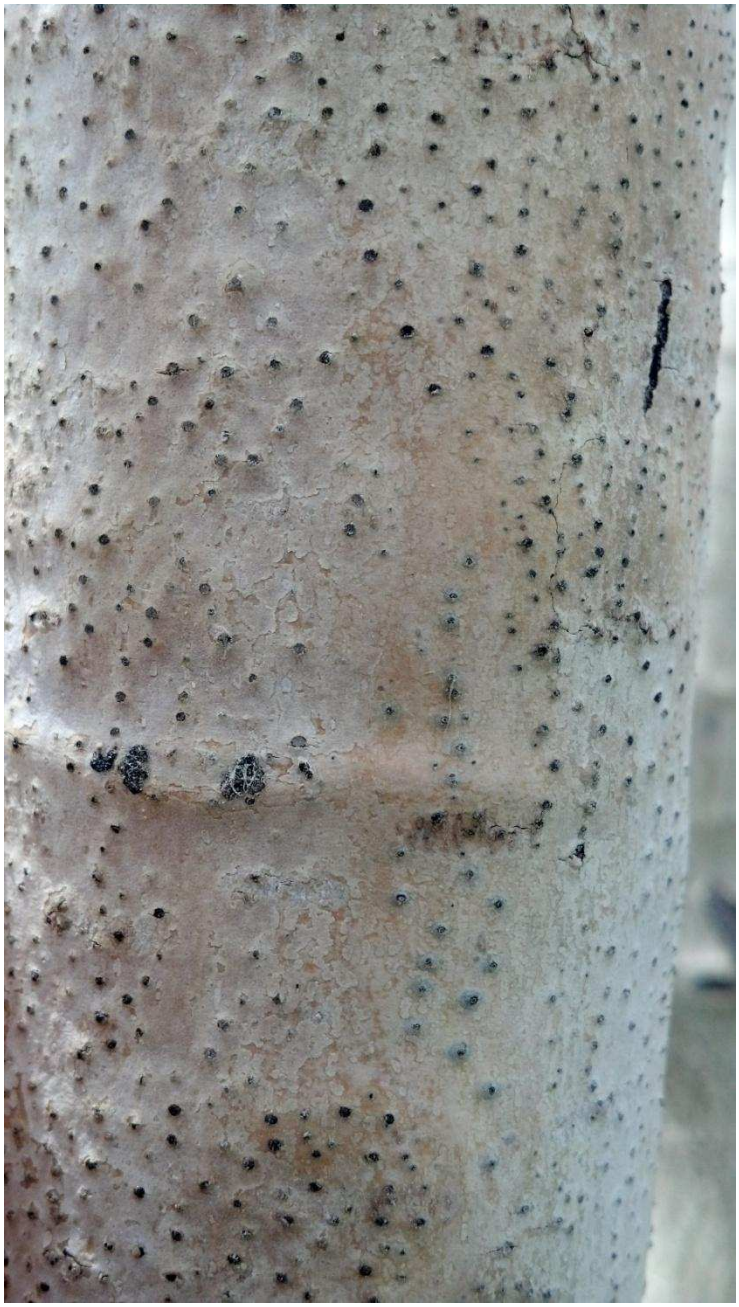

Figure 3.A4. An aspen trunk infected with Cytospora canker. Note the variable morphology of pycnidia formed on the same stem.

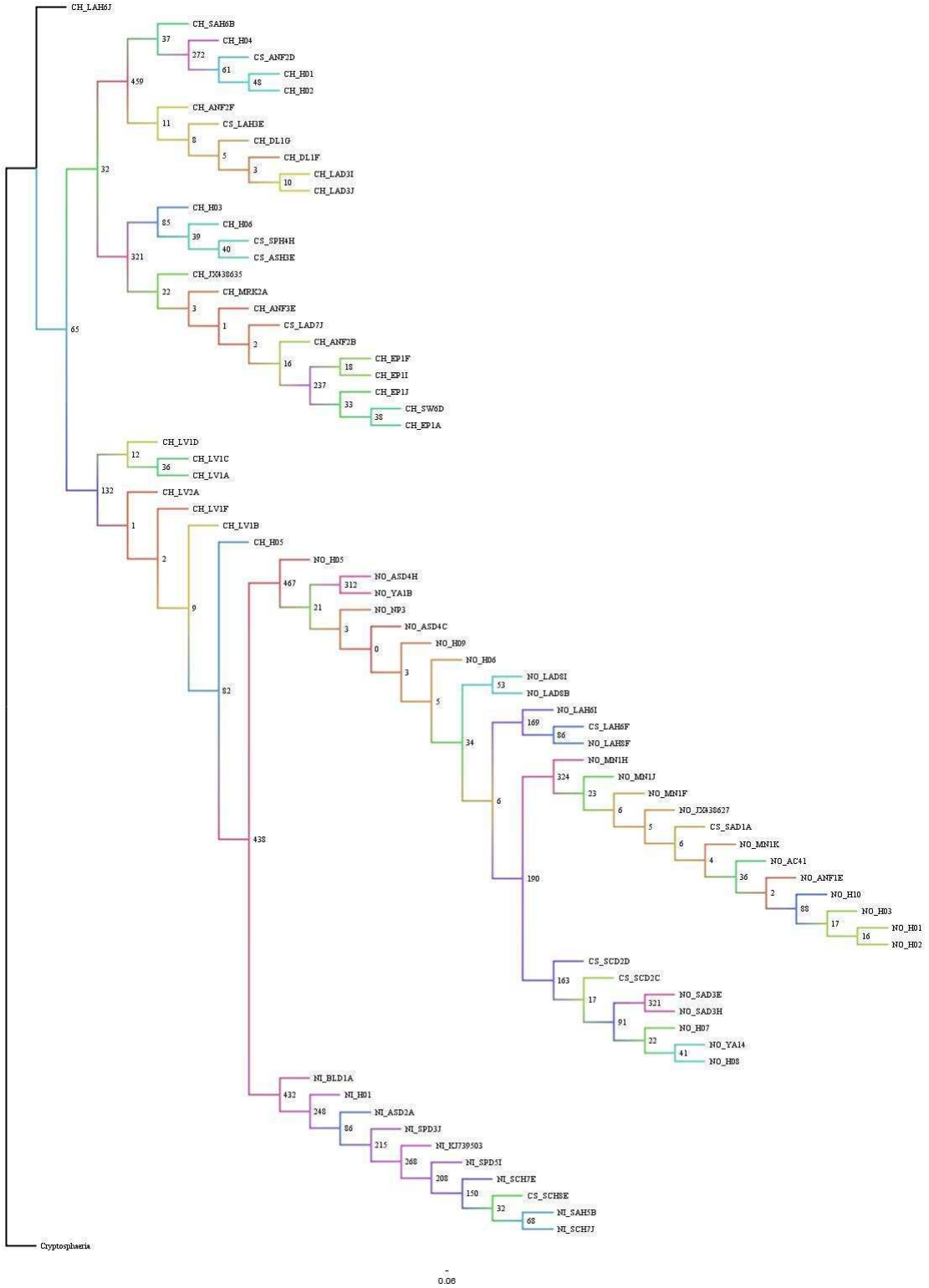

Figure 3.A5. A maximum parsimony (MP) phylogenetic tree, based on ITS sequences representing 67 isolates. Values are bootstrap values, with a maximum of 500 (100%).

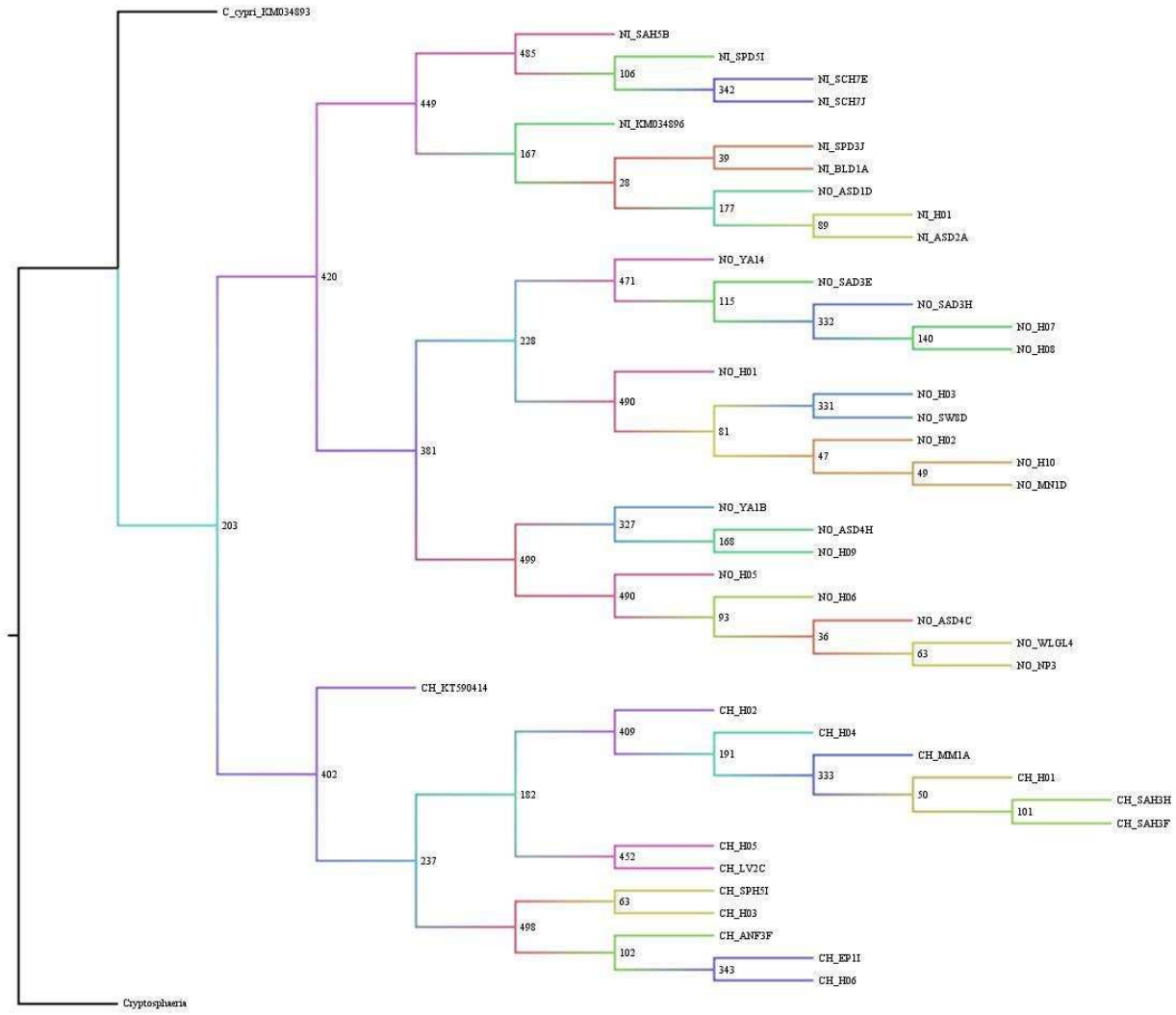

Figure 3.A6. A maximum parsimony (MP) phylogenetic tree, based on Bt sequences representing 45 isolates. Values are bootstrap values, with a maximum of 500 (100%).

Table 3.A1. All likely *Cytospora* isolates recovered from infected quaking aspen trees, collected from five states in the U.S.A.

Culture ID	Species	ITS	β-tubulin	Concateted Haplogroup	ITS Haplogrp name	Bt Haplogrp name	Tree ID	Site ID	State
AC 3-1	*C. notastroma*	**X**	**X**	CN_H05			AC3	AC	CO
AC 3-3	*C. notastroma*	**X**	**X**	CN_H05			AC3	AC	CO
AC 4-1	*C. notastroma*	**X**			NO_H20		AC4	AC	CO
ANF1B	*C. notastroma*	**X**	**X**	[Removed]			ANF1	ANF	UT
ANF1D	*C. notastroma*	**X**	**X**	[Removed]			ANF1	ANF	UT
ANF1E	*C. notastroma*	**X**			NO_H21		ANF1	ANF	UT
ANF1F	*C. notastroma*	**X**	**X**	CN_H10			ANF1	ANF	UT
ANF2B	*C. chrysosperma*	**X**			CH_H08		ANF2	ANF	UT
ANF2C	*C. chrysosperma*	**X**	**X**	CC_H01			ANF2	ANF	UT
ANF2D	*Cytospora sp.*	**X**	**X**		CS_H01	CS_H01	ANF2	ANF	UT
ANF2F	*C. chrysosperma*	**X**			CH_H15		ANF2	ANF	UT
ANF3B	*C. notastroma*	**X**	**X**	Ambig bp			ANF3	ANF	UT
ANF3C	*C. notastroma*	**X**	**X**				ANF3	ANF	UT
ANF3E	*C. chrysosperma*	**X**			CH_H09		ANF3	ANF	UT
ANF3F	*C. chrysosperma*		**X**			CH_H08	ANF3	ANF	UT
ASD1A	*C. chrysosperma*	**X**	**X**				ASD1	ASD	CO
ASD1B	*C. nivea*	**X**	**X**	CY_H01			ASD1	ASD	CO
ASD1D	*C. nivea*		**X**			NI_H09	ASD1	ASD	CO
ASD1F	*C. chrysosperma*	**X**	**X**	CC_H01			ASD1	ASD	CO
ASD1G	*C. nivea*	**X**	**X**				ASD1	ASD	CO
ASD1H	*C. nivea*	**X**	**X**	CY_H01			ASD1	ASD	CO
ASD2A	*C. nivea*	**X**	**X**		NI_H02	NI_H02	ASD2	ASD	CO
ASD2C	*C. nivea*	**X**	**X**	CY_H01			ASD2	ASD	CO
ASD2G	*C. chrysosperma*	**X**	**X**	[Removed]			ASD2	ASD	CO
ASD2H	Likely *Cytospora* sp.						ASD4	ASD	CO
ASD4B	*C. notastroma*	**X**	**X**	CN_H05			ASD4	ASD	CO
ASD4C	*C. notastroma*	**X**	**X**		NO_H11	NO_H11	ASD4	ASD	CO
ASD4F	Likely *Cytospora* sp.						ASD4	ASD	CO
ASD4H	*C. notastroma*	**X**	**X**		NO_H12	NO_H12	ASD4	ASD	CO
ASD4J	Likely *Cytospora* sp.						ASD4	ASD	CO
ASD5H	Likely *Cytospora* sp.						ASD5	ASD	CO
ASD6I	Likely						ASD6	ASD	CO

	Cytospora sp.								
ASD7A	Likely *Cytospora* sp.						ASD7	ASD	CO
ASD7D	Likely *Cytospora* sp.						ASD7	ASD	CO
ASD7G	Likely *Cytospora* sp.						ASD7	ASD	CO
ASD7I	Likely *Cytospora* sp.						ASD7	ASD	CO
ASD9B	Likely *Cytospora* sp.						ASD9	ASD	CO
ASD9C	Likely *Cytospora* sp.						ASD9	ASD	CO
ASD9D	Likely *Cytospora* sp.						ASD9	ASD	CO
ASD9E	Likely *Cytospora* sp.						ASD9	ASD	CO
ASD9G	Likely *Cytospora* sp.						ASD9	ASD	CO
ASD9H	Likely *Cytospora* sp.						ASD9	ASD	CO
ASD9I	Likely *Cytospora* sp.						ASD9	ASD	CO
ASD9J	Likely *Cytospora* sp.						ASD9	ASD	CO
ASH10B	*C. nivea*	**X**	**X**	CY_H01			ASH10	ASH	CO
ASH10C	*C. nivea*	**X**	**X**				ASH10	ASH	CO
ASH10E	*C. nivea*	**X**	**X**	CY_H01			ASH10	ASH	CO
ASH10G	Likely *Cytospora* sp.						ASH10	ASH	CO
ASH2I	*C. nivea*	**X**	**X**				ASH2	ASH	CO
ASH3D	Likely *Cytospora* sp.						ASH3	ASH	CO
ASH3E	*Cytospora sp.*	**X**	**X**		CS_H03	CS_H03	ASH3	ASH	CO
ASH3F	*Cytospora sp.*		**X**				ASH3	ASH	CO
ASH3I	*C. chrysosperma*	**X**	**X**	CC_H03			ASH3	ASH	CO
ASH5C	Likely *Cytospora* sp.						ASH5	ASH	CO
ASH5D	*C. notastroma*	**X**	**X**	[Removed]			ASH5	ASH	CO
ASH5F	*C. notastroma*	**X**	**X**	[Removed]			ASH5	ASH	CO
ASH5H	*C. notastroma*	**X**	**X**	[Removed]			ASH5	ASH	CO
ASH7I	Likely *Cytospora* sp.						ASH7	ASH	CO
ASH9A	Likely *Cytospora* sp.						ASH9	ASH	CO
ASH9B	Likely *Cytospora* sp.						ASH9	ASH	CO
ASH9H	Likely *Cytospora* sp.						ASH9	ASH	CO
BDSR1-2	*C. chrysosperma*	**X**	**X**	CC_H01			BDSR1	BDSR	CO
BDSR2-2	*C. notastroma*	**X**	**X**	CN_H01			BDSR2	BDSR	CO
BDSR2-3	Likely *Cytospora* sp.						BDSR2	BDSR	CO

BLD1A	*C. nivea*	**X**	**X**		NI_H03	NI_H03	BLD1	BLD	CO
BLD1F	*C. chrysosperma*	**X**	**X**				BLD1	BLD	CO
BLD1G	*C. chrysosperma*	**X**	**X**	CC_H01			BLD1	BLD	CO
BLD1H	Likely *Cytospora* sp.						BLD1	BLD	CO
BLD2A	*C. chrysosperma*	**X**	**X**	CC_H02			BLD2	BLD	CO
BLD2E	*C. chrysosperma*	**X**	**X**	CC_H02			BLD2	BLD	CO
BLD2J	*C. chrysosperma*	**X**	**X**	CC_H06			BLD2	BLD	CO
BLD3B	*C. notastroma*	**X**	**X**	CN_H05			BLD3	BLD	CO
BLD3D	*Cytospora sp.*		**X**				BLD3	BLD	CO
BLD3E	*Cytospora sp.*		**X**				BLD3	BLD	CO
BLD3I	*C. notastroma*	**X**	**X**	CN_H05			BLD3	BLD	CO
BLD3J	Likely *Cytospora* sp.						BLD3	BLD	CO
BLD4A	Likely *Cytospora* sp.						BLD4	BLD	CO
BLD4B	Likely *Cytospora* sp.						BLD4	BLD	CO
BLD4C	Likely *Cytospora* sp.						BLD4	BLD	CO
BLD4E	*C. chrysosperma*	**X**	**X**	CC_H02			BLD4	BLD	CO
BLD4F	*C. chrysosperma*	**X**	**X**	CC_H01			BLD4	BLD	CO
BLD4G	Likely *Cytospora* sp.						BLD4	BLD	CO
BLD4I	*C. chrysosperma*	**X**	**X**	CC_H01			BLD4	BLD	CO
BLD4J	Likely *Cytospora* sp.						BLD4	BLD	CO
BLH2C	Likely *Cytospora* sp.						BLH2	BLH	CO
BLH2D	Likely *Cytospora* sp.						BLH2	BLH	CO
BLH2E	Likely *Cytospora* sp.						BLH2	BLH	CO
BLH2F	Likely *Cytospora* sp.						BLH2	BLH	CO
BLH2G	Likely *Cytospora* sp.						BLH2	BLH	CO
BLH2J	Likely *Cytospora* sp.						BLH2	BLH	CO
BLH3A	Likely *Cytospora* sp.						BLH3	BLH	CO
BLH3G	Likely *Cytospora* sp.						BLH3	BLH	CO
BLH4A	Likely *Cytospora* sp.						BLH4	BLH	CO
BLH4E	Likely *Cytospora* sp.						BLH4	BLH	CO
BLH3B	Likely *Cytospora* sp.						BLH3	BLH	CO

BLH3D	Likely *Cytospora* sp.						BLH3	BLH	CO
BLH3E	Likely *Cytospora* sp.						BLH3	BLH	CO
BLH5A	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5B	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5C	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5D	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5E	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5F	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5G	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5H	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5I	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH5J	Likely *Cytospora* sp.						BLH5	BLH	CO
BLH6C	*C. chrysosperma*	**X**	**X**	CC_H03			BLH6	BLH	CO
BLH6E	*C. chrysosperma*	**X**	**X**	[Removed]			BLH6	BLH	CO
DG1-1A	*C. chrysosperma*	**X**	**X**	[Removed]			DG1	DG	CO
DG1-1B	*C. chrysosperma*	**X**	**X**	CC_H03			DG1	DG	CO
DG1-2A	*C. chrysosperma*	**X**	**X**	CC_H03			DG1	DG	CO
DL1C	*C. chrysosperma*	**X**	**X**	CC_H02			DL1	DL	CO
DL1E	*C. chrysosperma*	**X**	**X**	CC_H02			DL1	DL	CO
DL1F	*C. chrysosperma*	**X**			CH_H17		DL1	DL	CO
DL1G	*C. chrysosperma*	**X**			CH_H16		DL1	DL	CO
DL1H	*C. chrysosperma*	**X**	**X**	CC_H02			DL1	DL	CO
DL1I	*C. chrysosperma*	**X**	**X**	CC_H02			DL1	DL	CO
DL2B	Likely *Cytospora* sp.						DL2	DL	CO
DL3D	*C. notastroma*	**X**	**X**	CN_H01			DL3	DL	CO
DL3E	*C. notastroma*	**X**	**X**	CN_H01			DL3	DL	CO
DNF1B	*C. nivea*	**X**	**X**	CY_H01			DNF1	DNF	UT
DNF1C	*C. nivea*	**X**	**X**	CY_H01			DNF1	DNF	UT
EP1A	*C. chrysosperma*	**X**			CH_H10		EP1	EP	CO
EP1B	Likely *Cytospora* sp.						EP1	EP	CO
EP1C	Likely *Cytospora* sp.						EP1	EP	CO

EP1E	Likely *Cytospora* sp.						EP1	EP	CO
EP1F	*C. chrysosperma*	**X**			CH_H11		EP1	EP	CO
EP1I	*C. chrysosperma*	**X**	**X**		CH_H07	CH_H07	EP1	EP	CO
EP1J	*C. chrysosperma*	**X**			CH_H12		EP1	EP	CO
FC11	Likely *Cytospora* sp.						FC1	FC1	CO
FC1A	*C. chrysosperma*	**X**	**X**	CC_H06			FC1	FC1	CO
FC1C	Likely *Cytospora* sp.						FC1	FC1	CO
FC2A	*C. notastroma*	**X**	**X**	CN_H01			FC2	FC2	CO
FC2B	*C. chrysosperma*	**X**	**X**	CC_H06			FC2	FC2	CO
FC3B	*C. notastroma*	**X**	**X**	CN_H06			FC3	FC3	CO
FC4A	*C. chrysosperma*	**X**	**X**	CC_H03			FC4	FC4	CO
FC6	*C. chrysosperma*	**X**	**X**	CC_H03			FC6	FC6	CO
FC61	Likely *Cytospora* sp.						FC6	FC6	CO
FC61A	*C. chrysosperma*	**X**	**X**	[Removed]			FC6	FC6	CO
FC61B	*C. chrysosperma*	**X**	**X**	CC_H03			FC6	FC6	CO
FC62	*C. notastroma*	**X**	**X**	CN_H01			FC6	FC6	CO
FC63	*C. chrysosperma*	**X**	**X**	CC_H03			FC6	FC6	CO
FR1A	Likely *Cytospora* sp.						FR1	FR	CO
FR1B	Likely *Cytospora* sp.						FR1	FR	CO
FR1D	Likely *Cytospora* sp.						FR1	FR	CO
GW2A	*C. chrysosperma*	**X**	**X**	CC_H02			GW2	GW	CO
GW2B	*C. chrysosperma*	**X**	**X**	CC_H02			GW2	GW	CO
GW2C	*C. chrysosperma*	**X**	**X**	CC_H02			GW2	GW	CO
GW2D1	*C. chrysosperma*	**X**	**X**	CC_H01			GW2	GW	CO
GW2D2	*C. chrysosperma*	**X**	**X**	[Removed]			GW2	GW	CO
GW2E	*C. chrysosperma*	**X**					GW2	GW	CO
GW2F	*C. notastroma*	**X**	**X**	CN_H10			GW2	GW	CO
LAD10E	Likely *Cytospora* sp.						LAD10	LAD	WY
LAD10E	Likely *Cytospora* sp.						LAD10	LAD	WY
LAD10J	Likely *Cytospora* sp.						LAD10	LAD	WY
LAD1D	Likely *Cytospora* sp.						LAD1	LAD	WY

LAD2C	Likely *Cytospora* sp.						LAD2	LAD	WY
LAD2J	Likely *Cytospora* sp.						LAD2	LAD	WY
LAD3A	*C. notastroma*	**X**	**X**	CN_H03			LAD3	LAD	WY
LAD3B	*C. chrysosperma*	**X**	**X**	CC_H01			LAD3	LAD	WY
LAD3C	Likely *Cytospora* sp.						LAD3	LAD	WY
LAD3D	*C. chrysosperma*	**X**	**X**	CC_H01			LAD3	LAD	WY
LAD3E	Likely *Cytospora* sp.						LAD3	LAD	WY
LAD3F	Likely *Cytospora* sp.						LAD3	LAD	WY
LAD3G	Likely *Cytospora* sp.						LAD3	LAD	WY
LAD3H	Likely *Cytospora* sp.						LAD3	LAD	WY
LAD3I	*C. chrysosperma*	**X**			CH_H18		LAD3	LAD	WY
LAD3J	*C. chrysosperma*	**X**			CH_H19		LAD3	LAD	WY
LAD7C	Likely *Cytospora* sp.						LAD7	LAD	WY
LAD7I	Likely *Cytospora* sp.						LAD7	LAD	WY
LAD7J	*Cytospora sp.*	**X**					LAD7	LAD	WY
LAD8B	*C. notastroma*	**X**			NO_H19		LAD8	LAD	WY
LAD8E	Likely *Cytospora* sp.						LAD8	LAD	WY
LAD8I	*C. notastroma*	**X**			NO_H29		LAD8	LAD	WY
LAH10D	Likely *Cytospora* sp.						LAH10	LAH	WY
LAH10F	Likely *Cytospora* sp.						LAH10	LAH	WY
LAH10G	Likely *Cytospora* sp.						LAH10	LAH	WY
LAH10I	Likely *Cytospora* sp.						LAH10	LAH	WY
LAH1A	*C. chrysosperma*	**X**	**X**	[Removed]			LAH1	LAH	WY
LAH1B	*C. chrysosperma*	**X**	**X**	CC_H04			LAH1	LAH	WY
LAH1C	Likely *Cytospora* sp.	**X**					LAH1	LAH	WY
LAH1D	*C. chrysosperma*	**X**	**X**	CC_H01			LAH1	LAH	WY
LAH1E	*C. chrysosperma*	**X**	**X**	CC_H01			LAH1	LAH	WY
LAH1H	*C. chrysosperma*	**X**	**X**	CC_H01			LAH1	LAH	WY
LAH1I	Likely *Cytospora* sp.						LAH1	LAH	WY
LAH1J	Likely *Cytospora* sp.						LAH1	LAH	WY
LAH1J	Likely *Cytospora* sp.						LAH1	LAH	WY

LAH2A1	Likely *Cytospora* sp.						LAH2	LAH	WY
LAH2A1	Likely *Cytospora* sp.						LAH2	LAH	WY
LAH2A2	*C. chrysosperma*	**X**	**X**	CC_H01			LAH2	LAH	WY
LAH2A3	*C. chrysosperma*	**X**	**X**	CC_H01			LAH2	LAH	WY
LAH2B	*C. chrysosperma*	**X**	**X**	CC_H04			LAH2	LAH	WY
LAH3E	*Cytospora sp.*	**X**					LAH3	LAH	WY
LAH4F	Likely *Cytospora* sp.						LAH4	LAH	WY
LAH4G	Likely *Cytospora* sp.						LAH4	LAH	WY
LAH5C	Likely *Cytospora* sp.						LAH5	LAH	WY
LAH5J	Likely *Cytospora* sp.						LAH5	LAH	WY
LAH6B	*C. notastroma*	**X**	**X**	CN_H08			LAH6	LAH	WY
LAH6D	Likely *Cytospora* sp.						LAH6	LAH	WY
LAH6F	*C. notastroma*	**X**	**X**		NO_H28		LAH6	LAH	WY
LAH6G	*C. chrysosperma*	**X**	**X**	CC_H05			LAH6	LAH	WY
LAH6H	Likely *Cytospora* sp.						LAH6	LAH	WY
LAH6I	*C. notastroma*	**X**			NO_H23		LAH6	LAH	WY
LAH6J	*C. chrysosperma*	**X**			CH_H28		LAH6	LAH	WY
LAH7B	Likely *Cytospora* sp.						LAH7	LAH	WY
LAH8F	*C. notastroma*	**X**			NO_H24		LAH8	LAH	WY
LAH9C	Likely *Cytospora* sp.						LAH9	LAH	WY
LAH9F	Likely *Cytospora* sp.						LAH9	LAH	WY
LAH9G	Likely *Cytospora* sp.						LAH9	LAH	WY
LAH9J	Likely *Cytospora* sp.						LAH9	LAH	WY
LV1A	*C. chrysosperma*	**X**			CH_H22		LV1	LV	CO
LV1B	*C. chrysosperma*	**X**			CH_H23		LV1	LV	CO
LV1C	*C. chrysosperma*	**X**			CH_H24		LV1	LV	CO
LV1D	*C. chrysosperma*	**X**			CH_H25		LV1	LV	CO
LV1F	*C. chrysosperma*	**X**			CH_H26		LV1	LV	CO
LV1G	*C. chrysosperma*	**X**	**X**	CC_H05			LV1	LV	CO
LV2A	*C. chrysosperma*	**X**			CH_H27		LV2	LV	CO
LV2B	*C. chrysosperma*	**X**	**X**	CC_H05			LV2	LV	CO

LV2C	*C. chrysosperma*		**X**			CH_H09	LV2	LV	CO
LV2D	Likely *Cytospora* sp.						LV2	LV	CO
MKR1A	Likely *Cytospora* sp.						MKR1	MKR	CO
MKR1B	Likely *Cytospora* sp.						MKR1	MKR	CO
MKR1C	Likely *Cytospora* sp.						MKR1	MKR	CO
MKR22	*C. chrysosperma*	**X**	**X**	CC_H03			MKR2	MKR	CO
MKR22_EL	*C. chrysosperma*	**X**					MKR2	MKR	CO
MKR2A	*C. chrysosperma*	**X**			CH_H14		MKR2	MKR	CO
MKR2B	Likely *Cytospora* sp.						MKR2	MKR	CO
MKR2D	Likely *Cytospora* sp.						MKR2	MKR	CO
MKR2D	Likely *Cytospora* sp.						MKR2	MKR	CO
MKR2E	*Cytospora sp.*		**X**			CS_H05	MKR2	MKR	CO
MM1A	*C. chrysosperma*		**X**			CH_H11	MM1	MM	CO
MM1B	*C. chrysosperma*	**X**	**X**	[Removed]			MM1	MM	CO
MM1E	*C. chrysosperma*	**X**	**X**	[Removed]			MM1	MM	CO
MN1A	*C. notastroma*	**X**	**X**	CN_H01			MN1	MN	MN
MN1B	*C. notastroma*	**X**	**X**	CN_H01			MN1	MN	MN
MN1C	*C. notastroma*	**X**	**X**	CN_H01			MN1	MN	MN
MN1D	*C. notastroma*		**X**			NO_H18	MN1	MN	MN
MN1E	*C. notastroma*	**X**	**X**	CN_H01			MN1	MN	MN
MN1F	*C. notastroma*	**X**			NO_H26		MN1	MN	MN
MN1G	Likely *Cytospora* sp.	**X**					MN1	MN	MN
MN1H	*C. notastroma*	**X**			NO_H27		MN1	MN	MN
MN1K	*C. notastroma*	**X**			NO_H25		MN1	MN	MN
MNIJ	*C. notastroma*	**X**			NO_H18		MN1	MN	MN
NL2B	Likely *Cytospora* sp.						NL2	NL	UT
NL3B	*C. notastroma*	**X**	**X**	CN_H02			NL3	NL	UT
NL4B	*C. notastroma*	**X**	**X**	CN_H08			NL4	NL	UT
NL4C	*C. notastroma*	**X**	**X**	CN_H09			NL4	NL	UT
NP1	*C. notastroma*	**X**	**X**	CN_H09			NP1	NP	CO
NP2	Likely *Cytospora* sp.						NP1	NP	CO
NP3	*C. notastroma*	**X**	**X**		NO_H13	NO_H13	NP1	NP	CO
PF1	*C. notastroma*	**X**	**X**	CN_H07			PF1	PF	CO
PF2	*C. notastroma*	**X**	**X**	CN_H07			PF1	PF	CO

PF3	*C. notastroma*	**X**	**X**	CN_H07			PF1	PF	CO
RCKEP1A	*C. notastroma*	**X**	**X**	CN_H06			RCKEP1	RC	CO
RCKEP2A	*C. notastroma*	**X**	**X**	CN_H06			RCKEP1	RC	CO
RCKEP3A	*C. notastroma*	**X**	**X**	CN_H06			RCKEP1	RC	CO
SAD10E	Likely *Cytospora* sp.						SAD10	SAD	CO
SAD10F	Likely *Cytospora* sp.						SAD10	SAD	CO
SAD1A	*Cytospora sp.*	**X**					SAD1	SAD	CO
SAD1C	Likely *Cytospora* sp.						SAD1	SAD	CO
SAD1E	*Cytospora sp.*		**X**			CS_H04	SAD1	SAD	CO
SAD1F	Likely *Cytospora* sp.						SAD1	SAD	CO
SAD1H	Likely *Cytospora* sp.						SAD1	SAD	CO
SAD3A	Likely *Cytospora* sp.						SAD3	SAD	CO
SAD3D	*Cytospora sp.*		**X**				SAD3	SAD	CO
SAD3E	*C. notastroma*	**X**	**X**	[Removed]	NO_H15	NO_H15	SAD3	SAD	CO
SAD3H	*C. notastroma*	**X**	**X**	[Removed]	NO_H16	NO_H16	SAD3	SAD	CO
SAD5B	Likely *Cytospora* sp.						SAD5	SAD	CO
SAD5F	Likely *Cytospora* sp.						SAD5	SAD	CO
SAD5H	Likely *Cytospora* sp.						SAD5	SAD	CO
SAD5J	Likely *Cytospora* sp.						SAD5	SAD	CO
SAD9H	Likely *Cytospora* sp.						SAD9	SAD	CO
SAH3B	Likely *Cytospora* sp.						SAH3	SAH	CO
SAH3C	*C. chrysosperma*	**X**	**X**	CC_H01			SAH3	SAH	CO
SAH3D	*C. chrysosperma*	**X**	**X**	CC_H01			SAH3	SAH	CO
SAH3F	*C. chrysosperma*		**X**			CH_H12	SAH3	SAH	CO
SAH3H	*C. chrysosperma*		**X**			CH_H13	SAH3	SAH	CO
SAH3J	*C. chrysosperma*	**X**	**X**	[Removed]			SAH3	SAH	CO
SAH5B	*C. nivea*	**X**	**X**		NI_H04	NI_H04	SAH5	SAH	CO
SAH5E	Likely *Cytospora* sp.						SAH5	SAH	CO
SAH5H	Likely *Cytospora* sp.						SAH5	SAH	CO
SAH6B	*C. chrysosperma*	**X**			CH_H20		SAH6	SAH	CO
SAH6J	*Cytospora* sp.		**X**		CS_H06	CS_H06	SAH6	SAH	CO
SAH7E	Likely *Cytospora* sp.						SAH7	SAH	CO
SCD2C	*Cytospora sp.*	**X**	**X**				SCD2	SCD	CO

SCD2D	*Cytospora sp.*	**X**	**X**				SCD2	SCD	CO
SCD2E	Likely *Cytospora* sp.						SCD2	SCD	CO
SCD2F	*C. notastroma*	**X**	**X**	CN_H08			SCD2	SCD	CO
SCD2J	Likely *Cytospora* sp.						SCD2	SCD	CO
SCD5B	Likely *Cytospora* sp.						SCD5	SCD	CO
SCH10C	*Cytospora sp.*	**X**	**X**				SCH10	SCH	CO
SCH10D	Likely *Cytospora* sp.						SCH10	SCH	CO
SCH10F	Likely *Cytospora* sp.						SCH10	SCH	CO
SCH6C	Likely *Cytospora* sp.						SCH6	SCH	CO
SCH6H	Likely *Cytospora* sp.						SCH7	SCH	CO
SCH7A	Likely *Cytospora* sp.						SCH7	SCH	CO
SCH7B	Likely *Cytospora* sp.						SCH7	SCH	CO
SCH7C	Likely *Cytospora* sp.						SCH7	SCH	CO
SCH7E	*C. nivea*	**X**	**X**		NI_H05	NI_H05	SCH7	SCH	CO
SCH7H	Likely *Cytospora* sp.						SCH7	SCH	CO
SCH7J	*C. nivea*	**X**	**X**		NI_H06	NI_H06	SCH7	SCH	CO
SCH8D	Likely *Cytospora* sp.						SCH8	SCH	CO
SCH8E	*Cytospora sp.*						SCH8	SCH	CO
SCH8H	*Cytospora sp.*	**X**	**X**				SCH8	SCH	CO
SPD1B	Likely *Cytospora* sp.						SPD1	SPD	CO
SPD2C	Likely *Cytospora* sp.						SPD2	SPD	CO
SPD2I	Likely *Cytospora* sp.						SPD2	SPD	CO
SPD2J	Likely *Cytospora* sp.						SPD2	SPD	CO
SPD3J	*C. nivea*	**X**	**X**		NI_H07	NI_H07	SPD3	SPD	CO
SPD4A	Likely *Cytospora* sp.						SPD4	SPD	CO
SPD4B	Likely *Cytospora* sp.						SPD4	SPD	CO
SPD4C	Likely *Cytospora* sp.						SPD4	SPD	CO
SPD4D	*C. nivea*	**X**	**X**	CY_H01			SPD4	SPD	CO
SPD4F	*C. chrysosperma*	**X**	**X**	CC_H01			SPD4	SPD	CO
SPD4H	*C. chrysosperma*	**X**	**X**	CC_H01			SPD4	SPD	CO
SPD4I	Likely *Cytospora* sp.						SPD4	SPD	CO
SPD4J	Likely *Cytospora* sp.						SPD4	SPD	CO

SPD5F	*Cytospora sp.*	**X**	**X**				SPD5	SPD	CO
SPD5I	*C. nivea*	**X**	**X**		NI_H08	NI_H08	SPD5	SPD	CO
SPD7A	Likely *Cytospora* sp.						SPD7	SPD	CO
SPD7A	Likely *Cytospora* sp.						SPD7	SPD	CO
SPH2B	*C. chrysosperma*	**X**	**X**	CC_H04			SPH2	SPH	CO
SPH2C	*C. chrysosperma*	**X**	**X**	CC_H04			SPH2	SPH	CO
SPH3B	*Cytospora sp.*	**X**					SPH3	SPH	CO
SPH4C	Likely *Cytospora* sp.						SPH4	SPH	CO
SPH4D	Likely *Cytospora* sp.						SPH4	SPH	CO
SPH4E	Likely *Cytospora* sp.						SPH4	SPH	CO
SPH4F	Likely *Cytospora* sp.						SPH4	SPH	CO
SPH4G	Likely *Cytospora* sp.						SPH4	SPH	CO
SPH4H	*Cytospora sp.*	**X**	**X**		CS_H02	CS_H02	SPH4	SPH	CO
SPH4J	Likely *Cytospora* sp.						SPH4	SPH	CO
SPH5I	*C. chrysosperma*		**X**			CH_H10	SPH5	SPH	CO
SPH6D	Likely *Cytospora* sp.						SPH6	SPH	CO
SPH6J	Likely *Cytospora* sp.						SPH6	SPH	CO
SPH7I	Likely *Cytospora* sp.						SPH7	SPH	CO
SPH7I	Likely *Cytospora* sp.						SPH7	SPH	CO
SRES2	*C. notastroma*	**X**	**X**	CN_H05			SRES2	SRES	CO
SRES4C	*C. notastroma*	**X**	**X**	CN_H10			SRES4	SRES	CO
SW2P1	Likely *Cytospora* sp.						SW1	SW	CO
SW5B	*C. notastroma*	**X**	**X**	CN_H02			SW1	SW	CO
SW6D	*C. chrysosperma*	**X**			CH_H13		SW1	SW	CO
SW6P2	*C. notastroma*	**X**	**X**	CN_H03			SW1	SW	CO
SW6P3	*C. notastroma*	**X**	**X**	CN_H02			SW1	SW	CO
SW6P4	*C. notastroma*	**X**	**X**	CN_H02			SW1	SW	CO
SW6P5	*C. notastroma*	**X**	**X**	CN_H02			SW1	SW	CO
SW7C	*C. notastroma*	**X**	**X**	CN_H02			SW1	SW	CO
SW7C1	*C. notastroma*	**X**	**X**	CN_H03			SW1	SW	CO
SW7D	*C. notastroma*	**X**	**X**	CN_H03			SW1	SW	CO
SW8A	*C. notastroma*	**X**	**X**	CN_H03			SW1	SW	CO

Table 3.A2. Collection locations of *Cytospora* isolates included in this study.

Site name	UTM, Easting	UTM, Northing
LAD	472334.0	4570564.0
LAH	466252.0	4556893.0
MM	454539.8	4516146.8
BLD	271265.4	4451436.4
BLH	284246.0	4415173.0
DL	453162.3	4516086.7
ASD	343811.7	4326303.5
ASH	327989.8	4373583.0
SAH	390930.2	4293937.7
SAD	419397.4	4302439.3
SCD	446913.1	4217969.8
SCH	489489.9	4212748.6
SPH	447516.7	4301889.5
SPD	431745.2	4364322.1
BDSR	455823.8	4510856.2
SW	506267.1	4427201.7
FC	490496.1	4488533.0
FC	492362.9	4488272.9
FC	490619.1	4489467.5
FC	491143.9	4488214.7
RC	434456.2	4504205.5
AC	443265.3	4505539.1
DG	452653.4	4505719.7
FC	492152.6	4488363.5
EP	451597.6	4471953.1
LV	513507.0	4420844.1
SRES	337400.1	4446646.4
GW	298028.7	4381952.2
MKR	251878.0	4436740.9
NP	414451.5	4481497.5
PF	431237.9	4501222.4
YA	320137.3	4450162.0
ANF	90405.1	4520231.6
NL	-202946.1	4191778.6
DNF	-202946.1	4191778.6
WLGL	-66743.7	5424446.1
MN	1456060.3	5102951.6

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CHAPTER 4

THE ASPEN BARK BEETLE, *TRYPOPHLOEUS POPULI*, AS A POTENTIAL VECTOR OF CYTOSPORA CANKER (*CYTOSPORA* SPP.) ON QUAKING ASPEN (*POPULUS TREMULOIDES* MICHX.)

SUMMARY

The aspen bark beetle, *Trypophloeus populi*, is known as a stress-related damage agent on quaking aspen. In a previous study, we often found *T. populi* attacking host trees also infected with Cytospora canker. We wished to determine whether *T. populi* is a potential vector of Cytospora canker, and whether *Cytospora* inoculum could be recovered from adult beetles or gallery tissues. We did not recover any *Cytospora* isolates from 161 adult *T. populi* beetles cultured, and only two *Cytospora* isolates from 42 beetle galleries and seven adult aspen. We suspect that these isolates, cultured from two trees, were a result of a previous infection, as both host trees had extensive cankers as well as *Cytospora* fruiting bodies.

INTRODUCTION

In the western United States, two species of bark beetles (Solytinae) are known to attack quaking aspen (*Populus tremuloides*): *Trypophloeus populi* and *Procryphalus mucronatus* (Petty, 1977). Both are of the Cryphalini tribe, and are phloeophagus and monogamous (Wood, 1982). Identifying these insects generally requires a microscope. *T. populi* averages 1.5-2.1 mm and *P. mucronatus* averages 1.8-2.2 mm in length. A reliable feature for telling the two apart is the length of the antennal funicle and the shape of the antennal club; *T. populi* have a five-segmented

funicle, and a slender antennal club, the apex of which is narrowed. Members of *P. mucronatus* have a distinctly rounded antennal club (Wood, 1982).

Weakened or stressed aspen are preferred hosts of *T. populi*, although apparently healthy trees have also been reported to be mass-attacked by this insect (Petty, 1977; Stewart et al. 1979). Adults emerge in July-August and fly to new host trees, attacking the main stem and branches of the host from the lower bole to the crown (Petty, 1977; Stewart et al. 1979). Female beetles begin to excavate a cave type primary gallery and as the females emerge to remove boring dust, males copulate with them. The male may then either move on or follow the female into the gallery (Petty, 1977; Stewart et al. 1979). Territorial behavior occurs between males and females: a female searching for a potential gallery site may displace another female already constructing her primary gallery; any male protruding above a primary gallery entrance is a potential target for other, mate-less males (Petty, 1977; Stewart et al. 1979). Upon completion of the primary gallery, females lay an average of fourteen eggs either in a single mass or in two to three separate lobes in the primary gallery directly below the paper-thin outer bark (Petty, 1977; Stewart et al. 1979). Petty (1977) hypothesized that such a placement aids larval development, as heat from the sun could aid incubation, or allow air flow around the egg masses, preventing bacterial or fungal infestation. First-instar larvae construct meandering feeding tunnels, and pass through a second and third instar before excavating a frass-free pupation niche. Newly formed adults emerge and seek a new host tree (Petty, 1977; Stewart et al. 1979). All instar phases may overwinter, however Petty (1977) observed that survival was greater for second- and third-instars. Former brood trees can be identified by the characteristic cracking of the thin bark covering the primary and larval feeding galleries (Petty, 1977; Stewart et al. 1979).

A tree that has been host to *Trypophloeus populi* may weeks later become attacked by *Procryphalus mucronatus*, which is noted by Petty (1977). Unlike its cousin, *P. mucronatus* targets recently-killed host trees, particularly those with spongy, fermenting bark (Petty, 1977; Stewart et al. 1979). Host selection and mating behaviors are very similar to *T. populi*, with the exception that male *P. mucronatus* beetles help the females excavate the primary gallery, which is slightly larger (14 mm average length, vs 10 mm) than *T. populi*. Females lay an average of 16 individual eggs in niches along the primary gallery, and then cover each with boring dust. Larvae feed in a meandering pattern, and pass through two instar phases before pupating. All life stages except pupae and eggs may overwinter. Adults that have overwintered may emerge as early as April and May to search for new hosts. *Procryphalus mucronatus* produces 1.5- 2 generations per year (Petty, 1977; Stewart et al. 1979).

A third bark beetle known to occur on *Populus tremuloides*, though not in Colorado, is *Trypophloeus thatcheri*. Like *T. populi*, this species attacks bark tissue of dying aspen or black cottonwood (*Populus trichocarpa*) (Wood, 1982). Incidence of this beetle has been reported along the Pacific coast, from British Columbia to California (Wood, 1982). Another *Trypophloeus* species found in North America, *T. striatulus* (Mannerheim) occurs primarily in the boreal zone from Alaska to Nova Scotia and feeds on felt leaf willow (*Salix alexensis* (Andersson) Coville) (Furniss, 2004). Furniss frequently observed Cytospora cankers on stems infested by *T. striatulus*, although he found no evidence that the beetles vectored the fungus (*Cytospora* sp.) (2004).

Three other beetles often found on quaking aspen in Colorado include an ambrosia beetle, *Trypodendron retusum*, and two wood borer species, *Saperda calcarata* (Cerambycidae) and

Agrilus liragus (Buprestidae). The widely distributed aspen ambrosia beetle, *Typodendron retusum* (Cucurlionidae), attacks weakened aspen trees (Hinds & Davidson, 1972).

Petty, Stewart, and others in their investigations of *T. populi* (Petty, 1977; Stewart et al. 1979), observed that trees recently attacked by these beetles often displayed areas of orange tissue surrounding entrance holes. He successfully cultured the fungus, but did not identify it. This work, coupled with an aspen health survey conducted 2009-2010, in which we observed that aspen that had been attacked by bark beetles also had Cytospora canker, led us to speculate that *T. populi* is a vector for at least one species of *Cytospora*. The questions we wished to answer were: (1) do adult *T. populi* beetles carry spores or hyphae of one or more *Cytospora* species? (2) Does one or more *Cytospora* species occur in *T. populi* egg galleries?

MATERIALS AND METHODS

Emergence cages

Eight dying adult aspen trees having a DBH of 12.0 cm or greater were collected from five locations in Fort Collins, Colorado, and from a single location on the Pike National Forest in central Colorado. Three of the trees selected showed evidence of past beetle activity, such as minute exit holes (< 1 mm diameter), and small (2-3 cm) diameter patches of dead bark, which when removed revealed meandering feeding galleries. Potential source trees were identified and felled between June of 2012 and April 2014. Emergence boxes used in this study were constructed from wooden oriented strand board (OSB), and measured approximately 90cm on each side. The front of each box was covered with bronze screen, and a square piece of black woven weed barrier to exclude daylight. A glass mason jar was affixed over a circular hole over the bottom of each box, and was the sole source of light into each emergence box. Two to three

logs from the same tree were placed in each emergence box, and jars were checked for beetles weekly for a period of 6-8 months. Emerged beetles were placed in sterile, 1.0 mL plastic vials and stored in a -20 C freezer. All beetles were examined under a dissecting microscope to confirm the species, and tallied based on date of emergence and source tree. Other insects emerging from the source trees were noted, but not tallied.

Feeding Assay

Adult beetles were collected daily as they emerged, and were immediately placed in a paper bag with freshly-cut sections of young aspen (2-3 cm dbh), and the bag was then placed inside of a clear plastic box (45 cm x 25 cm x 20 cm). A dozen beetles were placed in each box, with a total of six boxes of beetles and aspen branches. Boxes were stacked out of direct sunlight in a laboratory at ambient (25 °C) conditions, and checked daily for evidence of beetle feeding.

Trapping Assay

It is unknown whether *T. populi* respond to a particular hormone lure, so we utilized three different lure types (ambrosia beetle lure, turpentine beetle lure, or none) placed in Lindgrenn funnel-type traps at two sites on the Pike National Forest in central Colorado in June, 2012. The two sites were selected based upon a previous study, conducted in 2009-2010 that indicated presence of dying trees and aspen bark beetles (Dudley, et al. 2015). At each site, a 278-meter (900 ft) transect was established, and one trap hung from the nearest adult aspen tree every 46 meters (150 ft) along the transect. For each trap placed along the transect, two more traps were established ten meters from the first trap, at an angle of 60° between them (Fig 1). A total of twenty-one traps were established at each of the two sites, in seven clusters of three traps. Each cluster included one trap without a lure ("none"), one trap with an ambrosia beetle-type lure (*Gnathotrichus sulcatus*), and one trap with a turpentine beetle-type lure (*Dendroctonus valens*).

Beetle lures were obtained from Synergy Semiochemicals Corporation (Burnaby, BC, Canada). No killing agent or liquid was used in collection cups. All traps were checked weekly for beetles June-September, 2012.

Isolation of beetles and beetle parts

A series of isolations were made from various beetle parts using frozen *T. populi* adult beetles obtained from emergence boxes. Either ½-strength potato dextrose agar (PDA) or ¼-strength PDA amended with two antibiotics (streptomycin and chlorothalonil at 100 mg/L) was used in all isolations. A variety of aseptic isolation techniques were utilized, including: whole beetle maceration; whole beetle maceration with surface disinfestation (using either a 10% bleach solution, or 70% ETOH); direct plating of beetle elytra (6 beetles); streaking of a plate with beetle elytra (6 beetles); vortex and streaking of an intact beetle in sterile water (50 beetles); maceration and plating of the macerate (50 beetles). In combination with these techniques, we also vortexed some whole adult beetles in sterile water and in a solution of sterile water and Tergitol™ 15-S-9 surfactant (The Dow® Chemical Company, Midland, MI), or sterile water and mild liquid castile soap (Dr Bronner's® Magic Soaps, Vista, CA). Three concentrations of Tergitol® were tested, including a 1:50 (0.02x) surfactant:sterile water solution (36 beetles), as well as a 1:10,000 (0.00001x) solution (18 beetles) and1:12,500 (0.000008x) surfactant:sterile water solution (24 beetles). Three concentrations of Dr Bronner's ® soap were tested, including a 1:50 (0.02x) sufactant:sterile water solution (24 beetles), a 1:100 (0.01x) sufactant:sterile water solution (14 beetles), and a 1:200 (0.005x) sufactant:sterile water solution (14 beetles); plating of beetle head capsule following immersion and vortexing in a 0.01x solution of castile soap in sterile water (8 beetles); plating of beetle head capsule following immersion and vortexing in a 0.005x solution of castile soap in sterile water (8 beetles) For each surfactant solution, one beetle

was placed in a sterile 1.2 mL screw-top microcentrifuge vial and placed in a shaker for 45 seconds, then a centrifuge for 30 seconds in order to dissipate excessive foam. The contents of each vial was streaked onto a plate of ½-strength potato dextrose agar (PDA), sealed with Parafilm M ® (Bemis Company, Inc. Oshkosh, WI), and placed in an incubation chamber at 25 °C for a period of up to three weeks. Cultures were examined for presence of *Cytospora* species, and a few non-Cytospora single-spore isolates were transferred to a 250-mL beaker with liquid media (potato dextrose broth). Beakers were placed in a shaking incubator for 5-6 days at 25 °C.

Once fungal tissue samples had grown to about 2 cm in diameter, they were extracted from the liquid medium using a centrifuge, placed in 2 mL plastic vials, and stored in a -4 °C freezer. DNA extraction was performed using the Invitrogen DNA extraction kit (Life Technologies, Grand Island, NY). Following extraction, nucleotide concentration of the samples was assessed using a Nanodrop© 2000 sensor (NanoDrop Products, Thermo Scientific, Wilmington, DE). Samples that contained at least 15 ng/µl of DNA were then used for polymerase chain reaction (PCR), using a MyCycler ™ Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA). Primers used in this study included ITS1 and ITS4, for the amplification of the 5.8S rDNA subunit. Successful PCR products were purified using a Roche High Pure PCR Product Purification Kit (Roche Diagnostics Corp., Indianapolis, IN), and DNA concentrations were again assessed using the Nanodrop© sensor. 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). All sequences were identified using the standard nucleotide BLAST Sequence Analysis Tool (Altschul, et al. 1990).

Isolation of gallery tissues

A series of isolations were conducted using discolored bark tissue in and around beetle galleries. Six individual galleries from seven of the eight attacked aspen trees (previously placed in emergence cages) were selected, and four pieces of bark tissue (1-2 mm^2) were removed from each gallery and plated onto a single plate of ½-strength potato dextrose agar (PDA). All forty-two plates were incubated in a laboratory under ambient conditions (25 °C) for a period of three weeks, during which they were examined for evidence of *Cytospora* species.

RESULTS

Emergence cages, feeding and trapping assays.

We collected 830 adult *T. populi* beetles and 450 adult *P. mucronatus* beetles from eight adult aspen trees over a period of eight to twelve months. The emergence period for *T. populi* lasted from May-July, and peaked in May (Figure 2). The emergence period for *P. mucronatus* lasted from June- August, and peaked in July (Figure 2). Under the artificial conditions in the lab, *T. populi* appeared to either undergo a second emergence period in the autumn, with 2 beetles emerging as late as January (Figure 2), or some of the adult beetles that emerged earlier in the spring may have tunneled back into the logs still in the cages and produced a second, false, emergence.

Of the 72 adult *T. populi* beetles placed in bins with freshly-cut aspen branches, only a single beetle appeared to feed on the aspen branches. All others died without feeding or producing entrance holes in the bark.

A single adult *T. populi* beetle was captured over the summer of 2012. A variety of insects were trapped each week, but none of them pertained to the focus of this study.

Isolation from beetle parts and plant tissues surround galleries.

Overall, 76% the 214 plates (representing 161 bark beetles) produced no fungi, and very little bacteria (Figure 2). No *Cytospora* isolates were recovered from any of the plates. The non-*Cytospora* fungal cultures obtained included *Chaetomium globosum* and *Clonostachys rosea*.

Of the seven aspen trees and 42 galleries examined and cultured, we recovered two *Cytospora* isolates from two galleries in two trees. These isolates were identified by morphology to be *C. chrysosperma*.

DISCUSSION

The results of this study suggests that the aspen bark beetle, *T. populi*, does not vector *Cytospora* species, or if it does, it is a poor vector. Although we recovered *Cytospora* isolates from tissues surrounding galleries on two aspen trees, we suspect that these trees were systemically infected with the fungus, as cultures were also made from numerous fruiting bodies found in clusters over most of the bark surface. Worrall et al. (2010) successfully isolated *C. umbria* from symptomless alder (*Alnus incana* ssp. *tenuifolia*) tree tissues, and thus it is possible that the *Cytospora* we isolated were, at that time, existing epiphytically.

Petty (1977) described an orange-colored canker on the aspen trees from which he collected *T. populi*. Although he did not identify the fungus, it seems likely that the canker he was describing was *Cytospora*. However, it is important to note that both *T. populi* and *Cytospora* spp. are secondary damage agents to quaking aspen, and generally only successfully colonize hosts trees experiencing some form of environmental stress (Bloomberg, 1962a & 1962b; Petty, 1977; Guyon, et al. 1996; Marchetti, et al. 2011). Cytospora is nearly ubiquitous in quaking aspen stands, especially those undergoing extensive dieback and mortality (Worrall, et

al. 2008; Marchetti et al. 2011; Dudley, et al. 2015). Thus, it may have been coincidental that the dying aspen on which the Petty's (1977) description of *T. populi* was based also had Cytospora canker. Alternatively, the beetles could also provide entry points to infection by *Cytospora* from the feeding and egg gallery construction.

We note that Furniss (2004) also hypothesized that *T. striatulus* (found on *Salix alaxensis* Colville) carried *Cytospora*, as he described frequently finding the two together on the same host. Upon examination with electron microscopy, however, Furniss showed that the few conidiospores present on *T. striatulus* adults were far too large (with a diameter of 8 μm) to be *Cytospora* conidia (approximately 3 μm) (Furniss, 2004). We did not investigate the possibility that *Cytospora* species could produce volatile compounds as it attacks a host tree, which could serve as an attractant to *T. populi*. This idea was likewise suggested by Furniss (2004) to occur between *S. alaxensis* and *T. striatulus*. However, because we could not consistently isolate *Cytospora* from the majority of host tissues (or adult beetles), an investigation of the purported semiochemicals found such a relationship would have to include those compounds produced by drought-stressed or otherwise dying quaking aspen trees.

In summary, we did not detect *Cytospora* on any of the adult aspen bark beetles we attempted isolations from, and recovered only two *Cytospora* isolates overall from gallery tissue, and which we suspect were a result of a previously-infected host tree. Thus, although multiple aspen health surveys have detected high incidence of both Cytospora canker and evidence of aspen bark beetles, the relationship between the two (if one exists) remains unclear.

FIGURES AND TABLES

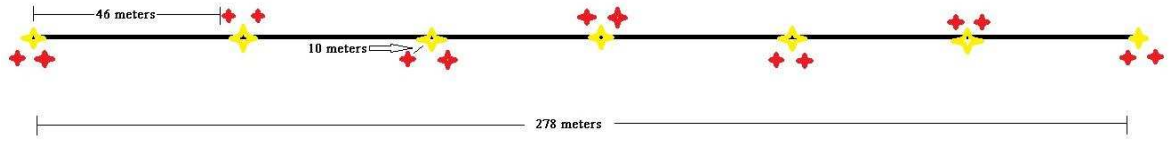

Figure 4.1. Schematic of Lindemann funnel traps placed at two sites on the Pike National Forest in central Colorado, June-September 2012. Yellow stars represent traps placed along the transect; red stars represent two additional traps placed 10 meters from the first trap. Each of the three lure treatments were assigned randomly to each triplet of traps.

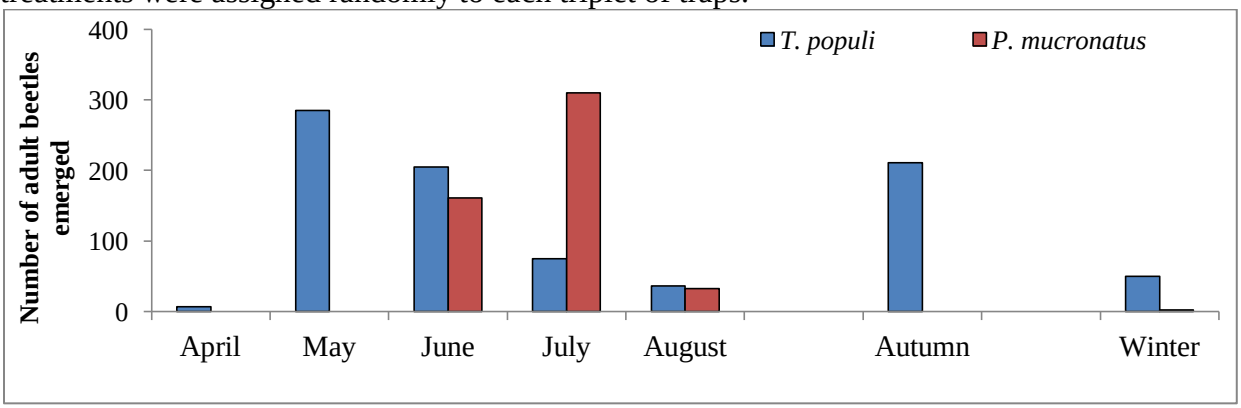

Figure 4.2. Numbers of adult *Trypophloeus populi* and *Procryphalus mucronatus* bark beetles emerging from eight adult (> 12 cm DBH) aspen trees placed in emergence cages, from June 2012-November 2014. Autumn includes the months September-November; Winter includes the months December-January. Monthly counts represent up to three years' worth of data.

Figure 4.3. Clockwise, from top left: Bark beetles that occur on quaking aspen (left) *Procryphalus mucronatus* and (right) *Trypophloeus populi*; Right: frass at gallery entries on a quaking aspen; a close-up of the head capsule of an adult *Trypophloeus populi* (note the elongated antennal club); adult T. populi beetles.

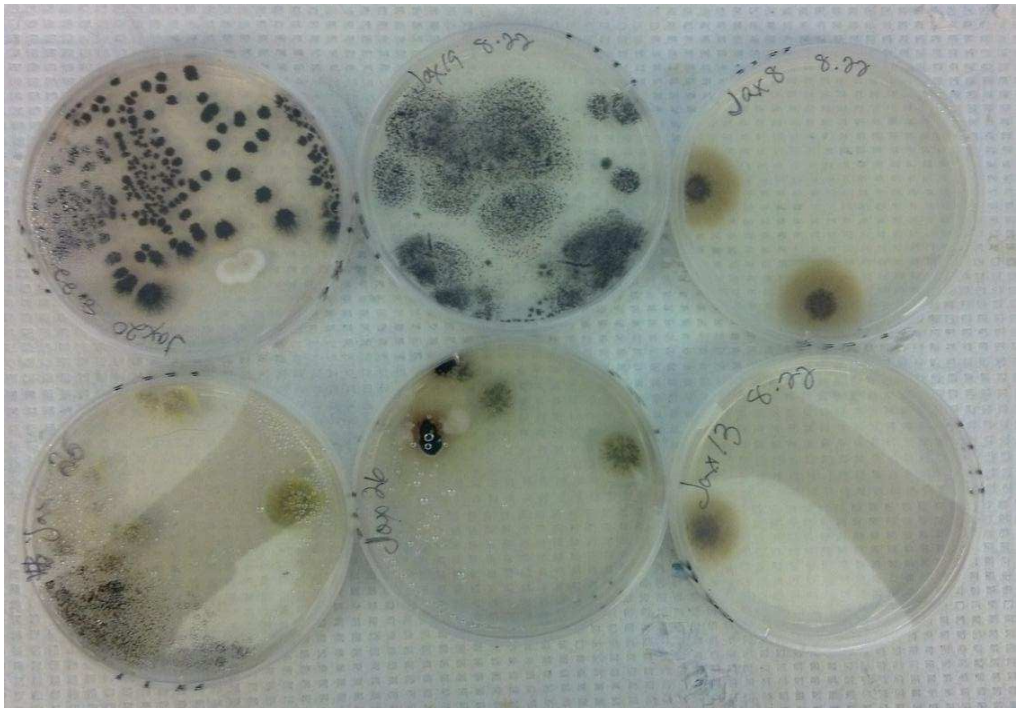

Figure 4.4. Photograph of isolates obtained from adult *T. populi* beetles, vortexed with 50 uL sterile water and streaked onto ¼-strength potato dextrose agar plus antibiotics.

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