

Allozyme Variation in Hemlock Woolly Adelgid (Hemiptera: Adelgidae) From the United States and China

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ABSTRACT The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is a major introduced pest of eastern hemlock, *Tsuga canadensis* (L.) Carrière. Hemlock woolly adelgid in the United States is anholocyclic and an obligate parthenogen, because no suitable primary host (on which sexual reproduction occurs in Asia) is reported for this adelgid in the United States. Allozyme analyses were conducted to assess the relatedness between three hemlock woolly adelgid populations in the eastern United States (Connecticut, New Jersey, and Virginia) and to compare them with adelgids from the western United States (Washington) and China. Significant hemlock woolly adelgid allozyme variation was found between nonadjacent trees within a site in the eastern United States. There was a small difference between the two northern and the southern populations despite the relative genetic uniformity between the sites across the allozyme loci evaluated. There were low numbers of protein alleles in the eastern populations, whereas the western and Chinese populations had additional alleles not found in the eastern populations. Both heterozygote excesses and deficits were detected as would be expected in populations that are not randomly mating. There were only a few abundant genotypes and they tended to be more homozygous than the rarer genotypes. The hemlock woolly adelgid population from China that is cyclically parthenogenic was more genetically diverse than the other hemlock woolly adelgid populations that are presumed to be obligately parthenogenic. Allozymes provide some useful information about genetic variation in hemlock woolly adelgid but seem to be less suited to assess actual genetic diversity and structure of populations because of the small size of the hemlock woolly adelgid.

KEY WORDS *Adelges tsugae*, genetic diversity, allozymes, parthenogenic

The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an aphid relative that feeds on hemlocks (*Tsuga* spp.). It was first described ≈80 yr ago from specimens collected from western hemlock, *Tsuga heterophylla* (Raf.) Sargent, originating from Eugene, OR; Fort Bragg, CA; and Vancouver, BC, Canada, in the Pacific Northwest (Annand 1924). It was collected some 30 yr later on the Atlantic seaboard in Richmond, VA, from eastern hemlock, *Tsuga canadensis* (L.) Carrière, stands and on the estate of a former avid plant collector (Souto et al. 1996, Stoetzel 2002). Although the hemlock woolly adelgid is capable of infesting all species of *Tsuga* native to North America, the eastern hemlock species *Tsuga canadensis* (L.) and *Tsuga caroliniana* Englemann are the only species that have experienced widespread mortality likely because the eastern species have limited resistance (McClure 1992), and there is a general lack of native natural enemies (McClure 1987). Currently, hemlock woolly adelgid is considered to be one of the most important pests of eastern hemlock and its potential impacts on the hemlock ecosystems have caused great

concern. The adelgid's effects on its hemlock hosts in its eastern range have been implicated in altering the forest landscape and may precipitate the rapid conversion of previously cool, clear, streamside hemlock sanctuaries into relatively warmer, open areas, which can alter stream conditions and dependent faunal guilds (Evans 2002). These openings also provide areas where rapidly colonizing hardwood species can invade and grow, further altering the local habitat (Orwig and Foster 2000).

Adelgids have complex lifecycles with polymorphisms that often include host alternation, where reproduction on primary hosts is both sexual and asexual and on secondary hosts is only parthenogenic. Under northeastern winter temperatures, the all-female overwintering generation reaches the adult stage in February and females begin laying eggs in March. As early as April, these eggs hatch into nymphs of one of two distinct adult forms: one form that remains on the host and a winged form that migrates off the hemlock, seeking a secondary host (McClure 1989). The thelytokous second generation form stays on its hemlock host and continues feeding during the late spring and early summer, and lays eggs that hatch around late

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Table 1. Collection information for *A. tsugae* collected from *Tsuga* sp.

Region	State	Town	Latitude, longitude	Elevation (m)	Mo/yr collected	Allozyme no. individuals	No. trees sampled	Code
Eastern United States	Connecticut	Hamden	41° 23.73' N, 072° 55.72' W	31	Aug./2000, April/2001	104	5	CT
Eastern United States	New Jersey	Haney Mills	41° 07.51' N, 074° 54.53' W	144	June/2000, March/2002	77	3	NJ
Eastern United States	Virginia	Blacksburg	37° 13.56' N, 080° 25.35' W	619	March/2002	120	3	VA
Western United States	Washington	Seattle	47° 39.30' N, 122° 18.40' W	15	July/2000, March/2002	40	2	WA
China	Sichuan	Kangding	30° 30.00' N, 101° 58.00' E	2,800	April/2002	47	5	CH

June to early July to become the source of the thelytokous overwintering generation (McClure 1989). Because no suitable secondary spruce host is reported for this adelgid in the eastern United States (McClure 1987), sexual reproduction is presumed to be absent in eastern states. This precludes genetic recombination among extant populations on the Atlantic seaboard. In addition, once the adelgid settles and inserts its mouth parts it does not move, this species only actively disperses as newly hatched crawlers. Consequently, it is likely that contiguous groups of diffusely distributed hemlock woolly adelgid are so genetically similar that they might be considered to be reproducing clonally. However, other factors can promote genetic diversity in geographically distributed populations. For example, variability can result if the founding parents arrived from multiple introductions or origins. Geographic variability can also result from uneven selection pressures across the broad range of this pest that result in the selection of different phenotypes present in the founding population or resulting from mutation. A model based on hemlock woolly adelgid population densities indicates that in a forested area of <1-ha mutation saturation (an average of a mutation every generation at every base pair in the entire genome) could occur (Butin et al. 2005). In addition, there is evidence that hemlock woolly adelgid has already evolved greater resistance to cold shock in the northern part of the range (Butin et al. 2005).

The distribution of hemlock trees in North America is disjunct, with two western hemlock species restricted to the Pacific Northwest and two eastern hemlock species distributed along the East Coast. Adelgid populations in the Pacific Northwest are generally less abundant than in the East, are genetically distinct from the eastern populations, and may be endemic to that region (Havill et al. 2006). Alternately, eastern populations of hemlock woolly adelgid are large, widely distributed in eastern hemlock habitats between 35° N and 45° N, and all share a single mitochondrial DNA haplotype (Havill et al. 2006). Over this range climatic conditions differ, and factors such as lower winter temperatures in the northeastern part of the current range can cause high overwintering mortality (Skinner et al. 2003, Shields and Cheah 2005). Evaluating heritable characters of the hemlock woolly adelgid along such north-south gradients is one way of deter-

mining their genetic diversity and estimating the number of distinct populations. Current research on hemlock woolly adelgid microsatellites is showing that there is almost no variation (out of five loci examined so far, one locus is polymorphic and has two genotypes; one genotype being rare) present in the populations from throughout the eastern United States but in the native range in Japan, where sexual reproduction occurs, genotypic variation is very high with multiple genotypes per site sampled (Annie Paradis and Nathan Havill, personal communication).

Heritable macromolecules such as allozymes are another method that can be used to provide estimates of large-scale measures of genetic diversity (Murphy et al. 1990), although the conventional methods typically require large volumes of sample per animal for obtaining broad measures of genetic diversity. Alternate methods developed for small arthropods (Herbert and Beaton 1993) can provide similar estimates using low volumes of gene derived enzymes or allozymes. Allozymes are physiologically important molecules reflecting large scale genetic differences in taxa and may be useful for identifying such differences among populations of hemlock woolly adelgid. Here, we report the relative genetic similarity of hemlock woolly adelgid populations collected from different trees within a single site and between three sites along the Atlantic Coast of the United States by using allozyme electrophoresis results. We also compare the eastern populations with one population from a coastal site in the Pacific Northwest and one population from northern China, which have been shown to be from genetically distinct ancestries (Havill et al. 2006). The goal was to determine whether allozyme variation existed at the different levels (within site, between sites, and between regions) and how useful they would be for understanding genetic diversity and population structure.

Materials and Methods

Hemlock woolly adelgids were collected in 2000, 2001, and 2002 from low to moderate density (10–20 adelgids per cm of ≤3-yr growth) sites, on eastern hemlock from three eastern states (Virginia [VA], New Jersey [NJ], and Connecticut [CT]) and on western hemlock from Washington (WA) (Table 1).

Additionally, in 2002, adelgids were obtained from *Tsuga chinensis* (Franchet) Pritzl collected in Sichuan Province, China (CH), to provide an out group. The adelgids from China were transported under permit to the Forest Service quarantine facility in Ansonia, CT. Slide-mounted voucher specimens have been deposited at the Yale University Peabody Museum (New Haven, CT).

At each collection site in the United States, five hemlock woolly adelgid infested branches were clipped from two to five nonadjacent hemlock trees whose branches did not touch. Different trees were sampled if a site was revisited in different years. This sampling scheme assumes that some clonal variation exists between trees at a site, as was found for a very sedentary parthenogenetic moth previously evaluated using allozymes (Menken and Wiebosch-Steeman 1988). Infested hemlock branches were put in zip lock polyethylene bags labeled with the site and tree number (material from multiple trees combined for the Chinese population) and kept moist and cool during transit. Upon arrival at the laboratory, the collected host material was divided, placed into double freezer bags, and stored at 5 and -70°C for later analysis. Material stored at 5°C was used as source of fresh live samples to conduct initial survey for allozymes, whereas material stored at -70°C provided additional stock for analyzing the most informative enzymes. All the adelgids (adults or large nymphs) used were removed from the terminal branch tips of current or previous year's growth and cleaned of wax before use. In successive electrophoresis runs of the same allozymes, adelgids were taken from different branch tips and/or trees (depending on the available live material) from each population being tested. Both the small size of adelgids and their susceptibility to damage during removal from hosts can lead to enzyme degradation and secondary products from digestive juices that affect the ability to resolve their allozymes. To prevent this, adelgids were removed from host material as needed and immediately homogenized.

Allozyme analysis was carried out using the Titan III cellulose acetate electrophoresis method developed for use with small aquatic invertebrates (Herbert and Beaton 1989) modified for adelgid chemistry. Adelgids were individually homogenized in the block wells of the Helena sample distribution system to minimize sample loss. After screening a variety of protein extracting systems, an aphid homogenizing buffer recipe was chosen that uses 10% sucrose in Triton X-100, a detergent that further stabilized enzymes and increased resolution (Puterka et al. 1993). Gels were run for 15 min at 200 V (2 mA per gel plate) and then stained using standard methods (Murphy et al. 1990, May 1992).

Seven enzymes were chosen that had good resolution and retention after an initial screening of 28 enzyme systems using 10–20 sample adelgids from CT and two buffer systems (Tris glycine, pH 8.0 and citric acid morpholine, pH 7.0): esterase (EST), fumerase (FUM), glucose-6-phosphate isomerase (GPI), malate dehydrogenase (NAD dependent) (MDH),

phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM), and triose-phosphate isomerase (TPI). The selection of only seven loci to evaluate was necessitated because limited numbers of adelgids were available from some populations; and, on average, only five enzymes produced resolvable products when using a single adelgid due in part to the insect's small size. Allozyme data for an average of 42 (minimum of 10) adelgids from each of the five populations were obtained for the seven enzyme systems. Allozyme linkage analysis was also not possible because of the inability to test all the enzymes on each individual insect. Two reference lanes of CT adelgids (VA when CT was sampled) were run as internal controls per cellulose acetate plate to ensure comparability among plates.

Putative enzyme loci resolved were scored using a protocol described in May (1992) and Sánchez and Cardé (1995) that considers a locus polymorphic if the mean frequency of the most common allele is ≤ 0.95 , labels the most common allele as 1, and assigns subsequent alleles in sequential number. Allele frequencies and observed (H_o) and expected (H_s) heterozygosities and deviations from Hardy-Weinberg equilibrium for the seven loci and five populations were obtained using Arlequin version 3.1 (Excoffier et al. 2005). Average heterozygosity, both observed and expected, also were obtained for each population over the seven loci by using Arlequin version 3.1 (Excoffier et al. 2005). Allele frequencies and average heterozygosities were compared using a Yate's corrected chi-square (Analytical Software 2003). A modified false discovery rate (FDR) method (Benjamini and Hochberg 1995) was used to determine the experiment-wide significance level to limit type 1 error because of multiple tests being performed. Between-tree variations in genotype frequencies were evaluated for adelgids from VA and data for two loci with more than one allele present. A Nei pairwise population matrix of genetic distance for all populations was calculated and used to run a principle coordinates analysis in GenALEX 6 (Peakall and Smouse 2006) to look for major patterns in relatedness between the populations. Data for only the first two coordinates are used.

Results

The allele frequencies and heterogeneity estimates for the seven polymorphic loci (EST, FUM, GPI, MDH, PGD, PGM, and TPI) assessed across all populations are shown in Table 2. A single allele was present in the eastern United States populations at the EST and PGD loci, whereas a second allele was only found in one population at the PGM and GPI loci. The Chinese population had two alleles present at four loci and three alleles at the remaining loci. At the FUM locus, two alleles were present in all populations, and an additional third allele was found only in the Chinese and western populations. Two alleles were present in all the populations at the MDH locus and in all but one of the United States populations (CT) for

Table 2. Relative allele frequencies and observed (H_o) and expected (H_s) heterozygosities at seven enzyme loci for five geographic populations of the hemlock woolly adelgid

Locus	Alleles gene diversity	Frequencies by pop (no. unresolved/n)				
		VA	NJ	CT	WA	CH
EST	1	0.994	1.000	1.000	0.375	0.308
	2	0.006	0.000	0.000	0.625	0.615
	3	0.000	0.000	0.000	0.000	0.077
		(0/84)	(4/70)	(7/69)	(0/40)	(3/16)
	H_o	0.012	0.000	0.000	0.000*	0.000*
FUM	H_s	0.012	0.000	0.000	0.469	0.521
	1	0.885	0.585	0.659	0.423	0.333
	2	0.115	0.415	0.341	0.404	0.542
	3	0.000	0.000	0.000	0.173	0.125
		(1/53)	(11/52)	(10/51)	(11/37)	(3/15)
GPI	H_o	0.231	0.829*	0.683*	0.423*	0.250*
	H_s	0.204	0.485	0.450	0.628	0.580
	1	1.000	0.980	1.000	0.923	0.944
	2	0.000	0.020	0.000	0.077	0.056
		(2/84)	(11/62)	(5/64)	(1/40)	(3/21)
MDH	H_o	0.000	0.000*	0.000	0.000*	0.000*
	H_s	0.000	0.038	0.000	0.142	0.105
	1	0.981	0.814	0.795	0.794	0.273
	2	0.019	0.186	0.205	0.206	0.727
		(0/52)	(4/47)	(1/45)	(3/37)	(3/14)
PGD	H_o	0.000*	0.372	0.364	0.412	0.182
	H_s	0.038	0.303	0.325	0.327	0.397
	1	1.000	1.000	1.000	0.921	0.833
	2	0.000	0.000	0.000	0.079	0.167
		(2/88)	(6/60)	(2/58)	(2/40)	(1/13)
PGM	H_o	0.000	0.000	0.000	0.000*	0.000*
	H_s	0.000	0.000	0.000	0.145	0.278
	1	1.000	1.000	1.000	0.842	0.821
	2	0.000	0.000	0.000	0.158	0.179
		(6/15)	(1/13)	(1/13)	(6/25)	(3/17)
TPI	H_o	0.000	0.000	0.000	0.000*	0.071*
	H_s	0.000	0.000	0.000	0.266	0.293
	1	0.977	0.922	1.000	0.847	0.525
	2	0.023	0.078	0.000	0.153	0.402
	3	0.000	0.000	0.000	0.000	0.025
Avg. H_o		(0/65)	(0/58)	(0/36)	(4/36)	(4/20)
	H_o	0.046	0.155	0.000	0.083*	0.550
	H_s	0.045	0.143	0.000	0.259	0.521
	SE	0.041	0.194	0.150	0.131*	0.150*
	SE	0.030	0.109	0.095	0.069	0.071
Avg. H_s		0.043	0.138	0.111	0.319	0.385
	SE	0.026	0.066	0.067	0.062	0.060

Observed heterozygosities followed by an asterisk (*) are significantly different from expected based on tests for deviations from Hardy-Weinberg equilibrium assessed using Arlequin version 3.1 (Excoffier et al. 2005). A modified FDR method (Benjamini and Hochberg 1995) was used to determine the experiment-wide significance level to limit type 1 error because of multiple tests being performed. The number unresolved are a count of the number of individuals that neither allele resolved. There was only one case of a null allele (VA at the TPI locus) where one case resolved and the other did not.

the TPI locus. The outlying Chinese population had an additional allele at the TPI locus that was not observed in any of the United States populations.

Gene frequencies were not significantly different at five of the seven loci among the eastern United States populations, with the most common allele at each locus (allele 1, Table 2) varying from 0.8 to 1.0. The alternate allele (allele 2) was found at a higher frequency at the EST locus in the WA population and at >0.3 in three of the four United States populations at the FUM locus. In the Chinese population, allele 2 was found at a higher frequency at the EST and FUM loci,

Table 3. Genotype frequencies at two polymorphic loci for adelgids from nonadjacent eastern hemlocks collected from Blacksburg, VA

Locus	Genotype	Genotype frequency (n)		
		Tree 1	Tree 2	Tree 3
FUM	11	0.90 (18)	0.18 (2)	0.96 (24)
	12	0.10 (2)	0.82 (9)	0.04 (1)
	22			
TPI	11	1.00 (25)	1.00 (21)	0.69 (22)
	12			0.31 (10)
	22			

and at about the same frequency as allele 1 at the TPI locus.

Observed heterozygosity was zero at two (PGD and GPI) of the seven polymorphic loci for all populations and at the PGM locus for all the United States populations (Table 2). In each case where the observed heterozygosity was zero for a locus, only the homozygous genotypes were present in that population and the second allele was found at very low frequency in only the WA and/or CH populations. At the TPI, FUM and MDH loci only the allele 1 homozygote and the heterozygote were observed in the eastern United States populations, with the exceptions of single individuals from the VA and CT populations that were homozygous for allele 2 at the MDH locus. The allele 3 homozygote was not observed in any population at the FUM locus and the allele 2 homozygote was also missing at the MDH locus from the WA population. Each of the populations were not in Hardy-Weinberg equilibrium at some of the seven loci but average observed heterozygosities were only significantly different from expected in the WA and China populations (Table 2).

Genotype frequencies varied significantly between adelgids collected from nonadjacent trees within the VA population for one (FUM) of the two polymorphic loci evaluated (Table 3). Only the heterozygous and one homozygous genotype (most common allele) were present for each locus at these sites. The second allele, in the heterozygous form, was not detected on all the trees and was present at higher frequencies than the allele one homozygote on some trees. Similar results were seen for other eastern United States sites but not presented here.

Principle coordinate analysis based on the calculated Nei distances between all the populations (Table 4) showed three distinct groups (eastern United States, western United States, and China) coinciding with their geographic origins (Fig. 1). The first

Table 4. Nei pairwise population genetic distances for HWA from five sites based on allozyme results for seven enzyme loci

	CH	CT	NJ	VA	WA
CH	0.000				
CT	0.209	0.000			
NJ	0.197	0.002	0.000		
VA	0.277	0.013	0.018	0.000	
WA	0.090	0.086	0.082	0.103	0.000

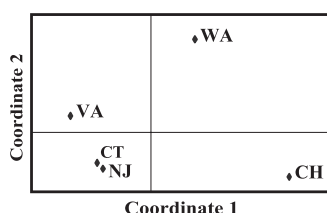


Fig. 1. Principle coordinate analysis using Nei pairwise population genetic distances based on allozyme results for seven enzyme loci.

and second coordinates explained 85 and 13%, respectively, of the variation. The CT and NJ populations were very similar, whereas the VA population was somewhat different based on Nei distances.

Discussion

Hemlock woolly adelgid populations from nonadjacent trees in an eastern United States population varied significantly in allozyme genotype frequencies. This is significant because there is only one mitochondrial DNA haplotype (Havill et al. 2006) and almost no microsatellite variation found in the eastern United States populations evaluated to date (Annie Paradis, personal communication). Similar allozyme results were found for a very sedentary parthenogenetic moth (Menken and Wiebosch-Steeman 1988); the clonal composition of populations on two trees in proximity differed significantly. This is not surprising because hemlock woolly adelgid is sedentary except in the crawler stage (limited active dispersal) and dispersal beyond a single tree is through passive wind dispersal or hitch-hiking of the crawlers on animals, vehicles or cargo. Thus, the allozyme clones present on even adjacent trees could differ in both presence and abundance. Even with our limited sampling of some sites our results are consistent with what is expected given the clonal nature of this insect in the eastern United States and the presence of cyclic sexual reproduction in China.

Populations of the hemlock woolly adelgid from the eastern United States were polymorphic at 25% of the 12 loci evaluated. This is higher than the 9% average and lower than the 42% maximum reported in a broad survey of pest aphids (Tomiuk and Wöhrmann 1980) and higher than the average value (11%) for other insects (Graur 1985). This could be because of the relatively small number of loci that could be completely evaluated and the low number of trees sampled, but that the CH population was polymorphic at all seven of the loci evaluated may indicate that polymorphic loci are more common in this adelgid, especially in areas where sexual reproduction occurs. This is consistent with what has been found for aphids, where about twice as many alleles and genotypes have been found in sexually reproducing than in asexually reproducing populations of the same species (Delmotte et al. 2002).

Surveys of the genotypic frequencies revealed that there were deviations from Hardy-Weinberg expect-

tations for a randomly mating population, as would be expected because the hemlock woolly adelgid is either obligately parthenogenetic (United States) or cyclically parthenogenetic. These deviations resulted from either heterozygote deficiencies (EST, FUM, GPI, MDH, PGD, PGM, and TPI) or excesses (FUM) depending on the locus and population. The heterozygote excesses only occurred in two eastern United States populations where sexual recombination is absent. These excesses are likely the result of selection for the most viable clonal genotypes (including new mutants) or population bottlenecks that occurred soon after introduction. There is mounting evidence that clones mutate rapidly and that some of this variation is maintained and adaptive (Loxdale 2008). The size of the adelgid population in the eastern United States is large enough that mutants should occur regularly as heterozygotes and adaptive selection has already been documented (Butin et al. 2005). Because both the populations that had heterozygote excesses were from New England (CT and NJ) selection for more cold tolerant clones may have directly (this enzyme itself involved) or indirectly (this enzyme is linked with genes involved) resulted in these excesses.

The predominance of homozygous genotypes at allozyme loci resulting in heterozygote deficiencies is common in aphids (Loxdale and Brookes 1989, Sunnucks et al. 1997, Simon et al. 1999) and more common in cyclical parthenogens than obligate parthenogens (Vorburger et al. 2003). Possible explanations for this pattern according to Delmotte et al. (2002) include null alleles, clonal selection, inbreeding, and allochronic isolation. Failure of individuals to produce a resolvable result was fairly common but it seemed to be directly correlated with the size and quality of the insects being used and occurred across all populations and for all loci. In addition, every time an electrophoresis run was setup the homogenized insects were used completely and the last allozyme locus may have received insufficient material resulting in no resolution so the data for that enzyme was excluded from the analysis. Other allozyme loci may have received insufficient material when large larvae were used in combination with adults and thus resulted in no resolution. There was only one case where a single allele copy was resolved for one individual (VA population at the TPI locus). It is unlikely that null alleles are the cause of heterozygote deficits because they should be equally distributed across alleles because they are probably the result of mutations that result in loss of enzyme function.

Clonal selection is a possible cause of heterozygote deficiencies for the same reasons that it could have resulted in homozygote deficits. Even in cyclically parthenogenetic populations there is evidence that intense selection occurs in the asexual generations that results in a few common genotypes (more often homozygous form) and only a few copies of rare genotypes (possibly because of mutation events) remaining in the population (Sunnucks et al. 1997, Vorburger 2006). This is consistent with the general-pur-

pose-genotype hypothesis that predicts few, broadly adapted, asexual genotypes should be present in fluctuating environments.

Inbreeding is also a possibility, not because it has been shown to actually occur but because of sampling limitations for some of the populations evaluated. The hemlock woolly adelgid obtained from both WA and CH was limited and not of the best quality (many dead because of predator feeding or conditions during shipping), and the CH material was all thrown together so source trees were not known. Thus, fewer clones may have been sampled than were actually present. Allochronic isolation may be possible in the CH population because hemlock woolly adelgid occurs over a large geographic range and sexual reproduction occurs but is unlikely to be a factor in the current analyses given the limited sampling of only one population.

There was a general lack of large genetic differentiation between the three eastern United States populations. This was not unexpected given that a single mitochondrial DNA (mtDNA) haplotype has been found in hemlock woolly adelgid in the eastern United States, and the only other occurrence of that haplotype was in a sample from southern Honshu, Japan (Havill et al. 2006). In addition, microsatellite variation is limited in the eastern United States (Annie Paradis, personal communication) where only parthenogenic reproduction occurs. The level of genetic variability expected in non-native taxa after their accidental introductions into a new area is a function of the number of genotypes introduced. For example, it was deduced that the accidental escape of *Lymantria dispar* (L.) in Massachusetts in 1869 was from a limited sample because of the few genotypic patterns and low variability observed among eastern North America populations by using allozyme analysis (Harrison et al. 1983) and later confirmed using mitochondrial DNA (Bogdanowicz et al. 2000). More recent evaluation of gypsy moth microsatellite loci from worldwide populations has shown that the genotypes present in the United States are a subsample of those found in the rest of the range (Keena et al. 2008). In hemlock woolly adelgid, there are no unique alleles present in the United States populations, and the genotypes present represent only a subsample of the genotypes found in the CH and WA populations, suggesting that there was a limited founder population from which it started or that strong clonal selection occurred since the introduction. Evidence that the introduction of the adelgid probably occurred with the planting of one or a few specimen trees collected from the same site in its native range (Souto and Shields 2000) further substantiates a limited introduction history for this insect.

There was clear genetic differentiation between the WA population and those in the eastern United States. This is consistent with mtDNA results that found western hemlock woolly adelgid to be distinct from those found on in the east (Havill et al. 2006). Also, during the collections off *T. heterophylla* in WA, western

hemlock woolly adelgid populations exhibited a behavior not reported previously for eastern populations in that adult and later instar nymphs were found feeding on the tree bole in addition to the usual feeding site at the leaf base (Young et al. 1995). Compared with the United States populations, the Chinese populations were even more genetically variable, which is not unexpected because cyclic sexual reproduction likely occurs at this site and a leading hypothesis suggests the adelgid has an ancestral origin in Asia (McClure 1987, Zilahi-Balogh et al. 2002).

These results suggest that allozymes are picking up more genetic diversity and structure in the eastern United States hemlock woolly adelgid populations than are the DNA methods that have been used to date. This is in spite of the inability to evaluate more than an average of five loci using a single insect and the clonal nature of this insect in the eastern United States. However, allozyme results were consistent with those obtained using mtDNA and did show that hemlock woolly adelgid populations that are cyclically parthenogenic are more genetically diverse than populations that are obligately parthenogenic. However, allozymes are not a good option for looking at genetic structure because of the limitations imposed by the small size of the insect.

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References Cited

- Analytical Software. 2003. Statistix for Windows user's manual. Analytical Software, Tallahassee, FL.
- Annand, P. N. 1924. A new species of adelgid (Hemiptera, Phylloxeridae). Pan-Pac. Entomol. 1: 79–82.
- Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57: 289–300.
- Bogdanowicz, S. M., P. W. Schaefer, and R. G. Harrison. 2000. Mitochondrial DNA variation among worldwide populations of gypsy moths, *Lymantria dispar*. Mol. Phylogenet. Evol. 15: 487–495.
- Butin, E., A. H. Porter, and J. Elkinton. 2005. Adaptation during biological invasions and the case of *Adelges tsugae*. Evol. Ecol. Res. 7: 887–900.
- Delmotte, F., N. Leterme, J.-P. Gauthier, C. Rispe, and J.-C. Simom. 2002. Genetic architecture of sexual and asexual populations of the aphid *Rhopalosiphum padi* based on allozyme and microsatellite markers. Mol. Ecol. 11: 711–723.
- Evans, R. A. 2002. An ecosystem unraveling?, pp. 23–24. In B. Onken, R. Reardon, and J. Lashomb [eds.], Proceedings: Hemlock Woolly Adelgid in the Eastern United States Symposium, 5–7 February 2002, East Brunswick, NJ. New Jersey Agricultural Experiment Station and Rutgers University, NJ.

- Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin version 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinformatics Online* 1: 47–50.
- Graur, D. 1985. Gene diversity in Hymenoptera. *Evolution* 39: 190–199.
- Harrison, R. G., S. F. Wintermeyer, and T. M. Odell. 1983. Patterns of genetic variation within and among gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae) populations. *Ann. Entomol. Soc. Am.* 76: 652–656.
- Havill, N. P., M. E. Montgomery, G. Yu, S. Shiyake, and A. Caccone. 2006. Mitochondrial DNA from hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. *Ann. Entomol. Soc.* 99: 195–203.
- Herbert, P.D.N., and M. J. Beaton. 1989. Methodologies for allozyme analysis using cellulose acetate electrophoresis. Helena Laboratories, Beaumont, TX.
- Herbert, P.D.N., and M. J. Beaton. 1993. Methodologies for allozyme analysis using cellulose acetate electrophoresis. Technical Bulletin, Helena Laboratories, Beaumont, TX.
- Keena, M. A., M.-J. Côté, P. S. Grinberg, and W. E. Wallner. 2008. World distribution of female flight and DNA variation in *Lymantria dispar* (Lepidoptera: Lymantriidae). *Environ. Entomol.* 37: 636–649.
- Loxdale, H. D., and C. P. Brookes. 1989. Use of genetic markers (allozymes) to study the structure, overwintering, and dynamics of pest aphid populations, pp. 231–270. *In* H. D. Loxdale and J. den Hollander [eds.], *Electrophoretic studies on agricultural pests*, Systematics Association, special volume no. 39. Clarendon, Oxford, United Kingdom.
- Loxdale, H. D. 2008. The nature and reality of the aphid clone: genetic variation, adaptation and evolution. *Agric. For. Entomol.* 10: 81–90.
- May, B. 1992. Starch gel electrophoresis of allozymes, pp. 1–27 and 271–280. *In* A. R. Hoelzel [ed.], *Molecular genetic analysis of populations: a practical approach*. Oxford University Press, Oxford, United Kingdom.
- McClure, M. S. 1987. Biology and control of hemlock woolly adelgid. *Bulletin of the Connecticut Agricultural Station* 851.
- McClure, M. S. 1989. Evidence of a polymorphic life cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). *Ann. Entomol. Soc. Am.* 82: 50–54.
- McClure, M. S. 1992. Hemlock woolly adelgid. *Am. Nurseryman* 175: 82–89.
- Menken, S.B.J., and M. Wiebosch-Steeman. 1988. Clonal diversity, population structure, and dispersal in the parthenogenetic moth *Ectoedemia argyropeza*. *Entomol. Exp. Appl.* 49: 141–152.
- Murphy, R. W., J. W. Sites, Jr., D. G. Buth, and C. H. Haufler. 1990. Proteins I: Isozyme electrophoresis, pp. 45–126. *In* D. W. Hillis and C. Moritz [eds.], *Molecular systematics*. Sinauer, Sunderland, MA.
- Orwig, D. A., and Foster, D. R. 2000. Stand, landscape, and ecosystem analyses of hemlock woolly adelgid outbreaks in southern New England: an overview, pp. 123–125. *In* K. A. McManus, K. S. Shields, and D. T. Souto [eds.], *Proceedings: Symposium on Sustainable Management of Hemlock Ecosystems in Eastern North America*, 22–24 June 1999, Durham, NH. U.S. Dep. Agric. Forest Service, Northeastern Research Station, Gen. Tech. Rep. NE-267. Radnor, PA.
- Peakall, R., and P. E. Smouse. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* 6: 88–295.
- Puterka, G. J., W. C. Black, IV, W. M. Steiner, and R. L. Burton. 1993. Genetic variation and phylogenetic relationships among worldwide collections of the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), inferred from allozyme and RAPD-PCR markers. *Heredity* 70: 604–618.
- Sánchez, V., and R. T. Cardé. 1995. Allozyme variability and genetic structure of *Compsilura concinnata* (Diptera: Tachinidae) populations in the northeastern United States. *Ann. Entomol. Soc. Am.* 91: 211–216.
- Shields, K. S., and C. A. S.-J. Cheah. 2005. Winter mortality in *Adelges tsugae* populations in 2003 and 2004, p. 354. *In* B. Onken, and R. Reardon (compilers), *Third Symposium on Hemlock Woolly Adelgid in the Eastern United States*, Asheville, NC. U.S. Dep. Agric., Forest Service, Forest Health Technology Enterprise Team. FHTET-2005-1. Poster (abstr.)
- Simon, J.-C., S. Baumann, P. Sunnucks, P.D.N. Hebert, J.-S. Pierre, J.-F. Le Gallic, and C.-A. Dedryver. 1999. Reproductive mode and population genetic structure of the cereal aphid *Sitobion avenae* studied using phenotypic and microsatellite markers. *Mol. Ecol.* 8: 531–545.
- Skinner, M., B. L. Parker, S. Gouli, and T. Ashikaga. 2003. Regional responses of hemlock woolly adelgid (Homoptera: Adelgidae) to low temperatures. *Environ. Entomol.* 32: 523–528.
- Souto, D. T., and K. S. Shields. 2000. Overview of hemlock health, pp. 76–80. *In* K. A. McManus, K. S. Shields, and D. T. Souto [eds.], *Proceedings: symposium on sustainable management of hemlock ecosystems in eastern North America*, 22–24 June 1999, Durham, NH. U.S. Dep. Agric. Forest Service, Northeastern Research Station, Gen. Tech. Rep. NE-267. Radnor, PA.
- Souto, D., T. Luther, and R. Chianese. 1996. Past and current status of HWA in eastern and Carolina hemlock stands, pp. 9–12. *In* S. M. Salom, T. C. Tigner, and R. C. Reardon [eds.], *Proceedings of the First Hemlock Woolly Adelgid Review*, 12 October 1995, Charlottesville, VA. U.S. Dep. Agric. Forest Service, Forest Health Technology Enterprise Team, FHTET 96-10. Morgantown, WV.
- Stoetzel, M. 2002. History of the introduction of *Adelges tsugae* based on voucher specimens in the Smithsonian Institution National Collection of Insects, p. 12. *In* B. Onken, R. Reardon, and J. Lashomb [eds.], *Proceedings: hemlock woolly adelgid in eastern United States symposium*, 5–7 February 2002, East Brunswick, NJ. New Jersey Agricultural Experiment Station at Rutgers, New Brunswick, NJ.
- Sunnucks, P., P. J. De Barro, G. Lushai, N. Maclean, and D. Hales. 1997. genetic structure of an aphid studied using microsatellites: cyclic parthenogenesis, differentiated lineages and host specialization. *Mol. Ecol.* 6: 1059–1073.
- Tomiuk, J., and K. Wöhrmann. 1980. Enzyme variability in populations of aphids. *Theor. Appl. Genet.* 57: 125–127.
- Vorburger, C. 2006. Temporal dynamics of genotypic diversity reveal strong clonal selection in the aphid *Myzus persicae*. *J. Evol. Biol.* 19: 97–107.
- Vorburger, C., M. Lancaster, and P. Sunnucks. 2003. Environmentally related patterns of reproductive modes in the aphid *Myzus persicae* and the predominance of two 'superclones' in Victoria, Australia. *Mol. Ecol.* 12: 3493–3504.
- Young, R. F., K. S. Shields, and G. P. Berlyn. 1995. Hemlock woolly adelgid (Homoptera: Adelgidae): stylet bundle insertion and feeding sites. *Ann. Entomol. Soc. Am.* 88: 827–835.

Zilahi-Balogh, G., L. T. Kok, and S. M. Salom. 2002. A review of world-wide biological control efforts for the family adelgidae, pp. 129–140. *In* B. Onken, R. Reardon, and J. Lashomb [eds.], Proceedings: hemlock woolly adelgid in eastern United States symposium, 5–7 February 2002, East

Brunswick, NJ. New Jersey Agricultural Experiment Station at Rutgers, New Brunswick, NJ.

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