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PLANTS, FUNGI, AND FREELOADERS: EXAMINING ENDOPHYTIC SPECIES RICHNESS CHANGES OVER THE GROWING SEASON OF *ARCEUTHOBIUM AMERICANUM*

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PLANTS, FUNGI, AND FREELOADERS: EXAMINING ENDOPHYTIC SPECIES RICHNESS CHANGES OVER THE GROWING SEASON OF *ARCEUTHOBIUM AMERICANUM*

by

Lucas D. Hampel

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This thesis has been accepted as conforming to the required standards by:

Cynthia Ross Friedman (Ph.D.), Thesis Supervisor, Dept. Biological Sciences

Naowarat Cheeptham (Ph.D.), Co-supervisor, Dept. Biological Sciences

Nancy Flood (Ph.D.), Committee Member, Dept. Biological Sciences

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ABSTRACT

Arceuthobium americanum, or the lodgepole pine dwarf mistletoe, is a tree parasite in northern Canada. The plant has negatively impacted the lumber industry, causing significant financial losses, particularly in the west. Recently, endophytic fungi have been found in this plant, which have been discovered to function in protecting the plant from surface pathogens. The current study aimed to examine the endophytic fungi in more depth, looking for changes in species richness over the plant's growing season and determining whether these richness changes differed between males and females. This information could yield a new management strategy, which would involve applying a surface pathogen spray at a time when the endophytic fungal diversity is lower and the plant is presumably more susceptible to pathogens.

Using pure culture techniques, endophytic fungi were isolated from surface sterilized *A. americanum* males and females weekly from the end of April to the beginning of September. Isolated fungi were characterized macroscopically to generate a database of morphologically unique forms that corresponded to individual species. Endophytic fungi that appeared consistently throughout the sampling period were sent to be sequenced by MACROGEN™ sequencing in Korea to determine their identities based on ITS rDNA sequences.

Throughout the study period, 47 distinct morphological strains of fungi were isolated. Endophytic fungal species abundance was found to increase throughout the growing season in both males and female *A. americanum* plants. There was no significant difference in the species richness between males and females, and the two groups shared 87.5% species similarity based on Sørensen's coefficient of similarity. Sequenced endophytes found in this study came from a diverse array of genera, including *Trichoderma*, *Serpula*, *Alternaria*, and *Tremella*, which may function as mutualistic symbionts within the plant.

Endophytic fungal species richness follows the trend shown by all other plants to date: it increases throughout the growing season in new aerial shoots. Future work should aim to further characterize endophytic communities using metagenomic techniques and comparing endophyte communities of *A. americanum* to those of the host tree to see if there is crossover between the two organisms.

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INTRODUCTION

ARCEUTHOBIUM AMERICANUM: A UNIQUE PLANT-PLANT PARASITE

Plants of the *Arceuthobium* genus are unique in that they are plant-plant parasites of a variety of host trees. The genus contains 42 different species, each of which infects a variety of trees from both *Pinaceae* and *Cupressaceae* (Hawksworth and Wiens 1996). Morphologically, *Arceuthobium* plants are small, evergreen herbs with haustorial tissues that permeate the host tree and reduce timber quality of the wood. The shoots (i.e., the portion of the plant growing out of the host) display the plant's reproductive status. Members of the genus are dioecious, meaning that the female (pistillate) and male (staminate) plant parts are found on separate individuals. Of particular import in northwestern Canada is the Lodgepole Pine Dwarf Mistletoe, *Arceuthobium americanum* Nutt. ex Engelm. This species has a complex reproductive cycle in which the female flowers develop over two years, culminating in explosive discharge of the seed at the end of the summer, while the male flowers grow and disperse their pollen on a yearly basis (Hawksworth and Wiens 1996). Seeds reaching a host remain exposed on the host branches over the winter before germinating in the spring. Typically, *A. americanum* infects two important lumber species present in British Columbia: *Pinus contorta* Dougl. ex. Loud. (lodgepole pine) and *Pinus banksiana* Lamb. (jack pine). The infection of these trees renders the plant a significant pest in western North America, causing losses in timber volume annually throughout the region (Drummond 1982; Shamoun et al. 2003).

Presently, there are a variety of management strategies in place to help mitigate the damage *A. americanum* inflicts on forests, but most of them aim to limit the spread of the parasite to neighbouring trees by selective logging of infected trees or by the subsequent planting of lumber trees resistant to *Arceuthobium* infection (Baranyay and Smith 1972). While these methods of control are capable of inhibiting the spread of infection in isolated stands, when infections become more widespread, they become harder to manage. New methods of control need to be found in order to mitigate any additional damage these parasites can cause to the industry.

ENDOPHYTIC FUNGI: A POSSIBLE TOOL FOR BIOCONTROL?

Recent research on *A. americanum* has revolved around the exploration of endophytic fungal symbionts within the plant (Martin et al. 2012). Endophytic fungi are organisms that live within the host plant's tissues. Greater than ninety percent of all plants are suspected to harbour symbiotic fungi that interact with the host in a mutualistic or commensal fashion (Rodriguez et al. 2009). Until recently, the phenomenon of plant endophytes in parasitic plants was poorly documented. A paper published by Martin et al. (2012) reported the extensive colonization of *Arceuthobium americanum* by endophytic fungal hyphae, a novel observation of endophytes within the genus. The presence of fungal endophytes could reveal a new avenue for the management of *A. americanum*. The study showed that the endophytes helped inhibit the growth of the surface pathogen *Cladosporium*, a mold fungus. It could be suspected that these endophytes play a role in helping the dwarf mistletoe resist pathogen infection. Loss of endophytes in the flowering plants *Quercus* (Wang et al. 2006), *Solanum melangena* (Narisawa et al. 2002) and *Zea* (Danielsen and Jensen 1999) can cause a marked decrease in the plants' ability to resist pathogen infections. The same could be true of *A. americanum*, which is also a flowering plant.

Martin et al. (2012) did not determine whether the fungal communities within *A. americanum* shoots changed over the growing season during shoot development. Previous studies have shown that endophyte communities within an individual plant can change over its lifespan (Faeth and Hammon 1997; Wearn et al. 2012). Using pure-culture techinques, the researchers were able to demonstrate the existence of an increasing trend in fungal endophyte species richness present within the host's tissues. If the endophytic communities in *A. americanum* do change in some fashion (e.g., species loss or gain), the observation would be the first for any parasitic plant currently known.

As mentioned, *Arceuthobium* species, including *A. americanum*, are dioecious (Hawksworth and Wiens 1996). The study by Martin et al. (2012) did not address whether the endophyte communities differed between genders. Previous research in another dioecious plant, *Antenarria dioica* showed that endophyte communities varied significantly between the sexes (Vega-Frutis et al. 2013). However, there has been very little research on endophytic fungi

in dioecious plants. Studies in this area will broaden and deepen our knowledge on the ecological interactions between *A. americanum* and its symbiotic partners, and provide more information that could help improve our understanding of endophytic fungal communities throughout the plant kingdom.

PURPOSE

The purpose of this project is to examine the endophytic fungi of *A. americanum* in more depth, looking for changes in species richness over the plant's growing season and differences between genders. Specifically, this project aims to 1) examine and characterize the endophytic fungi present within *A. americanum*; 2) determine if there are any significant differences in species richness between the genders with respect to their culturable endophyte communities; and 3) assess whether the communities of these endophytes change over the growing season (both as a whole and between the male and female plants). The results of such an exploration into the endophytic communities in *A. americanum* should reveal novel information about the complicated fungal assemblage residing within the plant, and could possibly yield a new management strategy based on endophytic organisms.

MATERIALS AND METHODS

SAMPLE COLLECTION

The methods used followed those of Martin et al. (2012). Samples of five growing *A. americanum* female shoots with two fruit generations and five male shoots were collected from five infected *Pinus contorta* (lodgepole pine) trees located near Stake Lake in the area of Kamloops, British Columbia, Canada (50°31'N, 120°28'W). The specific age of the shoots was selected based on the sheer abundance of female shoots available on trees in the region. Said female shoots were pruned so that only the first year shoots were used for culturing, thereby standardizing the age of the shoots between the male and female plants. Weekly collection began April 26 of 2015 and ended September 1 of 2015. Samples were taken from *A. americanum* shoots at eye level from all sides of the infected trees, and trees sampled were marked with flagging tape so that the same mistletoe plants could be repeatedly sampled. Shoots having damage or blemishes resulting from infection by other pathogens were avoided, reducing the probability that non-endophytic fungi (surface pathogens) were cultured, and increasing the likelihood that the organisms being cultured from the sterilized samples were endophytic in nature.

ENDOPHYTE CULTURING AND ISOLATION

Culturing and endophyte isolation and characterization occurred simultaneously each week over the collection period. The collected shoots were surface sterilized using 6% v/v sodium hypochlorite solution for 10 minutes to remove any surface organisms, after which each shoot was washed with sterile deionized water. Next, using a sterile razor blade, plant stems were cut longitudinally along each shoot and placed onto 100 mm (diameter) X 20 mm (deep) Petri plates (Fisherbrand™) with Potato Dextrose Agar, "PDA" (Sigma Aldrich) (Atlas 2010). The plates were incubated at 28°C for three weeks, being checked every three days for fungal growth. Fungi that grew from the sterilized tissue cultures was extracted and subcultured onto new PDA agar plates for isolation based on differences in morphology. The pure cultures were used to characterize the fungi based on macroscopic morphology.

ENDOPHYTE CHARACTERIZATION

Identification of genera present was made based on macroscopic morphology using references to the taxonomic fungal databases as defined by J. A. Stalpers (1978) and Kirk et al. (2008). Fungi were characterized based on their macroscopic morphology in pure culture, and were defined by their form (general colony shape), colony diameter, elevation of the colony, margin, surface texture, colour of the aerial and embedded colony, and architecture of the aerial mycelium. Pure-culture studies have been used extensively in order to observe endophytic fungi from a wide diversity of plant species (Faeth and Hammon 1997; Wearn et al. 2012; McPherson et al. 2013; Min et al. 2014). By using similar pure-culture methods supplemented with sequencing, a clear preliminary picture of the organisms present in *A. americanum* was obtained.

Colour classes were arranged into three broad, categories to sort the fungi, with more specific colour terms being used as defined by Ridgway (1912). There were three broad categories used in this study: Black, Cream/White, and Orange/Yellow. Particular groups of fungi have mycelial colours that are characteristic to a particular genus or species (Scurti et al. 1978; Iotti et al. 2002). In identifying colour categories, and by extension determining the colour of a particular isolate, one could be better able to elucidate the genera isolated from *A. americanum*. These broad categories were also used in the general sorting of the fungi found based on their morphology. The colours of multi-coloured colonies were recorded from the centre to the edge of the colony, separated by forward slashes (e.g., Saccardo's Olive/ Olive Ochre/ Cream).

Aerial mycelial mats were described using terminology adapted from Stalpers (1978). Terms for the mycelial mat were assigned to each pure culture:

a. absent: mycelium only submerged. The surface of the agar may be even or chamois-like.

b. downy: with fine, short, erect hyphae. The whole colony is usually transparent.

c. farinaceous: mealy, powdery.

d. granular: covered with minute grains.

e. silky: with long, radiating parallel hyphae or hyphal bundles, more or less prostrate, often glossy.

f. cottony: rather long, single mycelial hyphae spreading in all directions.

g. woolly: fairly long interwoven hyphae or groups of hyphae, somewhat matted, resembling woollen cloth.

h. floccose: small hyphal tufts, standing out from the agar or from the aerial mycelium.

i. plumose: mycelial tufts with short or long hyphae or groups of hyphae radiating from the central axis, often in fan-like arrangement.

j. pellicular or subfelty: covered with thin, low, coherent mycelium.

k. felty: cottony or woolly mycelium, which has become matted or packed; emerging hyphae absent.

l. velvety: a dense mat of erect, straight hyphae, usually short.

m. crustose: hyphae forming a solid, hard crust, usually dark brown (many Hymenochaetaceae) but sometimes cream or white (Radulomyces).

n. lacunose: mycelial surface depressed or indented.

o. zonate: with concentric bands or segments of different texture.

A random sampling of isolated fungi was sent to be sequenced by MACROGEN™ in Seoul, Korea. Of the 20 samples sent, 15 could be sequenced. Sequences were compared to the NCBI database to determine the identities of these fungi. The 97% sequence similarity in the ITS region between two sequenced samples has been conventionally used as the threshold to delineate two separate samples as the same species (O'Brien et al. 2005). Roles of these fungi were elucidated by searching the literature and their potential function(s) in *A. americanum* are explored in the discussion.

DATA ANALYSIS

Fugal species abundance was evaluated over the growing season in both males and females. The sum total of species present in males or females at each collection time was determined and plotted versus time. The time scale was set to ordinal dates ("day-of-year") using a perpetual Julian date calendar (non-leap year). Regression analysis was done using Minitab™, and a third order polynomial regression line was used to determine if there was a trend over time in species richness of the endophytes within *A. americanum* male and female flowers. The resulting values were compared to determine whether or not there were differences in the raw species abundance between the male and female *A. americanum* plants.

Species similarity between male and female *A. americanum* species was also examined. Using Sørensen's coefficient of similarity (Sørensen 1948). Species similarity was examined at each data point and overall to see how similar the communities inhabiting the dioecious plants really were. The equation for the coefficient is as follows [Equation 1]:

$$Coefficient\ of\ Community\ (Sorensen's\ coefficient) = \frac{2(s_{xy})}{s_x + s_y} \text{ x } 100\% \qquad \text{[Equation 1]}$$

where s_{xy} is the number of species the two groups share, s_x is the total number of species observed in males and s_y is the total number of species observed in females.

RESULTS

CHARACTERIZATION OF ENDOPHYTIC FUNGAL SPECIES IN *A. AMERICANUM*

Throughout the study period, 47 morphologically distinct fungal isolates were identified. The endophytic fungi identified had numerous distinct morphologies, and some isolates fell outside the three predetermined broad colour categories described in the methods. These outlier groups were placed under a fourth grouping, labelled “Variable” in Table 1. These fungi had a wide array of colours, including reds, greens, and blends of colours between the other colour classes (Table 1).

Table 1. Macroscopic morphologies of fungi isolated from *A. americanum*. Each isolate had a distinct two to three-letter name assigned in order to organize each morphologically distinct isolate.

Abbreviated name	Form	Size	Elevation	Margin	Surface	Aerial Mycelium	Colour (aerial)	Colour (Embedded)
(A) BLACK								
BP	Irregular	4.5cm	Convex	Undulate	Pitted, Wrinkled	Floccose, velvety	Black/ Brownish Olive	None/Black
BFF	Filamentous	0.8-1.1cm	Convex	Filiform	Dull	Velvety	Yellowish Olive	None
BC	Irregular	1.8cm	Raised	Undulate	Rough	Crustose	Black/Chrome/ white	None
BF	Circular	Fills Plate	Not Visible	Entire/ Filiform	Dull	Felty	Saccardo's Olive. Black at 3wk	Black/ Saccardo's Olive/ Cream. All Black at 3wk
FWB	Fills Plate	Fills Plate	Not Visible	NA	NA	Woolly, clumpy	White	Black patches
BS	Circular	Fills Plate	Raised	Entire/Filiform	Dull	Tufted, with granular, farinose powder	White Mycelia/powder	White/Black patches
BR	Circular/Rhizoid	7.9cm	Raised	Lobate	Dull	Felty	Black/ Saccardo's Umber	Black/ Saccardo's Umber/White
(B) CREAM/WHITE								
BM	Irregular	Fills Plate	Raised	Lobate, Filiform	Dull	Cottony	Cream buff/ White	Cream buff
CF	Circular	Fills Plate	Flat	Entire	Dull	Sparsley cottony to Farinaceous	Cream	None
CM	Irregular	1.75cm	Raised	Entire	Glistening	Absent, Small tuft in centre	Cream	None
CW	Circular	2.2cm	Raised	Entire	Dull	absent	Cream/ White	none
CWR	Irregular	3.6cm	Raised	Entire	Dull, wrinkled in centre	Absent, Farinaceous white ring near edge	Cream	None
PF	Circular	4.5cm	Flat	Entire/ Filiform	Dull	Downy	Pale Lemon yellow/ Maritus Yellow. Turned peach at 3 wk	None
PP	Irregular	2cm	Convex/ conical	Lobate	Pitted	Small, Felty around perimeter	Warm Buff	None
PU	Circular	5cm	Umbonate	Entire	Dull	Absent, Floccose tufts	Ochraceous Salmon/ Light Buff	None
PW	Circular	2.2cm	Raised	Entire	Wrinkled	Absent, Floccose tufts at apex	Light Ochraceous Buff/ Buff Yellow	None
SC	Circular	1.9cm	Umbonate	Entire/ Filiform	Dull	Absent w Dense Pellicular at apex	Sepia/ Cream	None
WBD	Circular	Fills Plate	Flat	Entire/ Filiform	Dull	Downy/absent at 3wk	Cream/ White	White, Mars Brown at 3wk
WCC	Circular	Fills plate	Flat	Curled	Dull	Cottony	White/ Cream	Cream
WD	Circular/ irregular	1.1cm	Raised	Entire	Dull	Downy	White	none
WF	Circular	Fills Plate	Convex	Filiform	Dull	Felty	White	None
WFC	Circular	4.8cm	Dull	Filiform	Dull	Densely Felty	White	None
WR	Circular	1.6cm	Umbonate	Entire/Filiform	Dull	Absent, floccose tuft at apex	Cream/ Yellow Ochre speckles/ Cream	None
WS	Circular	Fills Plate	Raised	Filamentous	Dull	Farinaceous/ downy	White	none
WU	Circular	0.7cm	Umbonate	Entire	Dull	Pellicular with floccose tuft	White	Cream

Abbreviated name	Form	Size	Elevation	Margin	Surface	Aerial Mycelium	Colour (aerial)	Colour (Embedded)
(C) ORANGE/YELLOW								
OC	Circular	3cm	Raised	Entire/ Filiform	Dull	Downy	Tawny/ Cream	None
ODF	Circular	7.2cm	Flat	Entire	Dull	Downy	Faintly Yellow Ocher	None
OF	Circular	2.9cm	Flat	Entire/ Filifrorm	Dull	Absent/ Pellicular	Xanthine Orange/ Cadmium Orange	Xanthine Orange/ Cadmium Orange
OG	Circular	2.4cm	Raised	Entire	Dull	Pellicular	Clay colour/ Deep Colonial Buff	None
OI	Irregular	2.2cm	Raised	Lobate, Filiform	Dull	downy	Ochraceous Orange	Ochraceous Orange
OL	Circular	7.2cm	Flat	Entire/Curled	Warty	Absent	Ochraceous Orange/ Pale Orange-Yellow	Ochraceous Orange/ Pale Orange-Yellow
OM	Irregular	5.5cm	Raised	Entire	Glistening	lacunose	Honey Yellow	Honey Yellow
OP	Irregular	5.3cm	Raised	Irregular	Dull	Farinaceous	White	Primuline Yellow/ Buckthorn Brown band 1cm from centre
OTW	Irregular	3.3cm	Raised	Entire	Wrinkled	Absent	Olive Ocher / Primuline Yellow	None
OW	Circular	2.1cm	Umbonate	Entire	Dull/Wrinkled	Absent	Yellow Ocher/Antimony Yellow/Dresden Brown	None
OWF	Irregular	3.9cm	Raised	Undulate, Filiform, Curled	Dull	Floccose, In spiky tufts, arranged in Curled pattern	White	Cadmium Yellow/ Light Orange-Yellow
YC	Irregular	2.8cm	Flat	Irregular/ Filiform	Covered	Cottony	Lemon Chrome	None
YF	Circular	5.6cm	Flat	Entire	Dull	Floccose centre/absent	Light Cadmium bands, Mustard yellow	None
YM	Irregular	0.6cm	Raised	Filiform	Dull	Absent	Buff Yellow, producing Yellow surfactants	Ochraceous Orange
YT	Circular	5.6cm	Flat	Entire	Dull	Floccose centre/absent	Light Cadmium bands, Mustard yellow	None
YW/YPR	Irregular	2.3cm	Raised	Entire	Dull, Wrinkled in centre	Pellicular, downy	Lemon Chrome, turns Russet at wk3	Yellow, turns Russet at wk3
(E) VARIABLE								
GI	Irregular	3.7cm	Raised	Undulate	Dull	Pellicular	Yellow-Green/ White margin	Alizarine Pink at 17 days
ORS	Circular	Fills Plate	Raised	Entire	Dull	felty centre, cottony outside with silky strands	White/ Warm Buff. Yellow Ocher at 2 weeks	Black/ Ferrugineous/ Cream colour
PD	Circular	3.9cm	Flat	Entire	Dull	downy	White mycelia, Phlox Pink/ White	Cream
RB	Circular	6.5cm	Raised	Entire	Dull	Felty	Mycelia Black with White surface	Honey Yellow
RC	Irregular	3.2cm	Raised	Lobate	Veined	Granular, with reflective droplets	Cameo Brown/ Light Congo Pink	Medal Bronze/ Buff Yellow
TCB	Circular	Fills Plate	Raised	Entire	Dull	Woolly, clumped	White mycelia, Amber Yellow surface/White	Black/ Deep Colonial Buff/ White

Of the 47 fungal isolates, 34 (72%) fell into the Cream/White and Orange/Yellow categories, while the rest fell into the other two colour classes, Black and Variable The macroscopic morphologies of the isolated endophytic fungi present within each of the classes were highly variable relative to the endophytes isolated previously by Martin et al. (2012), who found fungi with six aerial mycelial morphologies. By comparison, the present study had more than 13 different aerial mycelial morphologies.

Of the 20 isolates sent to be sequenced; 15 were returned with readable sequence data. A literature search was done to examine the general function these organisms performed in the environment (Table 2):

Table 2. Sequenced ITS region identities of endophytic fungal species isolated from *A. americanum*. BLAST searches were performed with a resolution value of 97% sequence similarity delineating a positive identification of a species.

Abbreviated Name	BLAST result	Ecological Characteristics
BF	*Alternaria*	Opportunistic pathogen endophyte in other plants, causing leaf blight. (Fisher et al. 1992)
BS	*Botryotinia/ Baudoinia/ Botrytis*	Latent pathogens of woody plants (Slippers and Wingfield 2007)
GI	*Nectria*	Pathogenic wood canker-causing fungus (Hopkins et al. 2009)
OF	*Sordariomycetes*	Highly diverse group of fungi, present in many environments. Some species are endophytic in nature. (Zhang et al. 2006)
ORS	*Galerina/Coprinus*	Saprotrophic fungi (Davey 2013; Noordeloos and Gulden 2012)
OTW	*Sordariomycetes*	Highly diverse, some endophytic (Zhang et al. 2006)
OWF	*Coniochaeta*	Fungal group that is pathogenic to trees. (Weber 2002)
PBR	*Alternaria*	Opportunistic leaf-blight pathogen endophyte in plants. (Fisher et al. 1992)
PD	*Lecythophora*	Wood soft rot fungi present in forest environments (Damm et al. 2010)
PP	*Anthostomella*	Saprotrophic fungus in a variety of plants (Cooke 1959)
PW	*Anthostomella*	Saprotrophic fungus in a variety of plants (Cooke 1959)

YC	*Serpula*	Dry rot fungi, commonly found in built timber structures and boreal forests (Eastwood et al. 2011)
YF	*Coniochaeta*	Fungal group that is pathogenic to trees. (Weber 2002)
YM	*Tremella*	Wood-inhabiting fungi mycoparasites (Vizzini 2007, Jeffries and Young 1994)
YPR	*Trichoderma*	Fungi with active cellulase enzymes. (Druzhinina et al. 2010)

Sequenced isolates were identified to the genus level, as further resolution can prove challenging and tends to require other techniques (e.g., DNA-DNA hybridization) to define species (O'Brien et al. 2005). What was sequenced and examined with a small literature search reveals that there is a high diversity of endophytes present within *A. americanum*. These endophytes came primarily from the colour groups Orange/Yellow, Black, and Variable. The fungi had a wide diversity of symbiotic syndromes with plants. Interestingly, there were a number of wood-inhabiting fungi that are pathogenic to trees.

ENDOPHYTIC SPECIES RICHNESS CHANGES BETWEEN MALE AND FEMALE PLANTS

Endophytic fungal species richness steadily increased over the growing season (Figure 1). A polynomial regression line of the third order was used because it fit the data better; the relationship between species richness and date was significant for both sexes (for males $F = 10.8$, $p = 0.0007$ and for females, $F = 6.8$, $p = 0.0051$). It should be noted that species richness declined in the latter part of the sampling period. Overall, richness did not differ substantially between male and female plants.

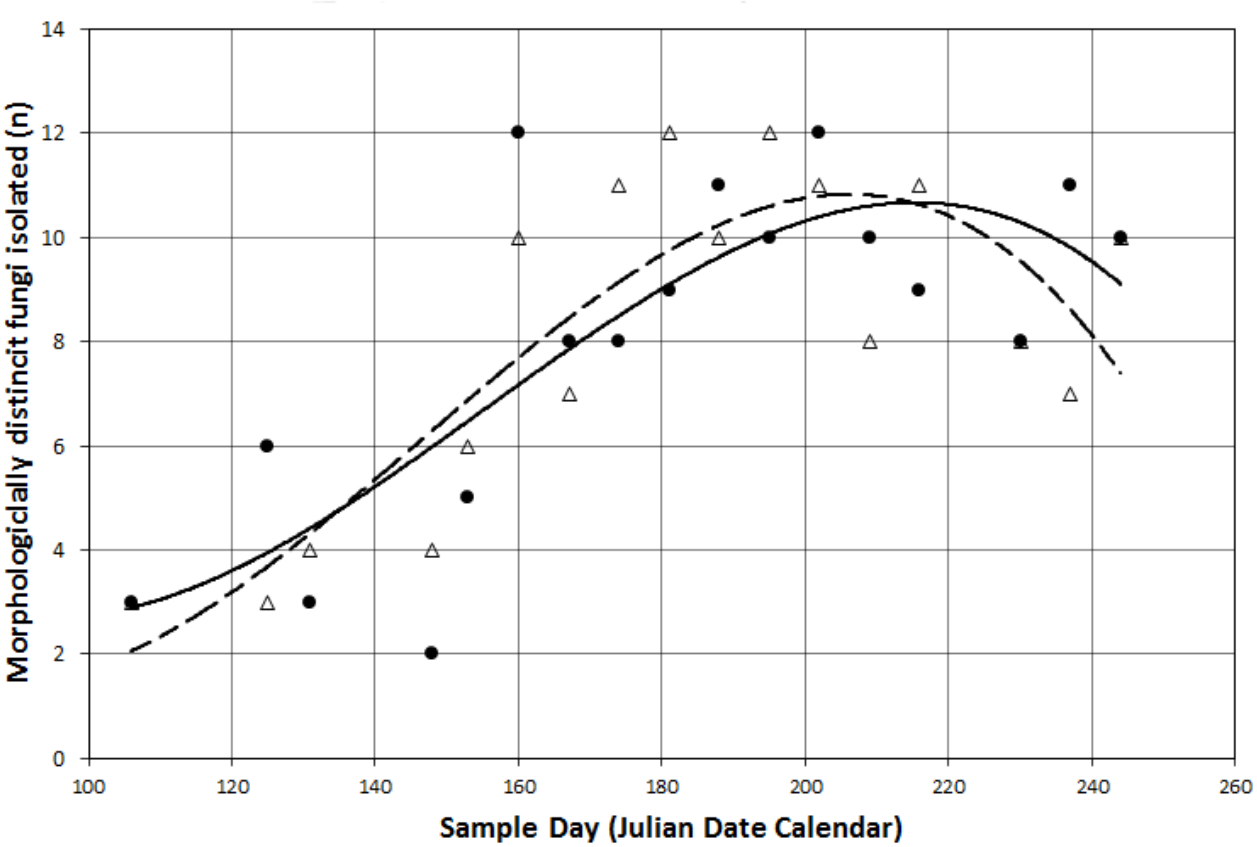

Figure 1. Endophytic species richness in male and female plants throughout the growing season. Open triangles denote male species richness and closed circles denote female species richness at each sampling point. Regression lines for males (dashed line, $R^2 = 0.71$) and females (solid line, $R^2 = 0.61$) were included.

As is evidenced in Figure 1, the change in the species richness in both the males and females of *A. americanum* was found to increase at roughly the same pace throughout the growing season. It should be noted that there is a switch between the male and female species richness in the latter portion of the growing season through July, with the female plants having an increased number of endophytes present through the mid-summer, but the observation is likely nonsignificant. Full regression analyses are presented in the Appendix, Table A1 and Table A2.

SIMILARITY MEASURES IN THE ENDOPHYTE COMMUNITIES OF MALE AND FEMALE *A. AMERICANUM*

The sum total of the male and female plants' endophyte isolates throughout the entire sampling period was compared using the Sørensen's coefficient. The male and female endophyte communities were 87.5% similar.

DISCUSSION

CHARACTERIZATION OF ENDOPHYTES REVEALS A HIGH DIVERSITY OF FUNGI PRESENT WITHIN *A. AMERICANUM* THROUGHOUT THE GROWING SEASON

The sequencing of a third of all isolated endophytes revealed that almost every morphologically distinct strain (12 of the 15 that were returned with readable sequences) was associated with a particular genus of fungus. In pure culture studies, a particular medium (in this case, potato dextrose agar) is usually only capable of supporting in-vitro growth of a fraction of the organisms present in a sample. This selection bias by a given medium, defined here as "culture bias", can cause a skewing of the observed richness of a community, leading to an underrepresentation of the members present in a particular sample (reviewed in Handelsman 2004). The high diversity of fungi observed here reinforces the idea that pure-culture studies can be used for examining fungi. Unlike in bacterial samples, pure culture techniques return a large proportion of fungal isolates without significant losses of individual isolates due to culture bias (Fröhlich and Hyde 1999; Lacap et al. 2003).

Compared to other plants such as *Plantago lanceolata*, *Rumex acetosa*, and *Cirsium arevense*, *A. americanum* had a reasonable number of morphologically distinct isolates (47 isolates in *A. americanum*, as compared to *P. lanceolata's* 55, *R. acetosa's* 36, and *C. arvense's* 25) (Wearn et al. 2012). The diversity of isolated fungi indicates that the plant experiences a wide array of ecological interactions, each of which may influence the health of the plant itself, or the health of the associated host. The fact that there are a number of latent plant pathogens within the mistletoe's tissues indicates that like other, more widely studied plants (*Theobroma cacao*, for instance), mistletoe is constantly bombarded by fungal pathogens, which threaten to invade its shoot tissues. These external, potentially pathogenic fungi could be inhibited by the internal fungal endophytes that have been previously described by Martin et al. (2012) as being able to inhibit plant pathogen growth. The fact that the plants are not immediately damaged by the pathogens could indicate that the mutualistic endophytes observed previously are constitutively present within the shoots of *A. americanum*, and may assist in inhibiting the growth of more pathogenic endophytes within the host tissues.

It is to be noted that Martin et al. (2012), revealed a number of different fungal genera living in *A. americanum*, including *Sydowia*, *Phoma*, *Phacidiopycnis*, *Davidiella*, and *Cladosporium*. These fungi had diverse morphologies, but most of them (*Sydowia*, *Phoma*, and *Phacidiopycnis*) had white aerial myceliao f varying densities. In the present study, the samples that were sent to be sequenced by MACROGEN™ in Korea did not include fungi with similar macroscopic morphologies, although there were fungi with similar morphologies observed throughout the sampling period (Table 1, isolate WFC and BM). It may be that some of the isolates in the present study are in fact the same genera that were isolated in the previous experiments, but were merely missed due to the sample selection procedure used here.

MALE AND FEMALE ENDOPHYTE COMMUNITIES EXHIBIT HIGH SIMILARITY

Sørensen's coefficient of similarity showed that that the male and female morphs of *A. americanum* were very similar with respect to their endophyte communities. The manner by which the mistletoe plant acquires its endophyte fungi might provide an explanation for the similarity. With the plant being present on the branches of its host and having continuous vasculature with its tree host, endophyte transmission between the pair of plants could be readily occurring through their connected vasculatures and the transmission of fluids from tree to parasite, which could result in a high degree of similarity between the fungal endophytes of the host tree and the parasitic *A. americanum* male and female plants. The host trees constitute a large "reservoir" of potential endophytic fungi, which could strongly influence the endophyte communities of the male and female plants.

Alternatively, *A. americanum* could be acquiring its endophytes through a more conventional mechanism. In forests, the acquisition of endophytic fungi by plants lower in the canopy can be strongly influenced by a "drip-down effect" from the branches and leaves of other trees nearby (Arnold and Herre 2003). *A. americanum* tends to be nestled on the branches of its host tree, or within the brooming structures that occur due to the parasite's infection of the tree. It might be that at least some of the various endophytic fungi (parasitic, wood-rot, saprophytic) could have ended up within *A. americanum* through this mechanism.

This is not to say that the communities of *A. americanum* and *P. contorta* are completely identical, or even that *A. americanum* only obtains its endophytes horizontally, either from external inputs (drip-down effects from the host tree's branches) or transmission from the vasculature of the host tree . Martin et al. (2012) observed that endophytic fungi emerged from the seed tissue of the plant. This indicates that *A. americanum* has endophytes that are vertically transmitted from parent to offspring. Vertically transmitted fungal symbionts can play very important roles in the development of their photosynthetic partner. In the reed species, *Phragmites australis*, endophytic fungi function in a unique way, helping to stimulate growth and proliferation of the host plant (Ernst et al. 2003). The grass *Festuca arundinace* has vertically-transmitted endophytes that help resist heavy metals within the soil by increasing the plant's ability to exude phenolic chelators into the earth (Malinowski and Belesky 1999).

A. americanum seeds, which are deposited on the host trees in the fall, are exposed to a number of stressors and challenges before infection occurs the following spring. As in *P. australis*, or *F. arundinace*, endophytic fungi within *A. americanum* could be helping facilitate this first challenging step in the plant life cycle, improving the ability of the plant to survive and thrive during the six or seven months before host infection.

The endophytes that were sequenced in this study could have similar effects to those seen in other plants. *Lecythophora, Trichoderma, Serpula, Botrytinia*, and many organisms from the *Sordariomycetes* were all found in the samples that were sent to be sequenced. Fungi in these groups play a diversity of roles. All are capable of breaking down cellulose, and cause a variety of rotting diseases in woody flora (Table 2). However, these organisms could be present in *A. americanum* as beneficial endophytes rather than pathogens.

During the initial stages of the *A. americanum* life-cycle, the seeds have to burrow through the thick, woody tissue of their tree hosts in order to proceed through their lifecycle. If these endophytes were present in the seed, they could break out when the seed germinates, softening the bark where the plant would subsequently invade the tree host, thereby improving the chance that the plant parasite will establish itself on the tree in question.

At maturity, *A. americanum* itself is a lignified plant, having a large amount of woody tissue within its stems and also in the haustorial tissues that infiltrate the host tree. These potentially pathogenic fungal endophytes within *A. americanum* could be infecting the parasitic plant itself, and are being repressed by the aforementioned symbionts that protected the plant from surface pathogen infection (Martin et al. 2012). A more interesting implication of the presence of these pathogens in the mature *A. americanum* plant could revolve around the maintenance of the plant-plant parasitic relationship. The parasitic plant could be hosting wood-degrading pathogens in order to improve its ability to infect the host tree; these lignin-degrading and necrotizing fungi could help maintain fluid flow from the host tree to the parasitic *A. americanum.* In conifers, lignification, the production of resins, and the synthesis of toxic phenolic compounds are all mechanisms by which a tree tries to prevent pathogens from infecting phloem tissues (Hudgins et al. 2004). When *A. americanum* infects a pine, the host's defence mechanisms will be initiated, and lignification of pine tissues surrounding the site of parasite invasion will occur. The wood rot endophytic fungi could help prevent the host tree from stopping the flow of fluids through its vasculature into *A. americanum*. The endophytes described here could represent a novel change of role from pathogens (as far as the host pine is concerned) to beneficial mutualists (for the dwarf mistletoe).

SPECIES RICHNESS SEEMS TO INCREASE OVER THE GROWING SEASON IN BOTH MALES AND FEMALES

Over the course of the growing season, the sum-total of endophytes present in male and female *A. americanum* plants was found to increase. However, there was a slight shift in species richness between male and female plants (from males having higher diversity at the beginning of the season to females having higher diversity at the end of the season). The shift, which was not statistically significant, could be attributed to random variation due to relatively low sample size for each data point ($n = 5$). Nonetheless, the trend is interesting and worth exploring in the future.

The results of the current study also indicate that *A. americanum* does follow common plant trends in endophyte species richness over its growing season (Thompson et al. 1993; Faeth and Hammon 1997; Mei et al. 2013). As other plants age and proceed towards senescence, a

number of important stressors will impact the microbial community associated with the plant tissues. Initially, when the plant is first beginning shoot growth, the number of endophytes present within the new shoots will be low merely due to the new tissue's lack of exposure to the environment (Arnold and Herre 2003). As was mentioned earlier, "drip-down effects" can strongly influence the species richness of endophytes within plants. Rainwater drainage from overhanging plant tissues causes young seedlings to be exposed to endophytes, allowing for the horizontal transmission of the fungi from one plant to another.

Due to the fact that *A. americanum* tends to infect the branches of its host tree, the acquisition of endophytes by the mistletoe could be facilitated through the drip down of water onto the plant. During the initial stages of seed dispersal and germination, *A. americanum* seeds are exposed to the environment. For up to year, the seeds will progressively make their way to the axis between a host tree's leaf (needle) and the bark of the tree (Hawksworth and Wiens 1996). As water passes through the branches above the seed and washes the seed closer to the base of the needle, the fungal spores present within the host tree's tissues could also fall down, coming into contact with, and potentially infecting, the mistletoe seed, and eventually the entire plant. Once the *A. americanum* infection is established, new shoots of mistletoes that are infecting the host could also acquire more novel endophytes from further drip-down from the surrounding canopy, causing the increased endophyte species richness depicted here.

More interesting would be the potential of an alternate mechanism of endophyte inoculation. When *A. americanum* infects its host, *Pinus contorta var. latifolia*, the small parasite's vasculature becomes continuous with that of its host. This means that there is continuous flow of nutrients and fluids between the two plants. It would therefore be feasible for endophytic organisms native to the tree to invade *A. americanum* plants "from below" so to speak rather than raining down "from above" from canopy drainage. In either case, endophytes will be accumulating over time as the new shoot tissue develops from the *A. americanum* mass located within the tree.

FUTURE WORK

Endophytic fungi in plants provide a variety of potentially beneficial roles to their plant hosts. In the present study, the endophytic species richness was examined in the male and female forms of *A. americanum* to determine whether species richness changed over time in this species, and if the species richness differed between the sexes. The results presented here regarding the diversity of endophytes present within mistletoe and the similarity between the endophyte communities of male and female plants opens a new avenue for further research regarding the functioning of the fungal symbionts within *A. americanum*. Future work could begin to explore the 12.5% difference in the Sørensen coefficient of community between males and females, and explore the small shift in species richness observed by the end of the growing season. These fungi could have biological roles that are specific to the particular sex of their associated plant host. In the dioecious plant *Antennaria. dimorpha*, it was observed that symbiotic fungi associated with the roots of the female plants were more abundant during the flowering season, and that the increase in mutualistic fungal symbionts in the roots allowed for increased nutrient uptake during the production of inflorescences (Vega-Frutis et al. 2013). Based on these observations in other plants, it could be reasoned that the slight differences between the sexes of A. americanum, with respect to their endophyte communities could also have implications for the health or virility of the male or female morphs. Examining fungal isolates specific to male or female plants would be useful in understanding these sex-specific interactions in the plant. An exploration into the presence of endophytes within the seeds of *A. americanum* would also be conducive to understanding aspects of the plant's relationship with fungi and their potential ability to assist in germination and proliferation of the mistletoe seeds.

More broadly, the project here was performed in pure culture, which, while reliable for fungi, still has culturing bias. Some species will be more abundant, and have a chance to crowd out slower-growing species on a plate. The species richness trends reflected here could be reinforced by using a more robust technique, like ITS sequencing of fungal symbionts. The usage of molecular techniques could also allow for comparisons of the endophyte communities in the host tree and the parasitic mistletoe plant. If there are similarities between the two groups, it could indicate horizontal transfer of endophytes between the plant and its host: a novel observation for parasitic plants worldwide.

CONCLUSION

Through this study, it was revealed that there was a high diversity of endophytic fungal symbionts housed within the tissues of *A. americanum*. Based on a literature search, the endophytes in question had a wide diversity of ecological roles, ranging from mutualists to pathogens to saprophytes. Species richness was found to be highly similar between both male and female plants, exhibiting 87.5% similarity in populations of endophytes grown from internal plant tissues. It was determined that, as in other plants, the endophytic species richness increased over the growing season in both male and female plants. These results do not shed much light on biocontrol implications of endophytic communities within *A. americanum*, but they do open up windows into aspects of the symbiotic fungal partners that have not been previously observed in a plant-plant parasitic interaction. Unveiling the fungal partner identities of the *A. americanum* plant could reveal novel metabolites that modulate pathogen growth within the plant or its associated tree host. These findings shed light on the biology of *A. americanum*, and provide researchers with a new picture of the world of endophytes within parasitic plants.

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APPENDIX

Table A1. Regression analysis of male species richness changes throughout the growing season in *A. americanum*.

SUMMARY OUTPUT: MALES					
Regression Statistics					
Multiple R	0.845088116				
R Square	0.714173923				
Adjusted R Square	0.64821406				
Standard Error	1.86937943				
Observations	17				
ANOVA					
	df	*SS*	*MS*	*F*	*P-Value*
Regression	3	113.5116436	37.83721453	10.82740142	0.000768249
Residual	13	45.42953287	3.494579452		
Total	16	158.9411765			

Table A2. Regression analysis of male species richness changes throughout the growing season in *A. americanum*.

SUMMARY OUTPUT: FEMALES					
Regression Statistics					
Multiple R	0.783229836				
R Square	0.613448976				
Adjusted R Square	0.524244894				
Standard Error	2.201137444				
Observations	17				
ANOVA					
	df	*SS*	*MS*	*F*	*P-Value*
Regression	3	99.95609786	33.31869929	6.876915935	0.005137652
Residual	13	62.98507861	4.845006047		
Total	16	162.9411765			