Ex situ Seed collection represents Genetic Variation Present in Natural Stands of Carolina Hemlock

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Abstact

Carolina hemlock (*Tsuga caroliniana* Engelmann) is a rare conifer endemic to the highlands of the southeastern United States, where it is experiencing significant decline as a result of infestation by the hemlock woolly adelgid (HWA, *Adelges tsugae* Annand). Since 2003, Camcore (NC State University) and the USDA Forest Service have been cooperating to conserve the genetic resources of Carolina and eastern (*T. canadensis* Carriére) hemlocks threatened by HWA. We employed amplified fragment length polymorphism (AFLP) molecular markers to: (1) compare the genetic diversity of a seed sample for *ex situ* gene conservation to the genetic diversity present in a broader foliage sample collected from natural stands, and (2) investigate the genetic relationships among the populations sampled. The results indicate that the *ex situ* collection did adequately represent the genetic variation present within the larger natural stand sample. The populations sampled were moderately genetically differentiated. Some of the populations with the highest genetic diversity were located along the eastern and southern edge of the species range.

Keywords

Adelges tsugae, *Tsuga caroliniana*, AFLP genetic analysis, gene conservation

Introduction

Carolina hemlock (*Tsuga caroliniana* Englemann) is a rare conifer species that grows at elevations of 600 to 1500 m along dry bluffs and rocky ridges of the Southern Appalachian Mountains in Virginia, Tennessee, Georgia, and North and South Carolina (Hum-

phrey 1989). A small number of populations can also be found growing at elevations of 100 to 600 m in association with isolated mountain ranges of the North Carolina and Virginia Piedmont (Stevens 1976). Historically, this species has been described as occupying dry, coarse, nutrient poor soils (Farjon 1990), although recent field studies have revealed that it is more broadly adapted to a greater variety of soil types than originally thought (Jetton et al. 2008a). Typical forest associates include *Pinus strobus* L., *P. pungens* Lamb., *Quercus* spp., *Acer rubrum* L., ericaceous shrubs such as *Kalmia latifolia* L. and *Rhododendron* spp., and occasionally eastern hemlock (*T. canadensis* Carriére) where the two species occur sympatrically in transition zones between their preferred habitats.

Across its geographic range, Carolina hemlock is beginning to experience significant decline due to the hemlock woolly adelgid (HWA, *Adelges tsugae* Annand), an exotic insect that has also caused widespread mortality among populations of eastern hemlock (McClure et al. 2001). The adelgid is native to the hemlock forests of eastern Asia, Japan, and the Pacific Northwest region of North America (McClure et al. 2001; Havill et al. 2006). It was first reported in the eastern United States in Richmond, Virginia in 1951 (Souto et al. 1996) and was likely introduced on nursery stock imported from Japan (Stoetzel 2002). Since then, HWA has spread to 18 eastern States from Maine south to Georgia, where it can kill trees in as little as four years, threatening to eliminate these ecologically important species from eastern forests (McClure et al. 2001).

The current integrated approach to the management of HWA includes silvicultural tools to maintain tree health and vigor, chemical controls in the form of systemic insecticides, biological control with adelgid predators imported from areas where HWA is native, and the *ex situ* conservation of hemlock genetic resources (Ward et al. 2004; Jetton et al. 2008b). A key factor to the success of gene conservation programs is that seed collections capture a representative number of alleles to adequately protect the gene pools of the species in question (FAO 2004). To do this, one must understand the population genetic structure of a species and how patterns of genetic diversity vary across the landscape (Erikson et al. 1993). For Carolina hemlock, Camcore addressed this issue in a study utilizing amplified fragment length polymorphism (AFLP) molecular markers to compare the genetic composition of seedlings grown from an *ex situ* seed sample with a broader population sample consisting of a foliage sample collected from trees in natural populations. The objectives of the study were to: (1) compare the genetic diversity of a seed sample collected from nine populations in 2003 for *ex situ* gene conservation to the genetic diversity present in a broader foliage sample collected in 2005 from 15 natural stands, and (2) investigate the genetic relationships among the populations sampled.

Materials and Methods

Provenance Seed and Foliage Collections: This study included 15 populations of Carolina hemlock in the Southern Appalachian region (Figure 1; Table 1). The *ex situ* seed sample consisted of seed collected in August and September 2003 from 64 mother trees representing nine of the populations located in North and South Carolina. Seed was collected from 3 to 10 mother trees per population, and each mother tree sampled within a population was located at least 100 m from the next. The natural stand foliage samples were made in all 15 populations in May 2005, covering the entire geographic range of Carolina

hemlock and encompassing 144 trees. In 13 of these provenances, foliage samples were made from the lower crown of 10 mother trees, located at least 100 m apart. At Dragons Tooth in Virginia, foliage was sampled from 11 trees, and at Tallulah Gorge in Georgia, foliage was collected from three of the four trees known to occur at the site.

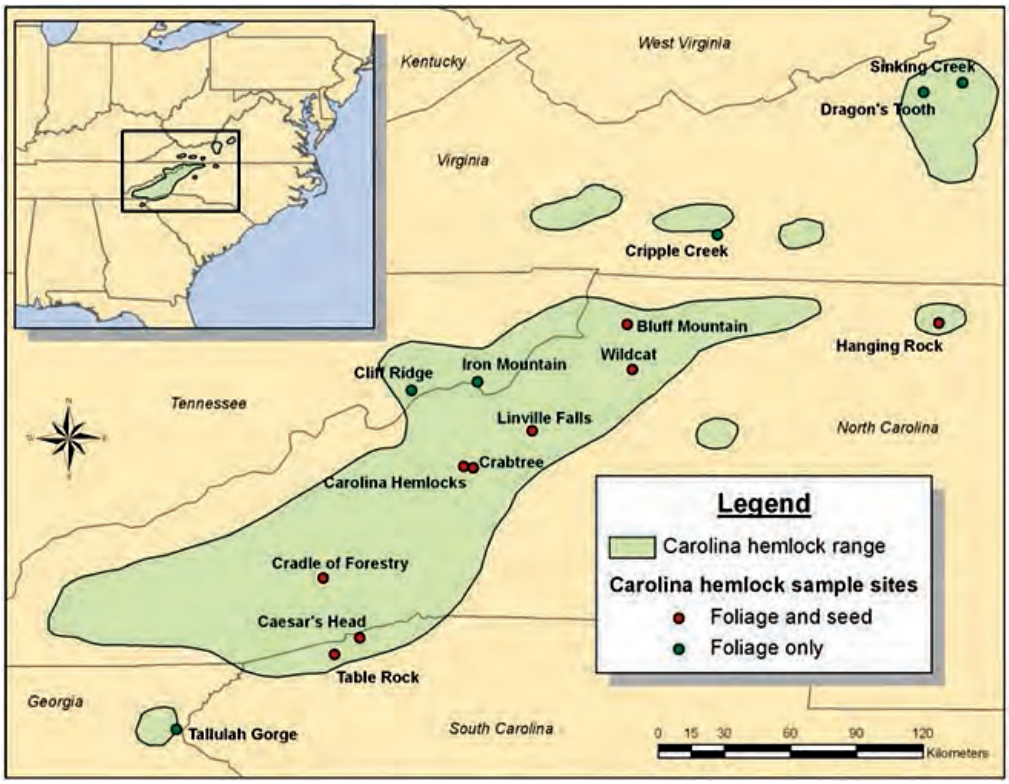

Figure 1. The range of Carolina hemlock and the provenances sampled for the molecular marker analysis.

Table 1. Carolina hemlock populations included in the molecular marker analysis, including coordinates, elevation and numbers of trees sampled for the *ex situ* conservation (seed sample) and from natural stands (foliage sample). Two seedlings were produced and sampled from each seed tree for the molecular marker study.

Population	County, State	Lat. (D.d)	Long. (D.d)	Elev. (m)	2003 Seed Sample Trees (#)	2005 Foliage Sample Trees (#)
Linville Falls	McDowell, NC	35.94 N	81.92 W	995	10	10
Table Rock	Pickens, SC	35.04 N	82.73 W	956	3	10
Carolina Hemlocks Cpgd	Yancey, NC	35.80 N	83.20 W	823	10	10
Caesar's Head	Greenville, SC	35.11 N	82.63 W	933	4	10
Cradle of Forestry	Transylvania, NC	35.35 N	82.78 W	1017	8	10
Wildcat	Watauga, NC	36.20 N	81.52 W	297	10	10
Hanging Rock	Stokes, NC	36.39 N	80.27 W	146	5	10
Bluff Mountain	Ashe, NC	36.38 N	81.54 W	1375	8	10
Crabtree	Yancey, NC	35.80 N	82.16 W	1132	6	10
Cripple Creek	Wythe, VA	36.75 N	81.17 W	766	*	10
Sinking Creek	Craig, VA	37.33 N	80.33 W	1009	*	10
Dragons Tooth	Roanoke, VA	37.37 N	80.17 W	852	*	11
Tallulah Gorge	Rabun, GA	34.73 N	83.38 W	410	*	3
Cliff Ridge	Unicoi, TN	36.10 N	82.45 W	671	*	10
Iron Mountain	Carter, TN	36.15 N	82.15 W	1503	*	10

DNA Extraction and Preparation

For the *ex situ* seed sample, two seedlings from each of 55 mother trees were grown from seed in the greenhouse and needles were harvested. For the natural stand sample, needles were harvested from each of the 144 foliage samples collected. Genomic DNA for all samples was extracted from needle samples using the DNeasy Plant Mini Kit (Qiagen, Chatsworth, California, USA). DNA concentrations were estimated using known concentrations of λ DNA on 1.5% agarose gels, and extracted DNA was stored at -80 °C for long term storage and at -20 °C when in use.

AFLP marker generation

Amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995) are molecular markers used in population genetics studies because they are reproducible, require no previous knowledge of genomic sequences, and generate a large amount of information (Glaubitz and Moran 2000). At the same time, they can be time-consuming and technically demanding, and are dominant markers with only two alleles (presence and absence of a given fragment), and therefore cannot be used to determine heterozygosity or homozygosity at a specific locus. The AFLP process involves five main steps: (1) digestion of the sample DNA with restriction enzymes, (2) ligation of adaptors to the restriction digest mix, (3) pre-amplification using polymerase chain reaction (PCR) of the ligated mixture, (4) selective PCR amplification of the pre-amplified fragments, and (5) separation and detection of the selective amplification fragments. For this study, AFLP reactions were performed on a PTC-100 thermal cycler (MJ Research, Waltham, Massachusetts). Restriction digestion and ligation were performed using the 5x reaction buffer, enzyme mix of *Eco*RI (a 6-base pair cutter) and *Mse*I (a 4-base pair cutter), adapter mix and T4 DNA ligase provided in the AFLP Template Preparation Kit (LI-COR Inc., Lincoln. Nebraska). Each adapter ligation reaction consisted of 12.5 µl of restriction digestion mixture, 12 µl of adapter mix and 0.5 µl of T4 DNA ligase. The ligase was then diluted with TE buffer. The restriction digestion, adapter ligation, pre-amplification, and selective amplification PCR steps generally followed the Myburg and Remington (2000) protocol. Because of the large genome size of conifers, pre-amplification was conducted using *Eco*RI+2/ *Mse*I+2 primers, *Eco*RI+AC and *Mse*I+CC. Selective amplification was performed using *Eco*RI+3/ *Mse*I+4 primers. Twenty-four primer combinations were screened across samples from nine Carolina hemlock and six eastern hemlock trees, with the combinations of *Eco*RI +ACC/*Mse*I+CCTG, *Eco*RI +ACG/*Mse*I+CCTG and *Eco*RI+ACT/*Mse*I+CCTG selected for their high levels of polymorphism. Each *Eco*RI primer was fluorescently labeled with a different label for multiplex fragment separation and detection on an ABI Prism 3100 capillary sequencer at the Iowa State University DNA Laboratory (Ames, Iowa). Peaks in the range of 50-560 bp were sized and binned, and then fragment presence called, using GeneMarker 1.51 (SoftGenetics, State College, Pennsylvania, USA). Peaks were tested for replication by comparing trace files from a subset of rerun samples, with 125 consistently amplifying markers selected across the three primer combinations.

AFLP DATA ANALYSES

The program AFLP-SURV version 1.0 (Vekemans et al. 2002) generated genetic diversity and population genetic structure estimates based on the approach of Lynch and Milligan (1994), which uses the average expected heterozygosity of the marker loci, or Nei's genetic diversity, as a measure of genetic diversity. This approach estimates allelic frequencies at each marker locus in each population, assuming they are dominant and have only two alleles (a dominant marker allele indicating the presence of a length fragment, and a recessive null allele indicating the absence of the fragment). These allele frequencies were generated by applying a Bayesian method with non-uniform prior distribution of allele frequencies, a general method that is expected to give the most accurate results (Zhivotovsky 1999). Hardy-Weinberg genotypic proportions were assumed, and 1000 permutations were run in each analysis of population genetic structure (F_{ST}) and genetic distance. We generated a neighbor-joining (NJ) dendrogram to visualize the relationships among provenances using the seed sample (*ex situ*) populations, including only one of the two seedlings from each mother tree. The seed sample populations were used rather than the natural stands because of more consistent fragment amplification across provenances. The Hanging Rock provenance was not included, however, because of an insufficient sample size, while the adjacent Table Rock and Caesar's Head provenances were combined into a single population. The dendrogram was constructed using the NEIGHBOR and CONSENSE components of PHYLIP 3.6 (Felsenstein 2005). Confidence estimates associated with the topology of the tree were assigned based on 1,000 bootstrap replicates, with the eastern hemlock samples included as an outgroup.

RESULTS

GENETIC DIFFERENTIATION BETWEEN THE FOLIAGE AND SEED SAMPLES

The F_{ST} genetic differentiation value between the foliage sample (natural stand population) and the seed sample (*ex situ* conservation population) was relatively small (F_{ST} = 0.037). Additionally, Nei's genetic difference between the two populations was small (0.018) and differences in the expected heterozygosity were minimal, although the percent of polymorphic loci was higher in the seed sample (Table 2). It is possible that some of the differences revealed in these analyses were the result of differences in sampling. However, a comparison of foliage samples and seedlings grown from seed collected from a single provenance, Crabtree in North Carolina, showed no genetic differentiation significantly different from 0 (F_{ST} = -0.001), and highly similar percentages of polymorphic loci (89.8 percent and 92.3 percent, respectively) and expected heterozygosity (0.353 and 0.329).

Table 2. Genetic diversity present within the foliage sample (natural stand) and the seed sample (*ex situ* conservation) populations, based on amplified fragment length polymorphism (AFLP) analysis.

Population	Trees (#)	Populations (#)	Polymorphic loci N	Polymorphic loci Percent	Expected Heterozygosity
Foliage (natural stands)	144	15	101	80.8	0.302
Seed (*ex situ* conservation)	110	9	103	90.4	0.331

Genetic Structure and Variation in the *Ex Situ* Seed Sample

Analysis of the *ex situ* seed sample showed moderate genetic differentiation among the populations represented (F_{ST} = 0.0584). The populations themselves showed relatively similar levels of genetic variation, with the exception of Linville Falls, which had a lower percentage of polymorphic loci and expected heterozygosity (Table 3). Interestingly, two populations with smaller sample sizes, Hanging Rock and Table Rock, each had 100 percent polymorphic loci; Hanging Rock is a disjunct population in the northern part of the range (Figure 1). Caesar's Head and Cradle of Forestry, both near the southern edge of the species distribution, also had relatively high polymorphism and heterozygosity.

Table 3. Genetic diversity present within the seed sample (*ex situ* conservation) populations, based on amplified fragment length polymorphism (AFLP) analysis.

Population	Trees (#)	Percent Polymorphic Loci	Expected Heterozygosity
Bluff Mountain	8	92.4	0.3309
Caesar's Head	5	97.1	0.3244
Carolina Hemlocks	10	92.4	0.3113
Crabtree	6	92.3	0.3291
Cradle of Forestry	8	95.8	0.3302
Hanging Rock	5	100	0.3801
Linville Falls	10	77.4	0.2655
Table Rock	3	100	0.3145
Wildcat	10	91.6	0.3289

Population pairwise genetic distance and genetic differentiation values (Table 4) tended to be smaller for populations of closer proximity. For example, both the genetic distance and the genetic differentiation between the southern Cradle of Forestry and Caesar's Head/Table Rock provenances was 0, as were these values between the northern Wildcat and Bluff Mountain provenances. At the same time, the Carolina Hemlocks

Table 4. Pairwise differences among Carolina hemlock seed sample (*ex situ* conservation) populations based on amplified fragment length polymorphism (AFLP) analysis. Upper diagonal: pairwise Nei's genetic distance. Lower diagonal: pairwise F_{ST} estimates.

	BM	CH/TR	CHCG	CT	CoF	LF	WC	EH
Bluff Mountain	.	0.011	0.013	0.007	0.004	0.030	0.000	0.107
Caesar's Head/Table Rock	0.029	.	0.000	0.049	0.000	0.013	0.012	0.154
Carolina Hemlocks C.G.	0.035	0.003	.	0.056	0.008	0.043	0.006	0.128
Crabtree	0.021	0.095	0.118	.	0.061	0.070	0.030	0.087
Cradle of Forestry	0.015	0.000	0.030	0.118	.	0.018	0.001	0.154
Linville Falls	0.072	0.042	0.099	0.141	0.044	.	0.016	0.198
Wildcat	0.000	0.035	0.031	0.061	0.016	0.044	.	0.131
Eastern hemlock	0.146	0.190	0.181	0.131	0.195	0.260	0.175	.

Campground provenance is more closely related to the Cradle of Forestry and Caesar's Head/Table Rock provenances than to the adjacent Crabtree provenance.

A consensus neighbor-joining dendrogram based on Nei's genetic distance (Figure 2) found statistical support (slightly more than ~50 percent of the 1,000 replicates) for a clustering of the two southernmost provenances, Cradle of Forestry and Caesar's Head/ Table Rock, with the central Linville Falls and Carolina Hemlocks provenances. The northern Wildcat population was clustered with this group, with the most northern population in the analysis, Bluff Mountain, clustered with this group with strong bootstrap support. External to this large cluster was the central Crabtree provenance.

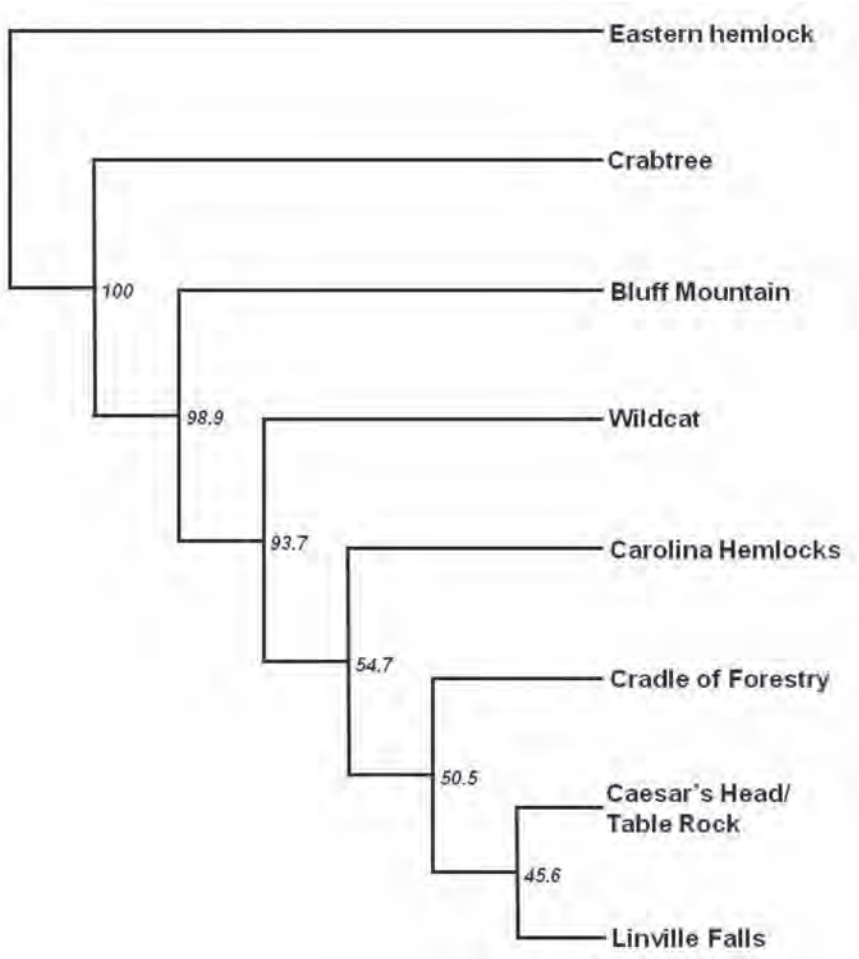

Figure 2. Consensus neighbor-joining dendrogram depicting Nei's genetic distances among the seven Carolina hemlock seed collection (*ex situ*) populations, with eastern hemlock used as an outgroup. The values represent the percent bootstrap support for the nodes over 1000 replicates; those above 50 percent are considered well-supported.

Discussion

Since 2003, Camcore (International Tree Conservation and Domestication, N.C. State University) and the USDA Forest Service Forest Health Protection have cooperated to conserve the genetic resources of Carolina and eastern hemlocks threatened by HWA (Jetton et al. 2008b). Utilizing an *ex situ* approach, seeds are being collected from across the geographic range of each species. Some seeds are placed in long-term cold storage while the rest are sent to other regions of the world with climates suitable for growing hemlock and where the adelgid does not occur. In this case, seeds are being sent to Chile, Brazil, and the Ozark Mountains of Arkansas, germinated, and out-planted into conservation reserves (seed orchards). The goal of this program is to maintain hemlock populations and seed reserves in perpetuity so that gene pools for both species survive and are available for restoration efforts once viable HWA management strategies are available.

Previous genetic conservation studies by Camcore with the Mexican and Central American pines indicate that, based on molecular markers, for species with low to moderate levels of genetic diversity, seed sampled from 10 to 20 mother trees per population in 6 to 8 populations distributed across a species' geographic range will capture alleles that occur at frequencies of 5% or greater (Dvorak et al. 1999). Our results here indicate that Carolina hemlock has overall moderate levels of genetic diversity for a conifer, and that our goal to collect germplasm from 10 mother trees per population in as many populations as can be located should be sufficient to obtain a genetically representative seed sample for *ex situ* conservation. This is supported by the finding that there was relatively little genetic differentiation between the 9 population seed sample collected in 2003 and the broader 15 population foliage sample collected in 2005.

Interestingly, we did find that the level of polymorphism in the *ex situ* seed sample was about 10% higher than in the foliage sample from the natural stands. One explanation for this might be that, by sampling two seedlings from each mother tree in the seed sample, greater pollen-parent contribution was captured relative to the foliage sample. Alternatively, this might also be the result of greater genetic variation in seedlings compared to mature trees, with some loss of variation expected as trees in natural stands mature and some are lost to selection.

Our results also show that some of the populations with the highest levels of genetic diversity are located along the eastern (Hanging Rock) and southern (Caesar's Head, Cradle of Forestry, and Table Rock) edges of the species' distribution. This suggests that the Pleistocene glacial refugia for Carolina hemlock may have been located southeast of the Appalachian Mountains, similar to the pattern we described for eastern hemlock using isozyme markers (Potter et al. 2008). This is supported by paleobotanical evidence, based on pollen sediments, that hemlocks were restricted to the Appalachian, coastal plain, and continental shelf regions of the southeast during the last maximum of the Wisconsinian glacial period (Davis 1981; Delcourt and Delcourt 1987; Prentice et al. 1991). However, additional work incorporating additional populations and using another molecular marker system, such as microsatellites, will be needed to further explore this possibility.

The results of molecular marker studies such as we present here are instructive to the design and success of current and future gene conservation strategies for Carolina and

eastern hemlock. The data on the genetic structure and variation among populations help us to understand how best to manage *ex situ* conservation plantings and seed reserves so that a broad and adaptive genetic base is maintained for breeding and restoration efforts. They can also tell us if small, fragmented populations such as the one found at Caesar's Head contribute unique alleles to the gene pool and if those genes might be captured in larger populations elsewhere. We can also use the data to better identify those populations that should receive priority for *in situ* protection (chemical and biological controls) so that high levels of genetic diversity are maintained in surviving populations that will be critical to the restoration of the species.

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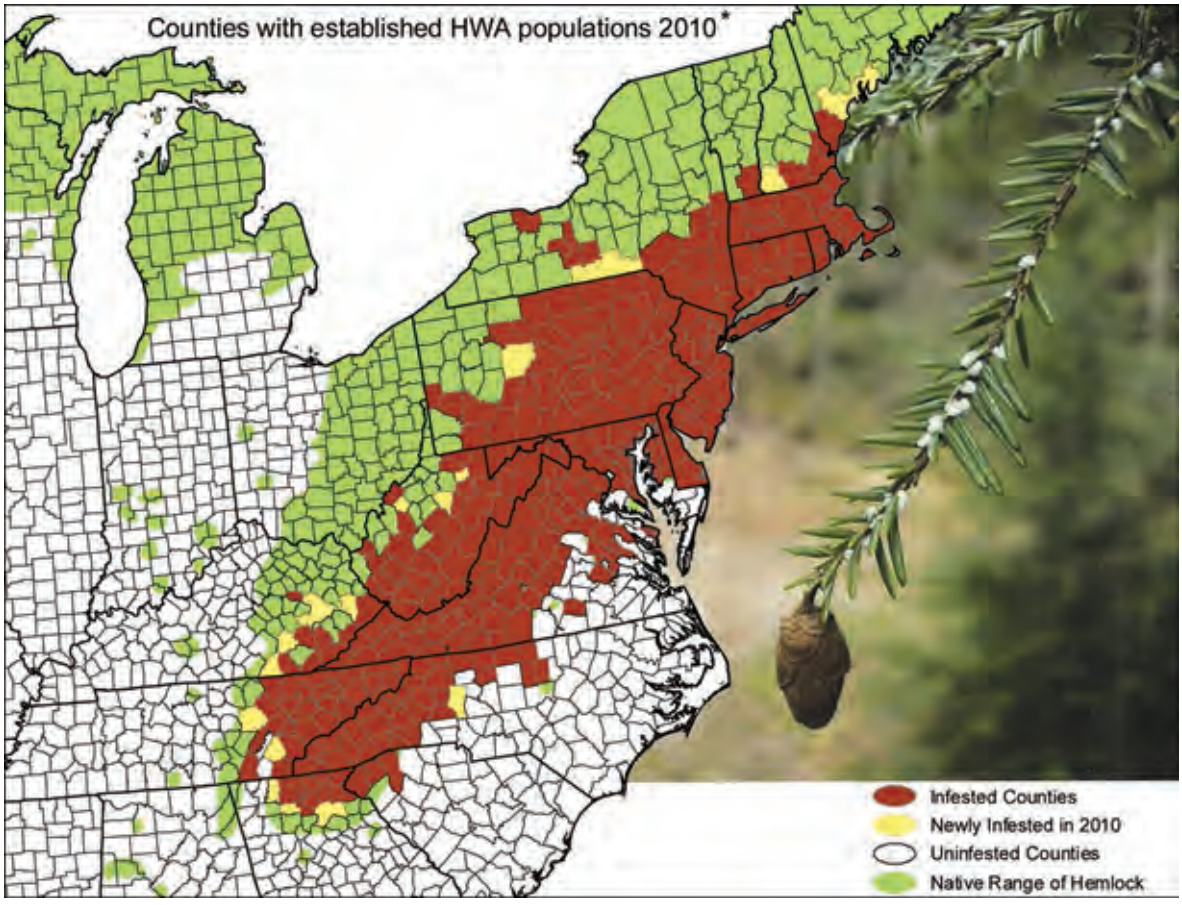

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***Cover map**: Produced by USDA Forest Service, 2010. The map depicts counties with established populations of hemlock woolly adelgid (HWA) that have been confirmed by state forest-health officials. The coarse nature (scale) of the map does not provide information below the county level; highlighted counties are not necessarily entirely infested with HWA.

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