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Insect Biodiversity, Herbivory, and Community Composition in American (*Castanea dentata*), Chinese (*Castanea mollissima*), and Hybrid Chestnut Trees

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Abstract

The American chestnut (*Castanea dentata*) was once a dominant tree in eastern North America before being nearly wiped out by the chestnut blight (*Cryphonectria parasitica*) in the early 1900s. Current restoration efforts focus on hybridizing the blight-resistant Chinese chestnut (*Castanea mollissima*) with the native American chestnut. While this approach may improve tree survival, it is still unclear whether hybrid chestnuts can support the same ecological relationships that made the American chestnut a keystone species. Insects play central roles in forest ecosystems as pollinators, herbivores, and decomposers. Without these interactions, hybridization may only provide a superficial solution. This study examines the variation in insect diversity across American, Chinese, and hybridized backcross chestnut tree stands at the American Chestnut

Foundation's Meadowview plantation. Insects were collected and analyzed across stands of different chestnut hybridizations using pyramid traps, pan traps, and Berlese funnel extraction. Leaf samples were also collected to analyze herbivory. Samples were processed in the NEMA lab at UNC Asheville to compare biodiversity, community composition, and leaf herbivory among treatments. This research links chestnut hybridization and insect community variables to examine whether hybrids can fill the ecological role once played by American chestnuts. Overall insect biodiversity did not differ significantly among chestnut treatments except in pan traps. However, Community composition varied among pan trap color and treatment type. indicating that color may influence capture patterns across treatments. The results provide insight into the impact (positive, negative, neutral) of Chestnut tree hybridization on insect biodiversity and community composition. These findings will inform restoration strategies by helping answer the larger question of whether forests planted with hybrid chestnuts will function like forests that existed before the chestnut blight decimated them.

Introduction

The American Chestnut tree (*Castanea dentata*) is a vital species, providing resources and employing unique niches, particularly in the eastern US. As a Keystone species, it not only provides essential resources for humans and wildlife but also supports the surrounding ecosystem [1]. Crucial to rural economies due to its rot-resistant wood, which is often used in construction and furniture. Its nuts are highly nutritious, calorie-dense, rich in vitamin C and antioxidants, and provide essential nutrients for the entire ecosystem [1]. Before the blight, American chestnut comprised roughly one-quarter to one-third of many Appalachian forests, dominating regions such as the Blue Ridge Mountains and the Cumberland Plateau [1, 2, 3].

The thriving population we once saw, however, is no longer what we see today. The fungus responsible for this decline is *Cryphonectria parasitica*, commonly called chestnut blight. Discovered in a New York zoo in 1904, it rapidly spread and by the 1960s had killed an estimated 4 billion trees. Today, American Chestnuts are common in Eastern US forests; however, almost all remaining individuals are stump sprouts that eventually succumb to the blight. The recovery of the chestnut population has proved difficult, as infection, dieback, and reinfection occurrences can persist for decades, with sprouts usually remaining small and rarely reaching reproductive maturity [3,4]. As a result, the species are considered endangered in their native ranges in Canada and several US states [1]. As a keystone species, its loss reshaped forest structure, mast production, and wildlife carrying capacity. Additionally, processes such as decomposition and nutrient cycling are disrupted, changing ecological dynamics [3,4]. Because of the strong and unique ecological niche the American Chestnut occupies, the restoration is a significant focus of forest recovery efforts.

One successful approach to this is backcrossing hybridization. For the American chestnut to persist and thrive in its natural environments, long-term resistance to the *Cryphonectria parasitica*

fungus is vital to restoring its ecological role [7]. Hybridization backcrossing presents the potential to do just that, restore the American Chestnut while still maintaining its native characteristics.

The backcross breeding program, initiated in 1989 by the American Chestnut Foundation, aims to transfer blight resistance from the Chinese chestnut into the American chestnut. The process introduces blight resistance from the Chinese chestnut (*C. mollissima*) into the American chestnut, followed by several generations of backcrossing to restore native traits. Trees that exhibit strong resistance and resemble the original species are selected in each generation, thereby maintaining both resilience and ecological compatibility. This entire process takes six generations [1,7].

While the breeding program has made progress, there remain gaps in understanding how hybridized chestnut influences interactions with the surrounding ecosystem, including associated fauna, vegetation dynamics, and forest structure [7]. Studies have begun to examine how hybridization influences ecological dynamics. Reed et al. (2024) found that chestnut hybridization affected invertebrate communities above- and belowground, indicating that hybrid restoration may alter forest ecosystem functioning [9]. Other studies found that hybridizations can alter forest carbon dynamics and affect overall structure and function [10,11]. While some research has examined the implications of hybridized trees and the potential changes with the associated environment, it is crucial to continue expanding our knowledge and deepening our understanding.

Insects, as central components of ecosystem function, provide valuable insights into the broader ecological implications of hybridization, making them reliable indicators of environmental change [12,13,14]. One way insects serve as bioindicators is by examining insect herbivory. Hybridization introduces genetic and chemical variation that can alter plant–insect interactions, making it vital to understand how these changes shape insect communities to determine whether hybrid chestnuts can genuinely fill the ecological role once occupied by the American Chestnut [15].

Introduced and hybrid plant species often interact differently with local insects because herbivores have not coevolved with their chemical and structural traits. This can lead to a form of “enemy release,” where non-native or genetically novel hosts experience reduced pressure from specialists and support different insect communities than native species [20, 25, 27]. Hybridization can also shift leaf chemistry and defensive traits, thereby altering how insects feed and which species can use the plant [26]. Because American, Chinese, and hybrid chestnuts vary in these traits, it is likely that they also differ in the insect groups they attract or support.

Genetic diversity in plants influences insect diversity and herbivory through bottom-up control, in which variation within and among tree species can shape specialization and feeding patterns [16, 17]. In a study by Kambach et al. (2016) found that insect herbivore species richness increased with tree diversity, emphasizing how greater plant diversity supports more complex multitrophic interactions [18]. These shifts in genetic makeup can alter which insect species occupy hybrid trees, potentially leading to differences in community composition even if overall biodiversity remains similar [15, 20].

Because of their high sensitivity to changes in plant chemistry and genetics, insects can serve as bioindicators to evaluate the ecological success of hybridization restoration efforts [16]. While progress has been made through hybrid breeding in restoring the American Chestnut, most current

research focuses on blight resistance and survival rates. This leaves the ecological outcomes of hybridization understudied, and without this knowledge, it is not possible to accurately assess the effectiveness and outcomes of this restoration approach [7,8].

Based on this, we predicted that the chestnut genotype would influence both herbivory and insect community composition. We did not expect significant differences in overall diversity across treatments, but we did expect shifts in which insect groups were present and in their abundance. Because hybrids share traits from both parent species, we also expected them to exhibit community patterns that fall between those of pure American and pure Chinese chestnuts.

This study aims to evaluate how hybridization influences insect biodiversity, herbivory, and community composition across American, Chinese, and hybrid chestnut trees to determine whether hybrids support insect biodiversity like that of native American chestnuts. Various collection and monitoring strategies were employed for Chinese, American, and hybrid chestnut genotypes to examine the effects of hybridization on community composition, herbivory, and biodiversity.

Methods

All field collections were conducted at the American Chestnut Foundation's Meadowview Research Farms in Meadowview, Virginia, a long-term breeding and restoration site for *Castanea dentata* and its hybrid lines. It contains more than 150 acres of orchards consisting of American, Chinese, and hybrid chestnuts at various stages of the breeding program. This site provides a controlled environment for growing, maintaining, and monitoring mature chestnuts [1]. For this study, sampling focused on four hybrid types: 100% American chestnut (100AC), 100% Chinese chestnut (100CC), 50% American/50% Chinese (50% AC/CC) hybrids, and ~60-75% American Chestnut (B352, ~60-75% AC) backcross lines.

Samples were collected between May and August 2024. Multiple different collection types were used, including pyramid traps, pan traps, and leaf litter samples. Leaf herbivory assessments were also examined. Field collections were labeled by treatment, date, trap type, and replicate (when applicable), then shipped overnight via express courier to the University of North Carolina at Asheville (UNCA) for laboratory processing and identification.

All samples were examined at the UNCA Zeis Laboratory under dissecting microscopes. Specimens were sorted, counted, and identified to the lowest possible taxonomic level (typically order) using external morphology. Data from all collections were entered and organized using Google Sheets, where specimen counts, diversity metrics, and percent-herbivory values were compiled for later analysis in RStudio.

All analyses were conducted in RStudio (Posit Cloud) using R version 4.5.1 [19]. The following packages were used: vegan (diversity indices, PERMANOVA, NMDS) [21], ggplot2 (visualizations) [22], car (Levene's test) [23], and emmeans (post-hoc tests) [24]. Before analysis, all community matrices were cleaned and transformed to remove missing data and ensure that species count columns contained numeric values. For each dataset, samples were grouped by treatment and,

when applicable, trap color. Significance thresholds were set at $\alpha = 0.05$, with marginal results noted at $0.05 \leq p < 0.10$.

All figures were generated using ggplot2, employing viridis color palettes for colorblind accessibility. Asterisks (*, **, ***) or above bars indicate significant post-hoc differences. NMDS ordination plots display 95% confidence ellipses for each treatment group. Results are presented as mean ± SE, and all statistical values (F, R^2, p, etc.) are reported in corresponding tables.

Pan Traps

Pan traps were used to collect small flying insects across four chestnut treatments. Sampling occurred on May 24, June 7, June 19, July 5, and July 22, 2024. For each collection date, a single set of colored bowls (white, yellow, green, and blue) was deployed for each treatment, yielding 16 pan traps per date (4 treatments × 4 colors). On July 22, 17 traps were recovered, totaling 81 samples over the season, including the one additional trap recorded that day.

Each pan consisted of a 12-oz plastic bowl mounted on a wooden post and filled with ~500 mL of water, with a few drops of unscented dish soap added to reduce surface tension. Traps were deployed in the morning and retrieved after 24 hours. The contents were poured through a fine-mesh sieve, rinsed with 70% ethanol, and transferred to labeled 50 mL conical tubes containing new 70% ethanol for preservation. Labels recorded treatment, trap color, and collection date.

In the laboratory, None of the B352 samples from the green trap arrived on July 5. Two of the yellow 50 AC/CC samples from July 22 had leaked during shipment. The number of individuals per order was recorded for each trap color × treatment combination for subsequent analyses.

For statistical analysis, a community matrix of order-level insect counts per sample was grouped by treatment and collection number. Shannon and Simpson diversity indices were run using the diversity function in vegan. The Shapiro-Wilk test was used to assess normality, and Levene's test was used to determine homogeneity of variance. One-way ANOVA was conducted on the Shannon and Simpson indices to compare the different hybridizations, independent of pan trap color.

Two-way ANOVAs were performed with treatment and trap color as factors to test for differences in biodiversity within each pan trap color. Tukey's HSD and Dunnett post hoc tests were used to identify pairwise differences among treatments. The Dunnett test was used to compare all treatments to 100 AC as the control. To test for overall community structure differences among trap colors and treatment, PERMANOVA (adonis, 999 permutations, Bray-Curtis dissimilarity). Betadisper was used to assess multivariate dispersion homogeneity and ensure that PERMANOVA results were valid.

Community structure was visualized using non-metric multidimensional scaling (NMDS) with Bray-Curtis distances (metaMDS in vegan). Bar plots were generated for Shannon and Simpson diversity (by treatment and color), with means ± SE, and NMDS ordinations with 95% confidence ellipses.

Leaf Litter

Leaf litter samples were collected from beneath chestnut trees for the four different treatments. Sampling occurred on 24 May, 25 June, and 22 July 2024. During each collection period, two replicate sets (A and B) were collected per treatment, yielding 8 leaf litter bags per date and 24 total samples across the season. Once samples were collected, the foliage was placed in a Ziplock bag and labeled with the hybridization date and set (A or B).

Leaf litter bags were shipped to the UNC Asheville laboratory and placed in Berlese funnels for 48 hours to extract organisms from the samples. Each bag was hand-mixed and put into the Berlese funnels so that it filled the 8.5' x 8.5' funnel without overflowing. Samples were kept under a heating lamp at 40 °C for 24 hours. Heat and light forced organisms downward through the litter into a 50 mL conical tube containing approximately 25 mL of 70% ethanol, and into vials containing 70% ethanol for preservation until identification.

Statistical analysis was performed by constructing a community matrix from order-level insect counts per treatment and sample ID. Shannon and Simpson diversity indices were calculated across the treatment groups, and one-way ANOVAs were conducted on both indices. A PERMANOVA was conducted (Bray–Curtis dissimilarity, 999 permutations) to examine community-level differences among treatments. Dispersion homogeneity was verified using betadisper. Data were visualized using bar plots (mean ± SE) and NMDS ordinations colored by treatment.

Pyramid Traps

Pyramid traps were used to sample ground and flying insects beneath the canopy of chestnut trees, representing four treatments. Two traps (replicates A and B) were deployed for each treatment, resulting in 8 traps in total.

Each trap consisted of a Pyramid Trap made up of 4 plastic panels forming a pyramid with a funnel at the top leading into a collection cup. Traps were installed in mid-May 2024 and remained in place through late August 2024, with 7 collections (56 samples in total). Trap contents were collected approximately every ten days.

After each collection, samples were transferred to labeled 50mL conical tubes containing 70% ethanol and shipped to the UNC Asheville laboratory. For identification and analysis, damaged or unidentifiable specimens were excluded.

For statistical analysis, a community matrix was constructed from order-level insect counts by treatment and sample ID (e.g., 100 AC 1 PY). Shannon and Simpson diversity indices were calculated for each treatment group, and one-way ANOVAs were performed to test for differences among treatments. Tukey HSD post hoc tests were used to identify pairwise differences, with significance indicated by asterisks on the diversity plots.

A PERMANOVA (Bray–Curtis dissimilarity, 999 permutations) was run to identify community-level differences among treatments. To assess dispersion uniformity, betadisper was used to ensure that the data remained uniform across groups. Data visualization included bar plots (mean ± SE) of Shannon and Simpson diversity indices, as well as NMDS ordination diagrams showing the treatment-level composition of insect communities.

Leaf Herbivory

Leaf herbivory was quantified from chestnut trees representing four treatments: 100% American chestnut (AC), 100% Chinese chestnut (CC), 50 AC/CC hybrids, and B352 backcross lines. Leaves were collected on 24 May and 24 June 2024. 10 leaves per treatment per date was the intended sample size; however, the actual sample sizes were: 24 May 2024: 100 AC (10 leaves), 100 CC (8 usable leaves), 50 AC/CC (10), B352 (10). 24 June 2024: 100 AC (9), 50 AC/CC (9), B352 (10), and no 100 CC sample collected on this date.

Each leaf was labeled with its treatment and its related leaf number (e.g., "100 CC 1 LH") and shipped to UNC Asheville for analysis. After the collection and arrival at the UNC Asheville laboratory, leaves were placed in a freezer at −18 °C for 24 hours to preserve and flatten them before imaging. To achieve shadow-free, consistent images, an examination box was built using a standard shoebox base with an internal LED light source and a paper-covered top for diffusion. A second open-bottom box was placed above the light source, and photographs were taken through a hole cut in the upper box using a 4th-generation iPad Air. Each leaf was laid flat on the illuminated surface between the boxes during imaging [9].

Images were analyzed using the LeafByte application to calculate the percentage of leaf area consumed by insect herbivory. When leaf edges were defoliated, the missing areas were estimated from the intact margin on the opposite side. The percentage of area missing was recorded as "Percent consumed" for each leaf. All measurements were recorded at the individual-leaf level and later averaged by treatment x date for statistical analyses.

Statistical analysis was performed using percent leaf area consumed as a measure of herbivory. Data were first checked for normality and homogeneity of variances using the Shapiro–Wilk and Levene's tests. Because residuals were not normally distributed ($p < 0.001$), non-parametric tests were used. A Kruskal–Wallis rank-sum test was run to evaluate overall differences in herbivory among chestnut treatments. Mean percent herbivory (± SE) for each treatment was visualized using bar plots created in R with the ggplot2 package.

Results

No significant differences in Shannon or Simpson diversity were found among chestnut treatments across most trap types. However, small trends appeared. Diversity tended to be slightly higher in the 100% Chinese chestnut (100 CC) traps than in the 100% American (100 AC) traps. When trap color was included as a factor in the pan traps, some color-specific differences emerged. Multivariate tests showed similar patterns. The PERMANOVA results suggested that overall insect community composition varied slightly among treatments, though not always at a statistically significant level. NMDS plots also showed that insect communities tended to cluster by treatment, with more apparent separation between the pure species (100 AC and 100 CC).

Pan Traps

Across treatments, alpha diversity did not differ significantly among chestnut hybridization types. Shannon diversity values ranged from 1.24 ± 0.14 in 100 AC to 1.43 ± 0.16 in 100 CC, with the 50 AC/CC (1.35 ± 0.11) and ~60–75% AC (1.31 ± 0.20) falling in between. Simpson diversity showed a similar pattern, ranging from 0.59 ± 0.07 to 0.64 ± 0.07 across treatments (*Figure 1*). The ANOVA test found no significant effect of treatment on Shannon diversity ($F_{3,15}$ = 0.292, p = 0.831) or Simpson diversity ($F_{3,15}$ = 0.105, p = 0.956). Both Shapiro–Wilk and Levene's tests verified normality and homogeneity of variance (all p > 0.15).

Dunnett's post-hoc tests were conducted using 100 AC as the control treatment for both Shannon (*Table 1*) and Simpson (*Table 2*) diversity indices. For Shannon's and Simpson's Diversity indices, no significant differences were observed among treatments compared to the control (all p > 0.25).

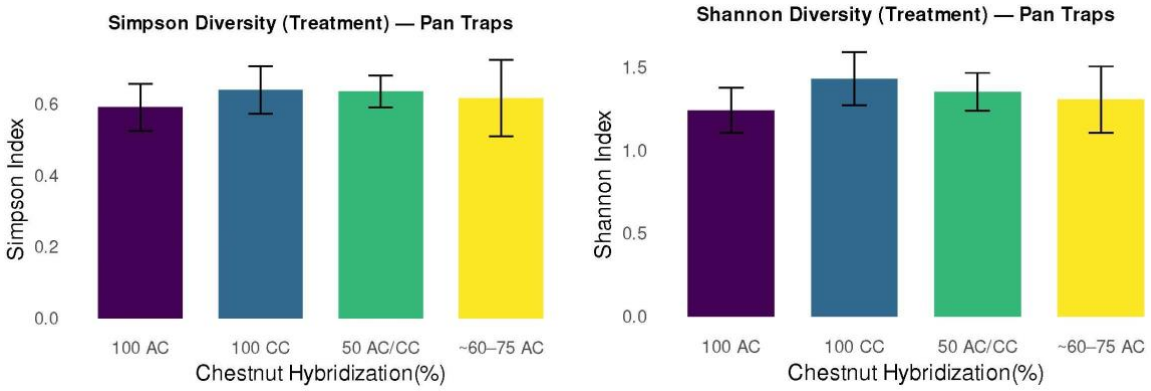

Figure 1. Mean Simpson and Shannon diversity indices (± 95% CI) across chestnut treatments. Bar plots show mean Simpson (left) and Shannon (right) diversity for insect communities collected from each chestnut treatment: 100% American chestnut (100 AC), 100% Chinese chestnut (100 CC), 50/50 hybrid (AC/CC), and the backcross hybrid (~60–75% AC; B352). Error bars represent 95% confidence intervals.

Table 1. Dunnett's test results for Shannon diversity comparing each hybridization concentration treatment to the 100 AC control.

Hybridizations percentage	Estimate	± SE	df	t.ratio	P - value
100 CC - 100 AC	0.813	0.119	58	1.530	0.3046
50 AC/CC - 100AC	0.192	0.119	58	1.609	0.2675
~60-75 AC - 100 AC	0.191	0.129	58	1.482	0.3287

Table 2. Dunnett's test results comparing Simpson diversity for each hybridization concentration treatment relative to the 100 AC control.

Contrast	Mean difference estimates	± SE	df	t.ratio	P. value
100 CC - 100 AC	0.1016	0.0534	58	1.902	0.1564
50 AC/CC - 100AC	0.1092	0.0534	58	2.045	0.1173
~60-75 AC - 100 AC	0.0809	0.0578	58	1.398	0.3733

Although overall alpha diversity did not vary with chestnut genotype, community composition differed among treatments. PERMANOVA based on Bray–Curtis dissimilarity indicated that species groups were not randomly distributed across treatments ($F = 5.12$, $R^2 = 0.121$, $p = 0.001$) as shown in *Figure 2*. Pairwise comparisons revealed that 100 AC differed significantly from 100 CC ($p = 0.001$) and from 50/50 AC/CC ($p = 0.001$), while other treatment pairs were not statistically distinct.

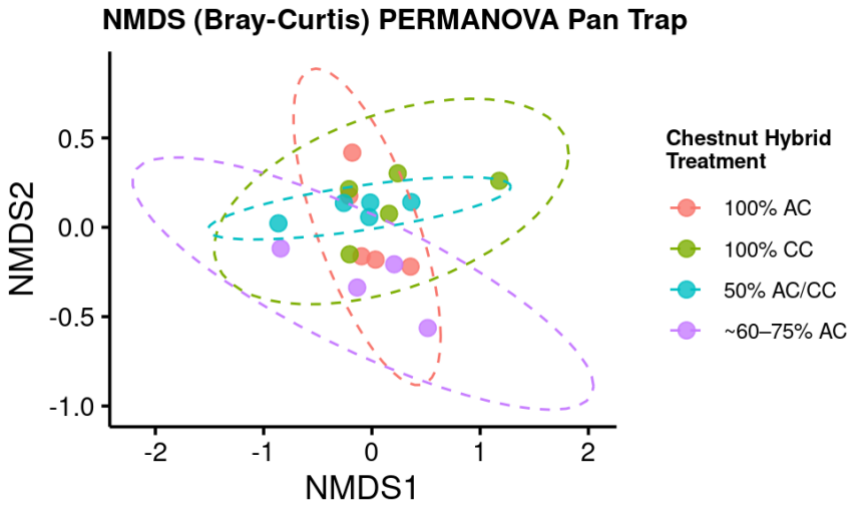

Figure 2. Non-metric multidimensional scaling (NMDS) ordination of insect community composition across chestnut treatments based on Bray–Curtis dissimilarity. Each point represents the insect community collected from an individual pan trap, grouped by chestnut treatment: 100% American chestnut (100 AC), 100% Chinese chestnut (100 CC), 50/50 hybrid (AC/CC), and the backcross hybrid (~60–75% AC; B352). Ellipses represent 95% confidence intervals around the centroid of each treatment group. The distance between points reflects the degree of difference in their species composition, as measured by Bray–Curtis dissimilarity.

A two-way ANOVA tested the effects of chestnut treatment and trap color on Shannon and Simpson diversity indices (*Table 3*). For Shannon diversity, there were no significant effects of treatment ($F_{3,58} = 1.12$, $p = 0.35$) or trap color ($F_{3,58} = 1.51$, $p = 0.22$), and no significant interaction between treatment and color ($F_{9,58} = 1.49$, $p = 0.17$). For Simpson diversity, treatment ($F_{3,58} = 1.64$, p

= 0.19) and trap color ($F_{3,58}$ = 1.64, p = 0.19) were also not significant, however, the treatment × color interaction approached significance ($F_{9,58}$ = 1.90, p = 0.07).

The mean Shannon index ranged from 0.67 ± 0.23 (100 AC – Green) to 1.34 ± 0.22 (~65-70 AC – Yellow), while the mean Simpson index ranged from 0.34 ± 0.13 (100 AC – White) to 0.68 ± 0.03 (100 AC – Yellow). Trap colors showed visually similar diversity patterns across treatments, with the highest mean values generally in Yellow traps and the lowest in White traps (*Figure 3*).

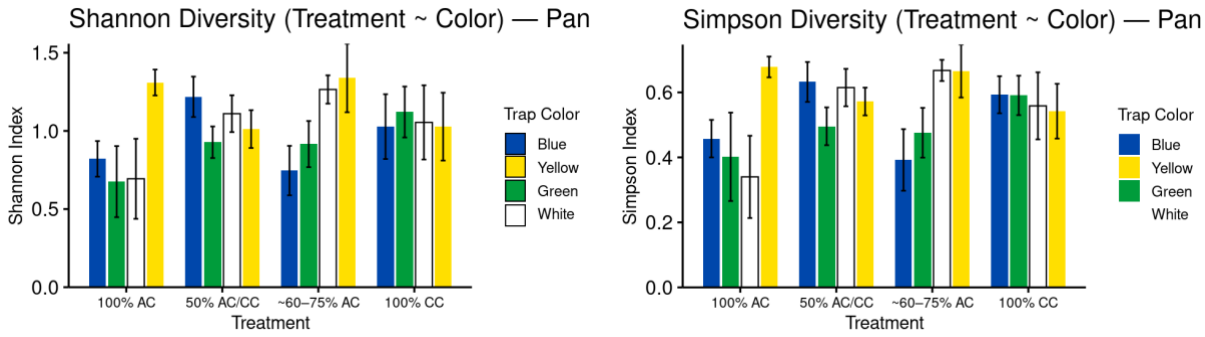

Figure 3. Mean (± SE) Shannon (right) and Simpson (left) diversity indices of insects collected from pan traps across four chestnut treatments: 100 AC (American chestnut), 100 CC (Chinese chestnut), 50/50 AC/CC (hybrid), and ~60–75 % AC (backcrossed hybrid). Trap color represents four different-colored pan traps: Blue (B), Green (G), White (W), and Yellow (Y). Bars represent mean diversity values per treatment, with error bars indicating the standard error.

Table 3. Results of two-way ANOVA testing the effects of chestnut treatment and trap color on insect diversity (Shannon and Simpson indices) collected from pan traps. Neither treatment nor color had a significant impact on Shannon or Simpson diversity (p > 0.05), although the treatment × color interaction for Simpson diversity approached significance (p = 0.07).

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Shannon Diversity					
Treatment	3	0.464	0.1546	1.120	0.348
Trap color	3	0.624	0.2079	1.506	0.223
Treatment × Trap color	9	1.853	0.2059	1.492	0.173
Residuals	58	8.007	0.1381	—	—
Simpson Diversity					
Treatment	3	0.1363	0.04544	1.642	0.190
Trap color	3	0.1361	0.04538	1.640	0.190
Treatment × Trap color	9	0.4735	0.05261	1.901	0.070
Residuals	58	1.6052	0.02768	—	—

Within individual trap colors, Shannon and Simpson diversity varied slightly among treatments but did not reach statistical significance overall (*Figure 4*). To further assess differences in insect community composition within each color, pairwise PERMANOVA tests were conducted using Bray–Curtis dissimilarity. Significant treatment effects were detected for blue and white traps. For blue traps, communities differed significantly between 100 AC and 100 CC ($p = 0.025$) and marginally between 100 AC and 50 AC/CC ($p = 0.089$). Similarly, white traps showed marginal differences between 100 AC and 100 CC ($p = 0.075$) and significant differences between 100 AC and 50/50 AC/CC ($p = 0.030$) (*Table 4*).

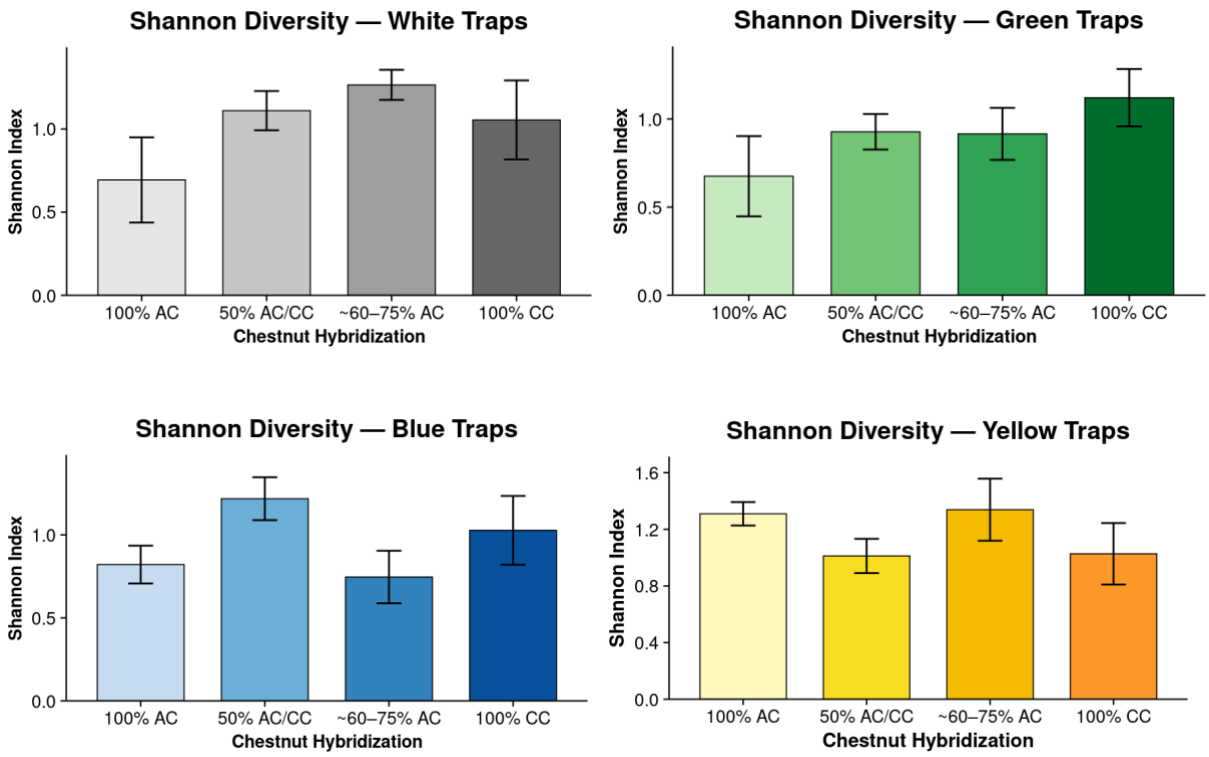

Figure 4: Mean (± SE) Shannon diversity of insects collected from pan traps across four chestnut treatments (100 AC, 100 CC, 50/50 AC/CC, and ~60–75% AC), separated by trap color (blue, green, white, and yellow). Bars represent mean diversity values within each color.

Table 4: Pairwise PERMANOVA results (Bray–Curtis dissimilarity) comparing insect community composition among chestnut treatments within trap colors, showing results with significance or marginal significance. Significance levels are shown as $p < 0.05$ (*), $0.05 \leq p < 0.10$ (marginal).

Trap Color	Comparison	F-value	R^2	p-value	Significance
Blue (B)	100 AC vs 100 CC	2.40	0.231	0.025	*
	100 AC vs 50 AC/CC	2.29	0.222	0.089	Marginal
White (W)	100 AC vs 100 CC	2.40	0.231	0.075	Marginal
	100 AC vs 50/50 AC/CC	2.85	0.263	0.030	*

PERMANOVA results (*Table 5*) revealed a significant overall effect of chestnut treatment and trap color on insect community composition ($F_{15,58} = 1.86$, $R^2 = 0.324$, $p = 0.001$), indicating that insect groups varied depending on both hybrid treatment and trap color. Dispersion tests confirmed homogeneity among treatments ($p = 0.25$) and slightly uneven dispersion among trap colors ($p = 0.089$).

When treatment and color were analyzed together, the interaction between the two factors did not significantly affect Shannon ($F_{9,58} = 1.49$, $p = 0.17$) or Simpson ($F_{9,58} = 1.90$, $p = 0.07$) diversity. NMDS ordination (*Figure 5*) shows partial clustering of communities by both treatment and color. Although overlap was evident among groups, the ordination suggested subtle compositional differences, particularly between pure American (100 AC) and pure Chinese (100 CC) chestnut plots.

Pairwise PERMANOVA comparisons (*Table 6*) revealed significant differences in community composition between 100 AC and 100 CC ($F = 5.12$, $R^2 = 0.121$, $p = 0.001$) and between 100 AC and 50/50 AC/CC ($F = 4.98$, $R^2 = 0.119$, $p = 0.001$). The comparison between 100 CC and 50/50 AC/CC was marginally significant ($F = 1.85$, $p = 0.061$). No significant differences were found between the remaining treatment pairs. Overall, these results show that insect communities in pure American (100 AC) and pure Chinese (100 CC) chestnuts differed compositionally.

Table 5. Results of PERMANOVA and betadisper analyses testing the effects of chestnut treatment and trap color on insect community composition (Bray–Curtis dissimilarity). The overall PERMANOVA model detected a significant interaction between treatment and trap color (p = 0.001). Betadisper tests confirmed homogeneous dispersion among treatments (p = 0.25) and slightly uneven dispersion among trap colors (p = 0.089).

Test	Factor(s)	df	F	R^2	p-value
PERMANOVA	Treatment × Trap Color	15, 58	1.86	0.324	0.001***
Betadisper	Treatment	3, 70	1.41	—	0.25
Betadisper	Trap Color	3, 70	2.26	—	0.089

Table 6. Pairwise PERMANOVA comparisons among chestnut treatments based on Bray–Curtis dissimilarity. Shows significant, marginally significant, and nonsignificant results (**p < 0.001, p < 0.01, p < 0.05, “marginal” = 0.05 ≤ p < 0.10, n.s. = not significant).

Comparison	F-value	R^2	p-value	Significance
100 AC vs 100 CC	5.12	0.121	0.001	**
100 AC vs 50/50 AC/CC	4.98	0.119	0.001	**
100 AC vs 60-75% AC	1.80	0.053	0.106	n.s.
100 CC vs 50/50 AC/CC	1.85	0.046	0.061	Marginal
100 CC vs 60-75% AC	1.60	0.046	0.109	n.s.
50/50 AC/CC vs 60-75% AC	1.14	0.033	0.301	n.s.

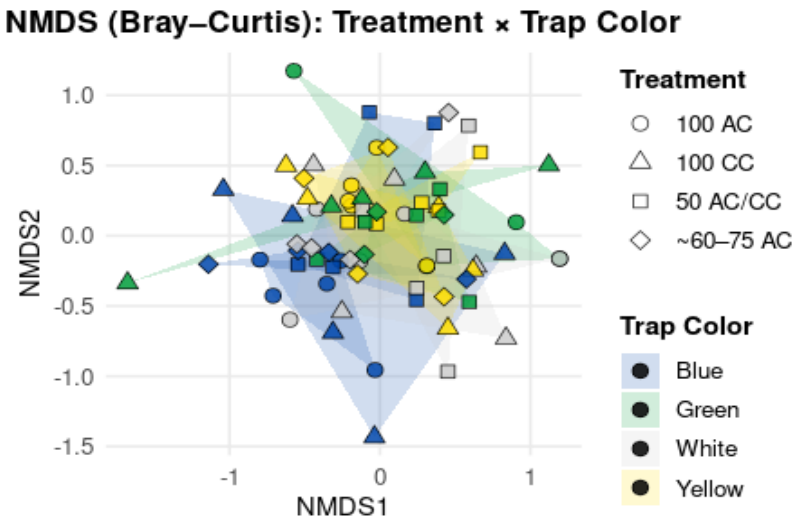

Figure 5. NMDS ordination (Bray–Curtis) illustrating the combined effects of chestnut treatment and trap color on insect community composition. Each point represents a sample, with shape denoting treatment and fill color denoting trap color. Shaded polygons indicate 95% confidence intervals around group centroids. The plot shows partial overlap among treatments, with some color-specific clustering, suggesting subtle differences in community composition influenced by both treatment and trap color.

Leaf Litter

A one-way ANOVA was used to test for differences in diversity among chestnut treatments. Neither Shannon ($F_{3,20} = 1.257$, $p = 0.316$) nor Simpson ($F_{3,20} = 0.556$, $p = 0.650$) diversity indices showed significant differences among treatments (*Figure 6*). Assumptions of normality and homogeneity of variance were met for both models (Shapiro–Wilk: Shannon, $W = 0.9426$, $p = 0.1867$; Simpson, $W = 0.9544$, $p = 0.3359$; Levene's test: Shannon, $F_{3,20} = 0.325$, $p = 0.807$; Simpson, $F_{3,20} = 0.054$, $p = 0.983$).

PERMANOVA using Bray–Curtis dissimilarity (999 permutations) detected no significant differences in community composition (*Figure 7*) among treatments ($F_{3,20} = 1.113$, $R^2 = 0.143$, $p = 0.352$). Betadisper analysis confirmed homogeneity of dispersion ($F_{3,20} = 1.038$, $p = 0.397$).

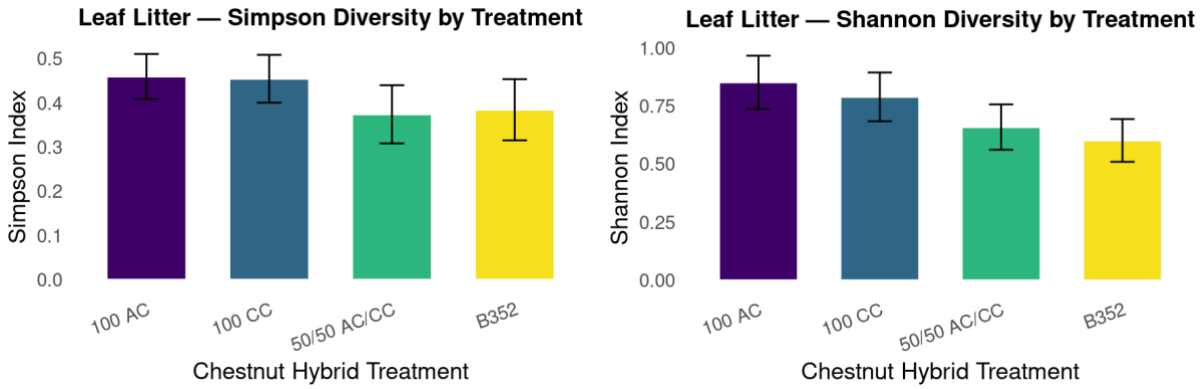

Figure 6. Mean (± SE) Simpson (left) and Shannon (right) diversity indices of insects collected from leaf litter samples across four chestnut hybridization treatments: 100% American chestnut (100% AC), 100% Chinese chestnut (100% CC), 50/50 AC/CC hybrid, and ~60–75% AC backcross hybrid. Bars represent mean diversity per treatment with standard error indicated. No significant differences were detected among treatments for either index (Shannon: $F_{3,20} = 1.257$, $p = 0.316$; Simpson: $F_{3,20} = 0.556$, $p = 0.650$).

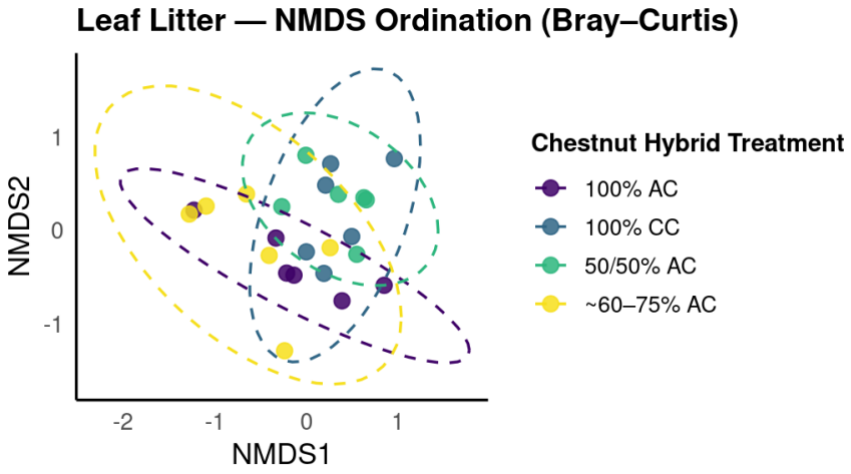

Figure 7. Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarity of insect communities collected from leaf litter across four chestnut hybridization treatments: 100% American chestnut (100% AC), 100% Chinese chestnut (100% CC), 50/50 AC/CC hybrid, and ~60–75% AC backcross hybrid. Each point represents one sample, and ellipses indicate 95% confidence intervals around group centroids. PERMANOVA showed no significant difference in community composition among treatments ($F_{3,20} = 1.113$, $R^2 = 0.143$, $p = 0.352$).

Pyramid Traps

A one-way ANOVA tested the effects of chestnut treatment on insects using Shannon and Simpson's diversity indices (*Table 7*). Both diversity metrics showed significant treatment effects, with Shannon diversity ($F_{3,53} = 3.05$, $p = 0.036$) and Simpson diversity ($F_{3,53} = 2.99$, $p = 0.039$) showing a similar pattern of difference among treatments. Post-hoc comparisons indicated that the ~60–75% American hybrid had higher Shannon diversity than the 50/50 AC/CC hybrid ($p < 0.05$). Simpson diversity also showed marginal differences between these same treatments (*Figure 8*).

Community-level analyses reflected these patterns. NMDS ordination (*Figure 9*) revealed partial clustering of insect groups by chestnut treatment, suggesting differences in community composition between hybrids and pure species. PERMANOVA results (Bray–Curtis dissimilarity) confirmed these trends, with significant overall differences among treatments ($p < 0.05$) and homogeneous dispersion among groups, indicating that treatment-related community variation was not driven by unequal sample spread.

Table 7. Results of one-way ANOVA testing the effect of chestnut hybrid treatment on Shannon and Simpson diversity indices from pyramid traps. Both indices showed significant differences among treatments ($p < 0.05$), indicating that insect diversity and evenness varied among chestnut types. Degrees of freedom (Df), sums of squares (Sum Sq), mean squares (Mean Sq), F-values, and p-values (Pr(>F)) are shown.

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Shannon Diversity					
Treatment	3	2.379	0.7931	3.053	0.036*
Residuals	53	13.767	0.2598	—	—
Simpson Diversity					
Treatment	3	0.580	0.1934	2.997	0.039*
Residuals	53	3.421	0.0645	—	—

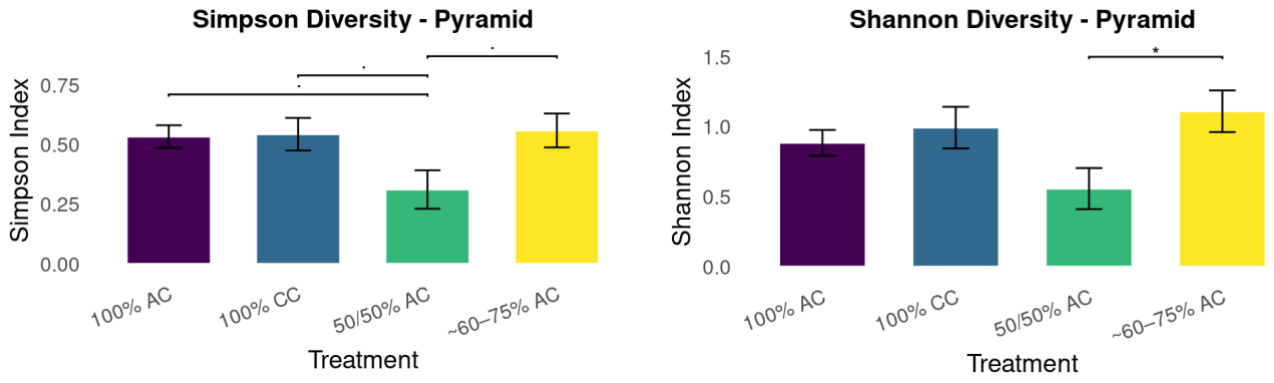

Figure 8. Mean (± SE) Shannon (right) and Simpson (left) diversity indices of insects collected from pyramid traps across four chestnut treatments: 100 AC (American chestnut), 100 CC (Chinese chestnut), 50/50 AC/CC (hybrid), and ~60–75% AC (backcrossed hybrid). Bars represent mean diversity values per treatment, with error bars indicating the standard error. Significant differences among treatments are denoted by asterisks ($p < 0.05$).

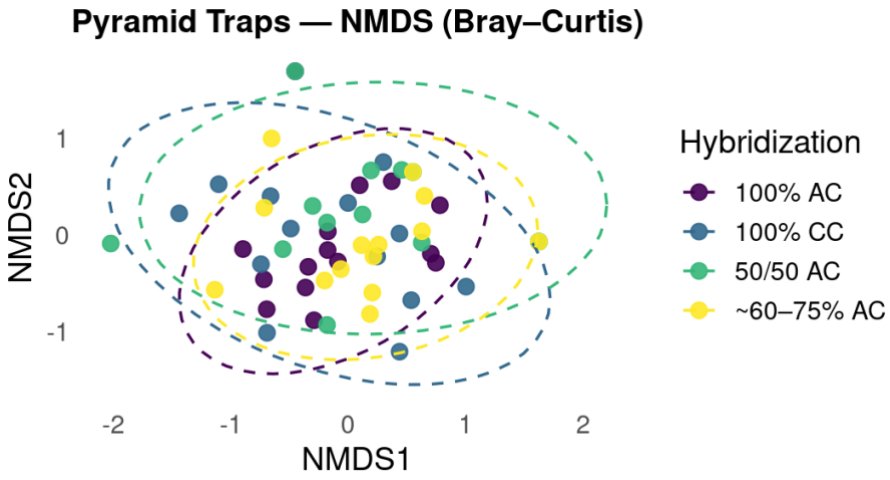

Figure 9. NMDS ordination (Bray–Curtis) showing clustering of insect communities collected from pyramid traps by chestnut treatment. Ellipses represent 95% confidence intervals around group centroids. The ordination indicates partial separation among treatments, with hybrid and pure species traps supporting distinct insect groups.

Discussion

Across all sampling methods, overall insect biodiversity did not differ significantly among chestnut treatments, suggesting that hybridization for blight resistance does not reduce total insect richness or evenness. However, community composition differed between pure American and Chinese chestnuts, with hybrid genotypes showing intermediate assemblages. These findings align with patterns described by Reed et al. (2024) and others, who similarly found that hybridization can shift insect assemblages even when overall diversity remains unchanged [9]. This type of compositional turnover is common in hybrid tree systems, where small genetic shifts in host plants alter the abundance and identity of associated insects [25,26,27].

Hybrid chestnuts appear capable of supporting greater insect biodiversity than their parent species, indicating that the reestablishment of this keystone tree is promising. At the same time, the observed shifts in species composition suggest that hybrid forests might not function exactly like historical American chestnut ecosystems. Foliar-trait variation is one factor that could contribute to those differences. Genetic differences among Chinese chestnuts shape their chemistry, including compounds such as tannins and phenolics, which can guide insect feeding habits and host preferences [28,15]. Insects are particularly effective bioindicators because they respond quickly to changes in plant chemistry, structure, and habitat conditions, making them valuable for detecting early, subtle ecological changes [12,14,29]. Comparable bottom-up effects of plant chemistry on communities have been observed in other forest-tree systems [17,18].

It's also important to consider how the study setting may have shaped these results. Because this work was conducted on a managed site, the chestnut trees grew into larger, canopy-forming individuals, which rarely occurs in natural environments for the American chestnut [3,4]. In those environments, stump sprouts typically die back before reaching maturity due to repeated blight infections and competition, limiting the amount of foliage and structure available for insects [3,4].

This difference in tree size and growth conditions likely contributed to some of the contrasts between our findings and those reported by Reed et al. [9], who conducted a similar study in natural forest settings. Their trees remained small and grew within mixed hardwood understories, and the insect communities they documented likely reflected the differences in available resources and space [9,12]. Seedlings generally attract a narrower range of insects; for this reason, a pattern is seen across many forest herbivore systems [12]. In contrast, the larger Meadowview trees offer more leaf area and a more stable canopy, which can support a broader or more structurally diverse insect community [12].

Additionally, morphological traits may further shape insect use of these hosts, for example, the Chinese chestnut, which often has more pubescence on the underside of its leaves, and leaf hairs can deter herbivores or harbor beneficial mites [28]. Even if overall diversity remains stable, shifts in composition can still affect ecological processes such as herbivory, nutrient cycling, and food-web stability.

Using multiple trap types (pan, pyramid, and Berlese) provided a broader and more complete view of biodiversity across chestnut types. Each trap offers insights into the niches occupied by specific insect groups. Flying insects, decomposers, and ground-dwelling insects each occupy

different niches, so examining insect guilds such as decomposers, herbivores, and pollinators will help us better link biodiversity patterns to ecosystem processes [12,14,30]. Thus, studying the ecosystem allows us to learn more about ecosystem dynamics, which would not be possible with a single trap alone. This strategy mirrors the advice of Montgomery et al. (2021), who argued that blending complementary techniques yields a more accurate assessment of biodiversity [30].

Future work should focus on the mechanisms underlying the community differences observed among chestnut hybrid types. Direct measurement of leaf chemistry and nutrient content could link insect patterns to shifts in leaf composition, including changes in compound composition and nutritional quality. Comparing insect groups by guild (e.g., pollinators, decomposers, herbivores, predators) would also give a clearer picture of how hybridization reshapes distinct ecological roles rather than just the overall community.

Examining order-level variation across treatment groups may reveal which insect groups are best adapted to their host tree's genotype and whether specific taxa respond differently in hybrid or pure-type chestnut trees. A longer-term study spanning seasons and many years would help determine whether these patterns are consistent or merely reflect the ebb and flow of seasonal cycles and shifting environmental conditions. Taken together, such strategies would provide a more holistic grasp of how hybrid and pure chestnuts engage with insect communities and how those linkages evolve as restoration saplings age.

Conclusions

This study investigated how hybridization between pure Chinese and American chestnuts affects leaf herbivory, insect biodiversity, and insect community composition using various sampling techniques. Overall, evenness and richness among treatments were similar, indicating that hybrid chestnuts can support a broad range of insect taxa, identical to those of their parent species. However, the community composition displayed noticeable shifts. Distinct groupings occur in pure American and pure Chinese chestnut trees, with the hybrid treatments falling in between.

These results suggest that while hybridization does not reduce overall biodiversity, it can shape which species are present and how insect communities are structured. Differences among trap types and colors highlight the importance of using multiple collection methods to capture the complete picture of insect diversity in forest restoration research.

Together, these results emphasize that restoring the American chestnut is not only about reestablishing trees but also about reintegrating the complex ecological factors that depend on them as a foundational species. Future studies that track insect communities over multiple seasons, quantify foliar chemistry, and examine functional guilds will provide deeper insight into how hybrid chestnuts influence ecosystem recovery and long-term forest health.

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