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Genetic Analysis of Geographically Distinct *Curculio sayi* Populations in the Northeastern United States

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Abstract

The American Chestnut was a tree of vital ecological importance to the forests of the eastern United States before the accidental introduction of the pathogenic chestnut blight which killed all adult trees. Efforts to reintroduce the historic American Chestnut tree to its native range using blight-resistant hybrids have also had the effect of increasing the range and numbers of an unwanted pest. *Curculio sayi*, the lesser chestnut weevil, is a specialized seed predator that not only damages the nuts through the oviposition of eggs but also through its inadvertent ability to introduce toxic fungi to the plant. As the population of its host plant has been increasing, so have *C. sayi* populations. Developing a strategy of population control of *C. sayi* stands to directly support the commercial chestnut industry and the successful reintroduction of this historical tree. Trapping and monitoring efforts have revealed divergences in reported phenologies in northeastern *C. sayi* populations as well as distinct morphological differences. Such differences could indicate a possibility of at least two diverging species. It is currently unknown how *C. sayi* was introduced or what role humans have had in its dispersal. Therefore, characterization of *C. sayi* phylogeography and the northeastern population structure would directly improve the efficacy of pest

management efforts. DNA barcoding analysis of the mitochondrial cytochrome *c* oxidase inhibitor subunit 1 (*CO1*) gene of two distinct populations of *C. sayi* showed that there is no phylogeographic signal present within the populations, indicating that these populations are not undergoing species divergence but are rather a product of recent shared ancestry, which suggests human-mediated movement. Although both populations were found to belong to the same species, when developing pest management strategies, the adaptation and spread capabilities of *C. sayi* should be considered to improve pest control efficacy.

Introduction

The American Chestnut, *Castanea dentata*, was historically the most prominent flowering tree in eastern North America, accounting for approximately 40-50% of the basal forest area in its native range which spanned over 800,000km^2 (Jacobs 2007). In the early 20th century, the bark pathogen *Cryphonectria parasitica*, commonly known as the chestnut blight, decimated the American Chestnut population, killing over four billion American Chestnut trees by the 1960s and causing the tree to be classified as endangered by Canada and many U.S. states (Jacobs 2007). Due to the superior carbon cycling and carrying capacity of the American Chestnut, conservation organizations such as The American Chestnut Foundation, as well as agricultural organizations, are making efforts to reintroduce American Chestnuts by hybridization and back-crossing with the Chinese Chestnut, *Castanea mollissima*, to produce a blight-resistant tree that is phenotypically indistinguishable from the American Chestnut (Clark et al. 2019). With the successful hybridization of the American and Chinese Chestnuts, the chestnut population is beginning to increase, with the number of chestnut farms growing hybridized chestnuts in the United States increasing 57% between 2012-2017 (Revord et al. 2021). Further, reintroduction methods are underway to increase natural American Chestnut populations to eastern deciduous forests (The American Chestnut Foundation Annual Report 2022).

However, as chestnut populations increase in the United States, so do populations of chestnut pests. *Curculio sayi*, the lesser chestnut weevil, is one of the most prevalent chestnut pests due to its capability for rapid emergence and dispersal (Filgueiras and Willett 2022). *C. sayi* populations emerge between September to October when the chestnut burrs open. The adults deposit their eggs in the burrs which in ten days hatch into larvae which burrow into the nut. The larvae spend two to three weeks feeding on the nut before emerging and dropping to the soil where they overwinter as larvae before pupating and overwintering once again, before emerging as adults the following fall (Lizotte 2020). Overall, this cycle takes two to three years to complete, with some *C. sayi* generations remaining within the ground for additional years (Filgueiras and Willett 2022). As *C. sayi* spends its larval stage in the chestnut

during peak harvest, growers run the risk of selling infested nuts and lowering customer confidence in their crops. The larval nut predation can also render the nut unviable and prevent it from sprouting. Further, *C. sayi* larvae spread the *Aspergillus* fungus during the pre-dispersal stage. *Aspergillus* fungi produce the diarrheagenic toxin emodin, rendering the nut harmful to human health and hindering the cultivation of new crops (Filgueiras and Willett 2022). Since *C. sayi* infestations can increase from 0% to 100% in less than two years (Filgueiras and Willett 2022), methods of effective pest control are desperately needed to prevent loss of revenue as well as to ensure the successful reintroduction of the historic American Chestnut.

Due to the functional extinction of the American Chestnut between the 20th and 21st century, not much is known about the chestnut weevil in the United States. Historic observations document two chestnut weevils, *C. sayi* and the greater chestnut weevil *C. caratrypes*, as worm-like larvae in the nuts of the American chestnuts that caused many harvests to be destroyed (Whitehead et al. 2018). Some observations on the phenology and life cycle of the weevil are also noted, with records of observations of these weevils laying their eggs in the chestnut burrs (Brooks 1929, Johnson 1956). More recent monitoring efforts have shown that the emergence times of *C. sayi* are directly correlated with cumulative degree days and that *C. sayi* emerge later in the season in more northern regions of the United States (Filgueiras and Willett 2022). However, some regions experience differing emergence times that are seemingly unexplained by the cumulative degree days. A study done on a *C. sayi* population in Rose, New York documented a single emergence late in the growing season around October that aligned with previous observations (Filgueiras and Willett 2022). An earlier study done on a Missouri population observed two emergences, one in May as the chestnut catkins bloomed and one in August as the chestnut burrs began to open (Keesey and Barrett 2008). This was originally thought to be due to the warmer conditions in Missouri allowing for an earlier emergence, but similar emergence times have been observed in areas of Michigan which share similar climate conditions to the New York population (Lizotte 2020). The success of various trapping methods varied between these populations, suggesting that there were differences in migration patterns between populations (Filgueiras and Willett 2022). Further, the Michigan populations were of lighter coloration and had longer setae than those collected from New York.

The differences in reported phenologies and distinct morphological differences between the Northeastern *C. sayi* populations indicate a possibility of at least two diverging species of chestnut weevil. Though the true weevil family, Curculionidae, is the second largest family of metazoans, many phylogenetic relationships remain uncharacterized (Shin et al. 2017, McKenna et al. 2015). It is currently unknown when the *C. sayi* species diverged, and there are no data to support the phylogenetic relationships of *C. sayi* at the species level. DNA barcoding analysis using the mitochondrial cytochrome *c* oxidase inhibitor subunit 1 gene (*CO1*) may help to detect

species divergence between the Rose, New York and Lansing, Michigan populations. *CO1* is a popular locus for DNA barcoding due to its role in energy metabolism which, under purifying selection, is highly responsive to environmental impacts. Further, *CO1* regions at the phylum level are relatively conserved (Zhang et al. 2019), but species in the order *Coleoptera* exhibit high rates of interspecific variance, making it a prime candidate for assessing population structures (Pentinsaari et al. 2016). The rate of mutation of the *CO1* gene is fast enough to distinguish between different species, but slow enough that the *CO1* sequence between two individuals of the same species will likely be identical (Ma et al. 2022). This results in the well-characterized barcode-gap based on pairwise genetic distances which is used to characterize the distribution of intraspecific genetic variance as well as variance between neighboring species (Vidya et al. 2022). Comparative phylogeographic information gained using DNA barcoding analysis will further aid in characterizing population structure and dispersal patterns of *C. sayi*, directly improving the efficacy of pest management efforts. Here, we examine *CO1* sequences of *C. sayi* populations from Rose, NY and Lansing, MI to determine potential species divergence of the chestnut pest.

Methods

Weevil Collection

Chestnut weevils were collected from two commercial chestnut orchards in Rose, NY and Lansing, MI. The Rose Valley Farm orchard is an organic chestnut farm containing mature (15+ years old) American Chestnut Hybrids. Traps were installed in the orchard at the beginning of May 2019 and samples were collected weekly from 2019 to 2020. Weevil samples from Michigan were provided by Dr. Deborah McCullough from Michigan State University who collected weevils from orchards in Lansing around the Michigan State University campus between 2019 to 2020. Both orchards contained a mix of mature American and Chinese Chestnut hybrids.

DNA Isolation and Amplification

Whole genomic DNA was extracted from 96 *C. sayi* leg tissue samples using the Promega Wizard SV® Kit and stored at -20 . Tissue samples were chopped finely before being added to the DNA digest solution to maximize DNA extraction. Polymerase chain reactions (PCR) were performed on 48 randomly selected samples to amplify the mitochondrial cytochrome *c* oxidase inhibitor subunit 1 (*CO1*) gene using universal primers LCO1490 and HCO2198 (Arias-Leclaire et al. 2018). Primers were diluted from a 100mM stock to a 10mM working solution and stored at -20 . GoTaq® Green Master Mix (Promega) was used as the dye agent and PCR products were checked for

successful amplification through gel electrophoresis using a 1% agarose gel made using GelGreen® Agarose Tabs™. The GoTaq® PCR protocol calls for 5μL DNA per PCR reaction, but the volume was increased to 7μL per reaction due to low DNA concentrations extracted from the samples. Amplified PCR products were sent to the Genomic Sciences Laboratory at North Carolina State University where they were sequenced in both directions. Contigs were assembled using Geneious® version R10 (Kearse et al. 2012) and ambiguous base pairs were visually identified and corrected using the original chromatograms when appropriate. The sample sequences were aligned using the ClustalW program in Geneious® to further correct ambiguous bases. Samples that appeared by eye to be very phylogenetically different were identified using BLAST within the program Geneious.

An additional alignment was created including mitochondrial *CO1* sequences available on GenBank from additional *Curculio* species. Using this alignment, we inferred a phylogenetic tree using a maximum likelihood method implemented in the program RaxML, a plugin available in Geneious®. Samples were assumed to be of different species when genetic variance was >2.0%, as is typically done when using barcode locus in coleopterans. DNA barcode gap analysis was performed using R Statistical Software (v4.0.3; R core team 2020) with the packages ape (v5.6.2; Paradis and Schliep 2019), pegas (v1.1; Paradis 2010), and BarcodingR (v1.0.3; Zhang et al. 2020).

Results

The universal primers LCO1490 and HCO2198 showed relatively low PCR success rate, with only 51.6% of samples successfully amplifying. This could be due to low initial DNA concentrations, as PCR success increased slightly when more sample DNA was added into the amplification reaction. *C. sayi CO1* sequences were 676 base pairs in length, consistent with other species in *Coleoptera* (Huang et al. 2020). In total, 47 out of the 48 samples sequenced by NC State returned high-quality sequences that could be used for genetic analysis. One sample did not result in clear sequence data, probably owing to PCR failure. Out of the 47 samples, two were found (via BLAST) not to belong to the *Curculio* genus, one belonging to *Eubulus parochus*, the hidden snout weevil, and one belonging to *Deroceras reticulatum*, the gray garden slug. The former means that one weevil from Michigan was mis-identified, while the latter result suggests that there were slug remains on the weevil leg. Within the remaining 45 samples, all showed <<2.0% divergence from each other, indicating that both populations are of the same species (Figure 1). There was high interspecific divergence among the *C. sayi* samples and the other *Curculio* species used in the alignment; *C. elephas*, *C. propinquus*, *C. nucum*, *C. venosus*, *C. sikkimensis*, and *C. betulae* (Figure 2).

Discussion

There was no phylogeographic signal detected between the New York and Michigan *C. sayi* populations and the sequences are interdigitated and <<2.0% divergent. This is strong evidence that they are both of the same species. Therefore, the difference in observed phenology and morphology between the populations are a result of regional adaptation rather than species divergence. Interestingly, the European chestnut weevil *C. elephas* has been reported to have variable emergence times not correlated to genomic differences (Menu 1993). It is hypothesized that *C. elephas* uses a staggered emergence pattern as a survival strategy to allow a single generation to emerge over multiple years, negating the risk of an entire generation emerging during a bad year and not being able to reproduce (Filgueiras and Willett 2022).

However, it is unlikely that the early emergence of *C. sayi* in Michigan is a similar adaptation, as the spring emergence aligns with the flowering of catkins rather than the opening of chestnut burrs, and there are no reports of *C. sayi* laying eggs in chestnut structures other than the burr itself. Previous studies have shown that the chestnut catkins produce organic volatile compounds that attract both male and female *C. sayi*, and the spring populations have been observed to feed on the catkins and disappear when the catkins decline (Lizotte 2020, Keesey et al. 2012). *C. sayi* have been observed to fly to surrounding chestnut plants up to 3 kilometers away in response to these volatiles, a migration which may be happening at a greater rate than originally thought (Keesey et al. 2012). It is possible that certain *C. sayi* populations have begun emerging in the spring in response to the flowering catkins as a way to identify and migrate to more fecund trees, increasing their chances of their larvae being able to sustain themselves on more robust chestnuts during their development.

Further, the fact that the sequences show no phylogeographic signal indicates that the NY and MI lineages are not divergent, rather, they share common ancestors from both populations. This indicates that the weevils have recently spread from a pool of potential propagules, rather than having existed on the landscape before, during, and after the demise of American Chestnuts. It is probable that *C. sayi* populations are being spread through the distribution of infested chestnut saplings to chestnut growers.

Conclusion

The use of DNA barcoding of the *CO1* gene allowed for the precise identification of the species of weevils sampled from New York and Michigan. While there was one weevil that was not within the *Curculio* genus, all other weevil samples were found to belong to the *C. sayi* species. Additionally, no *C. sayi* insects were found on non-chestnut plants during sampling, supporting *C. sayi* as a specialized predator that only targets chestnut trees. With *C. sayi* samples from larger regions of the eastern United

States, it may be possible to create a map of gene flow that can be used to track migration patterns, which combined with current phenological data, will help chestnut growers effectively target *C. sayi* infestation in their adult and larval stages, increasing the efficacy and success of pest management strategies.

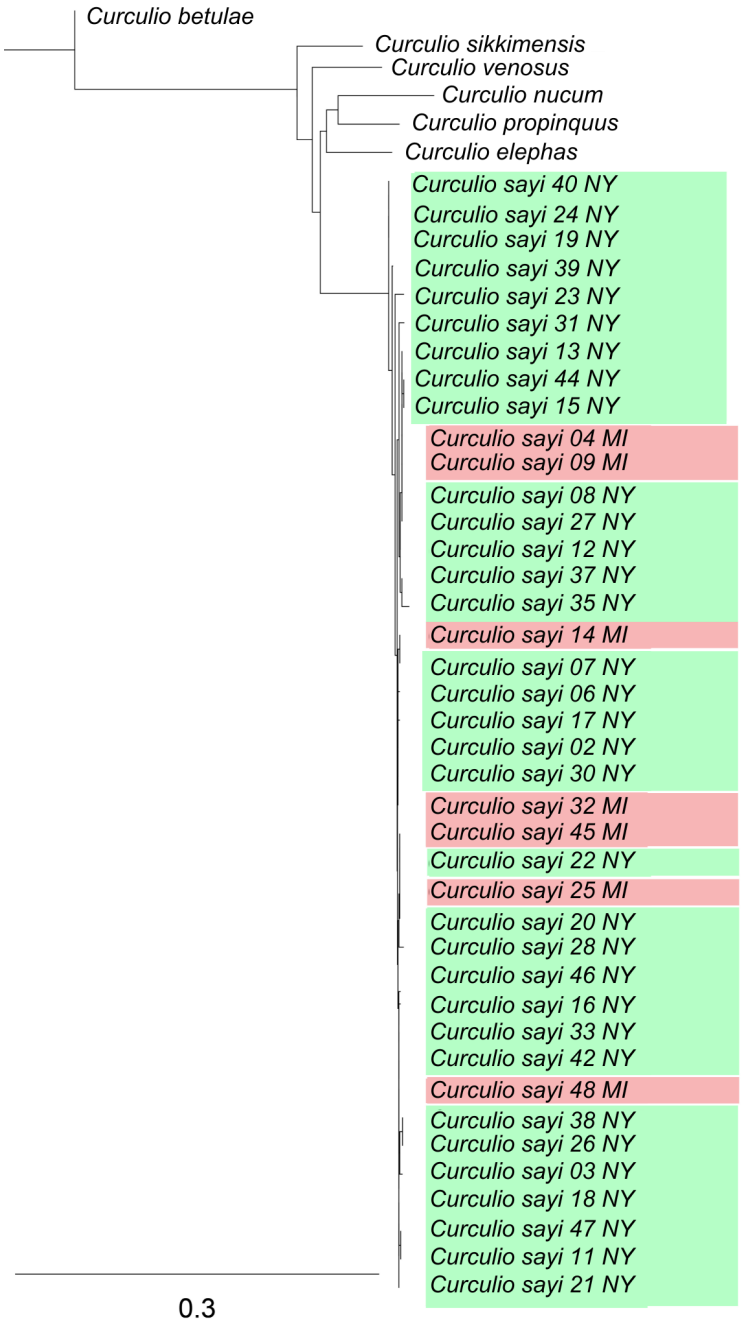

Figure 1. Phylogenetic tree for *Curculio* samples showing magnitudes of genetic divergence between species. *C. sayi* samples highlighted in green represent New York samples, whereas samples highlighted in red represent the Michigan samples. There

was no genetic difference found between the New York and Michigan *C. sayi* populations.

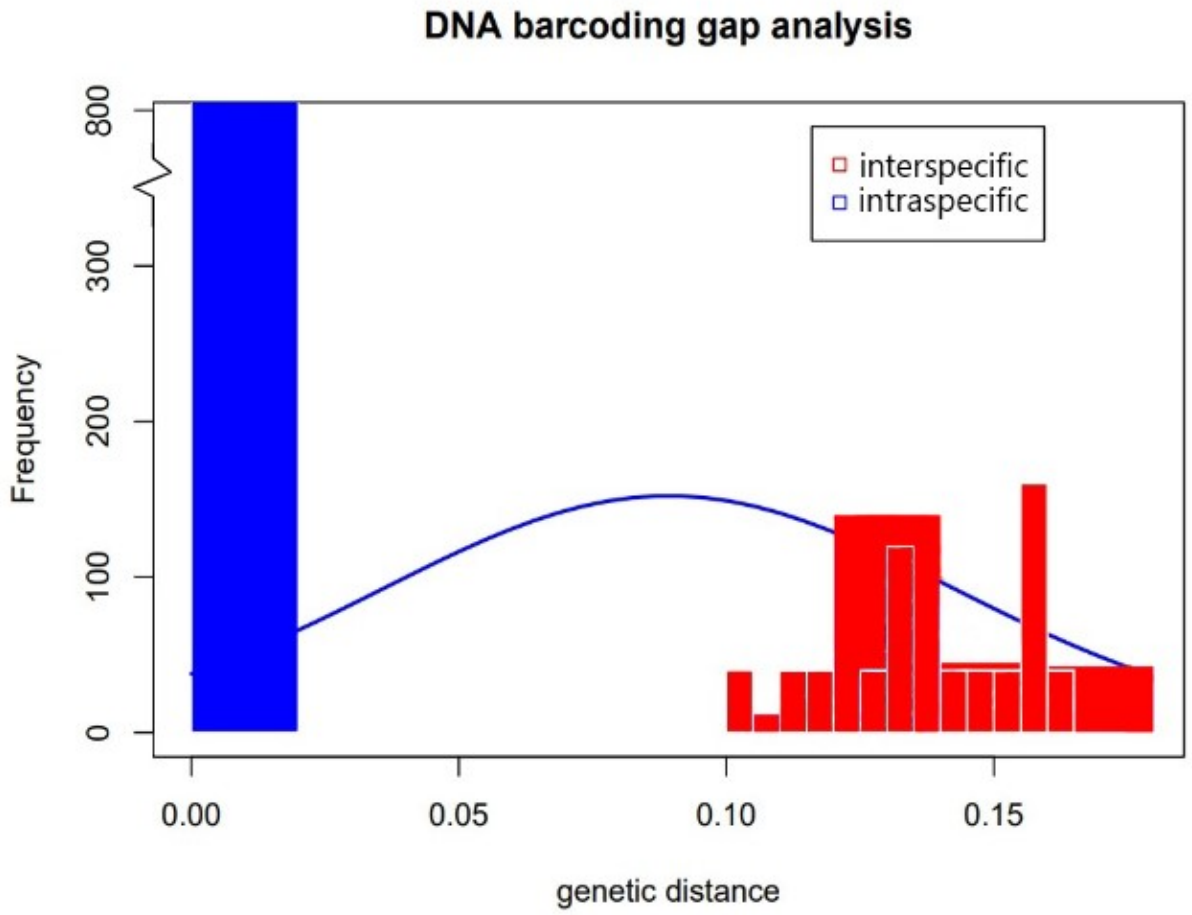

Figure 2. Barcoding gap for *Curculio* samples showing genetic difference between inter- and intraspecific samples. All tested *C. sayi* samples showed <<2.0% intraspecific variance and showed high interspecific variance with the other imported *Curculio* species.

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