MOLECULAR POPULATION GENETICS AND EPIDEMIOLOGY OF *CERATOCYSTIS FAGACEARUM*

A Thesis

by

KELLY LYNN IVORS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 1995

Major Subject: Plant Pathology

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ABSTRACT

Molecular Population Genetics and Epidemiology of *Ceratocystis fagacearum*. (December 1995)
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Chair of Advisory Committee: Dr. David N. Appel

The population genetic structure of *Ceratocystis fagacearum* was studied with genetic markers to address questions concerning the microgeographical spread and genetic variability of the oak wilt pathogen. Genetic diversity was assessed using restriction fragment length polymorphisms (RFLPs) and anonymous RFLP loci of the nuclear DNA (nuDNA). Results of the study revealed only 4 haplotypes existing in the 3 populations analyzed. Of the 3 populations studied, 2 were asexually expanding and the other population represented a sexually active disease center. Three polymorphic RFLP loci were found. Only one population, Willow Springs, had more than 1 allele present at the RFLP loci. Very low levels of variation (G = 1, 1, and 11.69) were detected in the nuDNA among 96 isolates, from 3 distinct populations, of *C. fagacearum*. Hypotheses are discussed to account for this low level of genetic variation.

DEDICATION

This thesis is dedicated to my parents, John William and Susan Mary Ivors, and to my late grandfather, Donald Russell Rockhill.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. David Appel for serving as chairman of my committee. His patience, advice, and support are sincerely appreciated and will always be remembered. It was with his encouragement that I decided to pursue an advanced degree in plant pathology, and for this I will always remember him fondly.

My sincere appreciation goes to my committee members, Dr. Bruce A. McDonald, Dr. J. Spencer Johnston, and Dr. Dan Wilson (of the USDA Forest Service), and the department head, Dr. Neal Van Alfen.

Special thanks to Dr. Charles M. Kenerley, who hired me as a lab technician after my assistanship ended which allowed me to continue working on my thesis in the evenings and on weekends. I learned so much from him and actually got my feet wet.

I would also like to thank Tom Kurdyla for his advice during the early stages of my research. I learned many valuable techniques from him. Thanks also goes to Dr. Phil Guthrie who constructed and screened a library of random, anonymous nuDNA fragments from *C. fagacearum* that I used for probes. Without his preliminary research I would have had no molecular tools with which to compare isolates. My research is largely based on his findings and I can not thank him enough for his hard work. Lastly I would like to thank my parents,

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INTRODUCTION

Oak wilt, caused by *Ceratocystis fagacearum* (Bretz) Hunt, is recognized as the most serious plant disease in Texas. Oak wilt was first discovered in Texas near Dallas in 1961 (9). Since then, the pathogen has been isolated from diseased oaks at numerous locations throughout 53 counties (personal communication, Dr. Jerral Johnson, Texas Agricultural Extension Service, TAMU, College Station, TX). The recent, intense development of oak wilt is responsible for enormous losses of live oak (*Quercus fusiformis* Small and *Q. virginiana* Mill), particularly in central Texas where live oak is a dominant hardwood species. This disease is currently one of the least understood of the serious pathogens affecting trees in the United States. More epidemiological research involving *C. fagacearum* is needed to provide better disease control.

Recently, a number of questions involving genetic variability have been raised for populations of *C. fagacearum*. These questions involve aspects of oak wilt such as the origins of the pathogen, the intensity and recent development of the Texas epidemic, the typical distances of pathogen transmission, and the relative importance of conidia and ascospores in new epidemics. Answers to these questions will provide a better understanding of the epidemiology of oak wilt, which includes local and long distance pathogen spread via connected roots and

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insect vectors, respectively. Origins of isolates which initiate infections may be identifiable and fungal associations between old and new infections may be made. This information relating origin of an isolate and subsequent spread may be used to formulate new strategies for disease control and improve those control practices currently in use. For example, with better knowledge of the genetic variability and spatial distribution of *C. fagacearum* in affected stands, placement of control trenches and intravascular injection of fungicides may be better utilized to provide preventative treatments for high risk individual trees or stands. Knowledge of the genetic structure of *C. fagacearum* populations may also suggest how quickly this pathogen is likely to evolve, and thus help predict how long a chosen method of control will last. Fungal populations with high levels of genetic variation are more likely to develop resistance to fungicides than populations with less variation (27). When populations of plant pathogens are exposed to sufficient selection pressure, the variants that can reproduce in the presence of fungicides will become predominant (13). Understanding the processes that affect genetic variation in populations of phytopathological fungi is imperative to develop adequate and proper methods of disease management (26). Evaluating the population genetic structure of plant pathogens provides useful information on the amount and distribution of genetic diversity within and between pathogen populations (27). This knowledge is needed in order to understand natural selection and host-pathogen coevolution

in plant pathosystems. Currently most pathosystems are studied on a macrogeographical scale (28). These studies evaluate genetic variation from a limited number of isolates collected from a wide geographical area. Only a few studies have involved individual populations. However, studies that encompass a limited number of isolates from large geographic areas are inadequate to provide data on plant pathogen population genetics. A more appropriate method is to sample individual populations by collecting large numbers of isolates on a microgeographical scale. Such is the case with research done by McDonald and Martinez (28) and McDermott et al. (25). Both studies successfully quantified genetic variation among single-location, pathogen populations of *Mycosphaerella graminicola* Fuckel and *Rhynchosporium secalis* Oudem, respectively. Oak wilt could be a good system for application of similar genetic methods. It is an important tree disease, has two distinct modes of transmission, and the importance of sexual reproduction in *C. fagacearum* on future populations has yet to be established.

Analysis of plant pathogen population genetics requires genetic markers "that are informative (polymorphic) and unambiguously scored" (27). RFLP (restriction fragment length polymorphisms) markers, based on electrophoretic separation of DNA molecules, have been applied in many studies of populations of pathogenic fungi over the past ten years. The research in this thesis deals with the application of RFLP markers to address questions concerning the microgeographical spread and

LITERATURE REVIEW

Epidemiology of *C. fagacearum*

Oak wilt damages most species of oaks, but live oaks (*Quercus fusiformis* Small and *Q. virginiana* Mill.) are most seriously affected in the central Texas live oak woodlands. Once a tree or stand of trees becomes infected, a reversal and inhibition of the disease process becomes very difficult. However, surrounding healthy trees can be protected and potential losses can be reduced if further fungal transmission is stopped (12). *C. fagacearum* spreads in two ways, by conidia transmitted through connected or grafted root systems or by spores vectored by sap-feeding nitidulid beetles (*Coleoptera: nitidulidae*) (3).

Ceratocystis fagacearum is a heterothallic Ascomycete, with two mating types required to produce the fruiting structures that characterize the sexual stage. This fungus produces two types of spores. Conidia, and more infrequently, ascospores in perithecia which are found exclusively on infected red and black oaks. Both spore types form underneath the bark on pads of mycelia called fungal mats (12). Conidia are abundantly produced. Perithecia and ascospores also may be present if either of the two compatible strains, designated 'A' and 'B', are transmitted from a mat of one mating type to one of the opposite type. The conidia serve as spermatia by fertilizing a mat of the opposite mating type. Nitidulid beetles (*Coleoptera : nitidulidae*) are uniquely suited for

long-distance fungal transmission with the ability to transport inoculum up to a mile or more from inoculum sources (3). Studies of conidia and ascospores associated with insects proved that this fungus can survive for extended periods on dead or dormant nitidulid beetles (38) and that conidia may remain viable after passage through the bodies of beetles (16).

Only a small percentage of diseased red or black oaks form fungal mats, and mat development is quite seasonal, occurring during cool, damp weather in winter and early spring. Conditions in Texas appear to be ideal for fungal mat formation beginning in October and extending through the month of May (3). Pathogen spread through root systems presumably occurs exclusively through movement of conidia via the continuous xylem system that exists between root-connected trees. Root connections may arise by grafting or by root sprouting from clonal motts, or both (2). Gibbs and French (12) state that the spread of the asexual stage (conidia) of *C. fagacearum* through connected root systems results in large stands of infected oaks and the majority of losses. Appel et al. (1) discovered that a majority of discrete, multiple infection centers consisted of only one sexual compatibility type. Kaufman (17) also found the same was true in West Virginia disease centers. In Texas the fungus is able to move up to 23 meters (75 ft) per year through groups of root connected live oaks, causing the loss of hundreds of trees (2). The degradation of the original grasslands in Texas along with the selective removal of other woody species has possibly enabled

the live oak to develop vast, homogeneous clonal populations due to the tendency of live oaks to produce root sprouts and maintain common root systems. Since live oaks in Texas appear to root graft readily in addition to some amount of clonal growth habits, pathogen spread through common root systems can be extremely serious and contagious. Epidemics of *C. fagacearum* in Texas may be responsible for more tree mortality than in any other portion of the disease range in the United States. The temporal climate of Texas and the influence of stand composition are thought to contribute to this intense epidemic (4).

Most control measures for oak wilt are based on transmission and general epidemiological data. Trenches dug to sever common root systems often fail and in areas where oak wilt occurs, new disease centers frequently appear. These outbreaks and failures to control the transmission of *C. fagacearum* are difficult to explain. Therefore, it is presumed that our understanding of oak wilt is not yet complete.

Markers to study fungal population biology

Addressing key co-evolutionary questions requires methods by which particular pathogen genotypes can be identified and followed in the population as a whole. Many genetic markers can be utilized for this purpose. These include: 1) markers subject to direct selection such as degree of aggressiveness and pathogenicity; 2) morphological markers such as growth rate or mycelial color; and 3) selectively neutral or near-neutral

markers such as isozymes, restriction fragment length polymorphisms (RFLPs), or RAPDs (random amplified polymorphic DNA) (7). RFLPs are based on specific differences in DNA sequences which can be detected through the use of restriction endonucleases that cut DNA at particular base sequences. These enzymes recognize specific base sequences in double-stranded DNA, called restriction sites, and catalyze the cleavages of specific phosphodiester bonds in or near that region. The various fragments formed can be separated (based on the molecular size of the fragments) by using agarose gel electrophoresis, yielding a distinctive banding pattern for each probe-enzyme combination. The rate at which the individual fragments move through the pores of the gel is a function of their length, with the shorter fragments moving faster than the longer ones. Each band corresponds to a restriction fragment whose molecular weight can be measured by comparison with DNA molecules of known molecular weight (37).

DNA sequence variation can either contribute to advantageous effects, detrimental effects, or no effect at all upon an individual organism. Genomes have the ability to withstand certain alterations in their nucleotide sequences. Survival through environmental or host related stresses can cause fungi to change their genetic composition. Nucleotide sequence variation can result from small or large alterations. Large changes are caused by deletions, additions, inversions, translocations, or rearrangements. Small changes are caused by point mutations (14). When one of these mutational events

occur, the genome will differ from an unaltered genome, thus yielding an RFLP.

Understanding variation in fungal populations is a complex task. First, the term population should be defined. A population is a group of interbreeding or potentially interbreeding individuals that occupies a certain space over a defined period of time (34). Only a few forces result in discernable changes in allele frequencies, such as migration, genetic drift, and sexual recombination. The size of the population is also significant to the maintenance of genetic variation. Large populations are more likely to contain variation than smaller ones. A more limited number of different gene combinations are expected to occur in populations with strict asexual reproduction, compared to populations that regularly reproduce sexually. A pathogen with an active sexual cycle can produce novel combinations of genes by recombination. Also, the frequency of the sexual stage as well as the necessity of the sexual stage as an obligate part of the pathogen's life cycle can have significant effects on the type and rate of genetic variation (5). The total number of alleles found in a population is not determined by sexual recombination, however recombination can strongly influence the distribution of these alleles and overall multilocus diversity (27). Mutation followed by genetic drift increase genetic variation between populations, whereas migration decreases variation between populations. In small populations, such as discrete disease centers of *C. fagacearum*, these forces have

greater effects (14). Of most critical importance to the dynamics of plant pathogens is the environment in which they exist and reproduce. This environment can be composed of both biotic factors (i.e. host plants) and physical factors (i.e. distribution of host plants and conditions conducive to pathogen reproduction) (5).

RFLPs were chosen for this study due to the nature and extent of the questions being addressed as well as the biology of the pathogen. RFLPs are informative (polymorphic) and unambiguously scored, unlike other traditional markers such as morphological markers or markers subject to direct selection (pathogenicity) (7). The existence of RFLP differences is independent of host genotype variation, often a problem when pathotype designations are used. RFLPs can also detect variation in coding and non-coding regions of DNA, hence they are much more variable than isozymes. Because RFLPs are determined by differences in DNA sequences, they are remarkably sensitive genetic markers (27).

Knowledge of RFLP variation in plant pathogenic fungi has grown substantially (30). However, sample sizes have been generally small and inadequate to quantify variation within individual populations. There have been several studies that used molecular markers to distinguish clones of fungal plant pathogens. One study involving two field populations of *S. sclerotiorum* (Lib.) de Bary on canola used DNA fingerprints to determine that the populations were genetically heterogeneous (i.e. composed of several clones) (19). This study

demonstrated that clones of *S. sclerotiorum* were distributed over long distances and that intact clonal genotypes can potentially be dispersed as ascospores (19). Using RFLPs, McDonald and Martinez (28) found considerable genetic variation within a population of *Septoria tritici* Roberge ex Desmaz in an individual wheat field. Most of these *S. tritici* clones were recovered several times with most clones highly clustered around certain sampling sites. McDonald et al. (29) also detected high levels of genetic and genotypic diversity in populations of *Stagonospora nodorum* (Berk.) Castellani and E. G. Germano. Surveyed fungal populations contained a large number of different genotypes distributed on a small scale. Different genotypes were found to occur on the same leaf, as separate lesions. Christiansen et al. (8) found RFLP markers that were efficient in characterizing powdery mildew isolates. Only three markers were necessary to differentiate twenty-eight isolates. Ueng et al. (41) used RFLPs to assess the amount and distribution of genetic variation in populations of *Phaeosphaeria nodorum* (E. Muller) Hedjaroude. Fingerprinting based on multilocus haplotypes also allowed them to follow 'marked' isolates in epidemiological studies. With DNA fingerprinting, Levy et al. (22) distinguished pathogenic clones of *Magnaporthe grisea* (Hebert) Barr (the causal agent of rice blast) from nonpathogenic clones. DNA fingerprinting involves the hybridization of a specific probe to highly variable repetitive DNA sequences. Doudrick et al. (10) were able to identify genetic variation within a single

urediniospore-derived culture of *Cronartium quercuum* (Berk.) Miyabe ex Shiria f.sp. *fusiforme*, using polymerase chain reaction. PCR is a highly sensitive and specific technique used for the amplification of nucleic acids. This study was the first to demonstrate genetic variation within the formae speciales of *C. quercuum*. Forster et al. (11) used restriction patterns of nuclear DNAs to distinguish isolates of *Phytophthora megasperma* Drechs. (Pm) into different formae speciales groups. Manicom et al. (24) used RFLPs with combinations of probes and restriction enzymes to detect differences at species, formae speciales, and isolate levels of a range of *Fusarium* species.

In asexual populations, polymorphic markers can be used to determine the numbers of genotypes present (30). Brown et al. (6) used RFLPs among isolates of *Erysiphe graminis* DC f.sp. *hordei* Marchal to test hypotheses about pathogen population structure, and discovered multiple groups of isolates consisting of single clonal lines. Epidemiological studies done on the tree root pathogen *Heterobasidion annosum* (Fr.:Fr.) Bref. (39) and *Armillaria mella* (Vahl:Fr.) Kummer (38), with isoenzyme patterns and RFLPs respectively, demonstrated that vegetative growth of single clones could be enormous, spreading over several hectares through the soil. In each infection center, the number of genotypes present indicated whether there had been single or multiple infections.

OBJECTIVES

The following experiments were designed to better understand the genetic make-up and variability among isolates of *C. fagacearum*. In turn, this understanding will hopefully allow the improvement of approaches to control oak wilt. Little consideration has been given to the microgeographical distribution and genetic variation of the oak wilt pathogen in Texas, and specifically, the epidemic levels in some areas and limited lack of distribution in others. For instance, the lack of oak wilt in the diverse east Texas piney woods remains a mystery. Some boundaries can be easily explained while others cannot (12). The genetic analyses of *C. fagacearum* populations have been hindered by inadequate knowledge of the reproductive biology, epidemiology, and by lack of genetic markers. Thus the origins of the pathogen and the intensity and recent development of the Texas epidemic are poorly understood. A greater knowledge of the pathosystem, such as sources of infection, and the rate at which the fungal population changes is needed. These objectives should provide useful evidence on mechanisms of disease transmission. The objectives of this research were to:

A. Determine the existing genetic variability throughout an asexually expanding population of the pathogen.

B. Determine and compare the existing genetic variability of two distinct, asexually

expanding populations of the pathogen.

C. Compare the genetic diversity present in Texas populations and a sexually expanding population in Minnesota.

MATERIALS AND METHODS

Study sites

Oak wilt centers in Texas live oak stands may be extensive, providing the opportunity to sample adequate numbers of isolates of the pathogen and test theories about population genetic variability. Texas oak wilt foci have been found to contain thousands of dead trees over areas as large as 80 hectares (12). In the present study, suitable study sites were surveyed and mapped, and isolations of the pathogen were made from selected areas within the study sites.

One site consisted of a small oak wilt focus at the Robinson Ranch located 10 kilometers west of Round Rock, Williamson County, Texas. This disease center, termed the 'Root Study', was a homogenous stand of diseased and healthy live oaks approximately 152.4 m^2 in size, and was estimated to have initiated in 1990 by a "breakover" from a previous trenching operation that failed to control the disease. On May 18, 1992, the site was surveyed and mapped on a 91.44 m x 91.44 m (300 ft x 300 ft) grid containing 36 sections. Each section was 15.24 m x 15.24 m (50 ft x 50 ft) in area. Tree removal for acquisition of roots was performed on May 19-20, 1992, using a bulldozer. Twenty isolates of *C. fagacearum* were collected in this disease center; two of the twenty isolates (labeled T10 and T14 for tree 10 and tree 14, respectively) were collected from bole samples removed from symptomatic trees. Thirteen isolates were collected from the roots of

asymptomatic and symptomatic trees, (labeled T3R1, T3R2, ..., T85R3 for tree 3 root 1, tree 3 root 2, and so on until tree 85 root 3). These thirteen isolates came from a total of 5 trees, with some isolates originating from different roots on the same tree. The remaining 5 isolates were collected from trench roots (labeled as: T1S16R1 meaning trench 1 section 16 root 1). Trenches were dug among symptomatic trees so that roots could be collected and assayed for the presence of *C. fagacearum*. Trenches were dug with a bulldozer equipped with a 1.2 m (4 ft) soil chisel. All trees and trenches sampled within this disease center were mapped (Figure 1).

The second site, called Willow Springs, is located on the same ranch within 0.8 km of the first site. It is estimated to have initiated in the late 1970s at point 'A' (Figure 2). The Willow Springs site is comprised of a nearly pure stand of live oak. Forty-two isolates of *C. fagacearum* were collected on a range of dates over a 36 month period (Table 1). All isolates were collected from bole samples of symptomatic trees, most located along the expanding disease perimeter. The perimeters of oak wilt foci in live oak are easily defined by a sharp contrast between the diseased, symptomatic, and asymptomatic trees (4). On live oak, the leaf symptoms are variable. The most common indication of infection is brown necrosis of the leaf veins, or veinal necrosis. Tip burn and marginal scorch are also common oak wilt symptoms. Attempts were made to collect isolates from older parts of this disease center. However those trees were either dead and no longer contained

the fungus, or were escape trees that never became colonized by the pathogen.

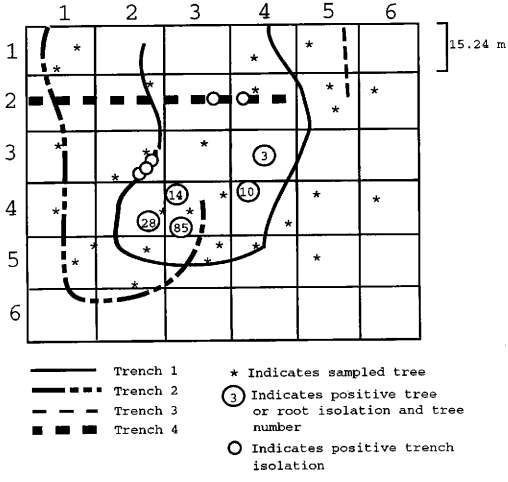

Fig. 1. Map of all trees and trenches sampled within the Root Study disease center. The site was 91.44 m x 91.44 m and contained 36 sections. Twenty isolates were collected from bole, root, and trench root samples. Isolates came from a total of 5 trees and 2 trenches.

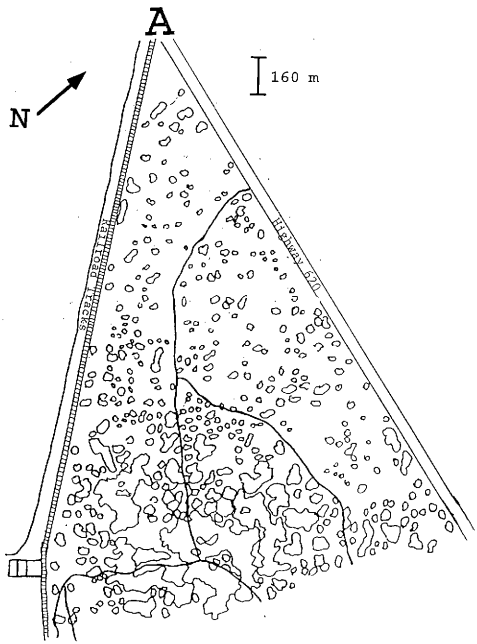

Fig. 2. The Willow Springs disease center. The letter 'A' marks the initiation point of infection.

TABLE 1. Collection dates of all Willows Springs isolates of *C. fagacearum* taken over a 36 month period

Willow Springs	
Isolate	Collection date
WS 604	Nov-90
WS 606	Nov-90
WS 607	Nov-90
WS 615	Jan-91
WS 847	Apr-92
WS 848	Apr-92
WS 851	Apr-92
WS 889	Jul-92
WS 891	Jul-92
WS 898	Jul-92
WS 901	Jul-92
WS 902	Jul-92
WS 903	Jul-92
WS 921	Jul-92
WS 923	Jul-92
WS 924	Jul-92
WS 925	Jul-92
WS 926	Jul-92
WS 948	Sep-92
WS 951	Sep-92
WS 955	Sep-92
WS 956	Sep-92
WS 963	Sep-92
WS 35	May-93
WS 36	May-93
WS 37	May-93
WS 39	May-93
WS 44	May-93
WS 55	May-93
WS 57	May-93
WS 60	Jun-93
WS 62	Jun-93
WS 63	Jun-93
WS 68	Aug-93
WS 69	Aug-93
WS 70	Aug-93
WS 71	Aug-93
WS 72	Aug-93
WS 73	Aug-93
WS 76	Oct-93
WS 79	Oct-93
WS 81	Oct-93

The Minnesota oak wilt focus is located in St. Paul, Minnesota. Two groups of isolates were collected from a fungal mat found on a red oak (*Quercus texana* Buckl. and *Q. marilandica* Muenchh.). Group 'One' consists of five isolations taken from a single perithecium, labeled Mn 1 through Mn 5. Group 'Two' consists of twenty-nine isolations taken from a different perithecium, labeled Mn 6 through Mn 34. This site represents a sexually active population that spreads from tree to tree via sap-feeding nitidulid beetles as well as through root connections formed by root grafting.

Fungal isolation

All Texas isolates of *C. fagacearum* used in this study were obtained by sampling boles and roots of symptomatic trees growing in the study sites described above. Bole samples consisted of excised sapwood from tangential, lower-trunk xylem pieces cut 5 to 10 mm deep with a hatchet at approximately 0.3 to 1 m above soil level. Root samples were obtained from the root systems of symptomatic trees by roguing these trees with a bulldozer. Both bole and root samples were processed in the laboratory by removing the outer and inner bark, chopping the tissue into small pieces, and surface sterilizing with 0.625% (bole samples) or 1.25% (root samples) sodium hypochlorite (Clorox). Individual wood chips were then plated on acidified-homemade-potato dextrose agar medium (APDA). The mycelium of *C. fagacearum* usually grew from the wood chips after three to five days of incubation. Fungal mat isolations, collected

from the study site in Minnesota, were obtained by probing perithecia that were produced on naturally spermatized fungal mats for ascospores, and then plating the spores on APDA. Isolated fungi were identified by microscopic examination of hyphal morphology, the appearance of typical *C. fagacearum* endoconidia, or by characteristic *C. fagacearum* mycelial-growth on agar. After isolation, each culture was hyphal-tipped to insure the growth of a single individual. All isolates were stored at 0°C as spore suspensions in glycerin and silica gel vials.

DNA extraction

Each isolate was grown in 50 ml of Sabouraud's broth (glucose 2 g, peptone 1 g, yeast extract 0.3 g, and distilled water 100 ml) for 7 to 10 days to produce sufficient material from which to obtain adequate quantities of purified DNA. Fungal tissue (0.1 g - 0.15 g) was harvested by centrifugation, lyophilized and the DNA extracted using a modified CTAB extraction procedure described by Kim et al. (18). The goal was to first inactivate endogenous nucleases and then separate DNA from protein and RNA. The resulting DNA concentration in solution was then measured with a fluorometer, (using Hoechst 33258 stain), for future digestion procedures.

DNA digestion, electrophoresis, blotting, and hybridization

Sequences of cloned DNA from *C. fagacearum* were used to probe certain regions of the genome for the presence of

polymorphisms at the DNA sequence level. Previously, a library of 124 random, anonymous nuDNA fragments from *C. fagacearum* was constructed for use as probes (20). CsCl-purified nuDNA from Texas isolate McLennan 1001 was digested with *Pst*I. Random fragments ranging in size from 2 to 10 kb were ligated into cloning plasmid vector pUC18 and used to transform *E. coli* strain DH5αMCR, following methods outlined in Maniatis et al. (23). The average size of *C. fagacearum* fragments in the library was approximately 5000 bp. The 124 anonymous DNA sequences were screened using a range of Texas, West Virginia, and Wisconsin isolates. Variations in the length of DNA fragments homologous to a labeled probe were scored as polymorphisms. The term restriction fragment length polymorphism is derived from this procedure (21). The RFLP locus refers to a sequence of DNA on a chromosome which has sequence homology to the DNA probe. The alleles at RFLP loci are the DNA restriction fragment size variants caused by insertions, deletions, or restriction enzyme recognition site changes along the portion of the chromosome where the probe hybridizes (30). The pattern of bands probed are dependent on the nature of the probe and the enzymes used to restrict the genome. After construction and screening of the nuDNA library of *Pst*I digestion fragments, seven informative probes representing seven RFLP loci were chosen for the construction of haplotypes for *C. fagacearum* isolates. Restriction endonucleases used for digestion were *Pst*I, *Hind*III, and *Eco*RI.

Five μg of each isolate's DNA was digested with the 3

restriction endonucleases. The restriction fragments were separated by electrophoresis on 0.8% agarose gels and transferred from the agarose to a Zetaprobe membrane. This capillary transfer method was developed for nylon membranes using NaOH as the transfer solution (35). The restriction endonuclease *Hind*III, a methylation sensitive enzyme, was used to insure that modified nucleotides were not occurring. The membrane was baked at 80°C for 2 hours and hybridized to a ^{32}P labeled probe by nick translation overnight in 250 mM disodium phosphate and 7% SDS at 60°C in a hybridization incubator. Following hybridization, membranes were washed twice for 1.5 hours at 60°C in 20 mM disodium phosphate and 5% SDS and then twice for 1.5 hours at 60°C in 20 mM disodium phosphate and 1% SDS. Membranes were placed on X-ray film with intensifying screens for periods up to one week, depending on the intensity of the radioactive signal on the membrane at -80°C. After development of autorads, all membranes were stripped by boiling in 0.01xSSC, 0.5% SDS for 30 minutes and then used for repeat hybridizations with other informative probes. Landry et al. (21) states that a few markers should be sufficient to quantify genetic variation for asexually propagated organisms like *C. fagacearum*.

Data analysis

These analyses were used to assess and compare genetic variability within and among populations: 1) mating type analysis, 2) number of alleles per locus, 3) contingency Chi-

square analysis (43), 4) multilocus haplotype analysis, 5) Nei's measures of genetic diversity and genetic distance (32), and 6) genotypic diversity (40).

Compatibility type analysis

Sexual compatibility of all isolates was determined by using a spermatization technique described by Kaufman (17). Isolates with unknown mating types were transferred onto potato dextrose agar (PDA) and cultured at 25°C for approximately ten days. Spore suspensions of known compatibility types were used to spermatize unknown isolates by pipetting 500 μl of a fresh spore suspension onto a PDA plate colonized with the unknown isolate. Production of fertile perithecia a few days after spermatization indicated a positive cross. Isolates that produced fertile perithecia when crossed with a known 'A' type were designated 'B' and vice versa. Perithecia can only be formed when opposite mating types are brought together and induced to anastomose (12). Two replications of each cross were made.

Allele frequencies

The frequency of an allele is a value that represents the proportion of that allele present among a group of individuals in a population. Information gathered in studies of population samples may be counted to calculate allele frequencies (14). Only a few forces result in discernable changes in allele frequencies, such as selection, migration, and genetic drift.

Frequencies of alleles present at each RFLP locus were computed for each population and for the total collection of isolates.

Multilocus haplotype analysis

Multilocus haploid-genotypes (here after referred to as haplotypes) were constructed by combining allelic data from individual RFLP loci. Each allele was assigned a number in order of decreasing frequency for the RFLP locus defined by each probe-enzyme combination. For instance, allele 1 was found most frequently, followed by allele 2 and so on. The numbers corresponding to the alleles at each RFLP locus were then combined in a fixed order. Each isolate was then characterized by a seven digit 'identifier', since seven probe-enzyme combinations were used. The resulting seven digit number for each isolate summarized which allele was present at each RFLP locus. For example, the most common allele at each RFLP locus would characterize the sequence 1111111 (28). Pairwise measures of genetic similarity were calculated for each haplotype. This calculation is formulated as the percentage of shared alleles at the seven RFLP loci. For example, the haplotypes 1111111 and 1111112 had the same alleles at six of the seven RFLP loci and therefore show 6/7 = 85.7% similarity.

Contingency Chi-square analysis

Direct-count allele frequencies may be compared among groups using contingency Chi-square analysis. Differences in

allele frequencies between locations were tested, using the χ^2 test for heterogeneity (43). Since, *C. fagacearum* is haploid, χ^2 values for each RFLP locus were calculated using the formula:

$$\chi^2 = N \Sigma\sigma^2 p_j / pj \qquad (1)$$

where N is the number of isolates and $\sigma^2 p_j$ and p_j are the weighted and mean variance of the frequency of the jth allele.

Nei's measure of genetic diversity

Nei's formula was applied to the allele frequencies at each locus to measure gene diversity:

$$H_i = 1 - J_i \qquad (2)$$

where H is Nei's measure of gene diversity and J is the probability that two randomly chosen alleles within the subpopulation 'i' are identical (32). The maximum possible value for H is 1.

Nei's measure of genetic distance

Hierarchical grouping of populations can be accomplished using a measure that estimates the amount of genetic divergence between populations. Nei's genetic distance is based on the probability that two alleles drawn at random from two separate subpopulations are identical, relative to the probability that

2 randomly chosen alleles from the same population will be identical. In this context, 'identical' means 'indistinguishable', not 'identical by descent'. The formula is:

$$D = -\ln I \qquad (3)$$

where D is the genetic distance and I is a measure of gene identity of two populations called the *normalized identity*. Nei defines the *normalized identity* (I), for a gene as:

$$I = \Sigma p_i q_i / \sqrt{(\Sigma p_i^2)(\Sigma q_i^2)} \qquad (4)$$

where p and q are the allele frequencies of the gene. Monomorphic loci are included in this analysis. Nei's measure is equal to 1 if the probabilities are equivalent, meaning the populations are identical. Nei's measure is equal to 0 when there exists a lack of genetic divergence between populations (32).

Genotypic diversity

Genotypic diversity was calculated using the method of Stoddart and Taylor (40):

$$G = \frac{1}{\Sigma [f_x \cdot (x/N)^2]} \qquad (5)$$

where G is genotypic diversity, N is sample size, and f_x is the number of genotypes observed x times in the sample. Values of genotypic diversity can range from the maximum value of N, the number of isolates in the sample population to 1, when all isolates are the same genotype. This equation may be used with some degree of confidence to reflect the degree at which asexual reproduction contributes to the genetic structure of a population in species with both an asexual and a sexual reproductive potential (40).

Gametic disequilibrium

Measures of gametic disequilibrium (42) were used to indicate the primary means of reproduction in distinct populations. Locus by locus comparisons for all possible pairwise combination were performed to measure gametic disequilibrium:

$$X_T^2 = \Sigma\Sigma(ND_{ij}^2/p_iq_j) \qquad (6)$$
$$d.f. = (k-1)(l-1)$$

where X_T^2 is the sum of the chi-square value, i and j are the alleles under consideration, p_i is the frequency of allele A_i at locus A, q_j is the frequency of allele B_j at locus B, D_{ij} is the disequilibrium coefficient for alleles A_i and B_j, N is the sample size, and k and l are the number of alleles at each locus respectively (42). Recombination of the loci will probably not occur if all of the population's genetic variation

exists as a few multilocus genotypes (30). This calculation measures the association between genes; if the genes are associated then non-random mating is occurring, and vice versa, if no association of the genes is found then the population is randomly mating.

RESULTS

Fungal isolates

All isolates screened for polymorphisms were identified as *C. fagacearum*; all isolates revealed morphological characteristics of *C. fagacearum*. A total of 96 isolates were analyzed in this study. Twenty isolates came from the Root Study, 42 isolates came from Willow Springs, and the remaining 34 isolates came from the Minnesota population.

Compatibility type analysis

Appendices I, II, and III display the composition of mating types found in the Root Study, Willow Springs, and MacDonald populations, respectively. All isolates collected from the Root Study and Willow Springs were compatibility type 'A'. Fungal mat isolations collected from the Minnesota site consisted of 41.2% compatibility type 'A' and 58.8% compatibility type 'B'.

Multilocus haplotype analysis

Seven random, anonymous nuDNA probes were examined for their ability to detect genomic variation among the populations of *C. fagacearum* being studied. Restriction endonucleases used for digestion were *Pst*I, *Hind*III, and *Eco*RI. Four of the probes (pCF79, pCF102, pCF124, and pCF219) hybridized to repetitive sequences and the other three probes (pCF9, pCF141, pCF189) hybridized to low/single-copy sequences. Appendices I,

II, and III show the allelic composition of the Root Study, Willow Springs, and Minnesota populations, respectively. Each digit in the haplotype corresponds to the allele present at each of the 7 RFLP loci identified by a specific probe-enzyme combination. Alleles were assigned numbers in the order of decreasing frequency in the total collection of 96 isolates. Among the 96 isolates studied, 3 polymorphic loci were found: pCF9/*EcoRI*, pCF79/*Hind*III, and pCF189/*Hind*III. Figures 3, 4, and 5 are autoradiograms that depict the different alleles present at these polymorphic loci in isolates of *C. fagacearum*. The majority of the variation detected appeared to result from small insertions or deletions (as seen in figures 3 and 4), rather than from gain or loss of restriction sites. Four haplotypes were found among the 96 isolates, or 3 populations, represented. Only one population, Willow Springs, consisted of more than one haplotype. Table 2 displays the allelic composition of these four haplotypes. The number of alleles per locus ranged from 3 to 1, the average being 1.71. Two of the RFLP loci had 3 alleles, one locus had 2 alleles, and the remaining four loci had only one allele present. Together, these 7 RFLP loci potentially could differentiate $(3^2)(2^1)(1^4)$ = 18 haplotypes. Table 3 shows the restriction fragment frequencies of the different alleles found at the 3 polymorphic RFLP loci. Only one population, Willow Springs, had more than one allele present at an RFLP locus. Pairwise measures of genetic similarity for each haplotype ranged from 85.71% to 57.14%. Haplotypes 'A' and 'B', and 'A' and 'D' were 57.14%

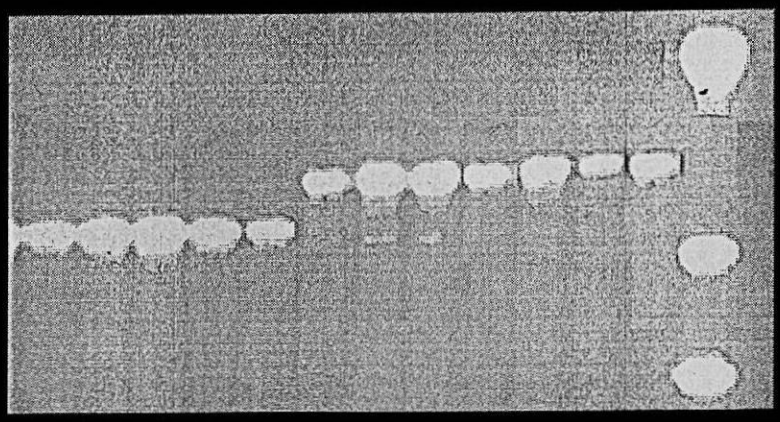

Fig. 3. Autoradiogram showing the 2 alleles present in isolates of *C. fagacearum* collected from 2 disease centers. This RFLP locus is defined by the restriction enzyme *Hind*III and the anonymous DNA probe pCF189. The last lane is a size marker consisting of lambda DNA digested with the enzyme *Hind*III.

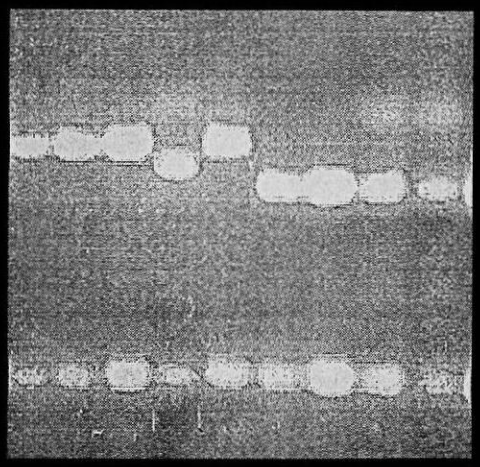

Fig. 4. Autoradiogram showing the 3 alleles present in isolates of *C. fagacearum* collected from 2 disease centers. This RFLP locus is defined by the restriction enzyme *EcoR*I and the anonymous DNA probe pCF9. The first 5 lanes contain DNA of isolates from the Willow Springs disease center. The last 4 lanes display allele 1 found in the Minnesota isolates.

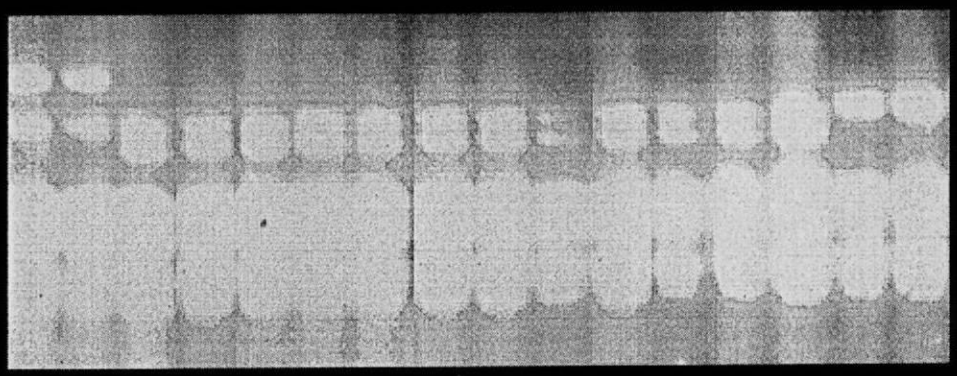

Fig. 5. Autoradiogram showing restriction fragment length polymorphisms among *Hind*III-digested DNA extracted from 16 *C. fagacearum* isolates. Hybridization with probe pCF79 showed 3 alleles present. Lanes 1 and 2 contain digested DNA from Willow Springs isolates, lanes 3 to 13 contain digested DNA from Root Study isolates, and lanes 14 and 15 contain DNA from isolates collected in the Minnesota population.

TABLE 2. Allelic composition and frequency of the four haplotypes found among the 96 isolates represented

Haplotype	Number of isolates	Location	RFLP locus						
			pCF 9-EcoRI	pCF 79-HindIII	pCF 102-HindIII	pCF 124-EcoRI	pCF 141-HindIII	pCF 189-HindIII	pCF 219-PstI
A	34	Mn	1	2	1	1	1	2	1
B	33	WS	2	1	1	1	1	1	1
C	20	RS	1	3	1	1	1	1	1
D	9	WS	3	1	1	1	1	1	1

Mn = Minnesota population

WS = Willow Springs population

RS = Root Study population

TABLE 3. Allele frequencies and measure of gene diversity for 3 polymorphic loci in *C. fagacearum* populations

RFLP Locus	Allele	Root Study	Willow Springs	Minnesota	Total	Nei's H
pCF9-EcoRI	1	100%	0%	100%	56.25%	0.5566
	2	0%	78.57%	0%	34.38%	
	3	0%	21.43%	0%	9.38%	
	N	20	42	34	96	
pCF79-HindIII	1	0%	100%	0%	43.75%	0.6397
	2	0%	0%	100%	35.42%	
	3	100%	0%	0%	20.83%	
	N	20	42	34	96	
pCF189-HindIII	1	100%	100%	0%	64.58%	0.4575
	2	0%	0%	100%	35.42%	
	N	20	42	34	96	

Total population includes allele frequencies from all 3 disease centers
Nei's H is based on diversity among all 3 populations

similar. Haplotypes 'A' and 'C', and 'B' and 'C' were 71.43% similar. Haplotypes 'B' and 'D' were 85.71% similar. All isolates from the Minnesota population and 9 isolates from the Willow Springs population shared the most alleles in common.

Contingency Chi-square analysis

Because such low levels of genetic variation were found and alleles were either fixed or non existent in the 3 populations studied, this calculation comparing the differences in allele frequencies is not necessary or useful. This measure therefore is not included or necessary for interpretation of the data.

Nei's measure of genetic diversity

Nei's genetic diversity index among the 96 isolates ranged from $H_{(North\ America)} = 0.4575$ to $H_{(North\ America)} = 0.6397$ per polymorphic locus (table 3). The other 4 loci had an H value of 0. The mean value for all 7 loci was 0.24. Since Willow Springs was the only diverse population, H was only calculated for this individual population with a value of 0.3368.

Nei's measure of genetic distance

Nei's D was used to calculate the amount of genetic divergence found between populations. At the pCF9/*EcoR*I locus, the Root Study and Minnesota populations were indistinguishable. The Root Study was also identical to Willow Springs at the pCF189/*Hind*III locus. All other D values were

0. Again, alleles were either fixed or nonexistent at the RFLP loci.

Genotypic diversity

Using Stoddart and Taylor's formula for genotypic diversity, the Root Study and Minnesota populations had a value for G = 1. This means that there was no diversity among isolates in these populations, or all of the isolates were of the same haplotype. The Willow Springs population had a G of 11.69, a rather low level of diversity considering the maximum possible value for G in this population was 42, or N. The normalized value for N was therefore 11.69/42, or 27.8% of its maximum possible value. The Willow Springs population was the only diverse disease center. When all 96 isolates were represented as 1 total population, G = 38.31, with a possible maximum of 96. The normalized value for N was therefore 38.31/96, or 39.9% of its maximum possible value.

Gametic disequilibrium

Again, because alleles were either fixed or nonexistent at the 7 RFLP loci analyzed, this measure to detect the amount of association between genes is not necessary or informative. Therefore it was not imperative to calculate pairwise gametic disequilibrium among RFLP loci by using measures of pairwise association as suggested by Weir (42).

DISCUSSION

Compatibility type analysis

Mating type analysis is one of the few genetic markers, besides RFLPs, that are currently available to study and observe the behavior of individual isolates of *C. fagacearum* in the field (4). Both mating types have been found in equal proportions in surveyed disease centers throughout the pathogen's distribution in the United States. However, both mating types are rarely isolated from the same individual tree (17). Any vector carrying ascospores would probably introduce both mating types into the tree. It has been shown that when both types are introduced, usually one type dominates (17). This phenomenon may be due to either chance or some form of competition favoring one compatibility type over the other. This scenario may have occurred in both the Root Study and Willow Springs pathogen populations. Only mating type 'A' was isolated at these locations. Also noted is the fact that both genotypes existing within the Willow Springs center were mating type 'A'. In contrast, it is expected that random mating of *C. fagacearum* populations will consist of equal numbers of mating type 'A' and mating type 'B'. Such was the case with the Minnesota population, which was a sexually expanding disease center. Of the isolates collected here, 41.2% were mating type 'A' and 58.8% were mating type 'B'. While these percentages are not exactly equal, they are sufficiently close to expected frequencies. A chi-square goodness-of-fit test between these

observed frequencies and the expected frequencies (50%) indicate that the computed value of χ^2 (3.0976) is less than the tabulated χ^2 value of 3.841 (with α = 0.05 and df = 1). Therefore these frequencies conform to the theory that *C. fagacearum* populations that randomly mate by sexual recombination consist of equal numbers of mating type 'A' and mating type 'B'.

Multilocus haplotype analysis

Root Study population

A sample of 20 isolates from the Root Study disease center contained only one genetic individual, haplotype 'C'. Presumably, the spread through this center was exclusively through connected roots, with no evidence for insect transmission. It is expected that plant pathogen populations lacking sexual recombination will consist of clonally propagated lines (26). The fungus was isolated from roots that were assumed to be connecting symptomatic and asymptomatic trees, indicating transmission was proceeding at the time of the collection. This disease center was the product of a 'breakover' from a previous trenching operation in 1990 that failed to control the disease. Either the trench did not sever all common root-connections between colonized and uninfected trees, or the trench was dug after the fungus had already spread through this part of the disease center. The latter is often the case because many trees newly infected with *C.*

fagacearum are asymptomatic and it becomes difficult to determine adequate placement of the trench so that breakovers do not occur. For these reasons, a 30 m barrier between diseased oaks and placement of the control trench is now recommended. Since only one genotype was observed in this disease center, it is possible that only one individual genotype actually initiated the breakover population. Other genotypes could have been vectored into the initial population, but may have been absent in the surrounding area of the breakover. Since this is a fairly new disease center, (it initiated in 1990), not enough time has elapsed for the generation of stable mutations. Mutations can introduce new alleles into a population at an assumed rate of the order 10^{-6}. Therefore one out of a million individuals will have a mutant allele for any particular gene (26). No genetic variability was observed in the Root Study.

Willow Springs population

A sample of 42 isolates from the Willow Springs disease center contained haplotypes 'B' and 'D' (Figure 6), which differed by a single allele at the pCF9/*EcoRI* RFLP locus. This is the first time that more than one genotype within a live oak disease center has been described. It had been hypothesized that each distinct, asexual reproducing disease center is initiated by a single inoculation event, possibly by a single isolate. Once the pathogen has infected a tree, it passes from that tree to the next tree by root connections, via the

continuous xylem system that exists between root-grafted trees. However, in light of this new data, insect vectors assume a more important role in the variability of *C. fagacearum* populations. These beetles are suspected of disseminating multiple genotypes during their course of several visits to fresh wound sites. We now believe that different genotypes exist within discrete clusters, or stands, of colonized trees (as seen in figure 6), with each cluster representing one single fungal clone.

Because of the predominance of asexual reproduction in many fungal pathogens (e.g. *C. fagacearum*) and the amount of time it takes for new alleles to be generated by mutations in a genome, the most logical hypothesis is to consider each genotype in the population as a distinct clone, with no sexual recombination among clones. Because sex is not occurring and each clone is different, the dynamics of population gene frequencies result from the differential growth rates among clones.

Minnesota population

A sample of 34 isolates from 2 groups of collections made from 2 perithecia from the same fungal mat contained only one genetic individual, haplotype 'A'. Mating type analysis of this population indicated that both mating types were found at this location and indeed, more than one genetic individual should exist in this population. However, the allelic data does not support this assumption. The discrepancy could

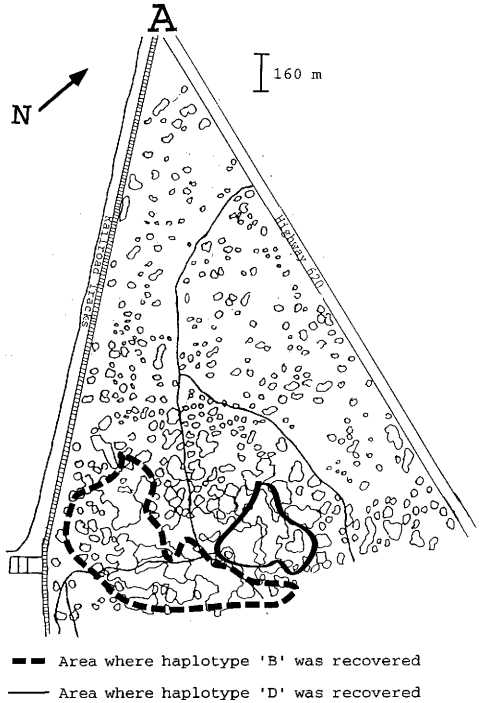

Fig. 6. Map of the distribution of haplotypes of *C. fagacearum* in the Willow Springs disease center. A sample of 42 isolates contained haplotypes 'B' and 'D'.

possibly be solved if more informative markers were found. RFLPs should be much more definitive than morphological or physiological characteristics. However, a previous study screened a large number of probe-enzyme combinations (437) and only detected 35 RFLPs (20). Because of the situation, this point should be raised: No group of genes is necessarily representative of the entire genome.

Measures of genetic variability

All values for G, (1, 1, and 11.69), are low and suggest that random mating was not occurring in these populations. The mean genetic diversity value among all 96 isolates for the 7 loci of $H_{(North\ America)}$ = 0.24 is comparatively high and might seem to contradict previously mentioned evidence for low levels of diversity in *C. fagacearum*. The same is true for the relatively high H value found within Willow Springs (0.3368). However, it should be mentioned that Nei's 'H' was based on only these 7 loci chosen to construct multilocus haplotypes. It does not reflect the other loci originally screened and rejected for lack of polymorphisms. Measures that calculated the Chi-square test for heterogeneity and gametic disequilibrium were not calculated because of the low levels of genetic diversity detected, and the fact that alleles were either fixed or non-existent at the RFLP loci. These measures would not be informative in this situation. Previously, a low amount of genetic variation was found in the nuDNA and mtDNA of *C. fagacearum* (20). Anonymous, randomly cloned DNA sequences

often detect a high degree of polymorphism in the nuclear DNA of fungi (28). It has been recently accepted that *C. fagacearum* has a restricted amount of variation in its nuDNA, compared to other plant pathogenic fungi that have been described using similar molecular markers (28).

It has been suggested that this fungus has only recently become a pathogen of oak in North America, due to the very low level of genetic diversity of *C. fagacearum* and its limited distribution in the United States (20). Two hypotheses can account for this low level of mtDNA and nuDNA variability. The first hypothesis is that *C. fagacearum* could have originated from a recent speciation event from a related fungus that was either saprophytic or weakly pathogenic to oaks. The second hypothesis is that *C. fagacearum* was only recently introduced into the United States and its limited genetic diversity is largely caused by a founder effect (20).

Under the first hypothesis, *C. fagacearum* could have originated from a closely related fungus that was either a saprophyte or weakly pathogenic to oaks. *C. fagacearum* has many closely related species, which include *C. adiposa, C. radicicola, C. paradoxa, C. moniliformis, C. coerulescens,* and *C. fimbriata*. These related species have been found existing throughout the world as saprophytes on many tree species and wood substrates. Previously these *Ceratocystis* species were grouped together based on ascospore morphology and conidiogenesis (33). Recently these species, except for *C. moniliformis*, were found to be closely related based on a

comparison of restriction patterns of ribosomal DNA (15). However, the related ancestor that may have given evolutionary rise to *C. fagacearum* has not yet been identified. It would be interesting to analyze and compare the nuDNA of these related species with that of *C. fagacearum*. Under the second hypothesis, the introduction of a small number of *C. fagacearum* individuals expressing a limited amount of genetic variation might have been too recent for neutral mutations in the nucleotide sequence of the nuDNA to stabilize. This scenario has been documented in other pathosystems. Support for the hypothesis that *Cryphonectria* (*Endothia*) *parasitica* (Murrill) Barr originated from China and was introduced into North America is based on recent RFLP analysis. A low amount of nuDNA diversity in U.S. isolates was found compared to isolates collected in China (31). It is now thought that *C. parasitica* in North America originated from a small founding population within the last 100 years. However, based on the current limited geographic range of oak wilt in the U.S., this founder effect hypothesis seems doubtful. Oak wilt has only been identified within the United States. It is possible, although unlikely, that *C. fagacearum* could have been introduced into North America from a distant location where the disease occurred and was never documented or where the fungus does not cause enough damage to be noticed. Both hypotheses conclude that a significant length of time is necessary for new alleles to be generated by mutations in the genome.

Knowledge of the genetic structure of *C. fagacearum*

populations may also suggest how quickly this pathogen is likely to evolve, and thus help predict how long a chosen method of control will last. Fungal populations with low levels of genetic variation, like these described populations, are less likely to develop resistance to fungicide treatments than populations with lots of variation. When fungal populations become exposed to sufficient selection pressure, and if the population varies genetically so that it can reproduce, the variants that reproduce in this presence of fungicides will become dominant (13). This low level of variation is a good indication that fungicide treatments should be effective in the control and management of this disease. In asexually expanding populations, like the Root Study and Willow Springs, where only a few genetic individual exist, fungicide treatments could be a successful management tool. However, trenching would be more efficient if the disease centers are large in size or in numbers of trees.

The sexual cycle of *C. fagacearum* does play a significant role in its population genetics. The dispersal of ascospores allows for different gene combinations of *C. fagacearum* to test the potential colonization of new host genotypes. Vegetative spread of this fungus is a method used to exploit environments already suited for growth of that genotype. Thus, the pathogen can increase its likelihood for survival by employing both asexual and sexual reproduction. The relative importance of the sexual cycle may largely be due to the fungus' need to initiate new disease centers before the

existing ones die, thus maximizing its chances for survival. The non-contiguous (disconnected) host distribution plays a role in this theory.

Genomic DNA markers, such as RFLPs, can be utilized in several study areas to complete many tasks. Range-wide genetic variation can be detected, characterization of within-stand diversity determined, and more characteristics of the basic biology of the pathogen derived. The genetic description of isolates will aid in the definition of vector relationships, disease spread and dispersal. Once the distribution of genetic variation has been determined among and between individual populations of this pathogen, the importance of the roles of traditional mechanisms whereby variation is generated can be established (7). For instance, the influence of sexual recombination, selection, genetic drift, migration, extinction, and mutation can be theorized regarding the overall population diversity of *C. fagacearum*. In order to understand the basis for adaptation of pathogens to the use of numerous types of resistance in economically important host plants, knowledge of pathogen structure and diversity is essential (13). It is further expected that this information could give rise to a better understanding of the evolutionary biology of *C. fagacearum*.

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TABLE 4. Composition of mating types and alleles present at RFLP loci in the Root Study

Isolate	Mating type	RFLP Locus						
		pCF 9-EcoRI	pCF 79-HindIII	pCF 102-HindIII	pCF 124-EcoRI	pCF 141-HindIII	pCF 189-HindIII	pCF 219-PstI
RS T10	A	1	3	1	1	1	1	1
RS T14	A	1	3	1	1	1	1	1
RS T3R1	A	1	3	1	1	1	1	1
RS T3R2	A	1	3	1	1	1	1	1
RS T3R3	A	1	3	1	1	1	1	1
RS T3R4	A	1	3	1	1	1	1	1
RS T3R5	A	1	3	1	1	1	1	1
RS T14R1	A	1	3	1	1	1	1	1
RS T14R2	A	1	3	1	1	1	1	1
RS T14R4	A	1	3	1	1	1	1	1
RS T14R5	A	1	3	1	1	1	1	1
RS T14R6	A	1	3	1	1	1	1	1
RS T28R3	A	1	3	1	1	1	1	1
RS T85R3	A	1	3	1	1	1	1	1
RS T1S16R1	A	1	3	1	1	1	1	1
RS T1S16R3	A	1	3	1	1	1	1	1
RS T1S16R4	A	1	3	1	1	1	1	1
RS T1S22R4	A	1	3	1	1	1	1	1
RS T4S9R4	A	1	3	1	1	1	1	1
RS T4S10R4	A	1	3	1	1	1	1	1

TABLE 5. Composition of mating types and alleles present at RFLP loci in Willow Springs

Isolate	Mating type	RFLP Locus						
		pCF 9-EcoRI	pCF 79-HindIII	pCF 102-HindIII	pCF 124-EcoRI	pCF 141-HindIII	pCF 189-HindIII	pCF 219-PstI
WS 604	A	3	1	1	1	1	1	1
WS 606	A	3	1	1	1	1	1	1
WS 607	A	3	1	1	1	1	1	1
WS 615	A	2	1	1	1	1	1	1
WS 847	A	2	1	1	1	1	1	1
WS 848	A	2	1	1	1	1	1	1
WS 851	A	2	1	1	1	1	1	1
WS 889	A	3	1	1	1	1	1	1
WS 891	A	2	1	1	1	1	1	1
WS 898	A	2	1	1	1	1	1	1
WS 901	A	2	1	1	1	1	1	1
WS 902	A	2	1	1	1	1	1	1
WS 903	A	2	1	1	1	1	1	1
WS 921	A	2	1	1	1	1	1	1
WS 923	A	2	1	1	1	1	1	1
WS 924	A	2	1	1	1	1	1	1
WS 925	A	2	1	1	1	1	1	1
WS 926	A	2	1	1	1	1	1	1
WS 948	A	2	1	1	1	1	1	1
WS 951	A	2	1	1	1	1	1	1
WS 955	A	2	1	1	1	1	1	1
WS 956	A	3	1	1	1	1	1	1
WS 963	A	2	1	1	1	1	1	1
WS 35	A	3	1	1	1	1	1	1
WS 36	A	2	1	1	1	1	1	1
WS 37	A	2	1	1	1	1	1	1
WS 39	A	2	1	1	1	1	1	1
WS 44	A	2	1	1	1	1	1	1

TABLE 5 continued

Isolate	Mating type	RFLP Locus						
		pCF 9-EcoRI	pCF 79-HindIII	pCF 102-HindIII	pCF 124-EcoRI	pCF 141-HindIII	pCF 189-HindIII	pCF 219-PstI
WS 55	A	2	1	1	1	1	1	1
WS 57	A	2	1	1	1	1	1	1
WS 60	A	2	1	1	1	1	1	1
WS 62	A	2	1	1	1	1	1	1
WS 63	A	2	1	1	1	1	1	1
WS 68	A	2	1	1	1	1	1	1
WS 69	A	2	1	1	1	1	1	1
WS 70	A	2	1	1	1	1	1	1
WS 71	A	2	1	1	1	1	1	1
WS 72	A	2	1	1	1	1	1	1
WS 73	A	2	1	1	1	1	1	1
WS 76	A	3	1	1	1	1	1	1
WS 79	A	3	1	1	1	1	1	1
WS 81	A	3	1	1	1	1	1	1

TABLE 6. Composition of mating types and alleles at RFLP loci in the Minnesota population

Isolate	Mating type	RFLP Locus						
		pCF 9-EcoRI	pCF 79-HindIII	pCF 102-HindIII	pCF 124-EcoRI	pCF 141-HindIII	pCF 189-HindIII	pCF 219-PstI
Mn 1	A	1	2	1	1	1	2	1
Mn 2	B	1	2	1	1	1	2	1
Mn 3	B	1	2	1	1	1	2	1
Mn 4	B	1	2	1	1	1	2	1
Mn 5	A	1	2	1	1	1	2	1
Mn 6	A	1	2	1	1	1	2	1
Mn 7	B	1	2	1	1	1	2	1
Mn 8	A	1	2	1	1	1	2	1
Mn 9	A	1	2	1	1	1	2	1
Mn 10	B	1	2	1	1	1	2	1
Mn 11	B	1	2	1	1	1	2	1
Mn 12	B	1	2	1	1	1	2	1
Mn 13	A	1	2	1	1	1	2	1
Mn 14	B	1	2	1	1	1	2	1
Mn 15	B	1	2	1	1	1	2	1
Mn 16	B	1	2	1	1	1	2	1
Mn 17	A	1	2	1	1	1	2	1
Mn 18	B	1	2	1	1	1	2	1
Mn 19	B	1	2	1	1	1	2	1
Mn 20	A	1	2	1	1	1	2	1
Mn 21	B	1	2	1	1	1	2	1
Mn 22	A	1	2	1	1	1	2	1
Mn 23	B	1	2	1	1	1	2	1
Mn 24	B	1	2	1	1	1	2	1
Mn 25	A	1	2	1	1	1	2	1

TABLE 6 continued

Isolate	Mating type	RFLP Locus						
		pCF 9-EcoRi	pCF 79-HindIII	pCF 102-HindIII	pCF 124-EcoRI	pCF 141-HindIII	pCF 189-HindIII	pCF 219-PstI
Mn 26	B	1	2	1	1	1	2	1
Mn 27	B	1	2	1	1	1	2	1
Mn 28	A	1	2	1	1	1	2	1
Mn 29	A	1	2	1	1	1	2	1
Mn 30	B	1	2	1	1	1	2	1
Mn 31	B	1	2	1	1	1	2	1
Mn 32	A	1	2	1	1	1	2	1
Mn 33	A	1	2	1	1	1	2	1
Mn 34	B	1	2	1	1	1	2	1

The bold bar separates group One from group Two.

VITA

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