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Speciation Proposal
May 15, 2018

Divergent selection and speciation of the invasive forest pest,
hemlock woolly adelgid (*Adelges tsugae*) in North America

I. Conceptual Framework:

The hemlock woolly adelgid (*Adelges tsugae*) is a hemipteran within the family Adelgidae, or conifer woolly adelgids. It is native to Asia with populations also found in western North America and the eastern United States. The ancestral species live across Japan and southern China. Adelgids are closely related to aphids, but consist of a relatively small family, made up of only about named 70 species (Footitt et al., 2009). Traditionally, the taxonomy of Adelgids has been based on morphologic characteristics such as shape and placement of plates located on the exoskeleton and dorsal wax glands (Footitt et al., 2009), but this proves to be difficult for a number of reasons. The first reason involves the small size of the insects; adults range from 0.4–2 mm and require microscopic examination. The second reason is that adelgids undergo complex lifecycles with up to five different morphological forms including crawlers (newly hatched adelgids), numerous nymph instars, and winged and nonwinged adults.

When groups of adelgids are morphologically similar, differences in their reproductive biology and host-use have been used to classify them into different species (Havill et al. 2006). Within their lifecycles, adelgids can utilize alternate coniferous hosts and can be either holocyclic, meaning they undergo a sexual generation in their lifecycle, or anholocyclic, meaning they are parthenogenic or asexual (Havill et al., 2007b). Species which alternate hosts are always holocyclic and utilize *Picea* (spruce) as their primary host where they complete the sexual generations of their lifecycle. During their asexual generations, they utilize a secondary conifer species, either *Abies*, *Larix*, *Pinus*, *Pseudotsuga*, or *Tsuga* (Havill et al., 2006). Anholocyclic females can produce both winged and nonwinged clones. Winged offspring will fly back to the primary host to complete its sexual generation (McClure, 1987) Some species are completely anholocyclic and only live on one of the previously mentioned secondary species, never completing a sexual generation on *Picea* (Havill et al., 2006).

A third, and the most recent means of distinguishing insect species, is through DNA barcoding, specifically mitochondrial DNA (Footitt et al., 2009 & Havill et al., 2006). Extracting, amplifying, and sequencing the ends of mitochondrial subunits can help characterize species by ancestral groups. Genetic differences may show up in species which are morphologically and or ecologically similar. In the case of adelgids, all three approaches, morphological, ecological, and genetic should be implemented in distinguishing and naming species.

In this study the hemlock woolly adelgid will be tested for speciation in its introduced ranges. Because geographic and ecological processes are two major factors which drive speciation, it is probable that the insect in North America is undergoing speciation. Insects in North America are no longer sharing genetic information with their sister species in Asia. They

are also inhabiting different niches on the different continents. Invasive species, such as this one, provide a suitable study for speciation because they are typically introduced as a small founder population, they become allopathically separated from their closest sister species, and they spread and reproduce rapidly in their new environments.

II. Background:

The hemlock woolly adelgid (*A. tsugae*) is named so because it uses *Tsuga* (hemlock) as its secondary host. Over wintering females self-fertilize and lay woolly egg sacks laid on hemlock branches hatch into “crawlers” in the spring. These newly hatched insects are the only mobile stage in the lifecycle, other than winged adults (Havill et al., 2007a). Crawlers settle on a needle and go through a number of nymph instars, or stages before becoming an adult in early summer. They then lay eggs and the insect goes through its second generation that year. Some of the eggs laid in this second generation will turn into winged adults which will leave in search of its primary host on a spruce species (McClure 1987). Here, winged adults will sexually reproduce in galls. Some offspring will remain on the spruce to undergo an anholocycle (asexual) generation while others will travel back to hemlock to undergo the anholocycle generation (figure 1). In Asia, there is a primary spruce species where the adelgid carries out its holocyclic generations, but in North America, there are no suitable spruce species, so the adelgid is completely parthenogenetic. Winged adults are still produced, but the eggs they lay both on hemlock and on native north American spruce die (McClure, 1987).

A. tsugae has a relatively large spread and is found on all nine species of Hemlock in their native ranges: China, Taiwan, eastern and western North America, Japan, India and Nepal (Havill et al., 2006). *A. tsugae* crawlers and nymphs feed on hemlocks by inserting a needle-like stylet into the vascular tissue at the base of a needle and feeds on nutrients from the xylem and phloem. Adelgids avoid older needles and prefer to feed on new growth (Young et al., 1995). In all of *A. tsugae*'s native range and some of its introduced range, native predators and genetically tolerant host trees keep the insect population in check. In these areas, hemlocks are not greatly affected by the presence of *A. tsugae*, unless the tree is stressed or unhealthy beforehand (Kohler et al., 2008).

One region where this does not hold true is in the eastern United States. Here, native hemlocks *T. canadensis* (eastern hemlock) and *T. caroliniana* (Carolina hemlock) are being greatly affected by the introduction of *A. tsugae*. There is no native predator in the region and native hemlocks have not evolved with any similar adelgid species. *A. tsugae* was first identified in the eastern US in the 1980s and it has since spread throughout all of the *T. caroliniana* range and most of the southern and western *T. canadensis* range. Trees impacted first lose needles, then limbs, and eventually die, often within just 4 years (McClure, 1991). This is concerning to conservationists because hemlock is a long-lived, shade-tolerant tree which can survive on steep

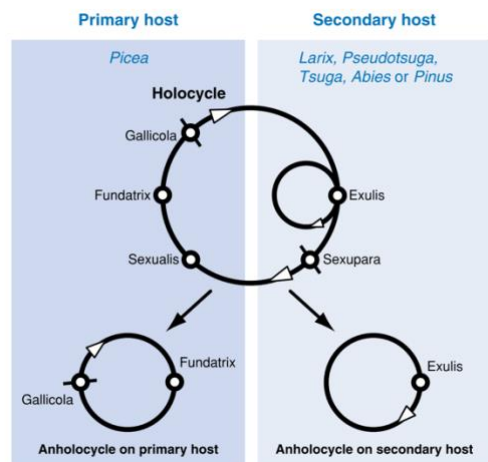


Figure 1: Adelgid lifecycle from Havill and Footitt, 2007a. Plain circles represent wingless generations and circles with bisecting lines represent winged generations.

slopes where other tree species cannot. The habitat hemlocks provide is also very unique and many organisms are dependent on it.

III. Objectives:

The purpose of this research will be to determine whether populations of hemlock woolly adelgid found in the western United States, have undergone speciation and whether adelgids in the eastern United States may be in the process of speciation. Specifically, I want to:

1. Repeat DNA barcoding conducted in previous studies but with more North American insects and Asian insects.
2. Conduct a common garden experiment populations from western north America, eastern north America and Japan.
3. Conduct a multivariate morphometric analysis to see if there are any morphological changes which have occurred since the species have been introduced.

IV. Experimental Plan and Preliminary Data:

1. Genetic divergence

There have been many experiments previously done which have given us preliminary data pointing towards speciation in *A. tsugae*. To understand how our experimental plan will further and improve this research, one must first understand the literature. Previous DNA barcoding done on adelgids and *A. tsugae* in particular have shown that there are three distinct lineages within *A. tsugae*. A study done by Footitt et al. in 2009 on 17 species of adelgids showed more genetic variation within *A. tsugae* than any other adelgid tested. Genetic variation within *A. tsugae* population was comparable to inter-species variation of other adelgids.

Another study (Havill et al., 2006) looked at the mitochondrial DNA of *A. tsugae* from 29 samples from the native ranges of all nine hemlock species. This included five samples from the eastern US, eight from the western US, one from Taiwan, six from china and nine from Japan. They found 16 different haplotypes with sequences that diverged from 0.07% to 6.7%. These haplogroups formed three main networks, one containing populations from China, one with the sample from Taiwan, and one containing the populations from Japan and North America. The last network could be further broken down into three subgroups: the most divergent containing the samples from populations from Japan sampled in higher elevations where a specific species of hemlock, *T. diversifolia*, grows, the second group consists of samples collected in western North America, and the third contained samples from eastern North America and lower elevations in Japan where a second species of hemlock, *T. sieboldii*, grows (figure 2).

I would like to further these studies by collecting more samples from the eastern and western north American populations, as well as from China, Taiwan, and Japan, and repeating

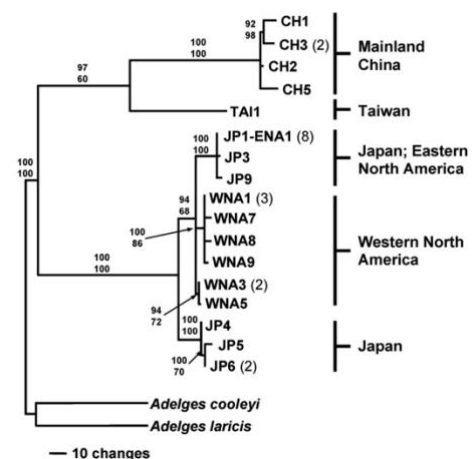


Figure 2: Bayesian analysis of mtDNA from Havill et al. 2006.

mtDNA analysis. With more samples, we may find greater haplotype divergence. DNA from samples will be extracted and mitochondrial regions will be PCR amplified. MtDNA haplotype networks will be constructed using the software TCS 1.20 (Clement et al. 2000).

2. Ecological divergence

The fact that insects collected in Japan in the natural range of *T. diversifolia* form a separate group from insects collected in the range of *T. sieboldii* suggests possible divergent selection between insects with different hosts (Havill et al., 2006). Not only does North American *A. tsugae* use different host species (in the west it feeds on *T. heterophylla* and *T. mertensiana* and in the east *T. canadensis* and *T. caroliniana*), North American *A. tsugae* does not utilize a *Picea* host species, at all, nor does it go through a sexual generation. Studies in cold tolerance have further shown ecological divergence by revealing a pattern of evolution occurring in eastern north American populations traveling northward into colder climates (Butin et al., 2005).

To further test this, I would conduct a common garden experiment with insects from the native range of *T. canadensis* in eastern North America, native range of *T. heterophylla* in Western North America, populations from the natural range of *T. diversifolia* in Japan, and populations from the natural range of *T. sieboldii* in Japan. Non-winged adults from these populations will be placed in nylon mesh cages on branches of their own hemlock as well as the other three species. After two weeks, branches will be examined for progeny.

3. Morphological divergence

In 1980, a detailed morphological analysis of balsam woolly adelgid, another invasive adelgid species, distinguished three separate groups in North America, suggesting it had been introduced three separate times (Footitt et al., 1980). Since I have seen no evidence of multivariate morphometric analysis done on *A. tsugae* in North America I would like to attempt it. Following the methodology in from Footitt and Mackauer's experiment on balsam woolly adelgids, I would like to look at *A. tsugae* from the two north American populations and the two Japanese populations. Non-winged adults will be collected and removed from the hemlock needles using a tweezer and microscope. Specimens will be mounted and body features will be counted and measured. A multivariate and cluster analysis will be conducted to show possible differences in morphological characteristics.

In summary, I believe there is evidence that A. tsugae should be broken up into at least two different species. By using these three methods, I will show that the western north American population has diverged from Japanese populations and that the eastern north American population is also in the process of diverging.

V. Broader impacts:

A major invasive pest threatening eastern forests is the hemlock woolly adelgid, *A. tsugae*. Forest structure and composition is the base of many ecosystems, and hemlock tree species are often considered "keystone" or "foundational" species. There are many organisms that rely on hemlock habitat and at least three hemlock-obligate bird species (Benzinger, J. 1994.) Accurate taxonomic classification of the invasive insect threatening these trees may help with further funding for conservation or research opportunities. If only the eastern N.A. population or a specific Japanese population is invasive, conservation efforts can be more concentrated on areas of need.

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