Mitochondrial DNA from Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Suggests Cryptic Speciation and Pinpoints the Source of the Introduction to Eastern North America

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ABSTRACT The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an introduced pest of unknown origin that is causing severe mortality to hemlocks (*Tsuga* spp.) in eastern North America. Adelgids also occur on other *Tsuga* species in western North America and East Asia, but these trees are not significantly damaged. The purpose of this study is to use molecular methods to clarify the relationship among hemlock adelgids worldwide and thereby determine the geographic origin of the introduction to eastern North America. Adelgids were collected from multiple locations in eastern and western North America, mainland China, Taiwan, and Japan, and 1521 bp of mitochondrial DNA was sequenced for each sample. Phylogenetic analyses suggest that the source of *A. tsugae* in eastern North America was likely a population of adelgids in southern Japan. A single haplotype was shared among all samples collected in eastern North America and samples collected in the natural range of *T. sieboldii* in southern Honshu, Japan. A separate adelgid mitochondrial lineage was found at higher elevations in the natural range of *T. diversifolia*. Adelgids from mainland China and Taiwan represent a lineage that is clearly diverged from insects in North America and Japan. In contrast to eastern North America, there is no conclusive evidence for a recent introduction of *A. tsugae* into western North America, where multiple haplotypes are found. Implications for hemlock woolly adelgid control, taxonomy, and plant–insect coevolution are discussed.

KEY WORDS invasive pest, molecular systematics, *Tsuga*

The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an introduced pest causing severe mortality of the two hemlock species native to eastern North America, *Tsuga canadensis* (L.) Carriére and *Tsuga caroliniana* Engelmann. The unchecked population growth on hemlocks in eastern North America is likely because of limited tree resistance and a lack of natural enemies in the introduced range (McClure 1987). The first record of *A. tsugae* in eastern North America was in 1951 in Richmond, VA (Stoetzel 2002), where it probably was introduced on exotic ornamental hemlocks. It has since spread extensively, causing the decline and death of forest and ornamental hemlocks from New England to the southern Apalachians (Ward et al. 2004).

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A. tsugae has been documented on all nine species of hemlock in their native ranges worldwide (Annand 1924, Takahashi 1937, Inouye 1953, McClure 1992, Montgomery et al. 2000), but it is considered a serious pest only on the two species in eastern North America. In Asia and western North America, *A. tsugae* causes little or no damage to trees. In Japan, *A. tsugae* is found on both *Tsuga sieboldii* Carriére and *Tsuga diversifolia* (Maxim.) Masters (Inouye 1953, McClure 1995); in mainland China on *Tsuga chinensis* (Franchet) Pritzel in Diels, *Tsuga forrestii* Downie, and *Tsuga dumosa* (D. Don) Eichler (Montgomery et al. 2000); in Taiwan on *Tsuga chinensis* var. *formosana* (Takahashi 1937); and in India (Ghosh 1975) on *T. dumosa.* The relationships among these populations of *A. tsugae* are unknown, and differences in the morphology and biology have not been examined.

Although the history of the introduction and spread of this insect in eastern North America is well-documented, it is unknown whether *A. tsugae* in western North America is native or introduced. Dreyfus (1889) was the first to report an adelgid feeding on *Tsuga.* He described adelgids collected from an exotic *T. canadensis* growing in Wiesbaden, Germany, and named the species *Chermes funitectus.* Chrystal (1916, 1922) later observed adelgids causing damage to isolated *T.*

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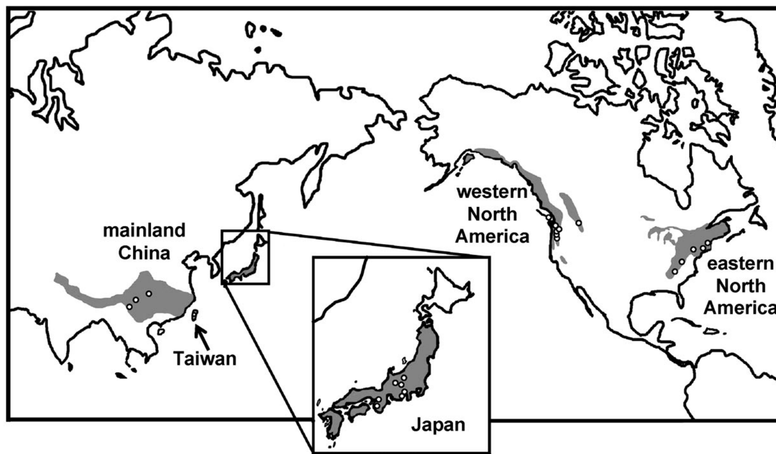

Fig. 1. Map showing the worldwide distribution of *Tsuga* (shaded area) with adelgid collection sites (open dots). The range of *Tsuga* is based on Little (1971), Hirokawa (1972, 1976), and Farjon (1990).

heterophylla trees in Vancouver, British Columbia, Canada. He identified these insects as *C. funitectus* based on the Dreyfus report. It is not clear, however, whether Dreyfus and Chrystal were looking at the same species because Dreyfus' description is vague, and according to Annand (1928) "would fit a number of species." In fact, Börner (1908), Marchal (1913), Cholodkovsky (1915), and Annand (1928) all suggest that Dreyfus may have misidentified the tree on which he collected his *C. funitectus.* To our knowledge, the first published record of *A. tsugae* in western North America is Chrystal (1916), and the first specimen was collected in 1907 from South Bend, WA, deposited in the U.S. National Museum of Natural History, and later identified by Louise Russell (M.E.M., unpublished data).

Taxonomy of Adelgidae is traditionally based on morphology, which is challenging because of their complex polymorphic life cycles and the paucity of distinct characters useful for species delineation. A single adelgid species can have up to seven distinct morphological forms in their multigeneration life cycle, and many adelgid species are described from only one or a few morphs (Blackman and Eastop 1994). Furthermore, because most species of adelgids migrate between two hosts, it can be difficult to assign the forms on both hosts to a single species. *A. tsugae* can be distinguished morphologically from congeneric species by the distribution and size of wax pores of first instars, the knobbed setae on the posterior end of the adult abdomen, and the distinct shape of the thoracic shield (Annand 1924). A detailed morphometric analysis of *Adelges piceae* (Ratzenburg), another invasive adelgid species, distinguished three groups in the introduced range, suggesting it had been introduced three separate times (Foottit and Mackauer 1980). A similar morphological study combined with the molecular data reported here would help resolve the taxonomic status of the different populations of *A. tsugae* worldwide.

Molecular methods have been very successful in helping to determine the invasion history of other introduced taxa (Downie 2002, Saltonstall 2002, Facon et al. 2003, Cognato et al. 2005). The purpose of this study is to determine the invasion history of *A. tsugae* using phylogenetic analysis of mitochondrial DNA sequences. We wish to identify the origin of the introduced lineages in eastern North America, determine the phylogenetic relationships and amount of genetic differentiation among native and introduced populations, and indicate whether the lineage in western North America is the result of human introduction or represents a natural colonization. This information will help prevent introduction of other non-native genotypes, and guide biological control and resistance breeding efforts.

Materials and Methods

We obtained 29 samples of *A. tsugae* from the native ranges of all nine hemlock species (Fig. 1), as well as two other *Adelges* species to include as outgroups. Samples were collected between 2002 and 2005 (Table 1). Live insects on branch tips were placed in 95% ethanol and shipped or carried to the Yale laboratory, where they were carefully removed from branches and stored in 95% ethanol at −20°C until DNA extraction. Slide-mounted vouchers have been deposited at the Yale University Peabody Museum and additional insects have been preserved in the Peabody Museum Cryolab.

Samples were dried of ethanol and ground in liquid nitrogen before DNA extraction. Total DNA was extracted using the Easy DNA extraction kit (Invitrogen, Carlsbad, CA) or the DNeasy tissue extraction kit (QIAGEN, Valencia, CA). DNA extraction from single insects resulted in reduced polymerase chain reaction (PCR) amplification success; therefore, each sample extraction consisted of five to 10 insects from a single branch tip.

Table 1. Collection data for samples of *A. tsugae* and outgroup species

Sample name	Host species	Collection information	Voucher accession no.
Adelges cooleyi	*Pseudotsuga menziesii*	USA; CONN; New Haven County; Hamden; 2 May 2002; Coll. N. Havill	YPM ENT 257584
Adelges laricis	*Larix* sp.	CHINA; Yunnan Province, Lijiang County, Bai Shui He; 1 June 2004; Coll. N. Havill, G. Yu	YPM ENT 257585
CH1	*Tsuga chinensis*	CHINA; Shaanxi Province; Ningshan County; Northwestern Agro-Forestry University, Houditang Forest Farm; 15 Oct. 2002; Coll. N. Havill, M. Montgomery, G. Yu	YPM ENT 257586
CH2	*Tsuga forrestii*	CHINA; Yunnan Province; Lijiang County; Bai Shui He; 24 Oct. 2002; Coll. N. Havill	YPM ENT 257587
CH3	*Tsuga dumosa*	CHINA; Sichuan Province; Baoxing County; Niba Gao Forestry Station; 12 April 2002; Coll. T. McAvoy, G. Zilahi-Balogh	YPM ENT 257588
CH4	*Tsuga chinensis*	CHINA; Sichuan Prov.; 15 Mar. 2004; Guza; Coll. T. McAvoy	YPM ENT 257589
CH5	*Tsuga chinensis*	CHINA; Sichuan Prov.; Kangding; 13 Mar. 2004; Coll. T. McAvoy	YPM ENT 257590
ENA1	*Tsuga canadensis*	USA; CONN; Litchfield, Co. Barkhampstead; 15 May 2002; Coll. L. Jones	YPM ENT 257591
ENA2	*Tsuga canadensis*	USA; WV; Randoph Co.; Elkins; Mon National Forest; May 2003; Coll. M. Seese	YPM ENT 257592
ENA3	*Tsuga canadensis*	USA; PA; Cumberland Co.; Carlisle; May 2003; Coll. L. Jones	YPM ENT 257593
ENA4	*Tsuga sieboldii*	USA; MASS; Suffolk County; Jamaica Plain; Arnold Arboretum; 23 June 2003; Coll. N. Havill	YPM ENT 257594
ENA5	*Tsuga canadensis*	USA; GA; Rabun County; Chattahoochee National Forest; 11 May 2004; Coll. M. Montgomery	YPM ENT 257595
JP1	*Tsuga sieboldii*	JAPAN; Osaka Prefecture; Yoshikawa, Toyono-cho; 9 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257596
JP2	*Tsuga sieboldii*	JAPAN; Osaka Prefecture; Nakahata, Takatsuki; 9 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257597
JP3	*Tsuga sieboldii*	JAPAN; Wakayama Prefecture; Kongo-buji Temple, Koya-san; 8 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257598
JP4	*Tsuga diversifolia*	JAPAN; Nagano Prefecture; Tashirobashi, Kamikochi, Azumi-mura; 10 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257599
JP5	*Tsuga diversifolia*	JAPAN; Gifu Prefecture; Norikura Skyline, Kamitakara-mura; 10 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257600
JP6	*Tsuga diversifolia*	JAPAN; Gifu Prefecture; Norikura Skyline, Kamitakara-mura; 10 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257601
JP7	*Tsuga diversifolia*	JAPAN; Yamanashi Prefecture; Mt. Fuji; first step, Fuji Subaruline; 12 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257602
JP8	*Tsuga diversifolia*	JAPAN; Nagano Prefecture; Shiojiri; Nagano Forestry and Forest Product Research Institute; 11 June 2004; Coll. N. Havill, S. Shiyake, G. Yu	YPM ENT 257603
JP9	*Tsuga sieboldii*	JAPAN; Shizouka Prefecture; Yugashima, Izu-shi; 19 Mar. 2005; Coll. S. Shiyake	YPM ENT 257604
TAI1	*Tsuga chinensis* var. *formosana*	TAIWAN; Taichung County; Tayuling; 23 Dec. 2004; Coll. H.Y. Wang	YPM ENT 257605
WNA1	*Tsuga heterophylla*	USA; WASH; Clark County; Vancouver; 5 May 2003; Coll. N. Havill	YPM ENT 257606
WNA2	*Tsuga heterophylla*	USA; OR; Clackamas County; Sandy; 17 decemner 2003; Coll. B. Willhite	YPM ENT 257607
WNA3	*Tsuga heterophylla*	USA; WA; Clallam County; Sequim; Dungeness National Wildlife Refuge; 27 April 2004; Coll. K. Ripley	YPM ENT 257608
WNA4	*Tsuga heterophylla*	USA; WA; King County; Vashon; 25 April 2004; Coll. K. Ripley	YPM ENT 257609
WNA5	*Tsuga heterophylla*	USA; WA; King County; Vashon; 25 April 2004; Coll. K. Ripley	YPM ENT 257610
WNA6	*Tsuga heterophylla*	USA; WA; Thurston Co.; Olympia; 13 April 2004; Coll. K. Ripley	YPM ENT 257611
WNA7	*Tsuga heterophylla*	USA; WA; Snohomish Co.; Edmonds; 30 Sept. 2004; Coll. K. Saltonstall	YPM ENT 257612
WNA8	*Tsuga heterophylla*	CANADA; British Columbia; Victoria; 4 Jan. 2005; Coll. G. Zilaghi-Balogh	YPM ENT 257613
WNA9	*Tsuga heterophylla*	USA; MT; Flathead Co.; Glacier National Park; 25 June 2005; Coll. K. Glover	YPM ENT 257614

Three mitochondrial regions were PCR amplified with primers designed using sequences of related aphidoid taxa obtained from GenBank (Table 2). The regions were 1) partial cytochrome *c* oxidase subunit II (COII) corresponding to positions 3023–3615 of the *Drosophila yakuba* Burla mitochondrial genome (GenBank no. X02340); 2) partial cytochrome *b* (cyt*b*) corresponding to *D. yakuba* positions 10908–11340; and 3) a region including partial NADH dehydrogenase subunit I (ND1), leucine tRNA (tRNA-leu), and partial large ribosomal subunit (16S) corresponding to *D. yakuba* positions 12666–12867.

Table 2. PCR primer sequences

Primer	Sequence
AdelCOIIF1	5′GCAGAAACCAATGCAATGAAC3′
AdelCOIIR1	5′CGTCCRGGAATTGCATCTATT3′
AdelCyt*b*F1	5′TATGTATTACCATGAGGACAAATATCA3′
AdelCyt*b*R1	5′AAAAATATCATTCTGGTTGAATATGA3′
AdelND1F2	5′TAAAACGAATTATYCCTAAAACWGA3′
AdelND1R1	5′GGTGTAAGCCAGGTTGGTT3′

F and R refer to the forward and reverse primers, respectively, for each of the gene region amplified.

For all three regions, 25-μl PCR reactions contained 1× PCR buffer (Applied Biosystems, Foster City, CA), 1.25 μg of bovine serum albumin, 3 mM $MgCl_2$, 250 μM each dNTP, 10 pmol of each primer, 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems), and 1 μl of template DNA (≈5 ng). Thermocycling conditions were 95°C for 5 min followed by 35–40 cycles of 1 min at 95°C, 1 min at 50°C, 1 min at 72°C, with a final extension of 72°C for 5 min. Amplification products were purified using the QIAquick PCR purification kit (QIAGEN). PCR products were sequenced in both directions by using the BigDye Terminator kit (Applied Biosystems) on an automated DNA sequencer (Applied Biosystems 3100) using the PCR primers and manufacturer's protocols. All sequences used in this study have been deposited in GenBank (accession nos. DQ256141–DQ256197). Sequences were edited using Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI) and aligned by eye. Gapped and ambiguous positions were excluded and identical haplotypes were collapsed for phylogenetic analyses.

A network can be a more appropriate way of depicting intraspecific gene genealogies than a bifurcating tree because of the potential for extant ancestral nodes and multifurcating relationships (Posada and Crandall 2001). We therefore examined the genealogical relationships among adelgid mitochondrial DNA (mtDNA) haplotypes by constructing a network based on the statistical parsimony method of Templeton et al. (1992), by using the software TCS 1.20 (Clement et al. 2000).

Phylogenetic analyses were performed by maximum parsimony (MP) by using Paup* 4.0b10 (Swofford 2003) and by Bayesian analysis by using Mr Bayes 3.1 (Ronquist and Huelsenbeck 2003). Incongruence among data partitions was tested using a partition homogeneity test with 1,000 replications as implemented in Paup*. MP analyses used the heuristic search option with tree-bisection-reconnection (TBR) branch swapping, collapsing zero-length branches, and equal weighting of all characters. MP bootstrap support was calculated using 1,000 replicates. Bayesian analysis was performed using a heterogeneous model of evolution that allowed for different parameters for each data partition. Using a complex and more realistic evolutionary model for multilocus data sets rather than a single, homogeneous model has been shown to result in more accurate and precise estimates of posterior probabilities (Castoe et al. 2004, Huelsenbeck et al. 2004). The most appropriate model for each partition was determined using hierarchical log-likelihood ratio tests as implemented by MrModeltest 2.2 (Nylander 2004), a modified version of the computer program Modeltest (Posada and Crandall 1998) that includes only the models currently available in MrBayes. Bayesian analyses used the default priors and consisted of two concurrent runs, with four chains, each with 1,000,000 generations, sampled every 1,000 generations. After visual examination of the plot of log-liklihood scores, a burn-in of 10,000 generations was used to discard trees generated before the MCMC had stabilized. To test for homogeneity of substitution rates across branch lengths, we applied a likelihood ratio test (Huelsenbeck and Rannala 1997) by using likelihood scores for the ingroup topology with and without enforcement of a molecular clock. Tests were performed using the combined data set and for each gene partition separately using the appropriate model of nucleotide substitution, as determined by the software Modeltest 3.7.

Results

The aligned data set was 1521 bp. Alignment of protein coding regions was unambiguous, and no gaps were needed, whereas the tRNA-leu/16S region required two gaps. Twenty sites in the gapped region were removed from subsequent analyses. All three PCR amplified regions were highly A-T biased, as is expected for mitochondrial DNA, and exhibited similar levels of polymorphism. COII, cyt*b*, and ND1/tRNA-leu/16S exhibited 82.5, 79.9 and 85.1% AT content; contained 103 (19.0%), 71 (16.4%), and 101 (18.5%) variable sites; and 66 (12.2%), 40 (9.3%), and 62 (11.4%) parsimony informative sites, respectively. Of the parsimony informative sites, 29 (17.3%) occurred at first codon positions, 10 (5.9%) at second codon positions, 119 (70.8%) at third codon positions, and 10 (5.9%) in the nonprotein-coding region.

Pairwise transition and transversion sequence distance was plotted versus total distance to check for evidence of saturation at first, second, and third codon positions for each partition (data available from first author). Only transitions at third codon positions showed evidence of saturation with the plot showing a clear plateau. The plateau occurred only for pairwise comparisons of ingroup to outgroup taxa, indicating that the relationships among ingroup taxa should not be influenced by saturation.

Haplotype sharing among samples is indicated in Table 3. In total, 16 haplotypes that differed by 0.07 to 6.7% sequence divergence (mean 5.0%) were found in our data set. The only haplotype shared across geographic regions, JP1-ENA1, was found in all eastern North American samples (ENA1–ENA5), and in samples collected in Japan on *T. sieboldii* (JP1 and JP2) and on an ornamental *T. diversifolia* (JP8). The mean distance between sequences from mainland China to the sample from Taiwan was 6.65% (range 6.58–6.72%), from mainland China to North America and

Table 3. Mitochondrial haplotype sharing among samples of *A. tsugae*

Haplotype	Samples included
CH3	CH3, CH4
JP1-ENA1	JP1, JP2, JP8, ENA1, ENA2, ENA3, ENA4, ENA5
JP6	JP6, JP7
WNA1	WNA1, WNA2, WNA6
WNA3	WNA3, WNA4

Shared haplotypes include samples with identical sequences across all mitochondrial regions included in this study and are named after the first sample from each region on the list. Haplotypes that only occur once are given the same name as the sample in which they are found (see Table 1). CH, China; ENA, eastern North America; JP, Japan; WNA, western North America.

Japan was 6.45% (range 6.25–6.72%), and from North American and Japan to Taiwan was 5.70% (range 5.39–6.25%).

Networks constructed using the software TCS set to a 90% confidence limit (CL) are shown in Fig. 2. A 90% CL allowed connections up to 24 steps and resulted in three unconnected networks with no loops and two multifurcations. The first network contained haplotypes found in samples from mainland China, the second network included samples from Japan and North America, and the single haplotype from Taiwan was not connected to either of these networks. A 95% CL (data not shown) allowed 17 steps and did not connect haplotypes JP4, JP5, and JP6 to the North American and other Japanese haplotypes.

The network including the samples from mainland China contained four haplotypes separated by a maximum of eight mutational steps. The network including the Japanese and North American samples contains three subgroups separated by 14–32 mutations. Samples collected in Japan from the native range of *T. diversifolia* contained three unique haplotypes (JP4, JP5, and JP6) and formed the most divergent subgroup, separated from the other haplotypes in the network by 30–42 mutations (2.0–2.8% sequence divergence). The second group consists of samples collected in western North America, which include six

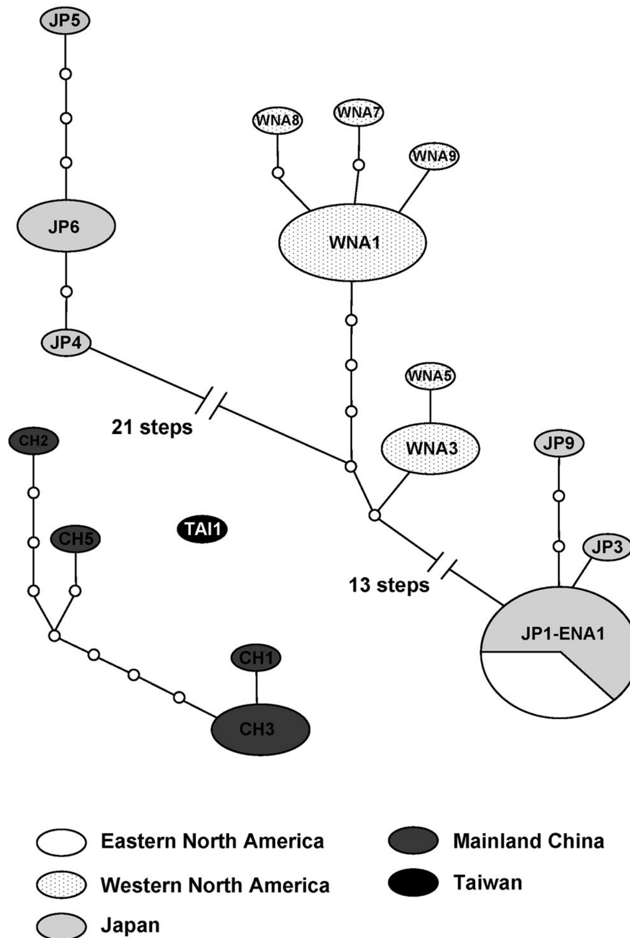

Fig. 2. TCS haplotype network showing genealogical relationships among hemlock adelgids. Haplotypes are connected with a 90% CL. The size of each oval is proportional to the frequency of the haploytpe in the analysis. White dots represent mutational steps separating the observed haplotypes and different shades represent the geographic origin of the haplotypes.

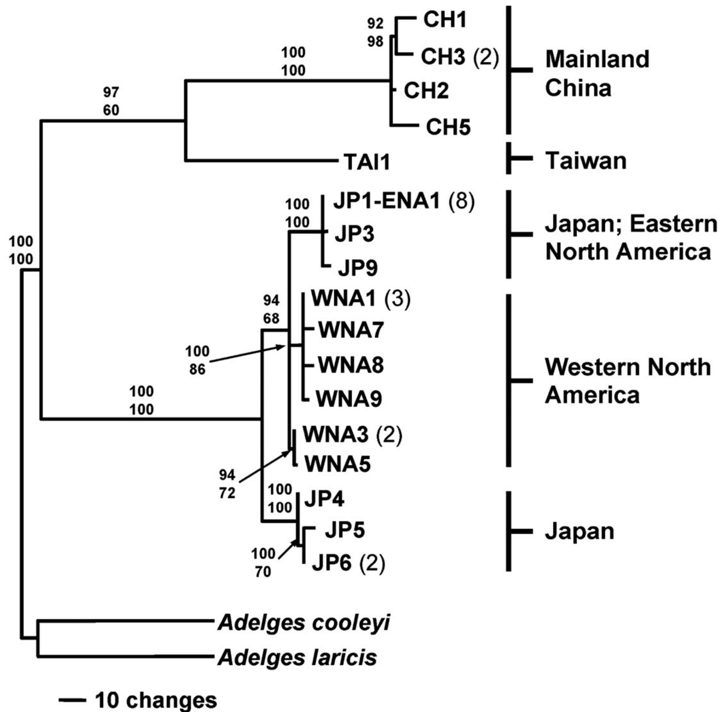

Fig. 3. Majority rule consensus tree resulting from the Bayesian analysis of 1504 bp of mtDNA for *A. tsugae* and two outgroups. Numbers of included samples for shared haplotypes are shown in parentheses (see Table 3). Clade support values for each node are Bayesian posterior probabilities on top and maximum parsimony bootstrap values on bottom. Brackets on the right indicate the geographic origin of samples.

unique haplotypes differing from each other by one to nine mutations (0.07–0.60% sequence divergence). The third group consisted of three haplotypes found in samples collected in eastern North America and from the native range of *T. sieboldii* in Japan.

For phylogenetic analysis, the data were separated into seven partitions. The first six partitions consisted of combined first and second codon positions, and third codon positions for the three protein coding genes, and the seventh partition consisted of tRNA-lea/16S. The partition homogeneity test indicated that the partitions did not indicate significant conflict ($P = 0.74$); therefore, the different regions were combined for MP analyses. For the Bayesian analysis, separate model parameters were assigned for each of the seven partitions. Details of the model can be obtained by contacting N.P.H.

The 50% majority rule consensus tree resulting from the Bayesian search is shown in Fig. 3. Maximum parsimony analysis of the combined data set resulted in four equally parsimonious trees with a length of 378 steps, a consistency index of 0.815, and a retention index of 0.878. These four trees differed only in the relationships among the western North American samples, and the strict consensus topology was identical to the Bayesian tree.

Adelgids clustered into two major clades. The first clade consisted of samples collected in mainland China and Taiwan, and the second clade consisted of samples collected in Japan and North America. The Taiwan sample clustered with the mainland China samples with high Bayesian posterior probability support but only moderate MP bootstrap support. Adelgids collected in Japan in the native range of *T. diversifolia* formed a separate clade from those collected in Japan in the native range of *T. sieboldii* and from North America. Adelgids found on *T. heterophylla* in western North America did not form a clear monophyletic group. A clade including all western North American haplotypes was not supported by MP boostrap analysis (52%) or by Bayesian posterior probability (46.8%). Likelihood ratio tests rejected clock-like behavior of substitution rates for the combined data set and for each gene partition (combined: $G = 142.4$, COII: $G = 33.2$, cytB: $G = 146.1$, ND1: $G = 70.1$; df = 15; $P < 0.01$).

Discussion

The relationships among mitochondrial lingeages uncovered for adelgids living on hemlock reveal previously unknown biogeographic patterns. The source of *A. tsugae* in eastern North America was very likely the lineage of adelgids living predominantly on *T. sieboldii* at low elevations in southern Japan. Adelgids collected in eastern North America from Massachusetts to West Virginia all share an identical haplotype, suggesting limited genetic variation in the eastern

North American insects. This low variation could have resulted either from a single introduction (perhaps of a single clone), clonal selection after introduction to the new environment, or fixation because of genetic drift. The single haplotype found in eastern North America also was found in samples collected in Japan at low elevations in the natural range of *T. sieboldii.*

The relationship among mitochondrial haplotypes from Japan suggests the occurrence of separate adelgid lineages with divergent evolutionary histories on the island of Honshu. *T. diversifolia* naturally occurs at higher elevations in more northern Honshu than *T. sieboldii* (Hirokawa 1976). Insects collected in the natural range of *T. diversifolia* form a separate clade from insects collected in the natural range of *T. sieboldii.* This finding suggests lineage-specific host specialization (Via et al. 2000); however, other possible explanations include different ecological preferences, separate clonal lineages with geographic structuring, or the presence of coexisting clonal and sexual lineages. In other aphidoid insects, sexual and asexual lineages can be entirely isolated because of the loss of the ability to form sexual offspring in the asexual lineage (Moran 1992), or they can experience some gene flow because of the ability of the asexual lineage to produce the occasional sexual form that crosses with the sexual lineage (Halkett et al. 2005). Other adelgid species such as *Adelges laricis* Vallot, *Adelges cooleyi* (Gillette), and *Adelges nordmannianae* (Eckstein) are known to exhibit both sexual and asexual lineages depending on the availability of suitable primary hosts (Blackman and Eastop 1994). In North America, *A. tsugae* does not host alternate or reproduce sexually, presumably because of the absence of a suitable primary host (McClure 1989). In contrast, *A. tsugae* in Japan is reported to have tigertail spruce, *Picea torano* (K. Koch) Koehne (=*Picea polita*) as a primary host where sexual reproduction is assumed to occur (Inouye 1953, Yukawa and Masuda 1996, Sato 1999). However, the extent and pattern of genetic structuring between sexual and asexual reproductive types in Japan are not known. Further rigorous experimental studies are needed to determine the ecological and evolutionary dynamics of *A. tsugae* in Japan and the implications for the introduced population in North America.

Adelgids collected in mainland China and Taiwan form a distinct and distant clade from insects collected in Japan and North America. Sequence divergence between the Chinese and Japanese clades (≈6.5%) falls in the same range as we find between other adelgid species (N.P.H., unpublished data), suggesting that these lineages could have diverged long enough to evolve substantial differences in their biology. Differences in host plant specificity, dispersal, phenology, cold hardiness, and susceptibility to natural enemies could all have significant implications for quarantine and control efforts. For example, Del Tredici and Kitajima (2004), found no adelgids on 38 *T. chinensis* seedlings planted in an arboretum in Massachusetts, whereas a comparable sample of *T. canadensis* seedlings was heavily infested. That adelgids are collected readily in China on *T. chinensis* suggests that the mainland Chinese adelgid lineage has different host preferences than the lineage currently present in eastern North America. More work is needed to examine other potential differences in the biology and ecology between Chinese and Japanese adelgids and to address the taxonomic status of the lineages found in mainland China and Taiwan.

Whether *A. tsugae* is endemic to western North America or the result of human introduction cannot be determined conclusively from our results. Although it is possible that the lineage present in western North America was introduced by human transport from an unsampled native lineage in Japan, it is also reasonable to contend that it originated from natural dispersal of an Asian lineage across the Beringian Corridor.

Aphidoid insects are poor dispersers, and intercontinental disjunctions that predate long-distance transfer by humans are likely because of migration and vicariance events in association with their host plants (von Dohlen et al. 2002). The current distribution of *Tsuga* is discontinuous with representatives in Asia and North America but not in Europe (Fig. 1). This conforms to a Pacific track biogeographic pattern exhibited by several other plant groups that probably results from diversification in Asia with subsequent episodes of *trans*-Beringian dispersion and vicariance (Donoghue and Smith 2004). According to fossil evidence (reviewed in Florin 1963, Lepage 2003), *Tsuga* was present in western North America as early as the Paleocene (60 million years ago), at which time there was likely exchange between North America and Europe across the existing North Atlantic land bridges. In more recent times, until the Pleistocene (1 million years ago), *Tsuga* was widely distributed throughout the Northern Hemisphere, and there was likely exchange of *Tsuga* between western North America and East Asia via the Beringian Corridor. At this time, there also could have been exchange of adelgids feeding on *Tsuga* between these regions.

Molecular dating of divergence times between clades would ideally involve assigning independent calibration points by using fossils or biogeographical events, but to our knowledge, these approaches are not available for *A. tsugae.* Other sources of error that inhibit meaningful molecular dating of this data set include heterogeneous substitution rates, topological uncertainty with regard to placement of western North American haplotypes, and unknown population dynamics, especially regarding allocation to sexual versus asexual reproduction. However, other types of evidence support the endemicity of the western North American lineage, including higher genetic variation than in eastern North America (six versus one haplotypes), suggesting in situ diversification, resistance of western *Tsuga* species to adelgid damage, and the presence of specialist natural enemies in western North America (Zilahi-Balogh et al. 2002). To more conclusively determine the evolutionary history of western North American *A. tsugae*, additional sampling and detailed population level analysis by using

fast-evolving nuclear regions, such as microsatellites, is necessary.

This mtDNA analysis of *A. tsugae* leads us to conclude that it was introduced to eastern North America from Japan, has divergent native lineages in Japan, mainland China, and Taiwan, and may be either indigenous or introduced to western North America. These results can be used to facilitate control efforts in several ways. For example, knowing the source of the introduction could help in predicting the extent of the spread of *A. tsugae* in eastern North America. A better understanding of the current distribution of this lineage in Japan may help to determine the ecological limits and therefore the potential introduced range in North America. If the adelgid lineage found in southern Japan is adapted to a warm, maritime climate, this could explain the high winter mortality of adelgids in northern New England (Shields and Cheah 2005).

The recognition of several distinct lineages indicates a need to identify and prevent the introduction of genotypes not already present in a region. Although it is not yet clear to what extent the mitochondrial lineages revealed in this study differ in their ecology and physiology, introduction of a new genotype into an area could potentially exacerbate or initiate a serious pest problem. To facilitate quarantine efforts, it would be possible to design PCR-based diagnostic tests to quickly and unequivocally distinguish the lineages uncovered in this study, given the presence of diagnostic sites that distinguish the majority of the clades. Such an assay would need to be validated by screening a larger number of samples throughout the geographic ranges of the lineages targeted by the test, and cross-checking population subdivision with nuclear markers.

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References Cited

Annand, P. N. 1924. A new species of *Adelges* (Hemiptera, Phylloxeridae). Pan-Pac. Entomol. 1: 79–82.

Annand, P. N. 1928. A contribution toward a monograph of the Adelginae (Phylloxeridae) of North America. Stanford University Press, Palo Alto, CA.

Blackman, R. L., and V. F. Eastop. 1994. Aphids on the world's trees: an identification and information guide. CAB International, Wallingford, United Kingdom.

Börner, C. V. 1908. Eine monographische Studie über die Chermiden. Arbeiten aus der Kaiserlichen Biologischen Anstalt für Land- und Forstwirtschatt. 6: 81–318.

Castoe, T. A., T. M. Doan, and C. L. Parkinson. 2004. Data partitions and complex models in Bayesian analysis: the phylogeny of gymnophthalmid lizards. Syst. Biol. 53: 448–469.

Cholodkovsky, N. A. 1915. Chermes injurious to conifers. Department of Agriculture Central Board of Land Administration and Agriculture, Petrograd, Russia.

Chrystal, R. N. 1916. The forest insect problem in Stanley Park. Proc. Entomol. Soc. Br. Columbia 9: 63–66.

Chrystal, R. N. 1922. The Douglas fir chermes (*Chermes cooleyi*). Bull. For. Commun. Lond. 4.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9: 1657–1659.

Cognato, A. I., J. H. Sun, M. A. Anducho-Reyes, and D. R. Owen. 2005. Genetic variation and origin of red turpentine beetle (*Dendroctonus valens* LeConte) introduced to the People's Republic of China. Agric. For. Entomol. 7: 87–94.

Del Tredici, P., and A. Kitajima. 2004. Introduction and cultivation of Chinese hemlock (*Tsuga chinensis*) and its resistance to hemlock woolly adelgid (*Adeles tsugae*). J. Arboric. 30: 282–287.

Donoghue, M. J., and S. A. Smith. 2004. Patterns in the assembly of temperate forests around the Northern Hemisphere. Phil. Trans. R. Soc. Lond. B Biol. Sci. 359: 1633–1644.

Downie, D. A. 2002. Locating the sources of an invasive pest, grape phylloxera, using a mitochondrial DNA gene genealogy. Mol. Ecol. 11: 2013–2026.

Dreyfus, L. 1889. Über neue Beobachtungen bei den Gattungen *Chermes* und *Phylloxera.* Tageblatt der Versammlung deutscher Naturforscher und Δrzte 61: 55–65.

Facon, B., J. P. Pointier, M. Glaubrecht, C. Poux, P. Jarne, and P. David. 2003. A molecular phylogeography approach to biological invasions of the New World by parthenogenetic thiarid snails. Mol. Ecol. 12: 3027–3039.

Farjon, A. 1990. Pinaceae: drawings and descriptions of the genera *Abies, Cedrus, Pseudolarix, Keteleeria, Nototsuga, Tsuga, Cathaya, Pseudotsuga, Larix,* and *Picea.* Koeltz Scientific Books, Königstein, Germany.

Florin, R. 1963. The distribution of conifer and taxad genera in time and space. Acta Hortic. 20: 783–791.

Foottit, R. G., and M. Mackauer. 1980. Morphometric variation between populations of the balsam woolly aphid *Adelges piceae* (Homoptera: Adelgidae) in North America. Can. J. Zool. 58: 1494–1503.

Ghosh, A. K. 1975. A list of aphids (Homoptera: Aphididae) from India and adjacent countries. J. Bombay Nat. Hist. Soc. 71: 201–225.

Halkett, F., M. Plantegenest, N. Prunier-Leterme, L. Mieuzet, F. Delmotte, and J. C. Simon. 2005. Admixed sexual and facultatively asexual aphid lineages at mating sites. Mol. Ecol. 14: 325–336.

Hirokawa, Y. 1972. Atlas of the Japanese flora: an introduction to plant sociology of East Asia. Gakken Co. Ltd., Tokyo, Japan.

Hirokawa, Y. 1976. Atlas of the Japanese flora II: an introduction to plant sociology of East Asia. Gakken Co. Ltd., Tokyo, Japan.

Huelsenbeck, J. P., and B. Rannala. 1997. Phylogenetic methods come of age: testing hypotheses in an evolutionary context. Science (Wash. DC) 276: 227–232.

Huelsenbeck, J. P., B. Larget, and M. E. Alfaro. 2004. Bayesian phylogenetic model selection using reversible jump Markov chain Monte Carlo. Mol. Biol. Evol. 21: 1123–1133.

Inouye, M. 1953. Monographische Studie über die japanischen Koniferen-Gallenläuse (Adelgidae). Bull. Sapporo Branch Gov. For. Exp. Stn. 15: 1–91.

Lepage, B. A. 2003. A new species of *Tsuga* (Pinaceae) from the middle Eocene of Axel Heiberg Island, Canada, and an assessment of the evolution and biogeographical history of the genus. Bot. J. Linn. Soc. 141: 257–296.

Little, E. L. 1971. Atlas of United States trees, volume 1, conifers and important hardwoods. U.S. Dep. Agric. Misc. Publ. 1146.

Marchal, P. 1913. Contribution à l'étude de la biologie des chermes. Ann. Sci. Nat. Zool. Biol. Anim. 18: 153–385.

McClure, M. S. 1987. Biology and control of hemlock woolly adelgid. Bull. Conn. Agric. Exp. Stn. 851: 1–9.

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 15: 82–86.

McClure, M. S. 1995. *Diapterobates humeralis* (Oribatida: Ceratozetidae): an effective control agent of hemlock woolly adelgid (Homoptera: Adelgidae) in Japan. Environ. Entomol. 24:1207–1215.

Montgomery, M. E., D. Yao, and H. Wang. 2000. Chinese Coccinellidae for biological control of the hemlock woolly adelgid: description of native habitat, pp. 97–102. *In* K. A. McManus, K. S. Shields, and D. R. 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, Newtown Square, PA.

Moran, N. A. 1992. The evolution of aphid life cycles. Annu. Rev. Entomol. 37: 321–348.

Nylander, J.A.A. 2004. MrModeltest. Program distributed by the author, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Posada, D., and K. A. Crandall. 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Ecol. Evol. 16: 37–45.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Saltonstall, K. 2002. Cryptic invasion by a non-native genotype of the common reed, *Phragmites australis,* into North America. Proc. Natl. Acad. Sci. U.S.A. 99: 2445–2449.

Sato, S. 1999. Galls on tiger-tail spruce made by *Aphrastasia tsugae* at Mt. Tsurugi. Gensei 73: 26.

Shields, K. S., and C.A.S.-J. Cheah. 2005. Winter Mortality in *Adelges tsugae* populations in 2003 and 2004, pp. 354–356. *In* B. Onken and R. Reardon [eds.], Third Symposium on Hemlock Woolly Adelgid in the Eastern United States, 1–3 February 2005, Asheville, NC. U.S. Dep. Agric.–Forest Service, Morgantown, WV.

Stoetzel, M. B. 2002. History of the introduction of *Adelges tsugae* based on voucher specimens in the Smithsonian Institute National Collection of Insects, p. 12. *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, New Brunswick, NJ.

Swofford, D. L. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer, Sunderland, MA.

Takahashi, R. 1937. Phylloxeridae of Formosa (Hemiptera). Trans. Nat. Hist. Soc. Formosa 27: 11–14.

Templeton, A. R., K. A. Crandall, and C. F. Sing. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132: 619–633.

Via, S., A. C. Bouck, and S. Skillman. 2000. Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. Evolution 54: 1626–1637.

von Dohlen, C. D., U. Kurosu, and S. Aoki. 2002. Phylogenetics and evolution of the eastern Asian-eastern North American disjunct aphid tribe, Hormaphidini (Hemiptera: Aphididae). Mol. Phylogenet. Evol. 23: 257–267.

Ward, J. S., M. E. Montgomery, C. A. Cheah, B. P. Onken, and R. S. Cowles. 2004. Eastern hemlock forests: guidelines to minimize the impacts of hemlock woolly adelgid. U.S. Dep. Agric.–For. Serv. Northeastern Area State and Private Forestry, NA-TP-03-04. U.S. Dep. Agric.–Forest Service, Morgantown, WV.

Yukawa, J., and H. Masuda. 1996. Insect and mite galls of Japan in color. Zenkoku-nouson-kyoiku kyokai, Tokyo, Japan.

Zilahi-Balogh, G.M.G., L. T. Kok, and S. M. Salom. 2002. Host specificity of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a potential biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). Biol. Control 24: 192–198.

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