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Investigating the Relationship Between Cryptococcus Fagisuga and Fagus Grandifolia in Great Smoky Mountains National Park

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INVESTIGATING THE RELATIONSHIP BETWEEN *CRYPTOCOCCUS FAGISUGA* AND *FAGUS GRANDIFOLIA* IN GREAT SMOKY MOUNTAINS NATIONAL PARK

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ABSTRACT - The high elevation beech gaps of the Great Smoky Mountains have become the killing front of beech bark disease. This insect/fungal pathogen was introduced into Nova Scotia in the late 1800's, and has since spread southward to the Southern Appalachians. In affected stands, mortality of beech stems frequently approaches 90 to 100 percent. We used inter-simple sequence repeats (ISSR) markers to assess the relationship between host genotype and degree of pathogen infection in beech trees in Great Smoky Mountains National Park. We used statistical analyses to test the relationship between stem diameter and degree of pathogen infection. We found no correlation between host genotype and degree of infection. We did find a significant positive relationship between stem size and degree of infection. Among three stem size classes, smallest stems (<1.5 cm) were least likely to be infected, while largest stems (>3.0 cm) were most likely to be infected. Implications for future studies are discussed.

INTRODUCTION

In recent years, populations of *Fagus grandifolia* Ehrh. (American beech) have been decimated across the species' range due to an insect mediated fungal pathogen complex known as beech bark disease (BBD). The insect component of this complex was accidentally introduced into Nova Scotia around 1890 by way of infected nursery stock, and has since spread westward and southward to cover much of the northern range of American beech (Ehrlich 1934, Houston et al. 1979) (Fig. 1). The woolly beech scale (*Cryptococcus fagisuga* Lindinger) infects beech bark, which then provides a pathway for infection by *Nectria coccinea* var. *faginata* Lohman, Watson, and Ayers or *N. galligena* Bresadola, two species of pathogenic fungi (Ehrlich 1934, Gavin and Peart 1993, Houston 1983). A recent molecular survey of *N. coccinea* var. *faginata* indicates that it may not be native to the United States, although the timing of its initial introduction is uncertain (Mahoney et al. 1999). The chronology of the disease has been well documented (Ehrlich 1934, Houston et al. 1979, Jones 1986). As this disease spreads throughout the range of American beech, average mortality of beech within a population is estimated to be

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as much as 85%, which has been partially attributed to past logging practices (Houston 1979). Historically, beech was not considered a valuable timber species in northern hardwoods and was often left behind in logged stands. This has resulted in the rise of beech as a major component in many northern forests. This overabundance of beech stems is thought to be a contributing factor in the widespread dispersal of beech bark disease. Damage incurred by logging practices is thought to have produced a niche for *C. fagisuga* on otherwise healthy stems (Houston 1982). Houston (1983) reported that although there are known predators of the beech scale, their effectiveness has been limited. Currently there is no known effective treatment for BBD.

Potential for genetic resistance

Resistance to BBD in North America was first studied by Houston (1982), who introduced *C. fagisuga* onto apparently resistant beech trees in aftermath forests to test susceptibility over a period of three years. On susceptible trees the insect was able to complete its life cycle and produce massive amounts of eggs. Alternatively, the insect failed to become established on stems that Houston perceived to be resistant. Based on these trials, Houston produced two hypotheses regarding the source of resistance in these trees: 1) the inability of overwintered insects to complete their life cycle on disease free trees may be a result of the presence of some toxin or lack of some necessary chemical in the tree itself; 2) the complete lack of infestation on some trees and consistently low levels of infestation on others may be the result of either a complete or partial anatomical barrier in these stems. In more recent work, Houston and Houston (1994,

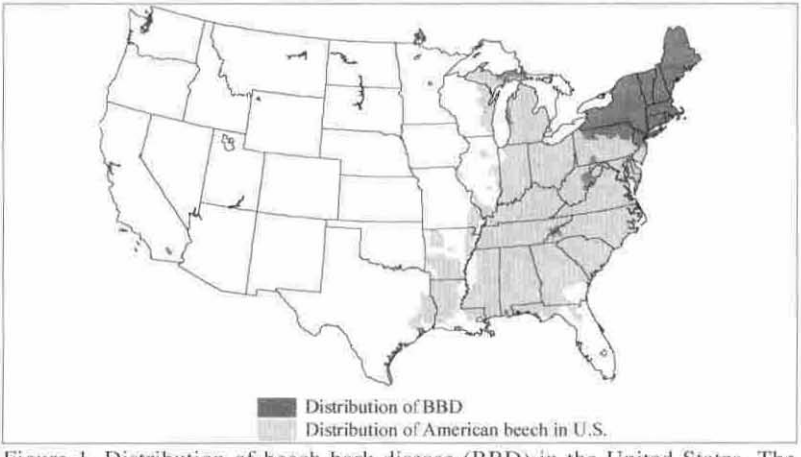

Figure 1. Distribution of beech bark disease (BBD) in the United States. The distribution of *Fagus grandifolia* in the United States is indicated in light gray. Counties in which BBD has been documented are in dark gray. The Great Smoky Mountains represent the southern-most occurrence of BBD. BBD data were provided by Martin MacKenzie, USFS.

2000) studied a third hypothesis: the inability of the scale insect to establish itself on some trees may be the result of genetic resistance.

The potential for resistance has been reported, although observed resistant trees are rarely recorded (Houston 1983). Houston and Houston (1994) observed resistance in less than 1% of all beech trees that they sampled. They also noted that resistant stems often occurred in discrete clumps or groups, suggesting the possibility of resistant clonal genotypes. At least for *F. sylvatica* L., resistance in planted orchards has been associated with genotype (Wainhouse and Deeble 1980). More recently, Krabel and Petercord (2000) found a correlation between beech scale infestation and the host tree genotype in *F. sylvatica*. For *F. grandifolia*, no known relationship between genotype and resistance has yet been found. Houston and Houston (1994, 2000) used isozymes in an attempt to identify resistant genotypes and found that in some instances individual stems sharing the same genotype could be either infected or not infected. However, they did report finding higher levels of observed heterozygosity in susceptible stands, suggesting some level of genetic differentiation between susceptible and resistance trees (Houston and Houston 2000). It is important to note that allozymes often do not display adequate levels of variation to resolve genetic identities in clonal populations (Cruzan 1998, Escaravage ET AL. 1998, Waycott 1998). Whereas allozyme studies are commonly limited to less than 10 polymorphic loci, studies using markers based on DNA variation can include many times that number of loci for the resolution of individual genotypes. In this study, we used inter-simple sequence repeats (ISSRs) to investigate the potential of genetic resistance to beech bark disease in *F. grandifolia* in Great Smoky Mountains National Park (GRSM). We sampled genetic variation within and among three sites to estimate the relationship between level of scale infestation and genotype in GRSM. Our results provide no evidence for genetic resistance, but do suggest a relationship between stem diameter and scale infestation.

METHODS

Study system

BBD was first noted in GRSM in 1993, and since initial discovery, areas of nearly complete stand mortality have been located (Blozan 1995). West of Clingman's Dome, high elevation beech forests are continuous along the North Carolina/Tennessee state boundary for several miles (Blozan 1995). This area is likely the most susceptible to BBD due to high densities of individuals within populations and relative proximity of populations to one another, and is feared to be the site of origin of BBD in the park (Blozan 1995). Evidence suggests that BBD is most prevalent in moist, shaded areas, and that larger trees in higher densities appear to be more susceptible (Gavin and Peart 1993). In

addition, high incidence of BBD has been reported for lower elevation areas where hemlock (*Tsuga canadensis* (L.) Carr) is common (likely due to high shade and moisture regimes) suggesting that BBD is not limited to higher elevation (Blozan 1995). However, the high density of stems and consistently moist conditions at higher elevation sites in GRSM create ideal conditions for BBD infestation and spread (Blozan 1995).

Sampling Methods

Data were collected from three sites, selected with the assistance of GRSM staff (Fig. 2). Two of these sites, Balsam Mountain, and Double Spring Gap, are above 1500 m in elevation and are representatives of "beech gap" forests, which are located within topographic gaps in areas otherwise dominated by spruce-fir forests. The third site, the Chimneys, occurs below 1100 m and is associated with *Tsuga canadensis*, *Acer rubrum* L., *A. saccharum* Marsh., *Halesia carolina* L., *Aesculus octandra* Marsh., and *Cornus alternifolia* L. Because stem densities vary greatly between high elevation and low elevation sites, different

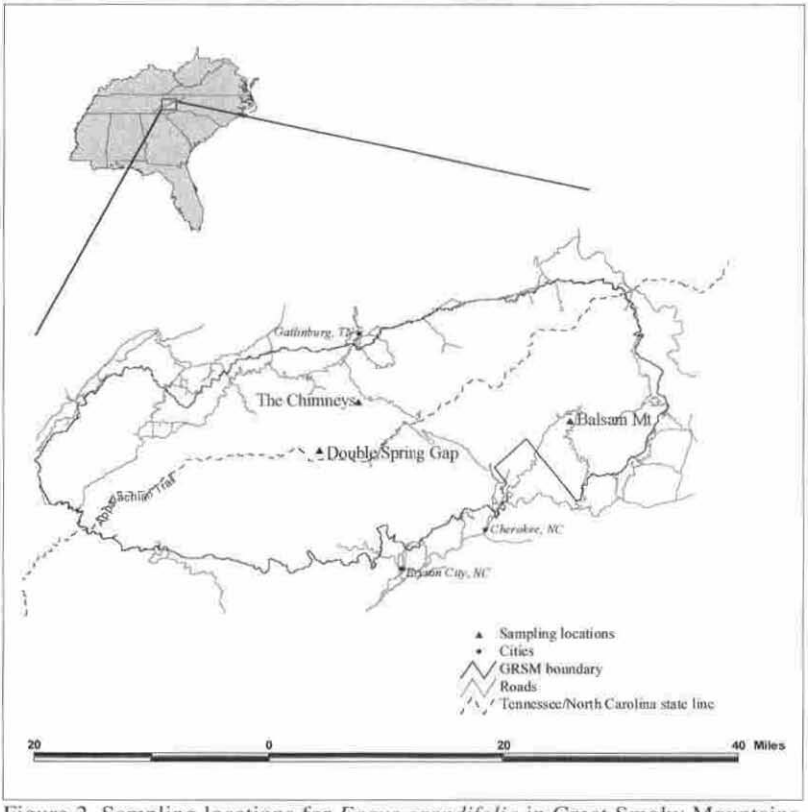

Figure 2. Sampling locations for *Fagus grandifolia* in Great Smoky Mountains National Park.

sampling strategies were applied to these two areas. At the Chimneys, a 10 m x 20 m plot was established, within which twelve, 1 m^2 subplots were randomly selected using a grid system and a random number generator. In each subplot, all *F. grandifolia* stems were sampled. At Balsam Mountain and Double Spring Gap, a 90 m transect was established, along which samples were collected at 10 m intervals. At each sampling interval the five closest stems were sampled and all sampled stems were mapped relative to plot boundaries.

Disease infection was assessed by estimating the percent cover of scale on the trunk of each tree (0 = none, 1 = < 25%, 2 = 26 – 75%, 3 = >75%). Stem diameter was recorded for all sampled stems, regardless of size class. For stems greater than 1.5 m tall, diameter at breast height (DBH) was recorded. For stems less than 1.5 m tall, stem diameter was measured at ground level using dial calipers. Leaf material was collected from each individual, placed in separate 1.5-mL microcentrifuge tubes and stored on ice while in the field. Upon returning to the lab, all samples were snap frozen in liquid nitrogen and stored at -70°C.

DNA Extraction

Total genomic DNA was extracted from each sample using a protocol adapted from Edwards et al. (1991) and modified by Martin and Cruzan (1999). Leaf material was ground in 100 mL of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) for 15 seconds using disposable grinders. An additional 400 mL of extraction buffer was added to this mixture and ground again. Each sample was then centrifuged at maximum speed for 2 minutes. Following centrifugation, 400 mL of supernatant was extracted and placed in a sterile 1.5 mL microcentrifuge tube, to which 400 mL of isopropanol was added. After standing at room temperature for 2 minutes, samples were centrifuged at maximum speed for 10 minutes. All liquid was then poured off, and the samples were allowed to dry. The remaining DNA was then resuspended in 100 mL of distilled water (dH_20). Each sample was cleaned by binding the DNA to DEAE-cellulose as described by Marechal-Drouard and Guillemaut (1995) and then stored at –20°C.

ISSR Protocol

One ISSR primer was selected from UBC primer set number 9 based on the brightness and consistency of the bands produced during screening. Single-primer reaction conditions were carried out in 15 mL reactions as follows: 2.5 mM $MgCl_2$, 200 mM dNTPs, 1 unit of Taq polymerase, 0.10 mM primer, and 0.5 mL DNA. The thermal cycler profile was adopted from Huang and Sun (2000) and is as follows: 1 cycle at 94°C for 5 minutes, followed by 45 cycles at 94°C for 5 seconds, 50°C for 45 seconds, 72°C for 1.5 minutes; and a final 7 minute extension at 72°C. PCR products were electrophoresed on 2% agarose gels in 1X

TBE buffer until bromphenol blue marker migrated 10 cm from the origin. Gels were stained with ethidium bromide and were documented digitally using Kodak 1D Biomax software. Bands with the same molecular weight were treated as identical loci. A data matrix was compiled in which band presence was scored as 1; band absence was scored as 0. Ambiguous bands were eliminated from the analysis.

Data efficiency

Varying numbers of loci (1 through 24) were randomly resampled to infer the effects of increasing the number of markers on the number of genets detected. The number of genets was calculated for one thousand replicates of each data set size and these data were used to examine the relationship between number of loci in the data set and the number of genets detected. We assumed that an asymptotic curve (i.e., the number of new genets detected approaches zero as the number of loci in the data set is reached) would indicate that adding additional loci would not result in an appreciable change in the total number of genets detected.

Genetic variation and estimates of resistance

Genetic structure was analyzed by calculating the number of ramets, number of genets, and number of polymorphic loci assayed. Ellstrand and Roose's (1987) proportion distinguishable (*PD*) was used to estimate clonal diversity within sites. Due to the small number of clones detected, statistical analysis of the relationship between genotype and level of infection was not possible. Thus, this relationship was qualitatively assessed. The hypothesis that scale coverage differs with stem diameter was tested using the row mean scores test statistic (Q_x) as calculated using SAS® software (SAS Institute, Inc., 1999). The results of this test were graphically visualized by constructing a mosaic plot using JMP® software (SAS Institute, Inc., 1989-2002).

Gene diversity within and among populations was estimated using the program Tools for Population Genetic Analyses (TFPGA, version 1.3, Miller 1997), which handles both codominant and dominant marker data. TFPGA uses Weir and Cockerham's (1984) methods for calculating Wright's *F* statistics where q_p is the amount of variation explained

Table 1. Sampling locations in Great Smoky Mountains National Park (GRSM). Three sites were sampled for genetic variation and clonal structure using ISSRs. A total of 85 trees were sampled. Site names are followed by abbreviations; elevation indicates the approximate elevation in meters; n = sample size (or number of ramets); G = number of genets; $PD = G/n$; P = percent polymorphic loci. As *PD* approaches 1.00, clonal structure decreases.

Site	Elevation	*n*	*G*	*PD*	*P*
Chimneys	1097	19	8	0.42	45.83
Balsam Mountain	1524	30	22	0.73	83.33
Double Spring Gap	1585	35	34	0.97	91.67

by differentiation of populations. We performed bootstrapping over loci to obtain variance estimates for these statistics. Since we did not have an estimate of the frequency of heterozygotes in our data, Hardy-Weinberg equilibrium was assumed.

RESULTS AND DISCUSSION

The objective of this research was to identify the relationship between incidence of *Cryptococcus fagisuga* and the genotype and diameter of its host, *Fagus grandifolia*, in GRSM. Due to loss of frozen leaf material because of freezer failure in the spring of 2000 and some generally problematic samples, the actual sample size was not equal to the numbers of samples originally collected. We assayed a total of 85 individuals across the three sites studied. Using one ISSR primer, we identified 24 loci, 23 of which were polymorphic. Locus resampling resulted in an asymptotic curve, indicating that we had obtained a sufficient amount of loci to detect the amount of variation present at these sites. Results are summarized in Table 1. The two high elevation sites (Double Spring Gap and Balsam Mountain) exhibited very little clonal structure ($PD = 0.97$ and 0.73, respectively), indicating that stand maintenance is dependent on seed production. This is inconsistent with previous hypotheses that high elevation beech gaps are maintained by clonal reproduction. Alternatively, the low elevation site (the Chimneys) was primarily clonal in nature ($PD = 0.42$), which is most likely a response to mortality caused by beech bark disease.

We found no evidence for genetic resistance, with clonemates expressing all levels of infection (from 0 to 3) by *C. fagisuga*. Previous work on *F. sylvatica* in Lower Saxony identified a correlation between beech scale infestation and the genotype of the host trees based on a single allozyme locus (Krabel and Petercord 2000). However, *C. fagisuga* is an introduced species in the United States, with its first introduction dating to about 1890. One hypothesis to explain the apparent absence of genetic resistance in *F. grandifolia* is a lack of time for such a relationship to evolve. To test this hypothesis, a thorough phylogeographic study is needed for both *Fagus* and *Cryptococcus*.

We found a positive correlation between DBH and level of *C. fagisuga* infection (Q_X=43.2302, $p<0.0001$), suggesting that larger stems are more susceptible than smaller ones (Fig. 3). In particular, 100% of stems greater than 3 cm DBH were infected to some degree, while smaller size classes contained a considerable percentage of non-infected stems. Although high levels of infection did not occur frequently, it is important to note that the majority of these occurrences were on stems greater than 3 cm in diameter. In contrast, stems less than 1.5 cm in diameter did not exhibit high levels of infection. The most likely explanation for this difference is the increased opportunity for

bark fissures and furrows as the tree grows in size. Such furrows supply a point of entry for *C. fagisuga*, which is then followed by *Nectria* spp., eventually resulting in tree mortality.

The occurrence of beech bark disease in the Great Smoky Mountains represents a disjunction from all other known occurrences, which are primarily limited to New England (Fig. 1). The reason for this disjunction is unclear. Perhaps the beech gaps of GRSM provide the most contiguous and most densely populated stands of *F. grandifolia* remaining across the species range. This hypothesis is supported by the rarity of BBD in low elevation stands of *F. grandifolia* in GRSM. The appearance of the disease in the beech gaps suggests dispersal along the ridge tops of the Southern Appalachian Mountains. However, there are no records of BBD to support this pattern. This raises the question of how the BBD complex would appear so far south without additional outbreaks occurring along the pathogen dispersal route. A better understanding of the population demographics of the disease is needed before this question can be answered. Additional questions that need to be answered include the effects of the disease on fecundity of *F. grandifolia* populations. Little or no information is available on seed mast production in stands at different stages of infection. Studies indicate that many aftermath stands are now recovering by clonal reproduction, resulting in thick stands of small,

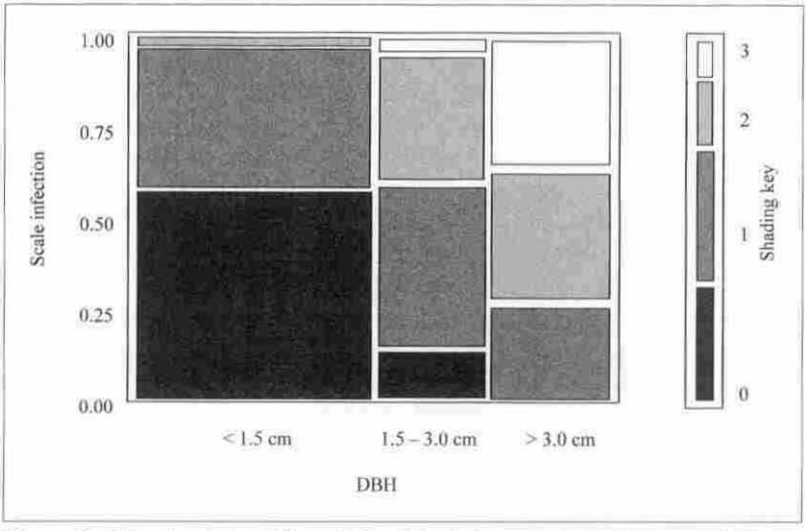

Figure 3. Mosaic plot of the relationship between stem diameter of *Fagus grandifolia* and level of infestation by *Cryptococcus fagisuga*. This plot represents the frequencies of combinations of two variables, in this case stem diameter and scale infestation. Each rectangle represents the joint probability of a particular combination of these two variables. The shading key indicates which color corresponds to each level of scale infestation (0 = none; 1 = < 25%; 2 = 25-75%; 3 = > 75%). In addition, the shading key indicates the overall probability of each level of infection based on all data collected.

genetically identical stems (personal communication, W. D. Ostrofsky). This not only changes stand species composition by limiting openings for the recruitment of other species, but also makes the stand more susceptible to future BBD outbreaks.

Because no genetic resistance has yet been identified in *F. grandifolia*, management decisions are limited. A number of recommendations have been made by previous researchers, most of which involve cutting the largest and most deformed stems to prevent further spread of the disease. Unfortunately, these are also often the most productive members of the population. In GRSM, the high elevation beeches represent the only hard masting species in these otherwise spruce-fir dominated forests, providing food for a large number of animal species. In order to protect these unique communities, more work is needed to understand the potential roles of genetic resistance and chemical defense to BBD. Work is currently underway to continue the search for genetic resistance using several different molecular techniques.

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