

Ecological Impacts of American-Chinese Chestnut Hybridization on Soil Arthropod Communities and Entomopathogenic Nematodes

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Abstract

In the first half of the 20th century, a fungal blight (*Cryphonectria parasitica*) decimated the American chestnut tree (*Castanea dentata*) population throughout the Eastern United States. Efforts to create a blight-resistant hybrid using backcross breeding techniques with Chinese chestnut trees (*Castanea mollissima*) to reintroduce this tree to its native range have shown promising results. In order to understand the ecological impacts of hybridization on belowground communities, we evaluated the abundance and biodiversity of soil arthropods and the presence of entomopathogenic nematodes (EPNs) in stands of chestnut trees with varying levels of American-Chinese hybridization. Arthropods were separated from leaf litter samples using the Berlese funnel method and manually quantified and identified. EPNs collected via soil probe were extracted using the white trap method. No differences were observed in overall arthropod abundance nor EPN presence between chestnut plots. However, the unhybridized American chestnut stand had greater species dominance in comparison to the 75% American chestnut plot as well as a greater number of arachnids, insects, and myriapods than the 94% plot. Our findings suggest that while the general abundances

of arthropods are unaffected by hybridization, backcross breeding may impact population composition among groups. Understanding the effects of chestnut hybridization on soil invertebrate biodiversity and abundance will provide context for future projects involving chestnut ecology as well as help identify potential pest species that may threaten the restoration of *C. dentata*.

Introduction

History of The American Chestnut

Prior to the 20th century, American chestnuts (*Castanea dentata*) accounted for 25%-50% of the Eastern United States hardwood forest canopy (Dalgleish et al. 2012). *C. dentata* played a significant role in native ecosystems, and at the time were valuable resources for timber production and construction industries (Kuhlman 1978). The fungus *Cryphonectria parasitica*, commonly known as chestnut blight, was discovered in New York for the first time in 1904 and effectively eliminated approximately 4 billion trees by 1960 (Jacobs 2007). The loss of this prominent species had wide-reaching economic and ecological effects. The sudden absence of the chestnut trees that previously made up a large portion of these forests fundamentally changed the dynamics of the biological community as other vegetation grew to fill in *C. dentata*'s niche (Jacobs 2007). Knowledge of the specific ecological role that the American chestnut historically fulfilled is limited in comparison to other species because *C. parasitica* infection spread throughout the U.S. before a modern understanding of forest ecology was implemented (Jacobs 2007).

In the 1980s, efforts to reintroduce the chestnut tree to American forests by creating a blight-resistant hybrid began. Chinese chestnuts were selected for backcross breeding due to their naturally high resistance to *C. parasitica*, and Chinese-American hybrid trees have shown promising results in terms of survival during reintroduction trials (Clark et al. 2019). Successful restoration of *C. dentata* hybrids to the chestnut tree's historic range is projected to have wide-reaching beneficial effects on the surrounding ecological community. These impacts include stabilizing food resources for foraging animals and increasing mammal presence responsible for the control of gypsy moth and lyme-carrying tick populations (Dalgleish et al. 2012). However, with the planting of these hybrids for nut farming and the return of the chestnut to American forests through conservation efforts, there comes a potential for unintended consequences. Studies have shown that this recent increase in chestnut tree farms has resulted in the reemergence of chestnut pests including the lesser chestnut weevil, *Curculio sayi* (Filgueiras and Willett 2022). In addition to the limited amount of research conducted on pests like *C. sayi*, there have been few to no studies thus far on the effect of the reintroduction of American-Chinese chestnut hybrids on underground organisms.

Soil Arthropod Biodiversity

Plants and soil biodiversity are inextricably linked. Subterranean animals aid in decomposition of organic matter and retention of moisture and nutrients within the soil necessary to plants' survival and soil fauna depend on the resources and shelter provided by plant roots for their own livelihood (Bagyaraj et al. 2016, Remelli 2020). These interactions keep the soil balanced and nutrient rich and results in a healthier ecosystem both above and below ground. These organisms can also cause damage to plants by consuming and parasitizing roots or leaving them susceptible to disease (Remelli 2020). Biodiversity within soil communities is important and indicative of soil health, as invertebrates' interactions with each other and their surrounding environment are essential to maintaining ecological balance (Bagyaraj et al. 2016; Remelli 2020). Soil biodiversity is positively correlated with plant diversity and nutrient retention and recycling (Wagg et al. 2014). By gaining an understanding of these interactions taking place in the soil surrounding American-Chinese chestnut hybrids, we can begin to examine how the reintroduction of these trees may be affecting our forests on both a micro and macro scale.

There are numerous arthropods that dwell under the surface of the earth. The most common taxa are insects, arachnids, myriapods, isopods, collembolans, diplurans, and proturans. The majority of these species live within the leaf litter layer of the soil where decomposing organic matter is most abundant. Soil arthropods can be split into two main groups based on their body size: microfauna and macrofauna (Coleman et al. 2004). Microfauna including Collembola and Acari are important to soil ecology mainly because of their roles in the food web as decomposers of organic material, predators of smaller invertebrates, and prey for larger animals (Remelli 2020). Macrofauna such as larger insects, arachnids, and myriapods inhabit similar niches to microfauna but with the additional ability to alter the structure of the soil. As these larger arthropods move through the earth, they mix the soil and create the pore space that is necessary for the survival of plant roots (Bagyaraj et al. 2016). As decomposers, both microfauna and macrofauna recycle nutrients from decaying plants and animals so that they can be taken up by roots and reintroduced into the larger ecosystem aboveground (Coleman et al. 2004).

Entomopathogenic Nematodes

Entomopathogenic nematodes (EPNs) are soil-dwelling roundworms that are obligate endoparasites of insect hosts. EPNs are found throughout the world in suitable soil environments and can vary in their insect host specificity. In general, these organisms have a free-living infective juvenile (IJ) stage and a reproductive adult stage within the host body. IJs must pass through the environment in order to locate and infect a host insect. Infection of the host occurs either through penetration of the cuticle,

penetration of intestine following entry from the mouth or anus, or penetration of the tracheal system through the spiracles. Once in the body cavity, IJs release symbiotic bacteria that kill the host before developing into reproductive adults (Askary et al. 2018). Multiple generations of nematodes may occur within the host before IJs emerge in search of a new insect to infect.

While the majority of research into EPNs has focused around the use of genera *Steinernema* and *Heterorhabditis* as biological control alternative to pesticide use, there is also potential for EPNs to be studied as soil ecology bioindicators (Askary et al. 2018; Campos-herrera et al. 2015). The IJ stage is non-feeding and host penetration is costly in terms of energy consumption, so it is important that nematodes only infect hosts that will be suitable for habitation and reproduction. Large EPN populations reflect healthy populations of their host insects (Askary et al. 2018). In addition, prior to infection, free-living IJs reside within soil pore space and are susceptible to changes in temperature and moisture as well as presence of toxic chemicals (Debraj 2022). Any of these factors that negatively impact nematode populations can result in changes to the stability of the larger ecological community.

Our study assessed the impact of genetic alteration of chestnut trees through hybridization on the populations of soil arthropods and EPNs to garner a clearer understanding of the ecological ramifications of chestnut reintroduction. We examined how differing levels of Chinese and American chestnut DNA was reflected in the overall number and diversity of arthropods as well as relative abundance between arthropod taxa and presence of EPNs in association with chestnut stands. No changes in these parameters between treatment groups could indicate a preservation of ecological integrity which would provide evidence to support the positive future effect of returning blight-resistant hybrid chestnut trees to American forests.

Methods

Experimental Design and Plot Characteristics

Treatment plots were located in Dupont Recreational Forest in Transylvania county of Western North Carolina. Dupont consists of 12,500 acres of coniferous, hardwood, and mixed temperate forest and was the site of experimental American chestnut tree planting during the late 2000's and mid 2010's. Treatment plots consisted of 100% American chestnuts, 94% American and 6% Chinese chestnuts, and 75% American and 25% Chinese chestnuts (table 1). The 100% unhybridized plot was located in a relatively flat densely forested area partially shaded by large surrounding trees. Trees planted within this site were more spread out than those of other sites and experienced a higher level of blight than the hybridized groups; the majority of the

chestnut trees in this plot were small resprouts undergoing continual growth suppression as a result of *C. parasitica* infection. The 94% American chestnut plot was located on a south facing slope with fewer large shading trees in comparison to the other two sites due to clearcutting of the area prior to chestnut planting. The 75% American chestnut plot was located in a flat and densely wooded area. In all plots, larger competing vegetation had been removed from the area directly surrounding chestnut stands. Leaf litter and soil samples were collected on four collection dates from May-August of 2023.

Table 1. Plot information on American-Chinese chestnut hybrid treatments in Dupont Forest.

% American Chestnut	% Chinese Chestnut	Plot Coordinates	Year Planted	Mean Height	Mean Diameter	Number of Trees
100%	0%	35.21939, -82.59705	2013	35.89 cm	6.98 mm	46
94%	6%	35.21192, -82.59662	2014	3.9 m	3.05 cm	62
75%	25%	35.21606, -81.59480	2009	4.27 m	5.2 cm	30

Methods Soil Arthropods

To assess arthropod diversity and richness, leaf litter samples were acquired during field collections from May-August 2023. Samples were taken by hand from sites corresponding to pyramid traps set up on each of the treatment plots. Four pyramid traps were associated with each chestnut plot collected during each visit to DuPont (n=48). The same locations surrounding the traps were used during each sampling and were distinguished using marking flags. The leaf litter samples were stored in one gallon ziplock bags before being processed in the lab.

Leaf litter samples were transported to the NEMA Lab within the University of North Carolina Asheville where they were mixed by hand and added to Berlese funnels in an amount that completely filled the 8.5" x 8.5" funnel. The samples were heated at 40°C for 48 hours to drive the majority of the organisms into a falcon tube collecting container containing approximately 25 ml of 70% ethanol where they were preserved until identification (Yahya et al. 2020). To quantify and sort arthropods to class, samples were identified by hand using a dissecting microscope and pipetted into glass vials. Arthropods were separated into groups Arachnida, Collembola, Insecta, Myriapoda, or

Protura. Evidence of mollusk presence was also recorded during sample sorting, but numbers were not high enough to influence statistical analysis.

Differences in arthropod abundance and diversity according to the Shannon and Simpson Diversity Indices were analyzed using a series of ANOVAs (Shannon 1948; Simpson 1949). A log transformation was performed to reduce skewing and account for non-normal distribution of data, and data with significant results were run through Tukey post-hoc tests. The significance level was set at $\alpha = 0.05$. All calculations and statistical analysis were performed using R statistical software version 2023.06 1+524 (R Core Team, 2021).

Methods EPNs

Soil samples were collected using soil probes to determine the association of EPN presence with chestnut hybridization (Filgueiras et al. 2023). Samples were extracted from two marked sites in proximity to each of four pyramid traps located in the experimental plots (n=96). Each sample consisted of the top 10-20 cm of three soil cores from the same site which were stored in 16 oz containers until lab analysis.

EPNs were extracted from soil samples using the white trap method (Orozco et al. 2014). Water was added to each sample to increase moisture content along with 10 *Galleria mellonella* to attract any IJs present in the soil. *G. mellonella* is a species of lepidopteran that is commonly used as an insect host to rear EPNs in its larval stage (Filgueiras and Willett 2021). The samples were allowed to rest for five days at room temperature without disturbance. Once inoculated, *G. mellonella* were removed from soil, externally sterilized using 10% bleach solution, separated according to color in order to prevent bacterial and fungal cross contamination, and added to white traps. The filter paper within the white traps were moistened with deionized water using a syringe and checked for fungal and bacterial growth every 1-2 days until EPNs began to emerge. Moisture levels were maintained within traps to facilitate EPN movement into water (Filgueiras, Willett 2021). Extracted EPNs were added to tissue flasks for storage.

Nematode data were run through Chi-squared tests, linear regression, and fisher tests to determine and analyze significant differences in presence between experimental plots. The significance level was set at $\alpha = 0.05$. All calculations and statistical analysis were performed using R statistical software version 2023.06 1+524 (R Core Team, 2021).

Results

Overall Arthropod Abundance

Five arthropod classes were identified during our sampling including Arachnida, Collembola, Insecta, Myriapoda, and Protura along with a small number of molluscs (figure 1). A total of 8,809 soil arthropods were collected. The plot containing unhybridized American chestnuts yielded a total of 4,331 organisms, followed by 2,738 in the 75% plot and 1,740 in the 94% plot. However, these overall abundances were not found to have any significant difference between plots ($F(1)=0.42$, $p>0.05$).

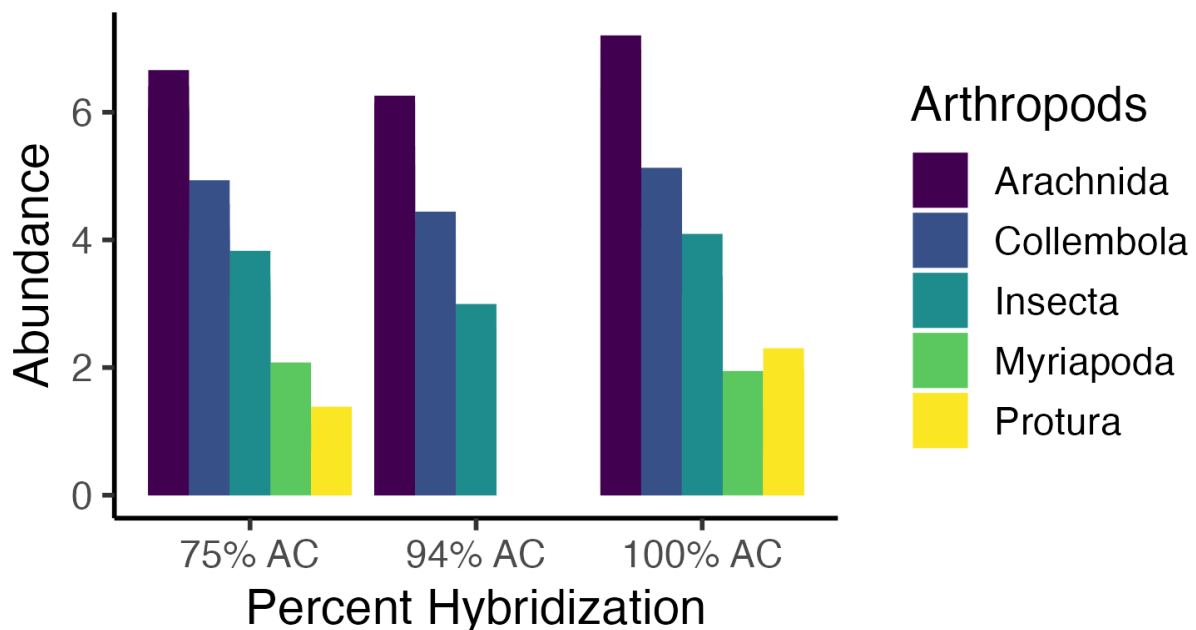


Figure 1. Soil arthropod abundances and distribution of taxa between plots of 75%, 94%, and 100% American-Chinese chestnut hybrids. Proturans collected at the 94% AC plot were not great enough in quantity to appear on the scale of this figure.

Arachnida Abundance

Arachnida made up the largest portion of arthropod samples collected during our study, accounting for 82.79% of all organisms identified. An ANOVA test used to compare average arachnid abundances found a significant difference between plots ($F(2)=5.59$, $p=0.03$) (figure 2). A Tukey HSD post-hoc test indicated that the difference was between the 100% and 94% plots ($p=0.02$), with the 100% stand having a significantly greater abundance of arachnids. No significant difference in arachnida

abundance was observed between the 94% and 75% nor the 100% and 75% plots ($p > 0.05$).

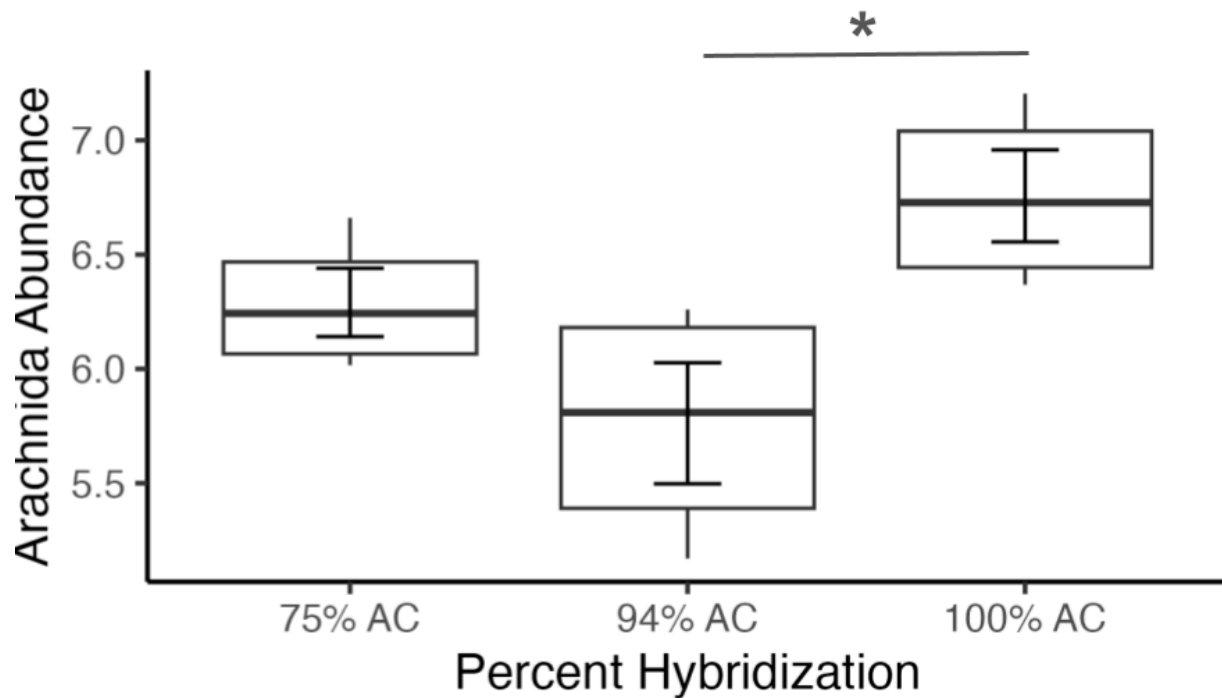


Figure 2. Distribution and variation of mean transformed arachnid abundance between plots of 75%, 94% and 100% American-Chinese chestnut hybrids. Middle lines represent median, error bars show standard error, asterisks show where significant differences lie between plots ($p < 0.05$).

Insect Abundance

Insects made up 3.54% of arthropod samples collected. An ANOVA used to analyze count data found significant differences in insect abundance among plots ($F(2)=5.78$, $p=0.02$) (figure 3). A post-hoc test detected the significance to lie between the 94% and 75% plots ($p=0.03$) and 94% and 100% plots ($p=0.05$), with the 94% plot having significantly lower abundance in both cases. No difference was discovered between the 100% and 75% treatment groups ($p > 0.05$).

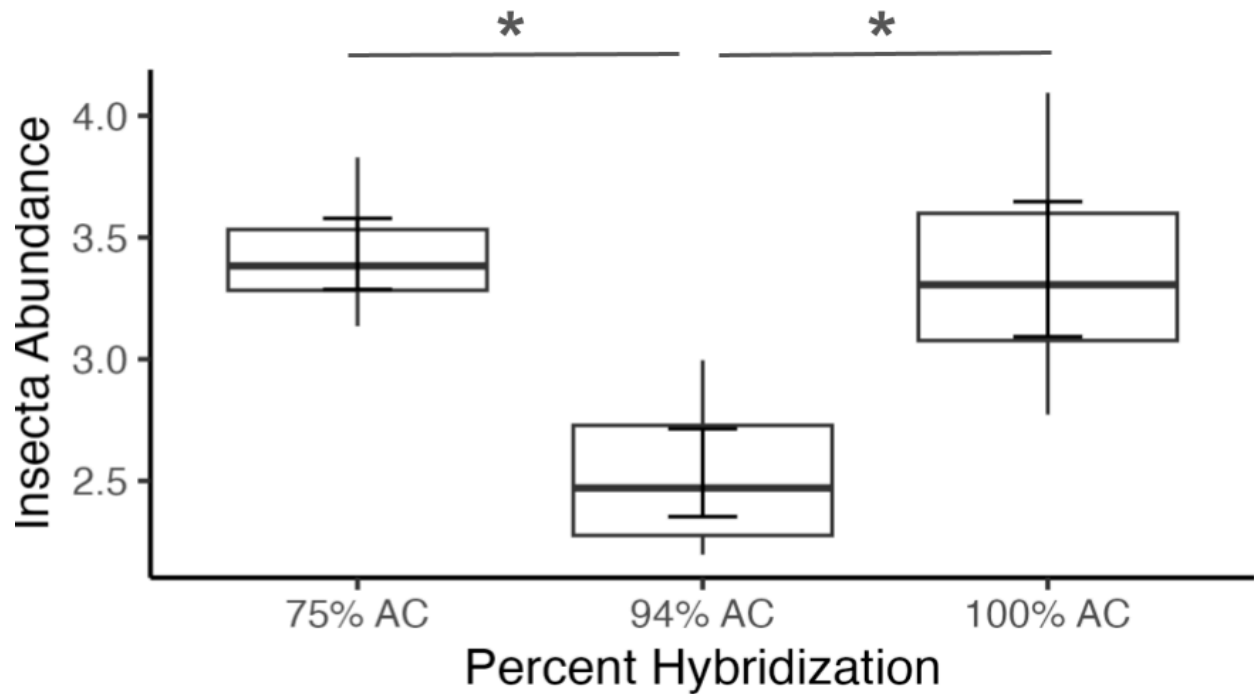


Figure 3. Distribution and variation of mean transformed insect abundance between plots of 75%, 94% and 100% American-Chinese chestnut hybrids. Middle lines represent median, error bars show standard error, asterisks show where significant differences lie between plots ($p < 0.05$).

Myriapoda Abundance

Myriapods were one of the least common groups identified from leaf litter samples, constituting only 0.31% of all soil organisms. An ANOVA comparing myriapoda abundances indicated significant differences among treatment groups ($F(2)=5.4$, $p=0.04$) (figure 4). A post-hoc tukey-test used found the 94% plot to have a significantly lower mean abundance than the 100% plot ($p=0.05$). No differences were found between the 94% and 75% nor the 100% and 75% plots ($p > 0.05$).

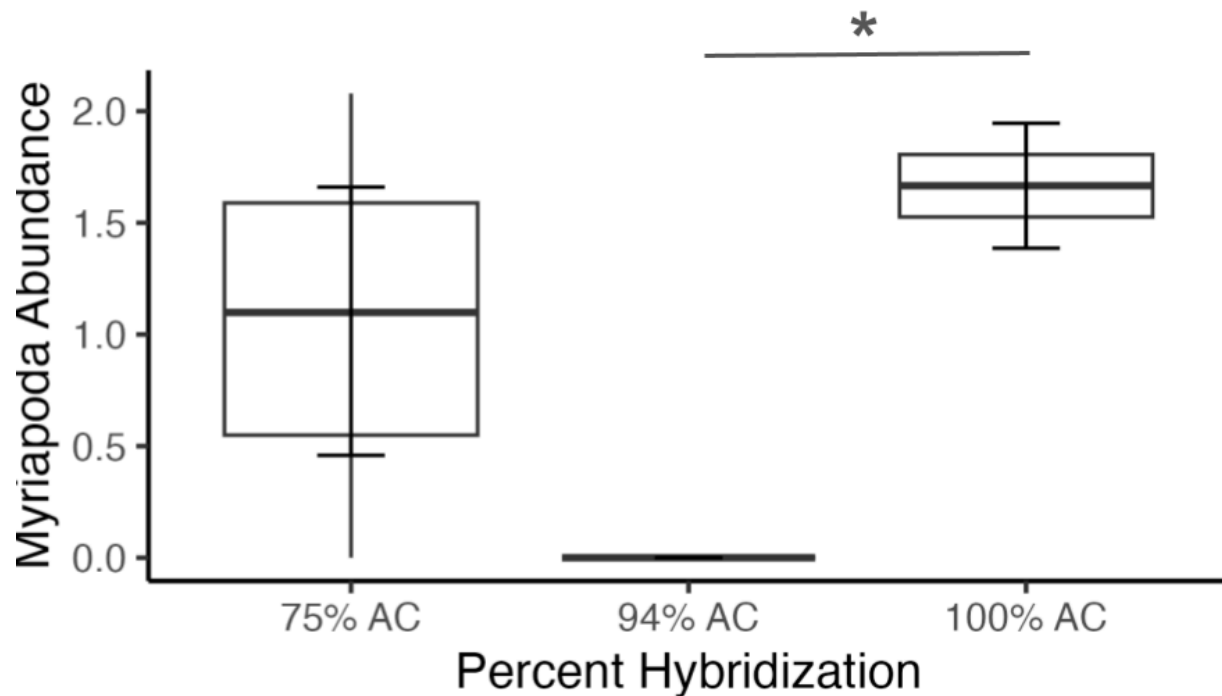


Figure 4. Distribution and variation of mean transformed myriapod abundance between plots of 75%, 94% and 100% American-Chinese chestnut hybrids. Middle lines represent median, error bars show standard error, asterisks show where significant differences lie between plots ($p < 0.05$).

Collembola and Protura had no significant difference in abundance between plots ($p > 0.05$). Mollusca did not have numerically viable or enough occurrences to run these tests.

Arthropod Diversity

Analysis revealed the Shannon Diversity Index scores for each treatment group as follows: 100% 0.892, 94% 0.674, and 75% 0.593. A higher index score indicates a greater amount of evenness and species richness in a community. An ANOVA performed comparing diversity between plots based on the Shannon Diversity Index scores of each chestnut stand discovered no significant differences between treatment groups ($p > 0.05$) (figure 5).

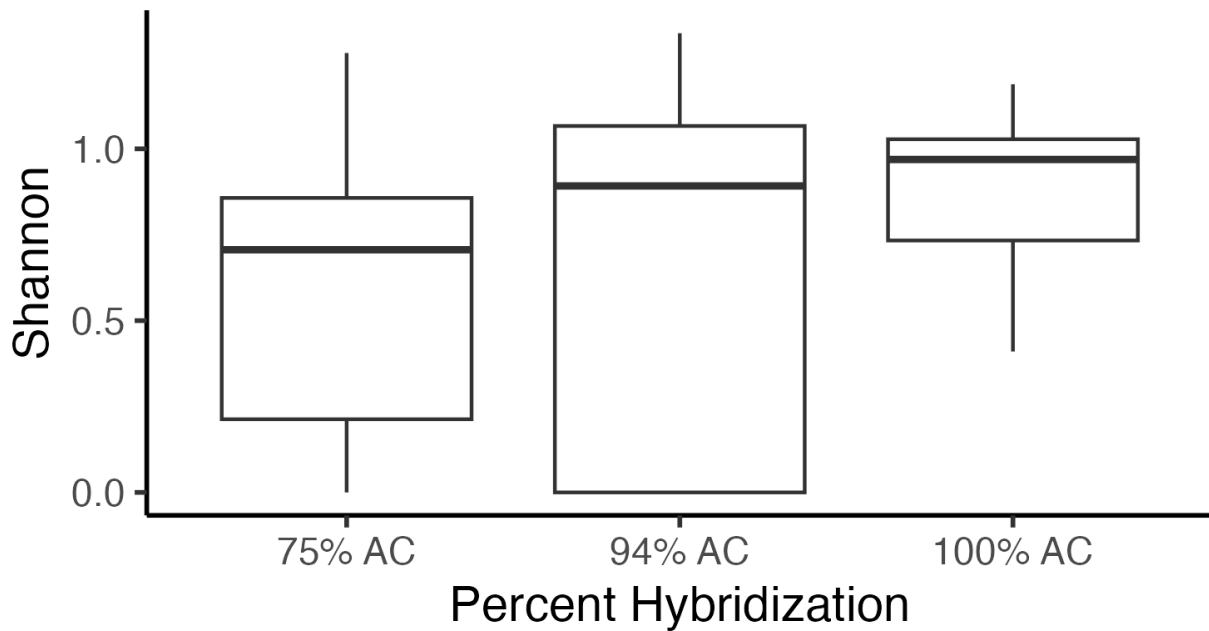


Figure 5. No significant differences in soil arthropod biodiversity based on the Shannon Diversity Index scores of 75%, 94%, and 100% American-Chinese chestnut plots.

Analysis revealed the Simpson Diversity Index scores for each treatment group as follows: 100% 0.525, 94% 0.371, and 75% 0.339. A higher index score indicates reduced biodiversity under the parameters of dominance and richness. An ANOVA comparing biodiversity using the Simpson Diversity Index discovered a significant difference among treatment groups ($F(2)=3.50$, $p=0.04$) (figure 6). A tukey-HSD test revealed the difference to be between the 100% and 75% plots ($p=0.04$).

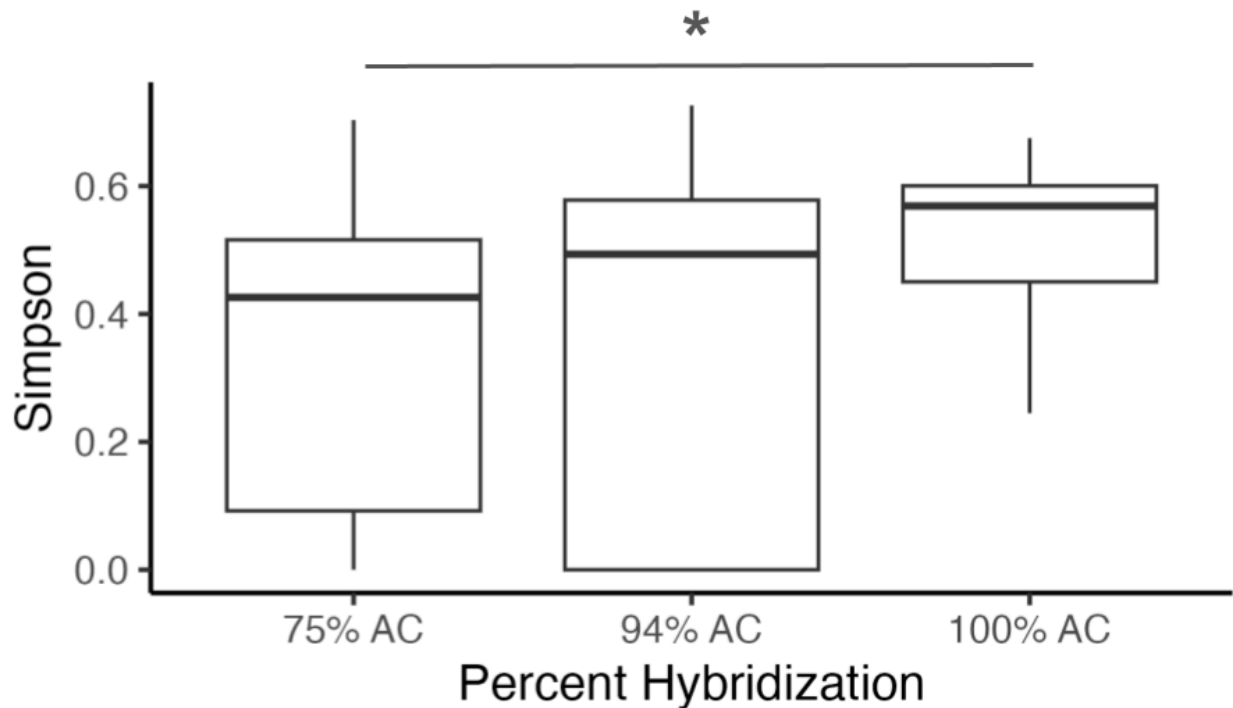


Figure 6. Differences in soil arthropod biodiversity between 75%, 94%, and 100% American-Chinese chestnut hybrids based on Simpson Diversity Index scores. Higher index scores indicate lower diversity. Asterisks show where significant differences lie between plots ($p < 0.05$)

EPN Presence

When looking for significant correlation with the independent variables and the presence of EPNs, no significance was found when using a chi-squared test of independence. After using both a linear regression to model the probability of nematode presence based on the combination of factors and a fisher test to analyze the association or independence between variables, no significant difference was found in EPN presence among any of the variables.

Discussion

Our analysis revealed no significant differences in overall abundance of arthropods nor presence of EPNs between experimental plots. Among arthropod groups, however, differences in abundance were observed in the 94% American chestnut plot in comparison to other treatments. The 94% plot yielded less arachnids, insects, and myriapods than the 100% plot (figures 2, 3, and 4). The 94% treatment also contained a smaller abundance of insects than the 75% plot (figure 3). Despite these

differences between specific taxa, overall arthropod abundance did not differ between treatments. The Simpson Diversity Index test revealed lower biodiversity within the 100% plot in comparison to the 75% plot (figure 6). However, no significance was found between experimental groups using the Shannon Diversity Index.

We used the Shannon and Simpson indices to measure diversity of arthropods within our experimental plots. While both of these tests are used to analyze biodiversity, each index relays different information about population dynamics based on its own parameters. The Simpson Diversity Index places emphasis on relative abundance and dominance of specific groups while the Shannon Diversity Index takes into account less common groups or rare species that would not affect the Simpson Index. Our analysis revealed a significant difference in biodiversity between the 100% and 75% plots according to the Simpson Index but not the Shannon Index. This result indicates that there is higher group dominance in the 100% plot versus the 75% plot but that the evenness of the ecological community is unaffected.

When assessing the reasons behind the differences in specific arthropod group abundance and biodiversity observed between chestnut stands it is necessary to consider the context of the surrounding environment and its ecological influence on soil organism populations. While chestnut trees made up the majority of the arboreal community on our experimental plots, other factors such as chestnut tree size and age, interactions from neighboring vegetation, terrain differences, and the physical and chemical characteristics of the soil (Ghiglieno et al. 2021) likely had as much or more impact on the areas' soil ecology as chestnut hybridization. Most of the differences in abundance seen in our experiment were between the 94% and 100% plots, with the 100% plot having greater numbers of certain arthropod groups including arachnida, insecta, and myriapoda. It is important to note the variations between these two plots to convey a more nuanced understanding of these results.

In the site containing hybridizations of 94% American chestnut to 6% Chinese chestnut, trees were larger, healthier, and made up a greater percentage of the vegetative cover than the trees in the unhybridized plot which was comprised almost entirely of resprouts experiencing continual growth suppression due to *C. parasitica* infection. 94% hybridized trees were, on average, 3.54 meters taller and had a greater average diameter by 2.35 cm than the unhybridized group. As a result, non chestnut plant cover faced less competition from chestnut trees in the 100% plot in comparison to the 94% plot and was subsequently more well established and overgrown by various other plant species. Multiple studies by Sanguankeo and León (2011), Chowdhury et al. (2022), and Yan et al. (2019) found that arthropod abundance and diversity were positively correlated with greater plant cover and diversity, and Elmquist et al. (2023) revealed a link between introducing novel crop plant species and increased arthropod richness. Plant community heterogeneity is known to promote soil fertility and decomposition rates as well as create more complex habitats to support a greater

number and diversity of belowground organisms (Yan et al. 2019). However, there is also evidence that different arthropod taxa may be attracted to differing levels of heterogeneity as shown in a study by Tripathi et al. (2005) on arthropod communities in silvopasture areas versus pure grass plots. Therefore, vegetative features of the plots unrelated to chestnut hybridization may have contributed to the greater abundance of certain arthropod taxa seen in the 100% American chestnut treatment group. External environmental factors may have also contributed to the difference observed in insect abundance between the 75% and 94% experimental plots. While the greater number of insects in the 75% plot may be related to Chinese-American hybridization, variation in site location and ecology also factor into the population composition of soil arthropods. The 75% plot was positioned in a more densely forested area and contained older chestnut trees than those in the 94% plot. Because the 75% American Chinese chestnut stand was planted in 2009, five years prior to the 94% hybrids planted in 2014, it is possible that soil communities have become more well established as a result of a longer period of non disturbance.

It is also important to note the terrain differences between chestnut stands. Both the 75% and 100% plots were located in relatively flat areas surrounded on all sides by the Eastern hardwood forest present in DuPont Recreational Forest. By contrast, the 94% American chestnut trees were planted on a south facing slope that had been clear cut previous to chestnut planting unlike the other two experimental plots. Temperatures were noticeably hotter when performing field collections on this plot in comparison to the other two likely due to its orientation in combination with a lack of canopy cover. It has been shown that a greater number and diversity of shade producing trees are associated with higher soil arthropod abundance and richness (Chowdhury et al. 2022). However, a study on the effect of tree thinning on soil organisms in coniferous stands found contrasting evidence where plots with intense thinning had the highest richness and abundance of arthropod communities and that biodiversity was not affected across treatment groups (Muscolo et al. 2021). High soil surface temperatures and exposure to UV radiation from sunlight are also connected with decreased biodiversity of subterranean arthropods (Huang et al 2021, Prather et al. 2020).

The 100% American chestnut plot was also characterized by the presence of several large ant colonies in the area which were not observed in the other two plots studied. Previous studies have shown that ant activity alters the surrounding soil by creating pore space and increasing the amount of several key nutrients including sodium (Sosa and Brazeiro 2012; Wang et al. 2017). An increase in plant and microbe biomass and richness has also been noted surrounding ant colonies, which in turn likely alters the community composition of larger belowground organisms (Sosa and Brazeiro 2012; Wang et al. 2017). Certainly, the greater number of ants within the unhybridized chestnut plot contributed to the larger abundance of insects sampled in comparison to

the 94% plot, but it is also possible that ant activity altered the community dynamics of other arthropods in the area.

Soil characteristics have an effect on the richness and abundance of subterranean organisms as well. Specific textures and types of soil along with nutrient and water content and thickness of leaf litter layer are known to impact the richness and abundance of arthropods they contain (Ghiglieno et al. 2021; Prather et al. 2020; Tripathi et al. 2005; Yan et al. 2019). Yahya et al.'s (2020) examination of populations of Acari (mites) and Collembola on different types of agricultural land found that numbers were impacted by nitrogen and carbon content, moisture levels, and aeration of the soil. Generally, both of these groups can be used as bioindicators because their presence is associated with healthier, more nutrient rich soils. Our analysis showed greater numbers of arachnida, a category which included both spiders and mites, in the 100% chestnut plot as opposed to the 94% plot. Collembola did not have any significant difference in abundance between groups. The marked higher levels of arthropod group abundance within the 100% plot may indicate higher soil quality which could be related to the presence of unhybridized American chestnuts or other ecological features of the area.

Given the inherent difficulty in controlling confounding variables within field sites and the role of additional ecological factors in soil invertebrate communities, our research provides some preliminary insight into the under researched topic on the impact of plant hybridization on soil organisms. The findings of this study suggest that while general abundance of arthropods and presence of EPNs are unaffected by hybridization, backcross breeding may impact population composition among groups. We observed greater arthropod group dominance in the 100% site versus the 75% site in addition to higher prevalence of specific arthropod groups in comparison to the 94% plot. These results could indicate an attraction of specific arthropod taxa to unhybridized trees or the environment they inhabit as shown previously in studies involving agricultural crop species (Elmqvist et al. 2023) and sites with varying habitat types (Tripathi et al 2005, Yan et al. 2019). Future studies may focus on further identification of these dominant groups found surrounding unhybridized *C. dentata* and the ecological interactions or habitat conditions which result in their presence. Further research on this topic may involve investigation into the effects of hybridization on nutrient cycling and soil health and long term monitoring of soil arthropod communities associated with chestnut hybridization.

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