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Tav Aronowitz
University of Vermont

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USING MULTIPLE METHODOLOGIES TO UNDERSTAND WITHIN SPECIES VARIABILITY OF *ADELGES* AND *PINEUS* (HEMIPTERA: STERNORRHYNCHA)

A Thesis Presented

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Tav (Hanna) Aronowitz

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Thesis Examination Committee:

Kimberly Wallin, Ph.D., Advisor
Ingi Agnarsson, Ph.D., Chairperson
James D. Murdoch, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

The species of two genera in Insecta: Hemiptera: Adelgidae were investigated through the lenses of genetics, morphology, life cycle and host species. The systematics are unclear due to complex life cycles, including multigenerational polymorphism, host switching and cyclical parthenogenesis. I studied the hemlock adelgids, including the nonnative invasive hemlock woolly adelgid on the east coast of the United States, that are currently viewed as a single species. I used multivariate morphometric analyses to identify morphological differences among hemlock adelgid lineages. With principal component analyses and MANOVA, the six lineages that were used in this study were found to be significantly different from each other. The findings of this project provide evidence for taxonomic designation of different hemlock adelgid lineages, which will hopefully inform regulation of these distinct lineages, as these distinctions between the lineages of hemlock adelgids could equate to other biological differences, ex. cold tolerance, host specialization, fecundity and dispersal ability. I also investigated the relationship between species *Pineus similis*, *Pineus abietinus* through phylogeny, genetic distances, life cycle and host species. This was done through using three mitochondrial (COI, COII, cytB) and one nuclear (EF1a) gene, in Maximum Parsimony, Maximum Likelihood and Bayesian analyses, along with genetic distance measurements. The *P. similis* and *P. abietinus* on *Pinus* could not be separated within the Bayesian analyses, and *P. similis* and *P. abietinus* on *Abies* had low calculated distance measurements (2.98%) compared to the average distance between species within the genus (28.07%). These two studies emphasize the current confusion within the Adelgidae family, and the results presented in this thesis stress the importance of using components of multiple species concepts to better understand the systematics of these lineages.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1 Adelgidae family

The Adelgidae family is situated within the Aphidoidea (Hemiptera: Sternorrhyncha) superfamily (Havill & Foottit, 2007). This family has a complex life cycle, including cyclical parthenogenesis, multigenerational polymorphism and host switching (Havill & Foottit 2007). Some confusion exists in the general classification of adelgids. In 1908, Börner revised the original taxonomy of this group when he split the genus *Chermes* into 8 genera and in Cholodkovksy's dichotomous key of these genera, all eight were defined by larval attributes (Cholodkovky 1915). This was disputed in Annand's monograph of North American Adelginae (1928), where he described only 2 genera within the group: *Pineus* Shimer with four pairs of abdominal spiracles on adults and *Adelges* Vallot with five. In 1968, Steffan attempted the first genera description of Adelgidae base on phylogeny by altering Börner's classification, using morphology, number of chromosomes and endosymbiotic bacteria (Havill & Foottit 2007). Even recently, there are discrepancies between different authors on which taxonomy method should be utilized, although Annand's taxonomy is the most commonly used in North America (Havill & Foottit 2007).

All adelgids have multigenerational polymorphic life cycles, while some have even more complexity with cyclically parthenogenetic and heteroecious life cycles as well (Havill & Foottit 2007, Fig. 1). This complex life cycle starts on a primary host, the *Picea* Miller spp., where it reproduces parthenogenetically as fundatrix phenotype. Fundatrix feed on first year *Picea* twigs, which alter the growth of the twig forming a gall. The adult fundatrix lays eggs of the next generation, gallicola. As the gallicola feed, the peripherally damaged plant tissue continues to become more damaged, adding to the gall which grows around individuals until the gallicola are engulfed in the gall. The nymphs go through 3 instars while inside the gall. Once the gallicola are mature, the galls dry out and the nymphs emerge and molt into winged adults. These new adults then fly off to find a suitable secondary host where they will lay eggs and die. The generations which live out their entire life cycle on the secondary host are called exulis, or the 'one who is banished' in Latin. There are two generations of exulis, sistentes and progredientes. The eggs laid by the gallicola are sistentes; they hatch on the secondary host in the early summer and aestivate until the late summer. In late summer, the sistentes begin to feed on new host growth before reverting to state of dormancy. They remain dormant until early spring and lay eggs in late spring. These eggs are progredientes, which are density dependent; apterous progredientes are sessile individuals which are prominent at low densities and sexuparae progredientes are winged individuals which are prominent at high densities (Sussky & Elkinton 2014). According to Sussky et al. (2014), the ratio of apterous and sexuparae progredientes depends on the density of its current generation, rather than the density of parental sistentes. The apterous progredientes complete development on the secondary host tree, resulting in the new sistentes generation. The sexuparae individuals also feed on secondary host and look identical to apterous progredientes until the final instars, when wing pads and wings develop. Once fully developed, the sexuparae migrates to a *Picea* tree, the primary host. They die after laying their eggs, using its roof-like wing positioning to protect the newly laid eggs. The sexualis go through 4 instars, develop as male or female and are the only adelgid generation to reproduce sexually. Each female sexualis lays a single egg on the *Picea* spp, which later hatches into a fundatrix. This explanation of the adelgid life cycle is based on descriptions from experts (Cholodkovsky 1912, Marchal 1913, Annand 1928, Havill & Foottit 2007) and terms used in the above description are those proposed by Marchal in 1913. Although other nomenclature has been created and used by various authors, this is the most common terminology.

1.2 Difficultly of Adelgidae systematics

Species within the Adelgidae family are defined by their multigenerational, polymorphic, cyclically parthenogenetic and host switching life cycle, along with the retention of the ancestral relationship to gymnosperms (Havill & Foottit 2007). The life cycle complexity is not consistent throughout Adelgidae (see Havill & Foottit 2007). Multigenerational polymorphic life cycles that reproduce asexually only are termed anholocyclic. They have lost the ability to migrate back to either the primary or secondary host and therefore utilize only part of its full ancestral life cycle (Havill & Foottit 2007). Holocyclic species have retained the 5 generation life cycle, switching from primary host to secondary host (Havill & Foottit 2007). There are still other species that have holocyclic populations and anholocyclic populations, the anholocyclic populations occurring where either the primary host or the secondary host does not occur (Havill & Foottit 2007).

The complexity of the Adelgidae life cycle has made systematics of this group very difficult (Annand 1928, Havill & Foottit 2007). It can be difficult to ascertain whether an anholocyclic grouping of adelgids are a population of an existing holocylclic species that do not have access to one of the two hosts, or whether it is its own distinct species (Havill & Foottit 2007). One example of this confusion includes *Pineus orientalis* Dreyfus and *Pineus pini* Gmelin (Havill et al. 2007). *Pineus orientalis* is a holocyclic species native to the Caucausus Mountains using *Picea orientalis* (Linnaeus) Link as a primary host and multiple *Pinus* Linnaeus spp. as secondary hosts (Havill & Foottit 2007). *Pineus pini* is an anholocylic species, which uses only *Pinus* spp as a host and exists in Europe where *Picea oreintalis* does not naturally occur (Havill & Foottit 2007). Although unusual for diagnosing species outside of this family, this distinction between existing on primary and secondary host species, along with geography, are the only traits separating these species. Havill et al (2007) found that some *P. orientalis* and *P. pini* have identical COI, COII, cytb and EF1a sequences. In addition, winged adults from this species complex have been found in Italy (in Covassi and Binazzi 1981; referenced in Havill et al. 2007) and Denmark (in Heie 19976; referenced in Havill et al. 2007), which are within the range of *P. pini*. These two pieces of evidence support the conclusion that the relationship between *P. orientalis* and *P. pini* is not completely understood (Havill et al. 2007).

There is an on-going discussion among experts in the field of adelgid biology about using the life cycle to determine species (Cholodkovsky 1915, Steffan 1964, Havill & Foottit 2007). There are plenty of examples, like the one stated above, of populations which are morphologically similar or seemingly identical but are designated species level because they are not known to migrate among hosts (Havill & Foottit 2007). Molecular evaluations of these groups may shed light on the evolutionary history, and inform the systematics of these species (Havill & Foottit 2007).

1.3 Hemlock Adelgids

1.3.1 *Adelges tsugae* distribution and description

Hemlock woolly adelgid, (*Adelges tsugae*, Annand), is native to Asia and western North America (Havill & Foottit 2007). The species has a relatively small native range due to its host specificity; *A. tsugae* uses species in the genus *Tsuga* Carrière as secondary hosts, and like all adelgids, are restricted to certain species in the genus *Picea* as a primary host (McClure 1989, Havill, Vieira & Salom 2014). In 1951 *A. tsugae* was found in Richmond, Virginia; this introduction is thought to have been from southern Japan (Havill et al. 2006). Currently *A. tsugae* is an invasive pest in approximately half of *Tsuga canadensis* (Linnaeus) Carrière range, about 9308 square kilometers and its limitation is speculated to be due to its low cold tolerance, as well as multiple other factors (Butin, Porter & Elkinton 2005).

1.3.2 *Tsuga* and *Adelges tsugae* significance

Hemlocks on the east coast of North America, both eastern hemlock (*Tsuga canadensis*) and Carolina hemlock (*T. carolinina*), are ecologically formative species. Hemlock is often thought of as a foundation species (Martin & Goebel 2013), creating and maintaining a significantly moist and cool microclimate compared to the surrounding environments. The soil in this micro-ecosystem becomes significantly more acidic then areas that lack regular deposits of hemlock needles (Ellison et al. 2005). Although slightly acidic soils (pH = 6.5) can be extremely beneficial to the majority of plants by allowing a greater accessibility to important minerals and phosphorous, a lower pH (4.0-5.0) creates a slightly toxic environment which allows only specialized organisms to colonize the area (Bickelhaupt unknown, Ellison et al. 2005). The change in soil chemistry and change in leaf litter has been seen to change ground dwelling arthropod communities (Rohr et al. 2009, Ingwell et al. 2012). The removal of hemlocks from ecosystems also dramatically changes the plant species composition; for example, black birch (*Betula lenta*) has started to replace eastern Hemlock in western Massachusetts where *A. tsugae* scare has cause salvage logging (Zukswert et al. 2014). This change from a coniferous to a deciduous canopy increases the light reaching the understory during the summer months (Zukswert et al. 2014).

Hemlocks also have some economic value; Hemlocks have little significance in logging operations, but the decline of Hemlocks due to *A. tsugae* has brought housing property values down (Holmes, Murphy, & Bell 2006).

1.3.3 Historic research of *Adelges tsugae*

Dreyfus presumably first discovered *A. tsugae* in 1889; although he used extremely colorful language, such as comparing the juvenile adelgid to an overturned boat, his description of the specimen's morphology and biology was very broad and could fit a variety of Adelgid species. Dreyfus named the species *Chermes funitectus*, because of the adults' large size. Very little can be extracted from this original report by Dreyfus: only the general description and the host tree the specimen was found on- *Tsuga heterophylla* (Rafiensque) Sargent (then called '*Abies canadensis*'). In Cholodkovsky's monograph of European Adelgids (1915) the species *Chermes funitectus* is mentioned as an adelgid which exists in Eastern Europe, though by its current name (*Adelges tsugae*) it hasn't been reported in Eastern Europe.

The next observation of a similar specimen was by Chrystal in 1916, 'The Forest-Insect Problem in Stanley Park'. Chrystal refers to the insect as "Western Hemlock *Chermes*", and based on the host tree alone Annand (1928) believed the specimens found by Chrystal (*Chermes funitectus*) were the same species he described on the western coast of North America in 1924. Annand noted that the genus *Chermes* should no longer be used due to the imprecise uses in the nomenclature (see Bulletin of Zoological Nomenclature below). In 1924, Annand described this species with a new name, *A. tsugae*, taken from a host tree *Tsuga heterophylla* in Eugene, Oregon. He stated in this description of *A. tsugae* that if there is a primary host of the species, it is unknown. Since then *A. tsugae* has been reported as a globally distributed species, having been found on all nine species of *Tsuga* and observed to complete a full holocyclic life cycle in parts of its range (in Takahashi 1937, referenced in Havill et al. 2006, in Inouye 1953, referenced in Havill et al. 2007, McMclure 1992, McMclure 1989, Montgomery et al 2000).

1.3.4 *Adelges tsugae* as an invasive insect

Adelges tsugae's current range includes most of *Tsuga*'s current range: mainland China, Taiwan, South Korea, Japan, western North America and eastern North America (Havill et al 2008, Havill, Montgomery & Keena 2011). Of this global distribution, only the population in eastern North America is considered to be invasive (Havill 2006). In its invaded range *A. tsugae* is a serious threat to *Tsuga* species, having caused mortality in some forest stands in 2-3 years (McClure 1990). Of the effected forests, tree mortality is faster in southern counties (Levy & Walker 2014, Sussky & Elkinton 2014, McClure 1996). As a sap-sucking insect, *A. tsugae* feeds on ray parenchyma tissue of young hemlock growth (Young, Sheilds & Berlyn 1995). These feeding habits, when occurring in high densities, reduce new growth of hemlock by reduced photosynthesis and water loss (Gonda-King et al. 2014)

Since the 1990s, biological control has been extensively studied as a method of control for *A. tsugae* on the east coast of North America (Cheah et al 2004, Havill, Vieira

& Salom 2014). During this time, no specialist fungal or parasitic biological control has been found to reduce the *A. tsugae* population (Havill, Vieira & Salom 2014). There has been some success with predator biological control (Cheah et al 2004). Of the more than five predator biological controls released in the east coast of North America, none have had the ability to fully control hemlock adelgids (Havill, Vieira & Salom 2014).

1.3.5 Recent research

Until recently, it was unclear whether *A. tsugae* found on western North American hemlocks were native to the Pacific Northwest, or if they were introduced along with *A. tsugae* found on the east coast (McClure 1987). *A. tsugae* clearly have a different relationship with the hemlock trees on the west coast as the populations are found in lower densities and have significantly lower impact on the trees than in the east (Havill et al. 2006, McClure 1989). Through genetic work of global populations of *A. tsugae*, Havill et al. (2006) found little evidence to suggest a recent invasion of *A. tsugae* to western North America. It is more likely that *A. tsugae* on the west coast of North America has been co-evolving with the hemlock species in the area for thousands of years (Havill et al 2006).

In the same study, Havill et al. (2006) found a large genetic difference between the global populations of *A. tsugae*. The mitochondrial DNA from samples within *A. tsugae* from mainland China and Taiwan differed from the samples from Japan at the same range as mitochondrial DNA of different species of adelgids (Havill et al. 2006). The study determined that more research is needed on the morphology, biology and ecology of *A. tsugae* to understand the true relationship between these adelgid populations.

1.4 *Pineus similis, Pineus abietinus*

Gillette (1907) described the species *Chermes similis* as an adelgid species with apterous females laying egg clusters, sometimes in the presence of galls on blue spruce in Colorado. Later, Annand (1928) placed this species in the *Pineus* genus with Gillette's (1907) apterous type specimen. Cumming (1962) published a more complete description of *Pineus similis,* introducing this species as anholocyclic, restrictively settling on the primary host. When studying *P. similis,* Cumming used specimens from northern North America: Manitoba, Saskatchewan, Alberta and British Columbia. Cumming (1962) found *P. similis* to have a seemingly four generations, fundatrices, winged gallicolae, apterous gallicoae, and apterous females, which do not live in new galls.

Johnson (1959) reported a previously unknown species of *Pineus* genus in Washington state. This species was descripted to be the first species within this genus to feed on true firs, attacking the trunk and branches of both *Abies amabilis* (Douglas) and *Abies grandis* (Douglas) (Johnson 1959). This species was officially described as *Pineus abietinus* by Underwood and Balch (1964). The specimens used to describe this species were collected from the bark of *Abies amabilis*, in Kitimat British Columbia and were recorded to be indistinguishable from specimens found in Washington. Underwood and Balch (1964) recorded *P. abietinus* to be anholocyclic on the secondary host, species in the *Abies* genus.

1.4.1 Current understandings of *Pineus similis* and *Pineus abietinus*

In 2006 adelgid specimens that were morphologically identical to *P. abietinus* were found on the bark of *Pinus monticola* Douglas trees in Oregon (Havill, personal communication, 10 February 2016). The existence of *P. abietinus* on a *Pinus* host had not been observed previously. When genetic analysis of COI was conducted, these newly discovered specimens were found to have identical COI sequences to *P. similis* (Havill, personal communication, 10 February 2016). This discovery revealed the possibility of a current misunderstanding in the relationship between *P. abietinus, P. similis* and these specimens found on the bark of *Pinus monticola*.

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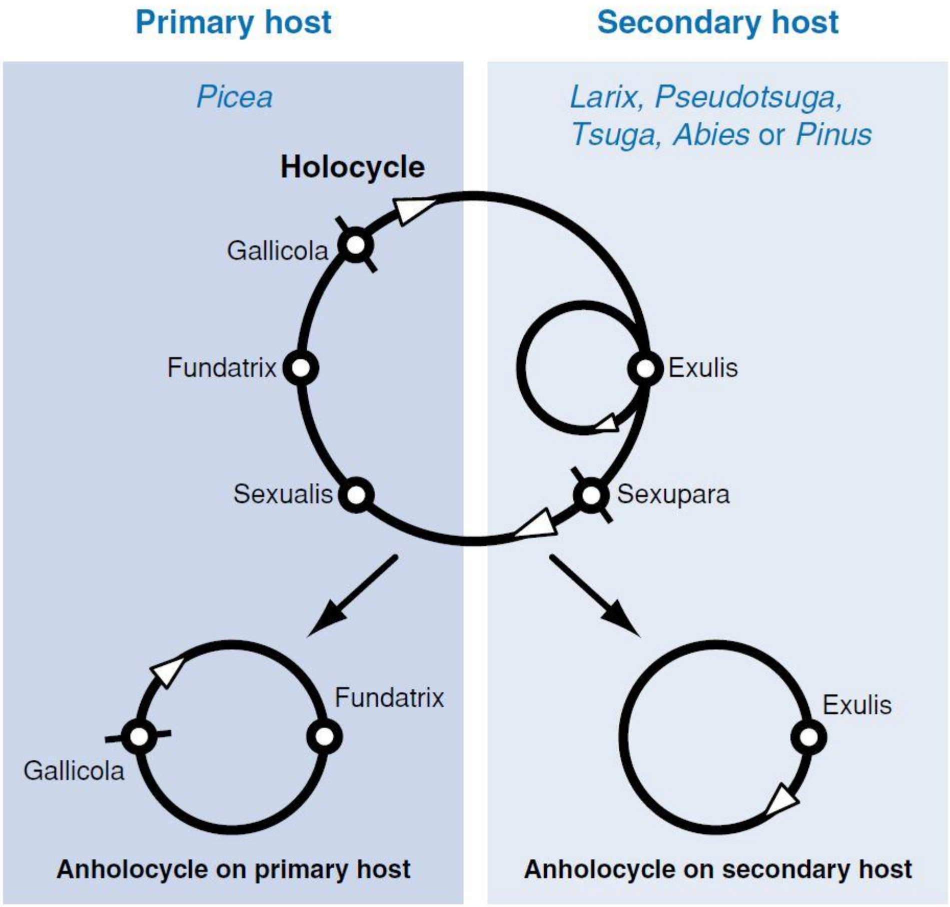
1.5 Figures

Figure 1. Description of Adelgid life cycle (Havill & Foottit 2007).

Figure 1.


Primary host
Secondary host
Picea
Larix, Pseudotsuga, Tsuga, Abies or Pinus
Holocycle
Gallicola
Fundatrix
Sexualis
Exulis
Sexupara
Fundatrix
Gallicola
Exulis
Anholocycle on primary host
Anholocycle on secondary host

CHAPTER 2: MORPHOMETRIC VARIATION AMONG GENETICALLY DISTINCT LINEAGES OF THE HEMLOCK WOOLLY ADELGID, *ADELGES TSUGAE* ANNAND (HEMIPTERA: ADELGIDAE)

2.1 Abstract

Hemlock adelgid, *Adelges tsugae* Annand, are non-native invasive forest insects in eastern North America, originating from Japan. They threaten the sustainability of two ecologically and economically important native trees species: eastern and Carolina hemlock. There are additional populations of hemlock adelgids native to China, Taiwan, Japan, South Korea and western North America. Within these populations there are eight distinct genetic lineages: one from central China, one from western China, two from Taiwan, two from Japan, one from Ulleung Island (South Korea), and one from western North America. These genetic distinctions provide evidence that the species designation *A. tsugae*, which places all eight lineages under the same description, does not reflect the diversity within the global hemlock adelgid distribution.

We used multivariate morphometric analyses to identify morphological differences among hemlock adelgid lineages. Using principal component analyses and MANOVA, the six lineages that were evaluated in this study were found to be significantly different ($p < 0.05$). The findings of this project provide evidence for taxonomic designation of different hemlock adelgid lineages that can inform regulation and biological control.

2.2 Introduction

Although Adelgidae (Hemiptera: Sternorrhyncha) has been studied for over 900 years, the first reference of adelgids in 1583, there is still much left unknown about this family (Annand 1928). Much of this confusion is due to their complex life cycle, which exists in all species in the family (Annand 1928). The complex life cycle presents itself in multiple ways: cyclical parthenogenesis, multigenerational polymorphy, and host switching (Havill & Foottit 2007). Although some adelgids species have lost cyclical parthenogenesis and host switching (Annand 1928), all contain multigenerational polymorphy, making it difficult to compare individuals between and within the same species.

Until 2016, hemlock adelgids were assumed to be one species with a global distribution (McClure 1987). Havill et al. (2016) reported eight genetically distinct lineages: western China, central China, Ulleung island, western North America, two in Taiwan, and two in Japan- on *Tsuga diversifolia* Masters and *Tsuga sieboldii* Carrière. In our study, we utilized multivariate morphometric methodologies to better understand if, and how, six of these eight genetically distinct lineages are manifested morphologically. These six, western China, Ulleung island, western North America, one in Taiwan, and the two lineages on *Tsuga diversifolia* and *Tsuga sieboldii*, predetermined lineages were used as groupings in the analyses.

A. tsugae is dramatically altering ecosystem functions as it spreads across hemlock forests in eastern United States. Understanding the species differentiation among populations worldwide is critical for management of these forested ecosystems. If these global populations are in fact separate species with distinct niches, including cold tolerance, fecundity and host use, the unintended introduction of individuals from Japan, western North America, Taiwan, China and Ulleung island into a new environment could be similarly disastrous to the hemlock populations and ecosystem function around the world.

This study aims to understand the potential differences in morphology between the genetically distinct *A. tsugae* populations. The results of this study will help to inform the systematics of *A. tsugae* populations.

2.3 Methods

2.3.1 Adelgid samples

Samples of adelgids that were collected between 1994-2015 from five different countries (Table 1) were individually slide-mounted in Canada balsam. Out of the eight genetic lineages previously found (Havill et al 2016), only specimens from western China, Ulleung island, western North America, one in Taiwan, and the two lineages from Japan on *Tsuga diversifolia* and *Tsuga sieboldii* were available for morphologic analysis. This left six lineages to be represented in this analysis. Specimens were grouped according to life stage and generation. Only 1st instar nymphs and adults were used because these stages have been established as being the most informative for distinguishing adelgid species (Blackman and Eastop 1994). Adelgid individuals have historically been classified as sistentes or progredientes (singular = sistens and progrediens) based on whether they do, or do not undergo an aestivation period, respectively (Havill and Foottit 2007). Morphological differences between sistentes and progredientes, such as the extent of sclerotization, stylet length, number of wax glands, and antennal length, have been noted in some adelgid species (see references in Havill et al. 2007), but these have not been reported for *A. tsugae* (Annand 1924, McClure 1989). *A. tsugae* is described as having two generations per year on hemlock: a generation of sistens individuals that hatches in late summer, aestivates for several months, overwinters, and lays eggs in the spring, and a generation of progrediens individuals that hatches in the spring and lays eggs in early summer (McClure 1989). Our samples were classified as sistentes or progredientes based on life cycle timing that has been documented for different regions (Veira et al. 2013, Joseph et al. 2011, Mausel et al. 2008, Kohler et al. 2008, Shiyake et al. 2008, Lamb et al. 2008). Temperatures from recorded life cycle timings were obtained, and compared to temperatures of locations that contained hemlock adelgids of the same genetic lineage. We used the minimum and maximum temperatures of each province from the Japan Meteorological Agency to estimate the generation of adelgids collected in Japan (retrieved 2016). For the Chinese specimens, unpublished data were used from a forest service scientist who recorded hemlock adelgid phenology (personal communication with Havill, September 2016). The Taiwanese hemlock adelgid lineages, for which the life cycle had never been recorded, were classified as sistentes or progredientes using the Chinese life cycle data, as they are assumed to have similar life cycle as the recorded adelgids of China. Any specimen collected in a timeframe which both sistentes and progredientes had previously been collected were taken out of the analysis. Each individual was therefore classified as a 1st instar sistens, 1st instar progrediens, adult sistens, or adult progrediens.

2.3.2 Morphometrics

We analyzed the morphology of these six distinct genetic lineages by measuring characters of 688 specimens (Table 1). Character measurements were selected based on a similar study of balsam woolly adelgid, *Adelges piceae* (Foottit et al. 1980, 1989). Images of each slide were captured using a Keyence VHX-2000 digital microscope (Keyence Corporation, Boston, Massachusetts, USA) and measurements were made using the VHX-2000 Communication software (add manufacturer details). Dorsal and ventral views were captured of each specimen. Eighteen morphological characters were measured on the most intact lateral side of each 1st instar specimen (Table 2 and Fig. 2), and 19 morphological characters were measured on adult specimens (Table 3 and Fig. 3). The accuracy measuring these characters was assessed by measuring one specimen ten times, on half hour intervals, throughout one day. Characters with a coefficient of variation lower than 0.035 were included in statistical analysis, following previous adelgid morphology research (Foottit & Mackauer, 1989). Only individuals for which it was possible to measure all characters were included in analyses.

2.3.3 Statistical analyses

We performed all analyses in R Version 3.3.1 (R Core Team 2014). We used the prcomp package (Sigg and Buhmann 2008) for principal components analyses (PCA), and base package for multivariate analysis of variance (MANOVA). The vegan package (Oksanen et al 2016) was used for visual representation of the principal component analysis. PCA and MANOVA analyses were run to test differences between sistens and progrediens generations across all lineages, and for differences among genetic lineages within each of the four groups: 1st instar sistens, 1st instar progrediens, adult sistens, and adult progrediens. The ellipses were based on standard deviation for each designated lineage grouping. P-values were calculated using Bonferroni correction when multiple significance tests were run on the same data set.

2.4 Results

The results of our morphological analyses support the six distinct genetic lineages of the species complex of hemlock adelgids (Havill et al. 2016). Although not all lineages were represented in each analysis, each lineage was shown to be statically significantly different from other lineages, with the exception of Ulleung island (see Tables in Appendix).

2.4.1 Morphological analysis

Differences among distinct genetic linages of 1st instar sistentes were tested using seven characters (N=62) (Table 2). The first two principal components accounted for 51.61% of the total variation (Fig. 4). The first principal component, which accounted for 30.24% of variation, consisted of only negative values (Table 4). The rostrum 3rd segment width (R3mw) and antenna 3rd segment length (Au) had the largest negative scores. The second principal component, which accounted for 21.40% of the variability, has low negative scores, with the exception of rostrum base (R4bw) and rostrum 4th segment length (R34L) which had high positive scores.

The MANOVA revealed the statistically significant differences of morphological traits among distinct genetic lineages of 1st instar sistentes. Four of the six distinct genetic lineages of 1st instar sistentes were different from each other in pairwise comparisons (Table 5). For these statistically significant pairwise comparisons of linages, F3, Tb3, dTs3, and Au were the defining characteristics (Table 6).

We used thirteen characters of 1st instar progredientes to test for statistical differences among distinct genetic lineages (N=121) (Table 2). The first two principal components accounts for 38.75% of the total variation (Fig. 5). The first principal component accounted for 25.82% of the total variation (Table 7). All the character scores for the first principal component had low negative values. The second principal component, which accounted for 12.93% of variation, had low negative scores except rostrum 4th segment base (R4bw) and rostrum 3rd segment width (R3mw) which were relatively high positive values.

The MANOVA found some of these lineages within the 1st instar progredientes analysis to be significantly different. A pairwise comparison of the lineages was done of the available lineages for 1st instar progredientes and four pairwise comparisons were found to be statistically different from each other (Tables 8). For the statistically significant pairwise comparisons of 1st instar progrediens lineages, R4bw, R4L, R3mw, F3, Tb3, F2, Tb2, dTs2, F1, F1w, Tb1, Au were defining characters (Table 9).

Seven characters were included in the analysis of adult sistentes (N=82) (Table 3). The first two principal components accounted for 61.32% of the variability (Figure 6). The first principal component accounted for 42.38% of variability and consisted of only positive scores (Table 10). Of these scores, the 3rd femur length (F3) was the highest score, with 2nd femur length (F2) and ovipositor length (Ov) as next highest measurements. The second principal component, which accounted for 18.94% of variability, consisted of relatively low positive scores, except for ovipositor length (Ov) which had a large negative score.

The MANOVA found these lineages within the adult sistentes analysis to be significantly different. A pairwise comparison was done of all available lineages and five of the ten pairwise comparisons were found to be statistically different from each other (Table 11). The characters R4bw, F3, F3w, F2, Tb1 and Au were found to be defining of the significant lineages (Table 12).

In the analysis of adult progredientes, four measurements were used (N=51) (Table 3). The first two principal components accounted for 85.12% of variability (Fig. 7). The first principal component, which accounted of 59.25% of variability, consisted of only negative scores (Table 13). Most of these scores were low, with exception of the 3rd femur length (F3) which had a large negative score. The second principal component, which accounted for 20.68% of variability, had relatively high positive scores for rostrum 4th segment base (R4bw) and 3rd femur width (F3w) but relatively a large negative score for antennal 3rd segment length (Aubw).

The MANOVA found these lineages within the adult progredientes to be significantly different. A pairwise comparison done on the available lineages and four of the six pairwise comparisons were statistically different from each other (Table 14). For these statistically significant pairwise comparisons of lineages, R4bw, F3, F3w, Au were found to be defining characters (Table 15).

2.4.2 Morphological groups

This study found each lineage, besides Ulleung, to have differences between at least one other lineage. Eight of the 15 lineage comparisons resulted in no morphological differences (Table 16). These morphological differences which separate the genetically defined lineages differ between lineage comparisons and life cycle stages (Appendix I, II, III, IV).

2.5 Discussion

Although systematics of multigenerational polymorphic species can be challenging to fully understand, incorporating morphology, genetics and geographic distribution can help clarify obscured boundaries of lineages. The results of this morphological analyses agree with the genetic lineages of hemlock adelgids found by Havill et al. (2006, 2016).

Differences observed in the morphology of the genetic lineage were not necessarily consistent between the two generations (Table 16). For example, western China is significantly different from the same lineages in both 1st instar sistentes and adult sistentes analyses. Whereas, the progredientes analyses between generations did not match up as well. Within the 1st instar progredientes analysis, western China was only significantly different from Taiwan. In the adult progredientes analysis, western China was not significantly different from Taiwan but was significantly different from *T. sieboldii* and western North America.

The significance level between lineages was not always seen through the generations. Although *T. sieboldii* was significantly different from Taiwan in both 1st instar progredientes and adult sistentes analysis, these lineages were not significant in 1st instar sistentes and adult progredientes. *T. sieboldii* was also not significant from western China in the 1st instar progredientes analysis but was significantly different in the rest of the analyses.

Although these significant morphological differences between lineages did not hold true throughout all four analyses, the analyses should not be considered contradictory. In the past, all known life cycle and 1st instar and adult generations of adelgids were considered important information and used in the description and identification of adelgids (Annand 1924 & 1928, Cumming 1962, Underwood & Balch 1964). Both instar and adult biology are independently important for defining and identifying a species, and thus do not necessarily have to tell the same story.

Each polymorphy which exists in the adelgid's multiple generations have distinct rolls in the life cycle. Although both sistentes and progredientes are categorized under the label of exules within adelgid biology, the stages have different existences. The sistentes have a period of diapause within the 1st first instar stage and in hemlock adelgids, sistentes are the generational stage to overwinter (Havill & Foottit 2007). The progredientes generation have no diapause and only exist a short period in the spring and early summer. Because of this, there are many traits of sistentes recorded to be distinct from progredientes (Havill & Foottit 2007). These two generational stages on the secondary host of hemlock adelgids are significantly different in morphology (Appendix V, VI) and represent distinct parts of the typical 5-year adelgid life cycle. The differences found in this study between the sistens and progrediens generations are not representative of a misunderstanding in the lineages, but rather both generations bring different and valid evidence to the existence of multiple distinct lineages within the hemlock adelgid species complex.

2.6 Conclusions

The results show a need for re-examination of the diversity of hemlock adelgids. Although not all generations were included in this study, the exules proved to be helpful in an examination of the morphological diversity within the six lineages included. Further research should include the two lineages that were not available for this study, central China and the second lineage in Taiwan, along with the inclusion of generations on the primary hosts, where it exists.

This research may also stimulate comprehensive policy for the species where it is invasive. Hemlock adelgids have been known to exist on the east coast of North America since 1951 (Stoetzel 2002), and since then caused damage on the eastern and Carolina hemlock population and eastern hemlock dominated ecosystems (Havill, Montgomery & Kenna 2011). This invasive population is an introduction from the lineage on *T. sieboldii* in Japan (Havill et al. 2016). In this study, there were four comparisons of lineages that were morphologically significantly different in at least three analyses, one of which were western North America and *T. sieboldii* (Table 16). This morphological evidence along with past genetic evidence (Havill et al. 2016), which both suggest the significant difference between these lineages, highlight the possibility for a negative outcome if the hemlock adelgid currently residing on the east coast of North America was transported to the west coast of North America or vice versa. Both these lineages currently existing in the United States adds additional potential for new introduction - although there is standard regulation when transporting plant matter to and from the United States (Canadian Border Agricultural Clearance Manual 2012), there is some regulation, but no check points when individuals transport plant matter across state lines (Don’t Move Firewood: State by State information 2017). This is cause for concern as hemlock adelgids have been introduced through logging operations (McClure 1990), and many other non-native insects have been accidently introduced by individuals crossing long distances with plant matter (Herms & McCullough 2013).

2.7 Acknowledgments

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2.9 Tables

Table 1. Description of samples for this study: sample sizes, geographic region and host species.

Host Species	Genetic Lineage by Geographic region	Sample Size			
		Adult		1st Instar	
		Progrediens	Sistens	Progrediens	Sistens
Tsuga chinensis *Tsuga dumosa* *Tsuga forrestii*	Western China	6	76	3	9
Tsuga formosana	Taiwan	3	33	144	17
Tsuga diversifolia	Japan lineage 1	11	5	24	2
Tsuga ulleungensis	Ulleung Island	0	12	8	0
Tsuga canadensis *Tsuga caroliniana*	Japan lineage 2, in eastern North America	31	39	18	35
Tsuga sieboldii	Japan lineage 2	8	65	26	27
Tsuga heterophylla *Tsuga mertensiana*	Western North America	21	23	28	10

Table 2. Character list of measurements taken on 1st instar hemlock adelgids.

Instar Adelgid		
Character number	Acronym	Character
1	R4bw	Rostrum 4th segment base Θ*ǂ
2	R4L	Rostrum 4th length Θ*ǂ
3	R3mw	Rostrum 3rd width Θ*ǂ
4	F3	3rd Femur length Θ*ǂ
5	F3w	3rd Femur width Θ
6	Tb3	3rd Tibia length Θ*ǂ
7	dTs3	3rd Tarsus length Θ*ǂ
8	F2	2nd Femur length Θǂ
9	F2w	2nd Femur width
10	Tb2	2nd Tibia length Θǂ
11	dTs2	2nd Tarsus length ǂ
12	F1	1st Femur length Θǂ
13	F1W	1st Femur width Θǂ
14	Tb1	1st Tibia length Θǂ
15	dTs1	1st Tarsus length
16	A1w	Antenna 1st segment width
17	Aubw	Antenna 3rd segment base
18	Au	Antenna 3rd segment length Θ*ǂ

(*) symbol indicates characters used in statistical analysis of sistens, (Θ) symbol indicates characters used in statistical analysis of progrediens, (ǂ) symbol indicates characters used in statistical analyses of both sistens and progrediens 1st instars.

Table 3. Character list of measurements taken on adult hemlock adelgids.

Adult Adelgid		
Character number	Acronym	Character
1	R4bw	Rostrum 4th segment base ϴ*ǂ
2	R4L	Rostrum 4th segment length
3	R3mw	Rostrum 3rd segment width
4	F3	3rd Femur length ϴ*ǂ
5	F3w	3rd Femur width ϴ*ǂ
6	Tb3	3rd Tibia length
7	dTs3	3rd Tarsus length ǂ
8	F2	2nd Femur length*
9	F2w	2nd Femur width
10	Tb2	2nd Tibia length
11	dTs2	2nd Tarsus length
12	F1	1st Femur length
13	F2W	1st Femur width
14	Tb1	1st Tibia length*
15	dTs1	1st Tarsus length
16	A1w	Antenna 1st segment width
17	Aubw	Antenna 3rd segment base
18	Aubw	Antenna 3rd segment length ϴ*ǂ
19	Ov	Ovipositor length*

(*) symbol indicates characters used in statistical analysis of sistens, (ϴ) symbol indicates characters used in statistical analysis of progrediens, (ǂ) symbol indicates characters used in statistical analyses of both sistens and progrediens adults.

Table 4. Character scores of the seven morphological characters to the seven principal components calculated from 1st instar sistens (N=62).

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
R4bw	-0.339	0.556	-0.187	0.6	0.419	-0.07	-0.032
R4L	-0.205	-0.142	-0.581	-0.094	-0.174	-0.624	0.415
R3mw	-0.514	0.563	0.2	-0.458	-0.405	0.059	0.035
F3	-0.28	-0.182	-0.636	-0.145	0.043	0.673	-0.092
Tb3	-0.424	-0.285	0.246	-0.405	0.701	-0.149	0.017
dTs3	-0.156	-0.115	-0.141	-0.01	-0.149	-0.344	-0.1
Au	-0.545	-0.475	0.321	0.487	-0.339	0.091	0.122
Relative % of variability	30.24	21.4	13.19	10.73	8.78	8.07	7.59

Table 5. MANOVA pairwise significance of 1st instar sistens lineages using seven morphological characters.

	Western China	*T. sieboldii*	Western North America	Taiwan
Western China	na	p<0.001* df: 7,34	0.0041* df: 7,7	0.0019* df: 7,11
T.sieboldii	--	na	0.0026* df: 7,35	0.0421 df: 7,39
Western North America	--	--	na	0.0565 df:7,12
Taiwan	--	--	--	na

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.008. The degrees of freedom are reported under the p-values: treatment, residual.

Table 6. MANOVA p-values of significant pairwise 1st instar sistens lineages using six morphological characters.

	R4bw	R4L	R3mw	F3	Tb3	dTs3	Au
T.sieboldii vs Western China df=40	0.4518	0.1924	0.898	0.0271	0.3365	0.0014*	0.1907
T.sieboldii vs Western North America df=41	0.1655	0.05458	0.0156	0.0001*	0.0002*	0.3041	0.0004*
Western North America vs Western China df=13	0.5281	0.7353	0.1050	0.2454	0.0316	0.1346	0.0129
Western China vs Taiwan df=17	0.0774	0.0322	0.0416	0.1146	0.2927	0.0076	0.1351

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.006.

Table 7. Character scores of the 13 morphological characters to the 13 principal components calculated from 1st instar progrediens. (N=121).

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13
R4bw	-0.223	0.591	-0.107	0.12	-0.275	0.036	0.037	-0.003	0.051	0.576	0.128	0.367	-0.105
R4L	-0.195	-0.049	0.0003	0.032	-0.104	0.343	0.429	0.309	-0.636	-0.262	0.104	0.258	-0.007
R3mw	-0.294	0.685	0.075	0.12	0.287	-0.091	-0.092	0.123	-0.031	-0.425	-0.109	-0.324	0.109
F3	-0.289	-0.132	-0.229	-0.016	-0.211	0.246	0.033	0.118	-0.138	0.381	-0.351	-0.662	0.016
Tb3	-0.372	-0.194	-0.017	-0.06	0.198	0.086	0.201	0.56	0.618	-0.02	-0.055	0.175	-0.058
dTs3	-0.121	-0.0456	0.023	0.053	-0.068	-0.016	0.086	-0.023	0.081	-0.045	0.791	-0.403	-0.411
F2	-0.318	-0.117	-0.336	-0.043	-0.535	-0.155	-0.52	0.049	0.003	-0.386	-0.028	0.164	-0.107
Tb2	-0.338	-0.137	-0.265	-0.166	0.252	-0.731	0.277	-0.118	-0.24	0.14	-0.006	0.046	0.0125
dTs2	-0.104	-0.061	-0.097	0.051	-0.129	0.028	0.017	-0.015	0.1	0.0289	0.393	-0.052	0.887
F1	-0.323	-0.15	-0.187	0.532	0.164	0.267	0.161	-0.601	0.157	-0.141	-0.102	0.11	-0.059
F1w	-0.118	0.166	0.1	-0.672	-0.275	0.139	0.375	-0.402	0.224	-0.204	-0.087	-0.031	0.007
Tb1	-0.32	-0.076	0.05	-0.415	0.474	0.3454	-0.487	-0.134	-0.193	0.179	0.167	0.133	0.0007
Au	-0.374	-0.176	0.831	0.159	-0.223	-0.187	-0.075	-0.065	-0.061	0.092	-0.084	0.004	0.041
Relative % of variability	25.82	12.93	8.77	8.07	7.15	6.7	5.65	5.17	4.7	4.35	3.91	3.67	3.13

Table 8. MANOVA pairwise significance of 1st instar progrediens lineages.

	Western China	*T.sieboldii*	Western North America	*T.diversifolia*	Ulleung	Taiwan
Western. China	na	0.11 df:13,5	x	x	x	$p<0.001$* df:13,64
T. sieboldii	--	na	0.0309 df:13,15	0.0282 df:13,16	0.5068 df:13,5	$p<0.001$* df:13,81
Western North America	--	--	na	0.0498 df:13,7	x	$p<0.001$* df:13,72
T. diversifolia	--	--	--	na	x	0.0022* df:13,72
Ulleung	--	--	--	--	na	0.0570 df:13,59
Taiwan	--	--	--	--	--	na

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.006. x values were used where the degrees of freedom were not sufficient to complete an analysis. The degrees of freedom are reported under the p-values: treatment, residual.

Table 9. MANOVA significance levels of significant pairwise 1st instar progredientes lineages using 13 characters.

	R4bw	R4L	R3mw	F3	Tb3	dTs3
T. sieboldii vs Taiwan df:93	0.0053*	0.0102	0.4449	p<0.001*	p<0.001*	0.0386
Taiwan vs *T. diversifolia* df:85	0.0051*	0.1552	0.3269	0.1853	0.4719	0.6327
Taiwan vs Western China df:76	0.0003*	0.0584	0.0006*	0.4719	0.4415	0.1224
Western North America vs Taiwan df: 84	0.1169	0.3545	p<0.001*	0.0091	0.1161	0.6551

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.006.

Continued - Table 9. MANOVA significance levels of significant pairwise 1st instar progredientes lineages using 13 characters.

	F2	Tb2	dTs2	F1	F1w	Tb1	Au
T. sieboldii vs Taiwan df:93	p<0.001*	p<0.001*	p<0.001*	p<0.001*	0.7818	0.0156	0.2976
Taiwan vs *T. diversifolia* df:85	0.8143	0.0961	0.9400	0.7134	0.0067	0.4288	0.1252
Taiwan vs Western China df:76	0.6759	0.8628	0.6366	0.3244	0.9005	0.8872	0.0041*
WNA vs Taiwan df: 84	0.0705	0.0055*	0.6835	0.1095	0.0336	0.0002*	0.1625

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.006.

Table 10. Character scores of the seven morphological characters to all seven principal components calculated from adult sistens (N=82).

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
R4bw	0.305	0.164	-0.812	-0.371	-0.285	-0.052	0.003
F3	0.577	0.229	0.428	-0.402	0.102	-0.294	-0.416
F3w	0.273	0.109	-0.306	0.393	0.764	-0.263	0.116
F2	0.461	0.139	0.236	-0.06	-0.07	0.314	0.778
Tb1	0.318	0.272	-0.044	0.549	-0.198	0.552	-0.422
Au	0.077	0.173	0.073	0.475	-0.519	-0.662	0.158
Ov	0.425	-0.886	-0.039	0.128	-0.106	-0.028	-0.069
Relative % of variability	42.38	18.94	14.55	7.93	7.02	5.07	4.11

Table 11. MANOVA pairwise significance of adult sistens lineages.

	western China	*T.sieboldii*	Ulleung	Taiwan	western North America
w. China	na	p<0.001* df:7,58	0.0073 df:7,35	p<0.001* df:7,46	p<0.001* df: 7,34
T.sieboldii	--	na	0.465 df:7,19	p<0.001* df:7,30	p<0.001* df: 7,18
Ulleung	--	--	na	0.3398 df:7,7	x
Taiwan	--	--	--	na	0.0782 df:7,6
WNA	--	--	--	--	na

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.007. x values were used where the degrees of freedom were not sufficient to complete an analysis. The degrees of freedom are reported under the p-values: treatment, residual.

Table 12. MANOVA significance level of significant pairwise adult sistens lineages using seven characters.

	R4bw	F3	F3w	F2	Tb1	Au	Ov
Taiwan vs *T. sieboldii* df: 36	0.1122	0.0005*	0.5764	0.0109	0.4217	0.5715	0.1092
T. sieboldii vs Western China df: 64	0.0002*	0.1493	0.0019*	0.0628	p<0.001*	0.0001*	0.2582
Taiwan vs Western China df: 52	p<0.001*	0.0237	0.0237	p<0.001*	p<0.001*	0.0076	0.1822
Western China vs WNA df: 40	p<0.001*	p<0.001*	0.0032*	p<0.001*	p<0.001*	0.7974	0.1050
WNA vs *T. sieboldii* df: 24	0.1282	0.0009*	0.2532	0.0005*	0.0232	0.5115	0.1870

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.006.

Table 13. Character scores of the four morphological characters to the four principal components calculated from adult progrediens (N=51).

	PC1	PC2	PC3	PC4
R4bw	-0.175	0.556	-0.602	-0.545
F3	-0.927	-0.033	0.352	-0.124
F3w	-0.199	0.521	-0.192	0.808
Au	-0.264	-0.647	-0.69	0.188
Relative % of variability	59.25	20.68	11.71	8.36

Table 14. MANOVA pairwise significance of adult progrediens lineage.

	T.sieboldii	western China	western North America	Taiwan
T.sieboldii	na	0.0003* df: 5,28	p<0.001* df: 5,36	0.0892 df: 5,25
w. China	--	na	0.0002* df: 5,14	0.0893 df: 5,3
WNA	--	--	na	0.0021* df: 5,11
Taiwan	--	--	--	na

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.008. The degrees of freedom are reported under the p-values: treatment, residual.

Table 15. MANOVA significance level of significant pairwise adult progrediens lineages using four morphological characters.

	R4bw	F3	F3w	Au
W. China vs *T. sieboldii* df: 32	0.0003*	0.5169	0.0102	0.0009*
WNA vs Taiwan df: 15	0.0457*	0.0038*	0.0434	p<0.001*
W. China vs WNA df: 18	p<0.001*	0.0007*	0.0006*	0.4023
WNA vs *T. sieboldii* df: 40	0.0048*	p<0.001*	0.0173	p<0.001*

The values with an asterisk represent instances where values are significant at a corrected alpha of 0.008.

Table 16. Summary of the findings in the study, showing if each lineage comparison was significantly different in each analysis.

	1st instar sistentes	1st instar progredientes	Adult sistentes	Adult progredientes
Western China vs Taiwan	x	x	x	0
Western China vs *T. sieboldii*	x	0	x	x
Western China vs western North America	x	0	x	x
Western China vs Ulleung	(--)	0	0	(--)
Western China vs *T. diversifolia*	(--)	0	(--)	(--)
Taiwan vs *T. sieboldii*	0	x	x	0
Taiwan vs western North America	0	x	0	x
Taiwan vs Ulleung	(--)	0	0	(--)
Taiwan vs *T. diversifolia*	(--)	x	(--)	(--)
T. sieboldii vs western North America	x	0	x	x
T. sieboldii vs Ulleung	(--)	0	0	(--)
T. sieboldii vs *T. diversifolia*	(--)	0	0	(--)
Western North America vs Ulleung	(--)	0	0	(--)
Western North America vs *T. diversifolia*	(--)	0	0	(--)
Ulleung vs *T. diversifolia*	(--)	0	0	(--)

X represents a significant result, 0 represents no significant result, and (--) indicates that this lineage comparison was not available for the analyses.

2.10 Figure legends

Figure 2. 1st instar hemlock adelgid, continuous appendage measurements.

Figure 3. Adult hemlock adelgid, continuous appendage measurements.

Figure 4. Principal component analysis of instar sistens lineages using seven morphological characters. The MANOVA found these lineages to be significant, $p<0.001$, N=62, DF= 21, 162.

Figure 5. Principal component analysis of instar progrediens lineages using fourteen morphological characters. The MANOVA found these lineages to be significantly different, $p<0.001$, N= 121, DF= 60, 495.

Figure 6. Principal component analysis of adult sistens lineages using seven morphological characters. The MANOVA found these lineages to be significantly different, $p<0.001$, N=82, DF= 28, 296.

Figure 7. Principal component analysis of adult progrediens lineages using four morphological characters. The MANOVA found these lineages to be significantly different, $p<0.001$, N=51, DF=28, 296.

Figure 2

Figure 3

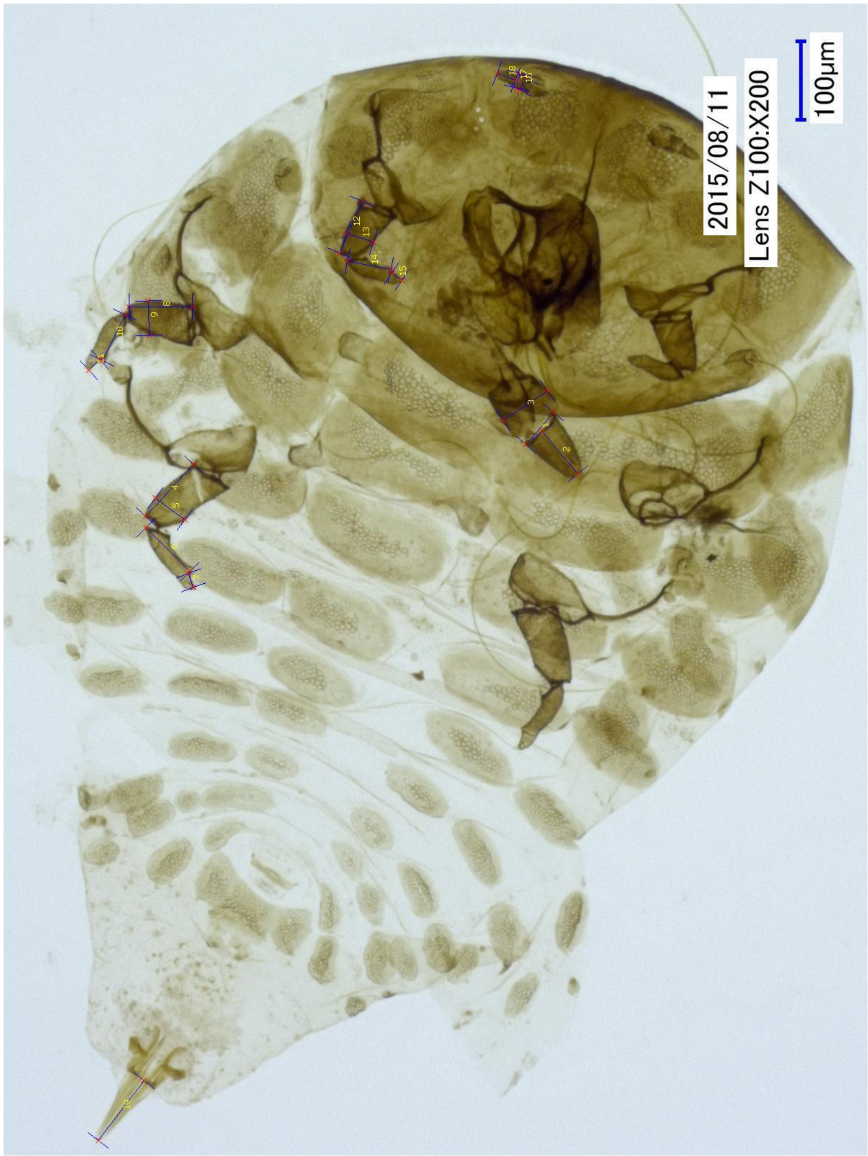
2015/08/11
Lens Z100:X200
100µm
1
2
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Figure 4

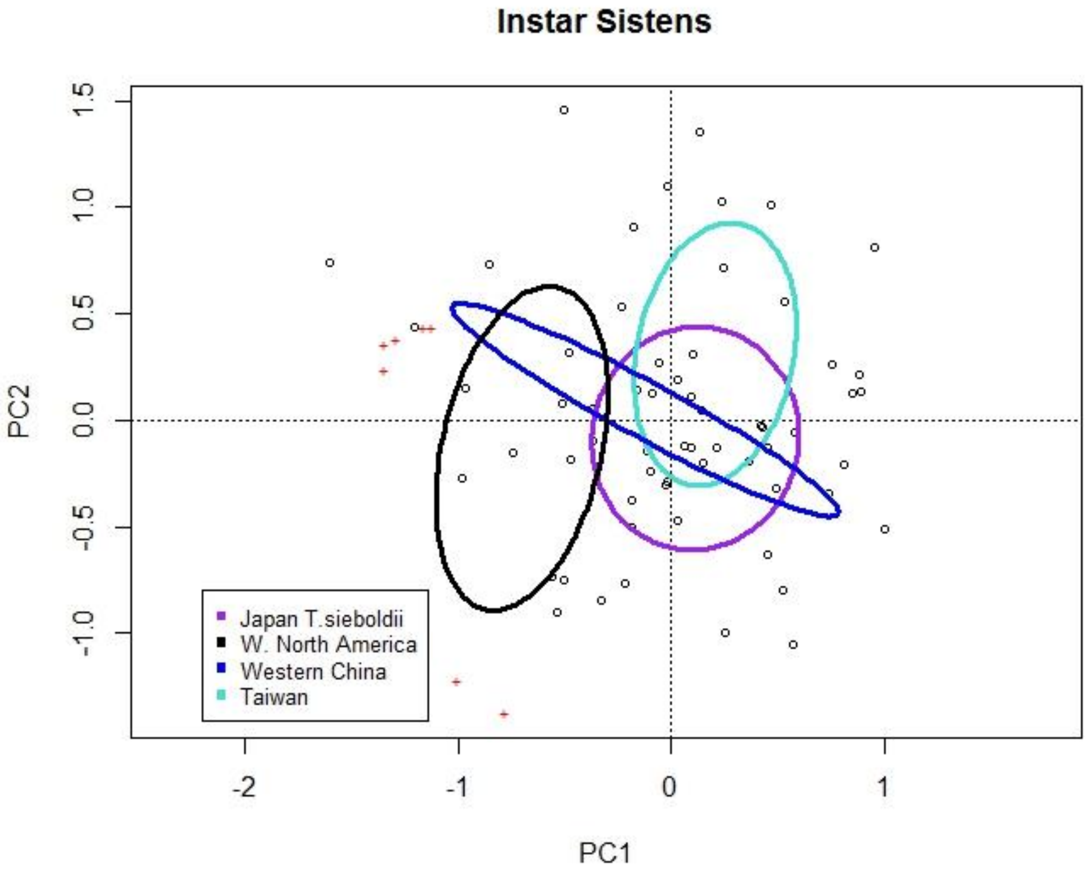
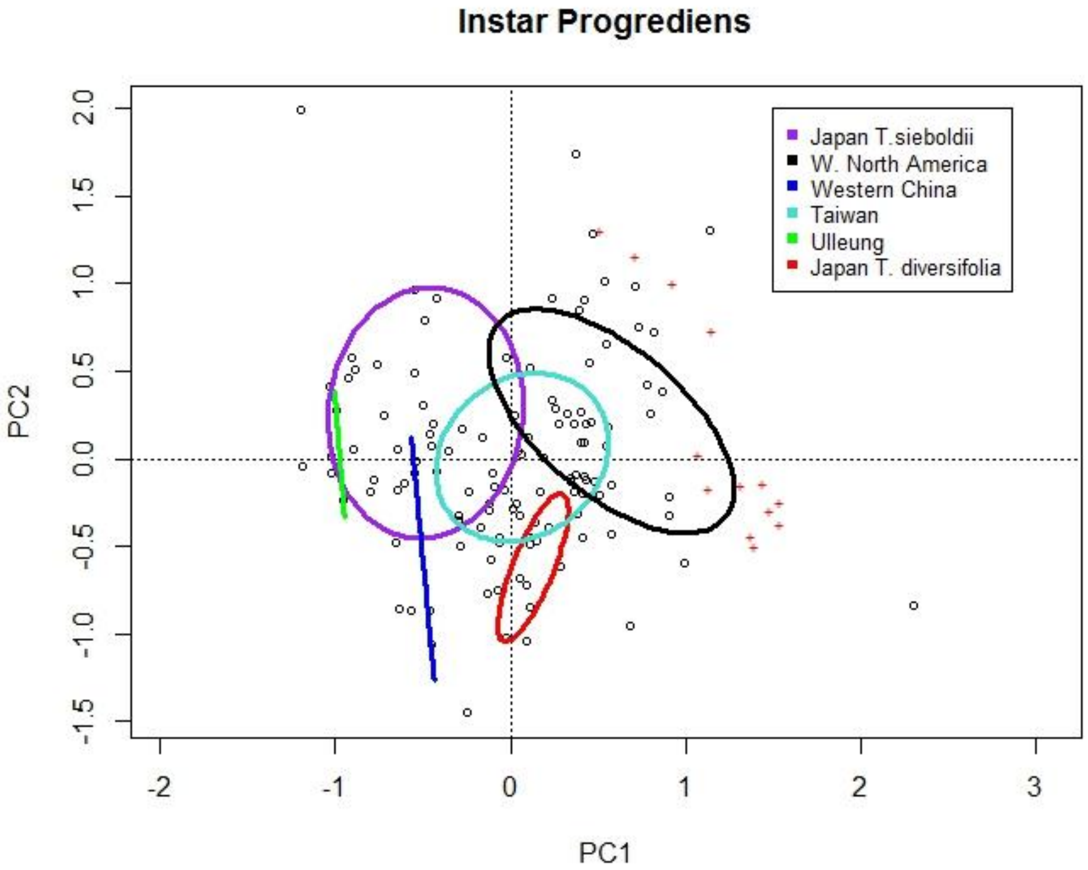

Instar Sistens
PC2
PC1
1.5
1.0
0.5
0.0
-0.5
-1.0
-2
-1
0
1
Japan T.sieboldii
W. North America
Western China
Taiwan

Figure 5


Instar Progrediens
Japan T.sieboldii
W. North America
Western China
Taiwan
Ulleung
Japan T. diversifolia
PC2
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1.0
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0.0
-0.5
-1.0
-1.5
-2
-1
0
1
2
3
PC1

Figure 6

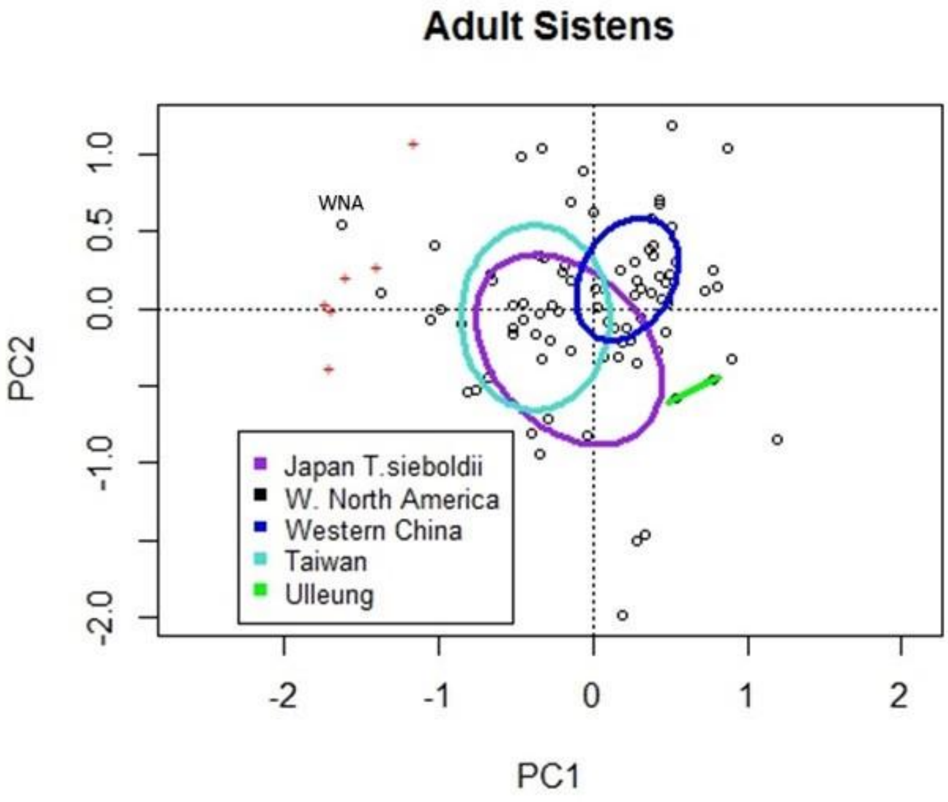
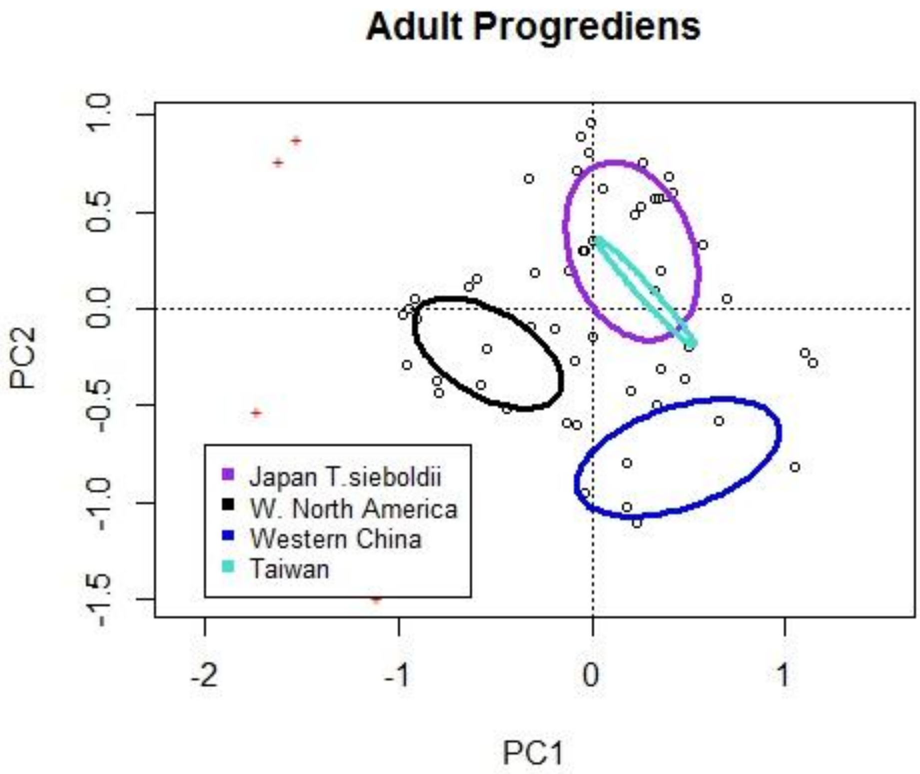

Adult Sistens
PC2
1.0
0.5
0.0
-1.0
-2.0
WNA
Japan T.sieboldii
W. North America
Western China
Taiwan
Ulleung
-2
-1
0
1
2
PC1

Figure 7



Adult Progrediens
PC2
PC1
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2
-1
0
1
Japan T.sieboldii
W. North America
Western China
Taiwan

CHAPTER 3: PHYLOGENETIC IDENTITY OF ADELGIDS (HEMIPTERA: ADELGIDAE) ON THREE HOST GENERA IN NORTH AMERICA

3.1 Abstract

Adelgids (Hempiera: Adelgidae) are host-specific insects closely related to aphids (Aphididae) and phylloxerans (Phylloxeridae). The holocyclic individuals alternate between spruce (*Picea*) primary hosts and secondary hosts in another conifer genus, while anholocyclic species complete their life cycle only on *Picea* or only on a secondary host genus. A recent molecular phylogeny of Adelgidae found that groups of species that feed on the same secondary host genus form divergent clades, indicating that switching to a different host genus is rare in their evolutionary history. The phylogeny also highlighted misunderstandings of the diversity within the family, probably due to their complex life cycles. In western North America, *Pineus similis* is described as being anholocyclic on *Picea* and *Pineus abietinus* is described as being anholocyclic on *Abies*. *Pineus abietinus* is also the only known *Pineus* species that uses a genus other than *Pinus* as a secondary host. Analysis of DNA sequence data from three mitochondrial genes and one nuclear gene showed that samples of *P. abietinus* collected from *Abies* spp. were very closely related to samples of *P. similis* collected from *Picea* spp. We also report the first records of *P. abietinus* collected from *Pinus monticola*, and show that while using the four genes previously mentioned they are phylogenetically indistinguishable from samples of *P. similis*. This suggests that some *P. similis* are capable of completing a holocycle by migrating to *Pinus monticola*, and points to a very recent secondary host switch from *Pinus* to *Abies*, the only example of a recent secondary host switch that has been observed in Adelgidae.

3.2 Introduction

Adelgids (Hemiptera: Adelgidae) form a small group of insects with 65 species in two genera, *Adelges* and *Pineus,* further separated into seven subgenera (Favret et al. 2015). They are host-specific and restricted to conifer host plants in the genera *Picea, Pinus, Abies, Tsuga, Pseudotsuga,* and *Larix* (Havill & Foottit 2007). Adelgid species can be holocyclic, having both sexual and parthenogenetic generations and host alteration between spruce (*Picea*) primary hosts where galls are formed, and secondary hosts in one of the other genera. Other species are anholocyclic, with only parthenogenetic reproduction and restricted to only *Picea* or only a secondary host genus (Havill and Foottit 2007). Species that are anholocyclic are hypothesized to have originated from holocyclic species (Havill & Foottit 2007). A recent phylogeny of Adelgidae found that species that feed on the same secondary host genus form divergent clades that originated in the Late Cretaceous to Early Tertiary, indicating that successful switching to a different genus is rare in their evolutionary history (Havill et al. 2007).

The complexity of the adelgid life cycle has led to some difficulty in circumscribing species (Havill & Foottit 2007). There has historically been a tendency to include life cycle and host plant identity in addition to morphology to delimit species (Annand 1928, Havill & Foottit 2007). This has resulted in species groups that include closely related holocyclic and anholocyclic members. For example, *Adelges (Dreyfusia) nordmannianae* (Eckstein)*,* is holocyclic on *Picea* and *Abies,* and *A. (Dreyfusia) piceae* (Ratzeburg) is anholocyclic on *Abies*. The shared generations of these species on *Abies* are morphologically very similar and DNA sequence data cannot distinguish them, and do not support them to be monophyletic groups (Eichhorn 1967, Havill et al. 2007, Foottit et al. 2009, Zurovcova et al. 2010).

We summarize new information about the phylogenetic identity of a pair of adelgid species native to North America by analyzing DNA sequence data. *Pineus similis* Gillette was described from galls on *Picea pungens* Engelmann (Gillette 1907). It has also been reported on *Picea engelmannii* Parry*, P. glauca* (Moench) Voss*, P. mariana* (Miller) Britton Sterns & Poggenburg*, P. sitchensis* (Bongard) Carriere*, P. rubens* Sargent*,* and *P. abies* Karsten (Cumming 1962, Carter 1975). It was described as being anholocyclic on *Picea* with alternating generations of fundatrices that settle at the base of buds and initiate gall formation, and winged gallicolae that emerge from galls and settle back on spruce to produce more fundatrices (Brown 1941, Cumming 1962, Carter 1975). Cumming (1961) also describes a small number of wingless progeny of the fundatrix that settle inside or outside of galls. Approximately 60 years later, Underwood and Balch (1964) described *Pineus abietinus,* the first anholocyclic species in the genus *Pineus* to be on *Abies*, rather than on *Pinus*. They described it from specimens settled on the bark of *Abies amabilis* (Douglas) Forbes and *A. grandis* (Douglas) Lindley.

In the course of accumulating DNA barcode data for adelgid species (after Foottit et al. 2009), it was found that COI DNA sequences from *P. similis* collected on *Picea* matched *P. abietinus* collected on fir, as well as newly-discovered *P. abietinus* samples collected on *Pinus monticola* Douglas. This study aims to better understand the species grouping of *Pineus similis* on *Picea* and *Pineus abietinus* on *Abies* and *Pinus* using DNA sequence data from additional mitochondrial and nuclear genes.

3.3 Methods

For outgroup taxa, we used one individual each from 11 other *Pineus* species, 12 *Adelges* species, and two members of the sister family Phylloxeridae (Table 17). Many of the outgroup samples and DNA sequences were the same as those reported in Havill et al. (2007). Unidentified *Pineus* sp. A NPH-2007 refers to Havill et al. (2007), and *Pineus* sp. A RGF-2008, B RGF-2008, D RGF-2008, and E RGF-2008 refers to Foottit et al. (2009). *Pineus similis* and *P. abietinus* specimens were collected between 1998-2016. All samples of *Pineus similis* were either fundatrices or gallicolae associated with galls on *Picea*. Samples of *Pineus abietinus* were collected from the bark of *Abies lasiocarpa*, *Pinus monticola*, *Pinus strobus*, or *Picea engelmanni* (Table 17). Most of the samples used have slide mounted vouchers at either the Yale Peabody Museum of Natural History, New Haven, Connecticut, USA (YPM) or the Canadian National Collection of Insects, Ottawa, Ontario, Canada (CNC).

DNA sequence data for three mitochondrial genes [cytochrome oxidae I (COI), cytochrome oxidae II (COII), cytochrome *b* (cyt*b*)] and one nuclear gene [elongation factor-1α (EF1α)] were generated for new samples using the methods described in Havill et al. (2007), except that DNA was extracted from individual adelgids using the Mag-Bind Blood & Tissue HDQ Kit (Omega Bio-tek, Norcross, GA). The EF1α intron was retained in the data sets for *Pineus* samples but excluded from phylloxerid and *Adelges* outgroup taxa because they could not be aligned. Sequences for each gene were aligned using ClustalW (Thompson et al.,1994) and concatenated. PartitionFinder 1.1.1 (Lanfear et al. 2016) was used to determine the most appropriate substitution model for the data.

Maximum likelihood (ML) and Bayesian analyses were performed for each gene separately and for the concatenated data matrix using a HKY+G model for 3rd codon positions of COI, COII, and cyt*b*, and GTR+I+G for all other codon positions. For maximum likelihood analysis, Garli 2.01 (Zwickl 2006) was run locally, with 5 search replications and 500 bootstrap replicates. The Bayesian analysis was run using Mr. Bayes 3.2.6 (Ronquist & Huelsenbeck 2012), with four heated Markov chains, and two runs of 10 million generations. Tracer v1.6.0 (Rambaut et al. 2009) was used to plot the log-likelihood scores versus number of generations to verify that the analysis had reached stabilization after discarding the first 25% of generations. Fifty percent consensus trees were visualized in Geneious 10.0.9 (Kearse et al 2012).

Maximum parsimony (MP) analysis was performed using PAUP* 4.0a152 (Swofford 2002) with tree-bisection-reconnection (TBR) branch swapping. Clade support was evaluated with 1000 bootstrap replicates with the same heuristic search conditions. Genetic uncorrected p-distances, number of nucleotide differences divided by the total number of nucleotides, among *Pineus* species was calculated using PAUP* 4.0a152 (Swofford 2002). Samples of *P. similis*, *P. abietinus* collected from *Abies*, and P. *abietinus* collected from *Pinus* were grouped for intra- and inter-specific comparisons.

3.4 Results

For outgroup taxa, sequences were included for all four genes in all except for COII in *Pineus* sp. B-rgf2008, and cyt*b* in *A. abietis*, *A. lariciatus*, and *A. laricis*. The final aligned and concatenated data set was 2321 base pairs long with insertions and deletions only in the EF1α intron. The MP analysis resulted in >500 equally parsimonious trees with a length of 2073 steps. Two of the five ML trees had a log likelihood score of -12661.05, and three had a score of -12661.31, showing convergence among replicates.

The topologies of the individual gene trees were consistent with each other and with the tree resulting from the concatenated data set. All of the trees placed the study group in a clade sister to the rest of the species in *Pineus*, with the exception of outlier *P. pinifoliae*. With this congruence in the analyses within the study group, the conclusions of this research will be based on the species tree, rather than the gene trees (Edwards et al. 2007).

The trees resulting from Bayesian, ML, and MP analyses agreed in topology. The 50% Bayesian consensus tree is shown in Figure 8, with clade support from all three analyses. The relationships among species in Adelgidae agree with those in Havill et al. (2007) with the exception of *A. cooleyi* and *A. tsugae,* whose placement within *Adelges* was unresolved in our analysis. We also added several additional taxa that were not included in Havill et al. (2007). These included the unidentified *Pineus* species from Foottit et al. (2008) [including *Pineus boerneri* (which was called *Pineus* sp. C)], and *P. pinifoliae*. The position of the latter species is unresolved within Adelgidae, but was placed in a basal position in *Pineus* with low support (<50%) in the best ML tree (not shown). The *P. similis* and *P. abietinus* samples formed a well-supported clade that was sister to the rest of the *Pineus* species minus *P. pinifoliae*.

Samples in the *P. similis-P. abietinus* clade were very closely related (Table 18). A sample of *P. abietinus* collected from *Pinus monticola* was in a basal position in the *P. similis-P. abietinus* clade with low support. Samples of *P. similis* and *P. abietinus* collected from *Pinus* were very closely related. The five samples of *P. abietinus* collected from *Abies* were monophyletic in the Bayesian tree (Figure 8), and formed a weakly-supported paraphyletic group in the ML tree (not shown). The single sample of *P. abietinus* collected from the bark of *Picea engelmanni* was in a basal position to the clade of *P. abietinus* collected on *Abies*.

The mean distance of species within *Pineus*, excluding the study group, was 28.07%. The distance between all specimens in the study group was 1.10%. The distance between *P. similis* and *P. abietinus* on *Pinus* is 0.51%, between *P. similis* and *P. abietinus* on *Abies* is 2.98% and between *P. abietinus* on *Pinus* and *Abies* is 2.32%.

3.5 Discussion and Conclusions

With the exception of *P. abietinus*, the relationships among species in Adelgidae in our results are consistent with past research of this family. While conducting a phylogenetic study on Adelgidae, Havill et al. (2007) found that groups of species that feed on the same secondary host genus form divergent clades, indicating that switching to a different host genus is rare in their evolutionary history. The unresolved placement of *A. cooleyi* and *A. tsugae* in our results do not affect this hypothesis since they are the only species known to utilize *Pseudotsuga* and *Tsuga*, respectively, as hosts.

The placement of *P. pinifoliae* at the base of Adelgidae or the base of *Pineus* is not surprising because it is morphologically unique within the genus in lacking gland facets in the dorsal plates of the head, thorax and first three abdominal segments (Annand 1928), and is the only member of the genus that has been placed in its own subgenus, *Pineodes*, based on its original description in 1926 (Börner 1926, Favret et al. 2015).

With a one nuclear and four mitochondrial genes included in these analyses, *P. similis* and the *P. abeitinus* on *Pinus* host cannot be separated. The distance between these groups were also found to be extremely low, 0.51%. This suggests that *P. similis* and the *P. abietinus* specimens on *Pinus* are conspecific, and that in some instances, *P. similis* and *P. abietinus* (*Pinus*) are capable of completing a holocycle. If this is the case, then it can be inferred that the holocyclic form of *P. similis* that migrates to *Pinus* is ancestral, because every other species within the Pineus genus uses Pinus as a secondary host (Havill and Foottit 2007).

There has been past research to test whether *P. similis* was in fact holocyclic. When *P. similis* was first described, Gillette (1907) did host selection tests by seeing if gallicolae would settle on *Pseudotsuga menziesii* as a potential secondary host. It was recorded to not be an acceptable host. Cumming (1962) also did host selection tests on this species, and found that *P. similis* gallicolae would not settle on *Pseudotsuga menziesii*, and only one individual settled on a *Pinus contorta* twig, but did not reproduce. *Pinus monticola*, *Pinus strobus,* or *Abies* spp. were not tested. More host selection tests should be done with *P. similis* gallicolae with these and related species to provide conclusive evidence for the existence of a holocycle.

The relationship between *P. abeitinus* found on *Abies* and *P. similis*- *P. abietinus* (*Pinus*) is less clear. These groups are clearly closely related with a mean pairwise nucleotide distance of 2.98%, but *P. abeitinus* found on *Abies* form a separate monophyletic group in the Bayesian analysis. One likely scenario is that *P. abeitinus* (*Abies*) is an anholocyclic descendent of the holocyclic form that arose after a secondary host switch from *Pinus* to *Abies*. This divergence is likely to have occurred very recently because of the short genetic distance. Through this hypothesis, *P. abietinus* (*Abies*) is a newly formed group which has started to separate from the *P. similis*- *P. abietinus* (*Pinus*) group.

Another possible scenario is that the same small clonal lineage stays on the secondary host, while the rest of the population has some gene flow through the *P. similis*- *P. abietinus* (*Pinus*) holocycle. It is therefore possible that all the specimens used in this study were from an isolated clonal lineage on a secondary host, and if we collected more specimens of *P. abietinus* from *Abies*, there could be a less distance between *P. abietinus* found on *Abies* and *P. similis*. If this scenario were true, this would be the first recorded case of a species within *Adelgidae* to have multiple secondary hosts in different subfamilies.

The single sample of *P. abietinus* from the bark of *Picea engelmanni* included in this analysis was basal to the *P. abietinus* (*Abies*) clade, but with low support (Figure 8). This points to the possibility that the ability to feed on the bark of *Picea engelmanni* might have mediated the host switch from *Pinus* to *Abies*. A holocyclic ancestor alternating between *Picea* and *Pinus* could have produced some individuals with the ability to settle on *Picea* bark, rather than on *Pinus* bark. Cumming's (1964) report of occasional "anomalies" in the life cycle of *P. similis,* where wingless individuals were found settled on *Picea* out of sync with alternating generations of fundatrices and gallicolae, could be evidence of this plasticity.

The results of this research shows that *Pineus similis* and *Pineus abietinus* form a grouping that is not fully expressed through the current taxonomy. The genetic distance and inability to resolve the relationship between these individuals from each other could point to these groups being forms of the same species. Sampling more individuals from each group would help to be sure there is not cryptic diversity that is being missed, as has been shown in other species in *Adelgidae* (Havill et al. 2016). In addition, transfer experiments from *Picea* to *Pinus monticola*, *Pinus strobus*, and *Abies* spp., and vice versa could test which *Pineus similis-Pineus abietinus* groups are capable of completing the holocycle.

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3.8 Tables

Table 17. Sample information. Vouchers deposited at the Canadian National Collection of Insects (CNC) or Yale Peabody Museum of Natural History (YPM). GenBank accession numbers are listed for DNA sequences; X represents genes not yet in GenBank.

Species	Voucher	Locality	Collector	Host plant	COI	COII	cytb	EF1a
Daktulosphaira vitifoliae Fitch	CNC#HEM054242	USA; California; Davis; 8 December 2004	J. Granett	*Vitis vinifera L*	KR041862	EF073121	EF073183	EF073221
Phylloxera caryaecaulis Fitch	CNC#HEM017791	CANADA; Ontario; Wolfe Island	M. Doyle	*Carya* sp.	EF073060	EF073122	EF073184	EF073222
Adelges abietis Linaeus	CNC#HEM053176	USA; Massachusetts; Jamaica Plain	N. P. Havill	*Picea abies* (L.) H. Karst.	EF073061	EF073123	--	EF073223
Adegles cooleyi Gillette	CNC#HEM053435	Poland	C. Bystrowski	*Pseudotsuga menziesii* (Mirb.) Franco	EF073065	EF073127	EF073185	EF073224
Adelges piceae Ratzeburg	CNC#HEM053155	USA; Maine; Owls Head	C. Donoghue	*Abies balsamea* (L.) Mill.	EF073085	EF073147	EF073194	EF073239
Adegles tsugae Annand	CNC#HEM053367	JAPAN; Osaka Prefecture; Nakahata	N. P. Havill, G. Yu, S. Shiyake	*Tsuga sieboldii* Carr.	EF073092	EF073154	EF073201	EF073243
Adegles glandulae Zhang	CNC#HEM053399	CHINA; Yunnan Province; Shangri-La	N. P. Havill, G. Yu	*Abies* sp.	EF073072	EF073134	EF073188	EF073229
Adelges japonicus Monzen	CNC#HEM050119	JAPAN; Hokkaido; Hitsujigaoka	K. Ozaki	*Picea jezoensis* (Siebold & Zucc.) Carr.	EF073073	EF073135	EF073189	EF073230
Adelges kitamiensis Inouye	CNC#HEM053415	JAPAN; Yamanashi Prefecture; Yamanaka	S. Shiyake	*Picea torano* (Siebold ex. K.Koch) Koehne	EF073102	EF073164	EF073210	EF073250
Adelges lariciatus Patch	CNC#HEM040004	CANADA; Alberta; Edson	E. Maw	*Picea glauca* (Moench) Voss	EF073075	EF073137	--	EF073231
Adelges laricis Vallot	CNC#HEM054132	SWITZERLAND; Delemont	N. P. Havill, M. Kenis	*Larix decidua* Mill.	EF073078	EF073140	--	EF073233
Adelges pectinatae ishiharai Inouye	CNC#HEM053389	JAPAN; Yamanashi Prefecture; Mount Fuji	N. P. Havill, G. Yu, S. Shiyake	*Abies veitchii* Lindley	X	X	X	X
Adelges pactinatae Cholodkovsky	CNC#HEM054110	POLNAD; Warsaw; Sowinski Park	N. P. Havill, C. Bystrowski	*Abies concolor* (Gordon) Lindley ex Hildebrand	EF073084	EF073146	EF073193	EF073238
Adelges sp B	CNC#HEM053359	CHINA; Yunnan Province; Lijiang	N. P. Havill, G. Yu	*Larix* sp.	EF073103	EF073165	EF073211	EF073251
Pineus armandicola Zhang, Zhong & Zhang	CNC#HEM053097	CHINA; Shaanxi Province; Huoditang Forest Farm	N. P. Havill, G. Yu, M. E. Montgomery	*Pinus* sp. (5-needle)	EF073106	EF073168	EF073212	EF073253
Pineus boerneri Annand	CNC#HEM061818	USA; Washington; Seattle	M.E. Montgomey, R. McDonald	*Pinus pinaster* Aiton	X	X	X	X
Pineus cembrae Cholodkovsky	CNC#HEM053433	POLNAD; Powsin	C. Bystrowski	*Pinus cembra* L.	EF073109	EF073171	EF073213	EF073254

Species	Voucher	Locality	Collector	Host plant	COI	COII	cytb	EF1a
Pineus coloradensis Gillette	CNC#HEM076222	USA; Washington; Beaver	G. Kohler	*Pinus monticola* Douglas ex D. Don	X	X	X	X
Pineus pini Macquart	CNC#HEM053442	POLAND; Ostrow Mazowiecka Forest District	C. Bystrowski	*Pinus sylvestris* L.	EF073114	EF073176	EF073216	EF073258
Pineus pinifoliae Fitch	15_037	USA; Idaho; Clark Fork	S. Kegley	*Pinus monticola* Douglas ex D. Don	X	X	X	X
Pineus similis Gillette	CNC#HEM026782	CANADA; British Columbia; New Denver	E. Maw	*Picea glauca* (Moench) Voss	X	--	--	--
Pineus similis Gillette	CNC#HEM026892	CANADA; British Columbia; Duhamel Road	R. G. Foottit	*Picea glauca* (Moench) Voss	X	--	--	--
Pineus similis Gillette	CNC#HEM054844	CANADA; British Columbia; Saanichton	C. Von Dohlen	*Picea sitchensis* (Bong.) Carr.	EF073116	EF073178	EF073217	EF073259
Pineus abietinus Underwood and Balch	CNC#HEM057990	USA; Oregon; St. Paul	G. Kohler	*Pinus monticola* Douglas ex D. Don	FJ502620	X	X	X
Pineus abietinus Underwood and Balch	CNC#HEM059802	USA; Idaho; Moscow	S. Lyons	*Pinus monticola* Douglas ex D. Don	X	X	X	X
Pineus abietinus Underwood and Balch	CNC#HEM061812	USA; Washington; Seattle	M.E. Montgomery, R. McDonald	*Pinus strobus* L.	KR036696	X	X	--
Pineus similis Gillette	CNC#HEM061820	USA; Washington; Seattle	M.E. Montgomery, R. McDonald	*Picea glauca* (Moench) Voss	KR043864	X	X	X
Pineus similis Gillette	CNC#HEM061825	USA; Washington; King County	M.E. Montgomery, R. McDonald	*Picea engemannii* Parry ex. Engelm.	X	X	X	X
Pineus similis Gillette	CNC#HEM070449	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR041314	X	X	--
Pineus abietinus Underwood and Balch	CNC#HEM076256	USA; Washington; North Seattle	G. Kohler	*Pinus monticola* Douglas ex D. Don	X	X	X	X
Pineus similis Gillette	CNC#HEM076303	USA; Idaho; Elk River	G. Davis	*Picea engemannii* Parry ex. Engelm.	X	X	X	X
Pineus similis Gillette	CNC#HEM076310	USA; Idaho; Elk River	G. Davis	*Picea engemannii* Parry ex. Engelm.	X	X	X	X
Pineus abietinus Underwood and Balch	CNC#HEM076317	USA; Montana; Darby	M. Church	*Abies lasiocarpa* (Hooker) Nuttall	X	--	--	--
Pineus similis Gillette	CNC#HEM076325	USA; Washington; Pomeroy	G. Kohler	Picea sp	X	X	X	X
Pineus abietinus Underwood and Balch	CNC#HEM076328	USA; Montana; Gallatin Gateway	M. Church	*Abies lasiocarpa* (Hooker) Nuttall	X	X	X	--
Pineus abietinus Underwood and Balch	CNC#HEM076329	USA; Montana; Gallatin Gateway	M. Church	*Abies lasiocarpa* (Hooker) Nuttall	X	--	--	--
Pineus abietinus Underwood and Balch	14-090	USA; Idaho; Dry Creek	S. Kegley	*Pinus monticola* Douglas. ex D. Don	X	X	X	X

Species	Voucher	Locality	Collector	Host plant	COI	COII	cytb	EF1a
Pineus abietinus Underwood and Balch	15-065	USA; Washington; Colville National Forest	D. Dickinson	*Pinus monticola* Douglas. ex D. Don	X	X	X	X
Pineus abietinus Underwood and Balch	15-193	USA; Idaho; Payette National Forest	L. Lowrey	*Abies lasiocarpa* (Hooker) Nuttall	X	X	X	X
Pineus abietinus Underwood and Balch	15-194	USA; Idaho; Payette National Forest	L. Lowrey	*Abies lasiocarpa* (Hooker) Nuttall	X	X	X	X
Pineus strobi Hartig	CNC#HEM053138	UDA; Connecticut; Bridgewater	N. P. Havill	*Pinus strobus* L.	EF073117	EF073179	X	EF073260
Pineus similis Gillette	CNC#HEM032874	USA; Idaho	E. Maw	*Picea engemannii* Parry ex. Engelm.	X	--	--	--
Pineus similis Gillette	CNC#HEM070450	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR042094	--	--	--
Pineus similis Gillette	CNC#HEM070451	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR033298	--	--	--
Pineus similis Gillette	CNC#HEM070453	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR041409	--	--	--
Pineus similis Gillette	CNC#HEM070454	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR038285	--	--	--
Pineus similis Gillette	CNC#HEM070456	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR040849	--	--	--
Pineus similis Gillette	CNC#HEM070457	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR044188	--	--	--
Pineus similis Gillette	CNC#HEM070458	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR044584	--	--	--
Pineus similis Gillette	CNC#HEM070459	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR045131	--	--	--
Pineus similis Gillette	CNC#HEM070460	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR040996	--	--	--
Pineus similis Gillette	CNC#HEM070462	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR040250	--	--	--
Pineus similis Gillette	CNC#HEM070463	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR043785	--	--	--
Pineus similis Gillette	CNC#HEM070464	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR032862	--	--	--
Pineus similis Gillette	CNC#HEM070466	CANADA; British Columbia; Salmon Arm	B. Bains	*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.	KR044341	--	--	--
Pineus similis Gillette	CNC#HEM076301	USA; Idaho; Elk River	G. Davis	*Picea engelmannii* Parry ex. Engelm.	X	X	X	X
Pineus similis Gillette	CNC#HEM076304	USA; Idaho; Elk River	G. Davis	*Picea engelmannii* Parry ex. Engelm.	X	X	X	X

Species	**Voucher**	**Locality**	**Collector**	**Host plant**	**COI**	**COII**	**cytb**	**EF1a**
Pineus similis Gillette	CNC#HEM076309	USA; Idaho; Elk River	G. Davis	*Picea engelmannii* Parry ex. Engelm.	X	X	X	X
Pineus sp A	CNC#HEM056294	JAPAN; Nagano Prefecture; Ina	S. Shiyake, M. E. Montgomery	*Pinus* sp.	X	X	X	X
Pineus sp B	CNC#HEM056254	JAPAN; Nagano Prefecture; Nagano	S. Shiyake, M. E. Montgomery	*Picea koyamai* Shiras.	FJ502626	--	X	X
Pineus sp D	CNC#HEM056259	JAPAN; Nagano Prefecture; Chino	S. Shiyake, M. E. Montgomery	*Pinus pumila* (Pall.) Regel	FJ502630	X	X	X
Pineus sp E	CNC#HEM053099	CHINA; Yunnan Province; Lijang	N. P. Havill	*Picea* sp.	FJ502632	X	X	X

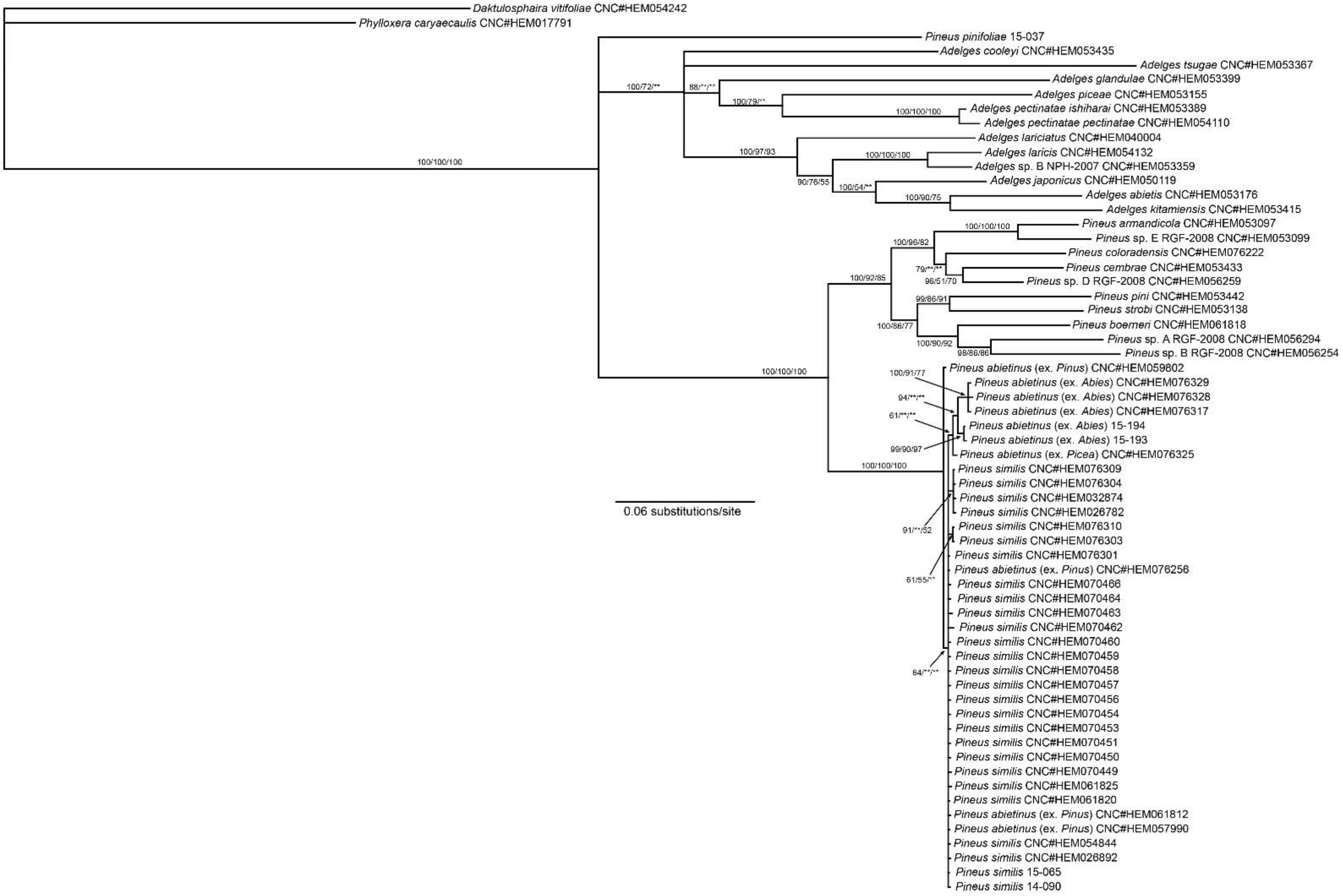
Table 18. Genetic distance (percent sequence divergence) for *Pineus* species in this study. Mean pairwise distances were calculated for species with multiple samples.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Pineus armandicola*													
2	*Pineus boerneri*	0.1813												
3	*Pineus cembrae*	0.1402	0.1634											
4	*Pineus coloradensis*	0.1410	0.1542	0.1370										
5	*Pineus pini*	0.2094	0.1722	0.1861	0.1796									
6	*Pineus pinifoliae*	0.2932	0.2958	0.3127	0.2734	0.2994								
7	*Pineus* sp A RGF-2008	0.1812	0.1484	0.1621	0.1654	0.1807	0.3280							
8	*Pineus* sp B RGF-2008	0.2075	0.1553	0.2033	0.1888	0.1828	0.3436	0.1579						
9	*Pineus* sp D RGF-2008	0.1431	0.1594	0.1178	0.1204	0.1708	0.2838	0.1694	0.1889					
10	*Pineus* sp E RGF-2008	0.1034	0.1883	0.1681	0.1578	0.2081	0.3128	0.1924	0.2065	0.1556				
11	*Pineus strobi*	0.1760	0.1568	0.1526	0.1546	0.1544	0.2855	0.1765	0.2111	0.1667	0.1817			
12	*Pineus similis*	0.2209	0.2110	0.2179	0.2053	0.2554	0.3071	0.2380	0.2481	0.2089	0.2299	0.2578		
13	*Pineus abietinus* (ex. *Pinus*)	0.1952	0.1800	0.1965	0.1768	0.2100	0.3104	0.2077	0.2144	0.1864	0.1992	0.2082	**0.0051**	
14	*Pineus abietinus* (ex. *Abies*)	0.2128	0.2164	0.2121	0.1966	0.2439	0.3116	0.2327	0.2406	0.2015	0.2277	0.2450	**0.0298**	**0.0233**

3.9 Figures

Figure 8. Bayesian 50% majority rule consensus tree of the concatenated data set. The three values shown for each clade are the Bayesian posterior probabilities, the maximum likelihood bootstrap support values, and the maximum parsimony bootstrap support values, in that order.

Figure 8.


Daktulosphaira vitifoliae CNC#HEM054242
Phylloxera caryaecaulis CNC#HEM017791
100/100/100
Pineus pinifoliae 15-037
100/72/**
Adelges cooleyi CNC#HEM053435
Adelges tsugae CNC#HEM053367
88/**/**
Adelges glandulae CNC#HEM053399
100/79/**
Adelges piceae CNC#HEM053155
100/100/100
Adelges pectinatae ishiharai CNC#HEM053389
Adelges pectinatae pectinatae CNC#HEM054110
100/97/93
Adelges lariciatus CNC#HEM040004
100/100/100
Adelges laricis CNC#HEM054132
Adelges sp. B NPH-2007 CNC#HEM053359
90/76/55
100/54/**
Adelges japonicus CNC#HEM050119
100/90/75
Adelges abietis CNC#HEM053176
Adelges kitamiensis CNC#HEM053415
100/100/100
Pineus armandicola CNC#HEM053097
Pineus sp. E RGF-2008 CNC#HEM053099
100/96/82
Pineus coloradensis CNC#HEM076222
79/**/**
Pineus cembrae CNC#HEM053433
96/51/70
Pineus sp. D RGF-2008 CNC#HEM056259
100/92/85
99/86/91
Pineus pini CNC#HEM053442
Pineus strobi CNC#HEM053138
100/86/77
Pineus boerneri CNC#HEM061818
100/90/92
Pineus sp. A RGF-2008 CNC#HEM056294
98/86/86
Pineus sp. B RGF-2008 CNC#HEM056254
100/100/100
Pineus abietinus (ex. Pinus) CNC#HEM059802
100/91/77
Pineus abietinus (ex. Abies) CNC#HEM076329
94/**/**
Pineus abietinus (ex. Abies) CNC#HEM076328
Pineus abietinus (ex. Abies) CNC#HEM076317
61/**/**
Pineus abietinus (ex. Abies) 15-194
99/90/97
Pineus abietinus (ex. Abies) 15-193
Pineus abietinus (ex. Picea) CNC#HEM076325
100/100/100
Pineus similis CNC#HEM076309
Pineus similis CNC#HEM076304
0.06 substitutions/site
Pineus similis CNC#HEM032874
Pineus similis CNC#HEM026782
91/**/52
Pineus similis CNC#HEM076310
Pineus similis CNC#HEM076303
Pineus similis CNC#HEM076301
61/55/**
Pineus abietinus (ex. Pinus) CNC#HEM076256
Pineus similis CNC#HEM070466
Pineus similis CNC#HEM070464
Pineus similis CNC#HEM070463
Pineus similis CNC#HEM070462
Pineus similis CNC#HEM070460
Pineus similis CNC#HEM070459
64/**/**
Pineus similis CNC#HEM070458
Pineus similis CNC#HEM070457
Pineus similis CNC#HEM070456
Pineus similis CNC#HEM070454
Pineus similis CNC#HEM070453
Pineus similis CNC#HEM070451
Pineus similis CNC#HEM070450
Pineus similis CNC#HEM070449
Pineus similis CNC#HEM061825
Pineus similis CNC#HEM061820
Pineus abietinus (ex. Pinus) CNC#HEM061812
Pineus abietinus (ex. Pinus) CNC#HEM057990
Pineus similis CNC#HEM054844
Pineus similis CNC#HEM026892
Pineus similis 15-065
Pineus similis 14-090

CONCLUSION

Though it is widely understood that adelgid species designation should represent biologically distinct lineages, informed by their evolutionary history, the complexity of their life cycle has made this difficult (Havill & Foottit 2007). In recent research, genetic analyses have been used to understand adelgid systematics without these past difficulties (Havill, Foottit & Dohlen 2007, Foottit & Havill 2009, Havill et al. 2006, Havill et al. 2016). This project has attempted to use multiple delimitation tools to better understand questioned species groups within the family Adelgidae. Using information on morphology, genetics, and life history we have added to the clarification of both the diversity of global populations of hemlock adelgids and the problematic species group *Pineus similis* and *Pineus abietinus*. Not being restricted by one species delimitation tool, we have gotten closer to understanding the relationship of individuals within these studies. We recommend using similar methods to better understand other problematic species groups within this complex family. Understanding this family by using multiple methodologies will help to fully comprehend adelgid diversity and their evolutionary history.

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APPENDICES

Appendix I. Measurements (μm ± SD) of the seven morphological characters of instar sistens hemlock adelgids, organized by lineage.

Variable	**1.Western China**	**2.Taiwan**	**3.*T .sieboldii***	**4.Western North America**
R4bw	29.36±1.74	27.00±3.73	28.50±3.85	32.63±5.48
R4L	33.31 ± 2.94^{2}	31.02 ± 1.75^{1}	32.13±2.35	34.03±2.19
R3mw	39.11 ± 1.28^{2}	35.35 ± 3.46^{1}	37.88 ± 4.57^{4}	43.71 ± 6.15^{3}
F3	44.17 ± 3.54^{3}	42.97±2.32	$42.43 \pm 2.40^{1,4}$	47.76 ± 3.36^{3}
Tb3	46.21 ± 6.70^{4}	47.84±3.88	47.52 ± 3.37^{4}	$51.92 \pm 1.50^{1,3}$
dTs3	$22.00 \pm 2.83^{2,3}$	19.44 ± 1.55^{1}	18.69 ± 1.92^{1}	19.78±1.76
Au	60.63 ± 6.70^{4}	62.89±2.76	61.74 ± 3.54^{4}	$67.45 \pm 2.81^{1,3}$

Superscript numbers describe which column that number was determined to be significantly different from, at an alpha level of 0.05

Appendix II. Measurements (µm ± SD) of the 13 morphological characters of instar progrediens hemlock adelgids, organized by lineage.

Variable	**1.Western China**	**2.Taiwan**	**3.*T. sieboldii***	**4.*T. diversifolia***	**5.Ulleung**	**6.Western North America**
R4bw	21.90±1.8^{2}	30.23±3.34^{1,3,4}	28.93±3.92^{1,2,4,6}	27.91±4.63^{2}	24.00±4.30	31.35±3.84^{3,4}
R4L	29.13±1.12	33.25±3.64^{3}	31.90±2.37^{2,4,6}	40.00±2.38^{3}	30.74±1.60	33.53±2.70^{3}
R3mw	30.30±2.07^{2}	39.60±3.84^{1,6}	39.26±5.31^{6}	39.35±5.37	33.39±4.46	43.72±4.98^{2,3,4}
F3	47.00±1.44	46.32±5.18^{3,6}	42.82±2.96^{2,4,6}	46.36±2.26^{3}	39.25±2.50	47.61±3.60^{3,6}
Tb3	50.33±4.07	52.01±4.76^{3}	47.47±3.29^{2,4,6}	52.22±3.31^{3}	46.30±1.11	53.18±4.34^{3}
dTs3	19.00±1.39	20.47±1.74^{3}	19.33±1.66^{2}	20.63±1.73	17.63±0.84	20.38±2.12
F2	43.77±2.27	44.76±3.21^{3}	40.83±3.94^{2,4,6}	45.15±2.51^{3}	38.47±1.94	46.05±3.36^{3}
Tb2	47.85±0.07	48.14±3.52^{3,6}	45.16±3.22^{2,4,6}	49.53±2.90^{3}	41.53±2.82	50.94±4.51^{2,3}
dTs2	20.37±0.51	19.45±1.66^{3}	17.94±1.54^{2,4}	19.84±1.46^{3}	16.96±1.13	19.12±1.91
F1	42.37±2.27	43.57±4.16^{3}	39.69±3.00^{2,4,6}	44.03±2.83^{3}	37.42±0.30	44.01±4.06^{3}
F1w	21.87±3.57	23.01±3.27^{4,6}	23.37±2.92	21.47±2.09^{2}	19.53±3.04	24.98±2.68^{2,4}
Tb1	43.17±5.38	46.25±4.32^{3,6}	44.60±3.47^{2,4,6}	48.18±3.11^{3}	40.46±2.61	50.30±4.54^{2,3,4}
Au	56.67±1.22^{2}	64.25±5.06^{1,3}	62.34±4.41^{2,4,6}	66.47±3.18^{3}	52.39±5.72	65.99±4.28^{3}

Superscript numbers describe which column that number was determined to be significantly different from, at an alpha level of 0.05.

Appendix III. Measurements (µm ± SD) of the seven morphological characters of adult sistens hemlock adelgids, organized by lineage.

Variable	**1.Western China**	**2.Taiwan1**	**3.*T. sieboldii***	**4.Ulleung**	**5. Western North America**
R4bw	43.16±7.26^{2,5}	41.45±10.43	55.39±12.96	39.00±2.69	57.27±14.91
F3	80.44±11.45^{2,4,5}	97.71±17.22^{1,3}	85.43±17.62^{2,5}	72.60±6.29^{1}	112.51±31.51^{1,3}
F3w	40.09±6.869^{3,4,5}	44.34±11.73^{1}	47.96±10.81^{1}	31.79±2.17^{1}	54.94±15.95^{1}
F2	69.92±9.50^{2,3,4,5}	79.16±15.58^{1,3}	75.62±13.41^{5}	63.16±5.41^{1}	96.14±26.17^{1}
Tb1	48.48±6.47^{1,3,5}	58.36±8.25^{1}	59.14±8.97^{1,5}	51.93±4.83	73.63±14.65^{1,3}
Au	20.69±3.99^{2,3}	27.74±13.81^{1}	24.64±4.49^{1}	27.06±2.75	28.38±6.95
Ov	73.96±12.70^{4}	76.87±20.44	72.55±16.45	44.91±11.67^{1}	73.75±25.15

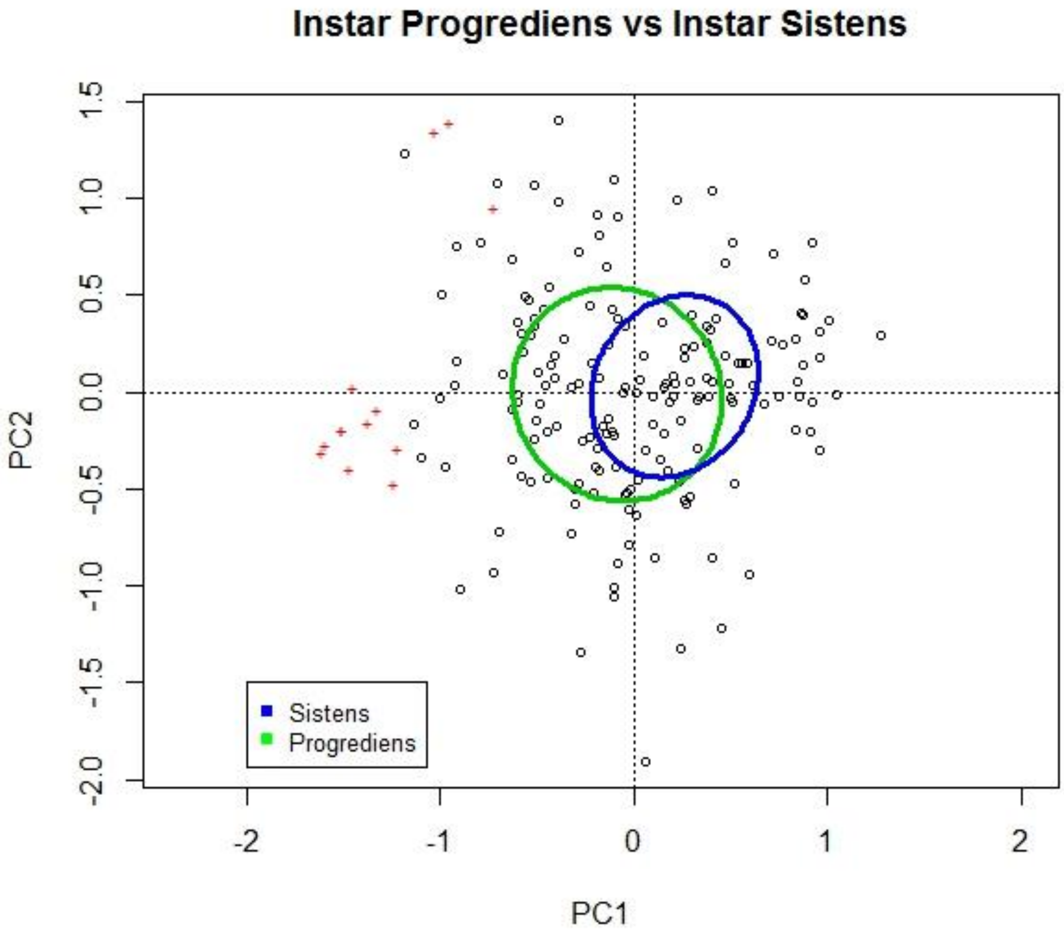
Superscript numbers describe which column that number was determined to be significantly different from, at an alpha level of 0.05.

Appendix IV. Measurements (µm ± SD) of the four morphological characters of adult progrediens hemlock adelgids, organized by lineage.

Variable	**1.Western China**	**2.Taiwan**	**3.*T. sieboldii***	**4. Western North America**
R4bw	$34.47 \pm 4.31^{3,4}$	42.60 ± 3.62^{4}	$44.41 \pm 5.39^{1,4}$	$50.14 \pm 5.52^{2,3}$
F3	80.23 ± 24.24^{4}	84.13 ± 5.06^{4}	76.60 ± 10.86^{4}	$112.24 \pm 13.09^{2,3}$
F3w	$32.30 \pm 6.27^{3,4}$	35.93 ± 5.08^{4}	$39.83 \pm 5.68^{1,4}$	$43.92 \pm 5.65^{1,3}$
Au	33.75 ± 7.15^{3}	22.40 ± 1.14^{4}	$22.42 \pm 6.69^{1,4}$	$35.53 \pm 4.40\text{A}^{2,3}$

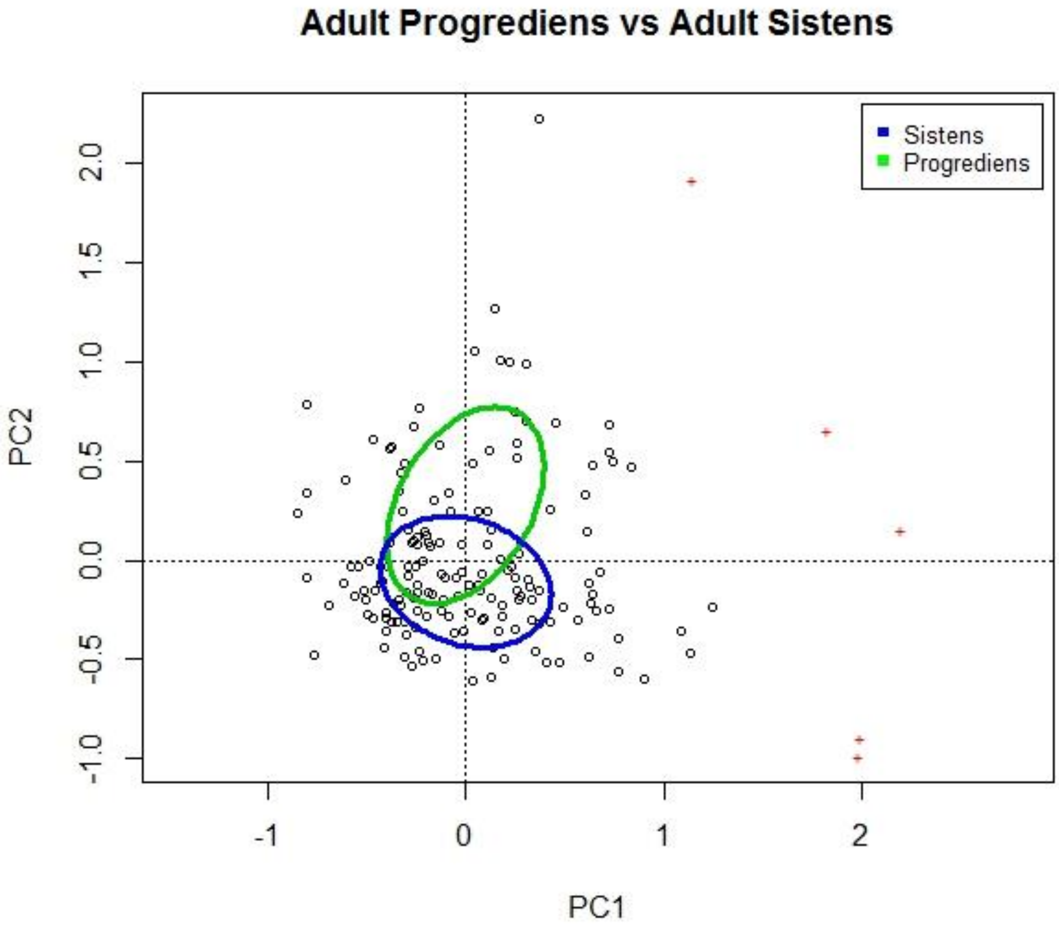
Superscript numbers describe which column that number was determined to be significantly different from, at an alpha level of 0.05.

Appendix V. Principal component analysis of 1st instar specimens, separated by generation.



MANOVA found generations to be significant, is p<0.001, N=171, DF= 65, 785.

Appendix VI. Principal component analysis of adult specimens, separated by generation.



MANOVA found generations to be significant, p<0.001, N=162, DF=30, 775.