AN ABSTRACT OF THE DISSERTATION OF

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Title: Genetic Variability in the Eastern Filbert Blight Pathosystem

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The heritability of resistance to eastern filbert blight, caused by *Anisogramma anomala*, in European hazelnut (*Corylus avellana*) was evaluated in the progeny of seven cultivars crossed in 12 combinations. Progeny produced by crossing 'VR6-28' with three susceptible cultivars segregated 1:1 for complete resistance to eastern filbert blight. Histograms of disease responses in progeny of the remaining six parents showed continuous distributions for all crosses examined, thus general and specific combining ability values were calculated for each parent. Based on general combining ability values, high levels of partial resistance were transmitted by 'Gem', 'Tonda di Giffoni' and 'Willamette'. Heritability of disease incidence, number of cankers, and proportion of wood diseased were calculated to be 0.21, 0.39, and 0.47, respectively, suggesting that hazelnuts with fewer, smaller cankers can be bred from partially resistant parents.

Variation in the ITS region was examined in 67 isolates of *A. anomala* collected from five geographically separated infection centers in Oregon and Washington. Sixty-six isolates yielded a PCR product 649 bp in length. Fourteen restriction sites, three of which were polymorphic, were found in the ITS region, but little sequence divergence was observed between isolates within (~2%) and among (~2%) infection centers. The ITS sequence of *A. anomala* was most closely related to those of diaporthaceous pathogens of woody plants.

Forty *A. anomala* isolates collected from seven geographic regions were examined for their ability to infect two European hazelnut clones: VR6-28, a selection that carries a single dominant gene for complete resistance, and Barcelona, a moderately susceptible cultivar. Two isolates, one from Ontario and one from Minnesota, caused small sunken lesions on 'VR6-28'. Isolates from all seven regions infected 'Barcelona'; there were no significant differences ($P > 0.05$) in isolate virulence among regions. Thirty-three isolates were examined with four RAPD primers. Based on cluster analysis of the RAPD products, some genetic variation attributable to geographic origin was apparent in the collected isolates. This observation, coupled with the fact that two isolates from different indigenous populations infected 'VR6-28', suggests the quarantine laws prohibiting the importation of hazelnuts from eastern North America to Oregon and Washington should be maintained.

GENETIC VARIABILITY IN THE EASTERN FILBERT BLIGHT PATHOSYSTEM

by

Nancy K. Osterbauer

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APPROVED:

Redacted for Privacy

Major Professor, representing Botany & Plant Pathology

Redacted for Privacy

Chair of Department of Botany & Plant Pathology

Redacted for Privacy

Dean of Graduate School

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Nancy K. Osterbauer, Author

CONTRIBUTION OF AUTHORS

Dr. K. B. Johnson was involved in the analyses of experiments and the review of each manuscript (Chapters 2, 3, and 4). Funding, and laboratory, greenhouse, and field space also were provided by Dr. Johnson. T. L. Sawyer maintained plant material in the greenhouse and field, and assisted with collection, analysis, and interpretation of field data (Chapter 2). Dr. S. A. Mehlenbacher provided plant material and laboratory space, and assisted with data analyses for the resistance heritability study and the pathogenicity study (Chapters 2 and 4). Dr. A. Liston participated in the experimental design of the systematics study, and the analysis and interpretation of results from that study (Chapter 3).

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DEDICATION

This dissertation is dedicated to my parents,

Gerald Eugene Osterbauer
(1935-)

and

Vivian Ann (Mellgren) Osterbauer
(1936-1995)

GENETIC VARIABILITY IN THE EASTERN FILBERT BLIGHT PATHOSYSTEM

CHAPTER 1
INTRODUCTION

Eastern Filbert Blight

Eastern filbert blight is an endemic canker disease on *Corylus americana* Marsh., the American hazel, in northeastern North America (9,27). The fungus causing this disease was first described in 1876 as *Diatrype anomala* Peck (27) and then renamed *Anisogramma anomala* (Peck) E. Muller in 1962 (24). Taxonomically, *A. anomala* has been placed in the Class Ascomycetes, Subclass Pyrenomycetes, Order Diaporthales, and Family Gnomoniaceae (3,24). Disease caused by *A. anomala* received no public notice until the late 1800s, when immigrants first introduced trees of *C. avellana* L., the European hazelnut, to eastern North America (9,27). In the early 1900s, Fuller (9) tried to establish commercial orchards of *C. avellana* in New York state, but discovered this species was highly susceptible to eastern filbert blight. Other growers had similar experiences (4,32). By 1921, entire orchards of *C. avellana* had been destroyed after becoming infected with *A. anomala* (4). Several sources claim eastern filbert blight prevented the establishment of a hazelnut industry on the eastern coast of North America (4,9,32).

In the late 1800s, Felix Gillet established a European hazelnut nursery in Nevada City, California (20), outside of the natural range of *A. anomala* and its native host *C. americana*. Gillet is credited with introducing over 20 European hazelnut cultivars to the western coast of North America, including 'Barcelona', the predominant commercial cultivar grown in Oregon and Washington (20). Since the introduction of European hazelnut in the late 1800s, the hazelnut

industry on the western coast of North America has grown to 12,000 ha (19). Currently, 100% of U.S. commercial hazelnut production takes place on the western coast of North America, with 98% of that production occuring in Oregon's Willamette Valley (19,38). Hazelnuts have a farm gate value of over $18 million per year in Oregon and the U.S. ranks fourth in the world in overall production (19).

A quarantine was established by the Western Plant Quarantine Board and its Canadian equivalent to prevent eastern filbert blight from being introduced into orchards in Oregon, Washington, and British Columbia on infected plant material (4). The board recommended that no nurseries or growers accept any hazelnut rootstock or cuttings from east of the Rocky Mountains. Despite the quarantine and these recommendations, the importation of plant material from eastern North America continued (38). In 1968, eastern filbert blight was observed on European hazelnuts grown in a commercial orchard near Woodland, WA (8). The significance of this finding, however, was ignored until several years later (34). By 1974, eastern filbert blight had spread throughout southern Washington and into Columbia County, Oregon (6,12,34). Despite extending the quarantine to include Washington state and Columbia County, Oregon, the disease continued to spread (34). It is now firmly established in the northern one-third of the hazelnut production area in Washington and Oregon (29) and is progressing southwards.

The life cycle of *A. anomala*, the pathogen causing eastern filbert blight, has been determined (10,11,16,30,36). Ascospores of this pathogen are disseminated by rain-splash and forceful ejection to neighboring trees in the spring (30,36). As new leaves begin to emerge, the ascospores land on the young vegetative shoots. There, the spores germinate, infect the new shoots, and colonize the vascular

tissue (10,16,30). Mycelial growth in the vascular tissue continues through the summer (10). After the infected tree undergoes a period of cold dormancy, fungal stroma containing the ascospores of the pathogen appear in sunken cankers on infected limbs (10). Expansion of these perennial cankers girdles the infected limbs, eventually killing the infected tree (36). The life cycle of *A. anomala* is usually 12-16 mo (11), although life cycles of 20-28 months have been observed (28).

Knowledge of the life cycle of this pathogen has allowed for the development of control methods for eastern filbert blight. Because of the perennial nature of this disease and its host, these control methods must be effective over an extended period of time. For example, many growers conduct disease surveys in their orchards to detect eastern filbert blight (15). When the disease is found, the growers remove and destroy the cankered limbs and trees (15,36). Some growers apply fungicides to trees during the period of new leaf emergence (15). Johnson et al (17) found that the broad spectrum fungicide chlorothalonil effectively protected new shoots from infection by *A. anomala* during the period of leaf emergence; the fungicides fenarimol and flusilazole also appeared able to protect new shoots from infection. Unfortunately, none of these control methods are 100% effective and some, such as fungicide treatments, are cost-prohibitive for hazelnut growers (15,17,36). In 1991, Mehlenbacher et al (22) reported that a single dominant gene for complete resistance to eastern filbert blight was found in an obsolete pollenizer 'Gasaway'. No disease symptoms have been observed on this cultivar in over 20 yr of field testing (S. A. Mehlenbacher, pers. comm.). In addition, Pinkerton et al (27) reported that several commercially acceptable cultivars had high levels of partial resistance to this disease. Thus, breeding cultivars that are resistant to eastern filbert blight is

considered the most durable, cost-effective form of control for this disease (7,15,18,27).

Variation in the Host and in the Pathogen

Species of *Corylus* exhibit a wide range of reactions to eastern filbert blight. In the early 1900's, Fuller (9) reported that *C. americana* was less susceptible to eastern filbert blight than *C. avellana*. He described *A. anomala* as a nuisance parasite on *C. americana*, its native host. Recently, Coyne (7) inoculated seven *Corylus* species with *A. anomala* to determine the species' reaction to eastern filbert blight. She found that some species, such as *C. cornuta* var. *californica*, the California beaked hazel, did not become infected when inoculated with ascospores of *A. anomala* whereas others, such as *C. jacquemontii*, the Indian tree hazel, were equally or more highly susceptible to eastern filbert blight than *C. avellana*. Those species that were resistant to eastern filbert blight are considered potential sources of new single, dominant resistance genes (7,15). A wide variety of disease resistance responses also have been detected within cultivars of *C. avellana*. For example, the pollenizer 'Daviana' is highly susceptible to eastern filbert blight (28). Numerous large cankers form on infected 'Daviana' trees, often killing the trees within 2 to 3 yr (20,28). Other cultivars, such as Tonda di Giffoni and Willamette, are more resistant to disease (28). Fewer, smaller cankers form on these trees and infected trees may remain in commercial production for several years. The single, dominant resistance gene found in the *C. avellana* cultivar Gasaway has been bred into new hazelnut releases which are intended to replace the highly susceptible 'Daviana' pollenizer (20). Efforts to breed a new, commercially acceptable cultivar that carries the 'Gasaway' resistance gene are

ongoing. It is believed that selection, evaluation, and deployment of this cultivar will take 20 to 30 yr (15,38).

Anisogramma anomala, the pathogen that causes eastern filbert blight, shares several traits with obligately biotrophic fungi that cause rust and mildew diseases (10,30,37). It has a limited host range (10,37), produces a self-inhibitor to spore germination and hyphal growth (37), and reproduces only on living host tissue (10,37). In addition, once a germ hyphae of *A. anomala* has successfully penetrated a host epidermal cell wall, the hyphae expands to form a vesicle similar to those produced by obligately biotrophic rusts (30). Although its infection biology is similar to obligately biotrophic rusts and mildews, *A. anomala* does differ from these fungi in some respects. It lacks an asexual life cycle (10,37) and apparently has a homothallic sexual mating system (10).

Many obligately biotrophic fungal pathogens have the ability to shift their virulence to overcome single, major host resistance genes (e.g. 1,18). This ability is so pronounced in some fungi that single, major resistance genes incorporated into new host cultivars last only 2 to 3 yr in the field (e.g. 1,18). *Anisogramma anomala*, a fungus that shares many similarities with these obligately biotrophic fungal pathogens, also may share this ability. The Oregon and Washington population of *A. anomala* was introduced relatively recently (8), suggesting this pathogen population is relatively homogeneous. The rate and mechanism by which variation is created in *A. anomala* is unknown. A rapidly evolving population may be indicative of the pathogen's ability to shift its virulence to overcome the 'Gasaway' gene for complete resistance. Breakdown of this resistance gene would represent a major setback for hazelnut breeders and growers.

Research Objectives

Only one single dominant, major gene for complete resistance to eastern filbert blight, the 'Gasaway' gene, has been identified (21), although similar genes may exist in other *Corylus* species (7). Unfortunately, many pathogens are able to shift their virulence to overcome major resistance genes (e.g. 1,18). Thus, there is a need for alternative sources of host resistance to eastern filbert blight. Partial resistance to this disease has been observed in several commercial cultivars of *C. avellana* (28). The level of partial resistance observed in these cultivars is apparently sufficient to change eastern filbert blight from a major to a minor disease. Unfortunately, like 'Gasaway', most cultivars with high levels of partial resistance are commercially unacceptable. In addition, the heritability of this partial resistance is unknown. Therefore, the first objective of this research was to quantify the heritability of partial disease resistance in these European hazelnut cultivars using regression analysis (14)(Chapter Two). Combining ability analysis (35) was used to identify those parents that transmitted high levels of partial resistance, as expressed by fewer, smaller cankers (25), to their progeny. Similar analyses have been used in the plum-*Xanthomonas* canker (40), the pear-fire blight (31), and other pathosystems (5,41).

The genetic homogeneity and potential rate of genetic change of the Oregon and Washington population of *A. anomala* was evaluated by examining intraspecific variation in the internal transcribed spacer region of the nuclear ribosomal DNA (Chapter Three). Variation in the internal transcribed spacer region of the nuclear ribosomal DNA has been detected within several fungal species (23,25,33,44). O'Donnell (25) was able to differentiate *Fusarium sambucinum* populations into three distinct groups based on variation in the internal transcribed

spacer region. The two pathotypes of *Leptosphaeria maculans*, the cause of blackleg disease on crucifers, also may be distinguished by differences in their internal transcribed spacer regions (23,44). In addition, the phylogenetic relationship of *A. anomala* to other fungi, including members of the Diaporthales, was evaluated with this data.

The third objective of this research was to examine genetic and pathogenic variation within isolates of *A. anomala* collected from eastern North America and from Oregon and Washington (Chapter Four). Because *A. anomala* was introduced to Oregon and Washington, it is likely that greater intraspecific variation may be present in isolates of *A. anomala* from within its indigenous range. This variation could pose an additional threat to Oregon and Washington hazelnuts in the form of more aggressive isolates or isolates capable of overcoming the 'Gasaway' gene for complete resistance. To identify pathogenic variation in these isolates, two European hazelnut clones, 'Barcelona', a moderately susceptible cultivar, and VR6-28, a selection that carries the 'Gasaway' gene, were inoculated with ascospores of each isolate and the disease responses on each clone compared. Relative genetic variation in these isolates also was investigated using the random amplification of polymorphic DNA (RAPD) technique (43). This technique is well-suited for examining genetic variation in *A. anomala* because no prior sequence knowledge of the organism is required and very small quantities of DNA are required (43). Other researchers investigating genetic variation in obligately biotrophic parasites have chosen the RAPD technique for similar reasons (e.g. 13,18). In addition, the RAPD technique has proven very effective for detecting population-level differences within a species (2,13,18,38,41,43). For example, researchers were able to differentiate isolates of *F. oxysporum* f. sp.

vasinfectum, the cause of cotton wilt, by pathogenicity group and by region of origin using the RAPD technique (2).

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CHAPTER TWO
ANALYSIS OF RESISTANCE TO EASTERN FILBERT BLIGHT IN *CORYLUS AVELLANA*

N.K. Osterbauer, T.L. Sawyer, S.A. Mehlenbacher, and K.B. Johnson

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Abstract

The genetic control of resistance to eastern filbert blight, caused by *Anisogramma anomala*, in European hazelnut (*Corylus avellana*) was evaluated in the progeny of seven cultivars crossed in 12 combinations. The progeny were subjected to inoculation with *A. anomala* in the greenhouse and in the field. Three disease responses, or components of resistance, were measured: disease incidence, number of cankers, and proportion of wood diseased. In both the greenhouse and the field, progeny produced by crossing 'VR6-28' with three susceptible cultivars segregated 1:1 for complete resistance to eastern filbert blight, confirming a previous report that 'VR6-28' is heterozygous for a single, dominant resistance gene. Histograms of disease responses in progeny of the remaining six parents showed continuous distributions for all crosses examined. Consequently, these parents were analyzed for general and specific combining abilities for each disease response. In the field, general and specific combining ability were both significant for all disease responses ($P < 0.05$), with general combining ability having twice the magnitude of specific combining ability. These results suggest these disease responses are controlled by additive gene action in the cultivars examined with non-additive gene action being of some importance. Based on general combining ability values, high levels of partial resistance were transmitted by the pollen parents 'Gem' and 'Tonda di Giffoni' and the seed parent 'Willamette'. The progeny of 'Willamette' x 'Gem' had the highest levels of partial resistance after the first exposure period in the field, with an average proportion of wood diseased of 0.15; the progeny of 'Willamette' x 'Tonda di 'Giffoni' showed similar levels of resistance (proportion of wood diseased = 0.23). In contrast, the

cross 'Ennis' x 'Tonda Gentile delle Langhe' exhibited an average proportion of wood diseased of 0.54. Heritability of disease incidence, number of cankers, and proportion of wood diseased were calculated to be 0.21, 0.39, and 0.47, respectively, for this set of nine crosses after the first exposure period in the field. This suggests it will be possible to use partially resistant parents to breed for hazelnuts with fewer and smaller cankers.

Introduction

Eastern filbert blight is a serious disease of European hazelnuts (*Corylus avellana* L) grown commercially in Oregon and Washington (2,4,20). This disease was first detected in this area in 1970, when it was found in a commercial orchard located near Woodland, WA (4). It is now firmly established in the northern one-third of Oregon's Willamette Valley where 98% of United States hazelnut production occurs (2,20). Eastern filbert blight is caused by *Anisogramma anomala* (Peck) E. Muller, an ascomycetous fungus indigenous to the eastern United States (18). This fungus invades new, vegetative shoots in the spring and, after a period of tissue maturation and cold dormancy, forms stromata within a perennial canker (8,21,27). Expansion of these cankers leads to girdling of branches and limbs, resulting in canopy dieback and tree death in 4-10 yr.

Several control methods for eastern filbert blight have been examined, the most effective of which has been fungicide applications (9,19,27). To completely prevent infection, the trees must be protected for a period of 2 mo following leaf emergence each spring. This requires multiple fungicide applications resulting in high costs for hazelnut growers (8). Cultural control methods, such as pruning,

are only partially effective, thus limiting their value to growers (27). Consequently, breeding commercially acceptable, eastern filbert blight-resistant cultivars is viewed as the most desirable method to control this disease (3,10,13,19).

In 1991, Mehlenbacher et al, reported that 'Gasaway', an obsolete pollenizer cultivar, carried a single dominant gene for complete resistance to eastern filbert blight (13). This gene has since been bred into new, more commercially acceptable germplasm releases (12). In several other pathosystems, however, resistance controlled by a single dominant gene can be overcome by a virulence shift in the pathogen (e.g. 9). Therefore, identifying new sources of resistance to eastern filbert blight and determining the heritability of this resistance is desirable.

The susceptibility of several cultivars and clones of *C. avellana* and of other *Corylus* species to infection by *A. anomala* has been evaluated previously (3,13,19). In addition to 'Gasaway', several other cultivars and *Corylus* sp. have shown nearly complete resistance to infection (3,19). Unfortunately, most of these resistant cultivars and species are commercially unacceptable. These resistant species and cultivars are being used by hazelnut breeders to breed resistance to eastern filbert blight into new cultivars (10,11). The objective of this study was to evaluate the heritability of resistance to eastern filbert blight in seven European hazelnut cultivars.

Materials and Methods

Plant material. Crosses were made between selected cultivars of *C. avellana* in 1991, as described previously (28). Trees of seed parents were emasculated in early December of 1990 prior to female flower

receptivity and enclosed in wooden frames covered with white polyethylene to exclude wind-borne pollen. Pollen was collected from male parents and stored in vials in the freezer until needed. In February, when the flowers of the seed parents were fully receptive, they were pollinated by touching pollen onto the surface of the stigmatic styles. The seed parents selected for this study were 'Ennis', a cultivar highly susceptible to eastern filbert blight, 'Willamette', a moderately susceptible cultivar, and 'Casina', another moderately susceptible cultivar (19). Pollen parents were 'Tonda Gentile delle Langhe', a highly susceptible cultivar, 'Tonda di Giffoni', a moderately susceptible cultivar, 'Gem', a resistant cultivar (19), and 'VR6-28' ('Riccia di Talanico' x 'Gasaway'), a selection that carries a single dominant gene for complete resistance (12,13).

Nuts from the crosses were collected in mid-August of 1991 and stratified at 4C until the following spring (28). After germination, the nuts were planted in a mixture of peat and vermiculite (1:1, v:v) in 3 x 3 x 5 cm cells and fertilized periodically with a water soluble fertilizer (20:20:20, N:P:K). After 6 wk, the seedlings were transplanted to a mix of peat, sand, and pumice (1:1:2, v:v:v) in 3 x 3 x 3 cm pots and treated with a slow release fertilizer (14:14:14, N:P:K). Seedlings for the greenhouse study were used immediately in experiments and maintained in 3 x 3 x 3 cm pots. Those for the field study were transplanted to 4 L pots and grown through the summer and fall of 1992 before being transferred to the Washington State University Experiment Station in Vancouver, WA, to overwinter. In March of 1993, prior to the first period of exposure in the field, the trees were transplanted to 8 L pots containing a mix of sandy loam,

peat, sand, and pumice (1:1:1:2, v:v:v:v). Uninoculated trees from each cross were maintained at the Oregon State University Botany & Plant Pathology Field Laboratory located east of Corvallis, OR, a location outside the current range of eastern filbert blight (19). Two-yr-old trees of the parent cultivars were exposed to *A. anomala* in the field as well. These trees were obtained from local nurseries and planted in 8 L pots containing the same soil mix and fertilizer as used for the progeny populations.

Experimental design. The greenhouse experiment was arranged in a completely randomized design and trees inoculated on two different sets of inoculation dates. Because only a limited number of seedlings were available from some crosses, the number of seedlings used per cross on an inoculation date ranged from a low of four seedlings to a high of 41; the mean number of seedlings inoculated per cross per date was 11. No seedlings were available from the cross 'Ennis' x 'Gem' for the greenhouse study. Data collected from the seedlings included disease incidence, total number of cankers, total length of diseased wood, and total seedling length. The latter measure was included in order to calculate proportion of wood diseased. Absence of infection in symptomless trees was confirmed by microscopic examination of hand sections taken from inoculated tissues (7,19,27).

The field study was arranged in a randomized complete-block design with three replications. Each replication included 15 trees from each cross. Nine trees of each parent were included in each replication. Incidence of disease, total number of cankers, total length of diseased wood, and total tree length were measured for each tree.

Inoculation. Diseased hazelnut branches exhibiting cankers and mature stromata of *A. anomala* were collected in November of 1991 and 1992, from a commercial orchard located near Boring, OR. Cankers collected in 1991 were used as a source of inoculum for the greenhouse study. Cankers collected in 1992 were used to inoculate trees in the first year of the field study. For the second year of the field study, diseased branches were collected from an abandoned orchard near Damascus, OR, in November of 1993.

In the greenhouse study, a small drop of a latex grafting compound (Farewell Industries, Wenatchee, WA) was used to mark susceptible tissue behind the apical bud on a single shoot of an 8-week-old seedling one day prior to inoculation (7). After the latex dried, the seedling was inoculated by dipping the shoot in a suspension of *A. anomala* ascospores (1×10^7) plus 0.02% activated charcoal for 3 sec (7,27). Inoculated and non-inoculated (control) seedlings were placed in an intermittent mist chamber that applied mist for 15 sec every 30 min. Seedlings remained in the mist chamber for 5 days. Shoot inoculations were repeated three times over a period of 3 wk for each set of inoculation dates, beginning on 2 June 1992 for the first set and 20 July 1992 for the second set. After inoculation, the seedlings were moved to a greenhouse bench and maintained for 12 wk in a controlled environment (18C day, 13C night). After this time, seedlings were moved to an unheated greenhouse at the North Willamette Research and Extension Center, Aurora, OR, to cycle through a natural period of cold dormancy; this is required for symptom expression and canker development (27). Disease responses were measured in June of 1993.

In the field study, 1- to 2-yr-old trees were inoculated as described previously (19) by exposing the potted trees under elevated, wire-mesh platforms (3.3 x 4.6 m) that held diseased hazelnut branches. Ascospores were released from stromata contained within the cankers during periods of rain and high humidity. The first period of exposure was from 12 March to 29 May 1993, and the second from 17 March to 31 May 1994. In 1993, a supplemental sprinkler system was installed on each platform to enhance spore release during the exposure period and to irrigate the trees over the summer. Each spring, gutter-type traps were placed under each platform to collect spore wash from the disease branches overhead. The concentration of ascospores in the wash was estimated as described previously (19,27). Disease responses were measured on the trees 16 mo after each exposure period. The trees were maintained in pots until the end of the second exposure period, then were transplanted into a field of sandy loam soil located on the experiment station.

Data analyses. Values for disease incidence, number of cankers, and proportion of wood diseased were summarized for each cross. Number of cankers could not be summarized for the second year of the field study because of merging of cankers from the previous year. For crosses that involving the pollen parent 'VR6-28', the incidence of disease in individual seedlings was subjected to chi-square (χ^2) analysis using Yates' correction factor (5) to determine if the progeny segregated 1:1 for susceptibility and complete resistance. For all crosses, frequency distributions of the proportion of wood diseased and the number of cankers on individual trees were constructed. In those crosses where individual trees exhibited a continuous range of disease responses, the parent cultivars were subjected to combining ability analysis.

General combining abilities for six parents (excluding 'VR6-28') were calculated using the disease responses measured in the greenhouse seedlings and in trees from the field study (25). General combining ability measures the average performance of a parent in a series of crosses for a particular trait and is considered a measure of additive gene action (6,25). General combining ability was determined by comparing the mean value of the progeny of a parent for a particular disease response to the grand mean for that disease response averaged across all crosses used in the analysis. For each disease response, the specific combining ability values for each cross were calculated by subtracting the observed disease response from the expected disease response (25). The expected disease response for each cross was obtained by adding the general combining ability estimates of the parents to the grand mean for that disease response. For example, if the grand mean for number of cankers was 2.5, and the general combining ability estimates for the seed and pollen parents were -1.0 and +0.5, respectively, the expected value for the progeny would equal 2.0. Specific combining ability is indicative of non-additive gene action (6,25). In the greenhouse study, disease incidence, mean number of cankers, and mean proportion of wood diseased for the missing cross 'Ennis' x 'Gem' were estimated by a least squares method (26). Disease response variables were subjected to analysis of variance using parent cultivars as the independent variables to determine the significance of general and specific combining abilities in each study. The model statement for this analysis was:

$$X_{AB} = X' + G_A + G_B + S_{AB} + E$$

where X_{AB} is the mean progeny disease response for cross A x B, X' is the grand mean for all crosses, G_A and G_B are the general combining

abilities of the seed and pollen parents, respectively, S_{AB} is the specific combining ability, and E is the residual error (24,25).

Estimates of heritability for disease incidence, number of cankers, and proportion of wood diseased were calculated using data collected after the first exposure period in the field as described by Hartl (6). Data from crosses with 'VR6-28' were excluded in this analysis. The mean disease responses for each cross were regressed on the midparent disease responses; the midparent disease response equaled the average of the two parental mean values for that disease response. The regression coefficient is considered an estimate of heritability for that disease response (6).

Results

In the greenhouse, 78% of the seedlings from all 12 crosses inoculated with *A. anomala* ascospores showed symptoms of eastern filbert blight (Table 2.1). Diseased seedlings had an average of one canker per seedling and a mean proportion of wood diseased of 0.36.

In the field, potted trees were exposed to an average of 2.3×10^7 ascospores/m^2/wk from 12 March to 29 May 1993. During the second exposure period, from 17 March to 31 May 1994, trees were exposed to an average of 1.2×10^7 ascospores/m^2/wk. Eighty-two percent of the potted trees became diseased after the first exposure period (Table 2.1). Diseased trees exhibited an average of five cankers per tree and a mean proportion of wood diseased of 0.29. After the second exposure period, 84% of the potted trees were diseased and diseased trees exhibited an average proportion of wood diseased of 0.40.

For trees of the parental cultivars, an average disease incidence of 59% was observed in the field (Table 2.2). Diseased trees averaged

Table 2.1. Mean incidence and severity of eastern filbert blight measured on the progeny of 12 crosses of seven European hazelnut cultivars inoculated with *Anisogramma anomala* in the greenhouse and in the field.

Controlled Cross (Seed x Pollen)	Greenhouse			Field Year One			Field Year Two[a]	
	Incidence (%)	Mean # of cankers	Proportion of wood diseased (SD)[b]	Incidence (%)	Mean # of cankers	Proportion of wood diseased (SD)	Incidence (%)	Proportion of wood diseased (SD)
Ennis x TGDL[c]	93	0.9	0.45 (± 0.27)	98	7.8	0.54 (± 0.23)	100	0.60 (± 0.27)
Ennis x TDG[d]	100	0.9	0.46 (± 0.30)	94	6.7	0.36 (± 0.21)	95	0.49 (± 0.22)
Ennis x Gem	----	----	----	95	5.8	0.35 (± 0.23)	100	0.49 (± 0.25)
Ennis x VR6-28	33	0.4	0.24 (± 0.26)	58	3.6	0.16 (± 0.20)	55	0.23 (± 0.27)
Will.[f] x TGDL	98	1.2	0.52 (± 0.19)	100	7.7	0.54 (± 0.23)	100	0.64 (± 0.28)
Will. x TDG	97	0.8	0.38 (± 0.26)	86	4.6	0.23 (± 0.17)	87	0.40 (± 0.28)
Will. x Gem	82	0.6	0.29 (± 0.28)	81	3.4	0.15 (± 0.13)	94	0.30 (± 0.25)
Will. x VR6-28	45	0.4	0.22 (± 0.21)	42	1.4	0.05 (± 0.08)	47	0.08 (± 0.13)
Casina x TGDL	84	1.0	0.34 (± 0.30)	95	6.5	0.36 (± 0.19)	95	0.55 (± 0.27)
Casina x TDG	75	0.8	0.35 (± 0.22)	100	7.7	0.39 (± 0.19)	100	0.51 (± 0.24)
Casina x VR6-28	38	0.4	0.21 (± 0.20)	41	1.6	0.06 (± 0.12)	31	0.11 (± 0.20)
Casina x Gem	100	0.8	0.46 (± 0.17)	94	6.5	0.33 (± 0.22)	97	0.44 (± 0.24)

Table 2.1. Continued.

[a] Mean number of cankers was not calculated for field year two because of merging of cankers from the previous year.
[b] Standard deviation.
[c] Pollen parent 'Tonda Gentile delle Langhe'.
[d] Pollen parent 'Tonda di Giffoni'.
[e] No value was obtained.
[f] Seed parent 'Willamette'.

two cankers per tree and a mean proportion of wood diseased of 0.14. After the second exposure period, mean disease incidence increased to 65% and mean proportion of wood diseased to 0.21. No disease was observed on 'VR6-28' (Table 2.2). Disease was observed on trees of the remaining six cultivars. All three disease responses were significantly lower in cultivars Gem and Tonda di Giffoni ($P \leq 0.05$) than in cultivars Ennis, Casina, and Tonda Gentile delle Langhe (Table 2.2). Mean number of cankers and mean proportion of wood diseased were significantly lower in 'Willamette' ($P \leq 0.05$), as well, although a high incidence of disease was observed (77%)(Table 2.2).

Table 2.2. Mean incidence and severity of eastern filbert blight measured on seven cultivars of European hazelnut inoculated with *Anisogramma anomala* in the field.

Cultivar	Field Year One			Field Year Two	
	Disease Incidence (%)	Mean # of cankers	Mean Prop. of wood diseased (SD) [a]	Disease Incidence (%)	Mean Prop. of wood diseased (SD)
Ennis	90	4	0.22 (± 0.15)	100.0	0.38 (± 0.20)
Willamette	77	2	0.07 (± 0.07)	86.7	0.18 (± 0.11)
Casina	83	4	0.23 (± 0.15)	96.7	0.39 (± 0.20)
TGDL[b]	87	3	0.32 (± 0.18)	82.2	0.36 (± 0.25)
TDG[c]	41	1	0.04 (± 0.08)	37.0	0.02 (± 0.03)
VR6-28	0	0	0.00 (± 0.00)	0.0	0.00 (± 0.00)
Gem	30	2	0.08 (± 0.15)	50.0	0.13 (± 0.23)
LSD[d]	24	2	0.07	25	0.14

[a] Standard deviation.
[b] 'Tonda Gentile delle Langhe'
[c] 'Tonda di Giffoni'
[d] Least Significant Difference.

Complete resistance. In the greenhouse and field studies, progeny from crosses with the pollen parent 'VR6-28' were examined for inheritance of a single dominant gene for complete resistance. For the greenhouse study, disease incidence in seedlings combined across all three crosses did not deviate significantly from the expected ratio of one non-diseased (resistant) seedling for each diseased (susceptible) seedling (pooled χ^2 = 2.96 with 1 df)(Table 2.3)(13). Similar results were obtained for the first year of the field study and the second year of the field study (pooled χ^2 = 0.01 with 1 df, and pooled χ^2 = 0.95 with 1 df, respectively)(Table 2.3)(14). The ratio of diseased to non-diseased trees changed from 1.2:1 to 2.2:1 for cross 'Casina' x 'VR6-28' from the first year of the field study to the next (Table 2.3). Four trees that were diseased the first year were symptomless the following year. Three diseased trees from the first year of the field study were missing the following year. For all three crosses combined,the progeny did not deviate significantly from a ratio of one diseased offspring to one non-diseased offspring (χ^2 = 4.22 with 1 df)(Table 2.3).

Partial resistance. The frequency distributions for proportion of wood diseased for all 12 crosses examined in this study are shown in Figure 2.1. The histograms show a continuous range of disease responses observed in the progeny from nine crosses, suggesting resistance in these crosses may be controlled quantitatively. The progeny from crosses with certain parents (e.g. 'Willamette') had consistently low levels of disease with small standard deviations (Figure 2.1 and Table 2.2), whereas the progeny of other parents (e.g. 'Tonda Gentile delle Langhe') showed a much broader range of responses. Crosses with parent 'VR6-28' had skewed segregation patterns with a large number of trees having no disease. This is consistent with the presence of a single

Table 2.3. Segregation analysis for incidence of eastern filbert blight in progeny populations of the resistant European hazelnut selection VR6-28 and three susceptible cultivars.

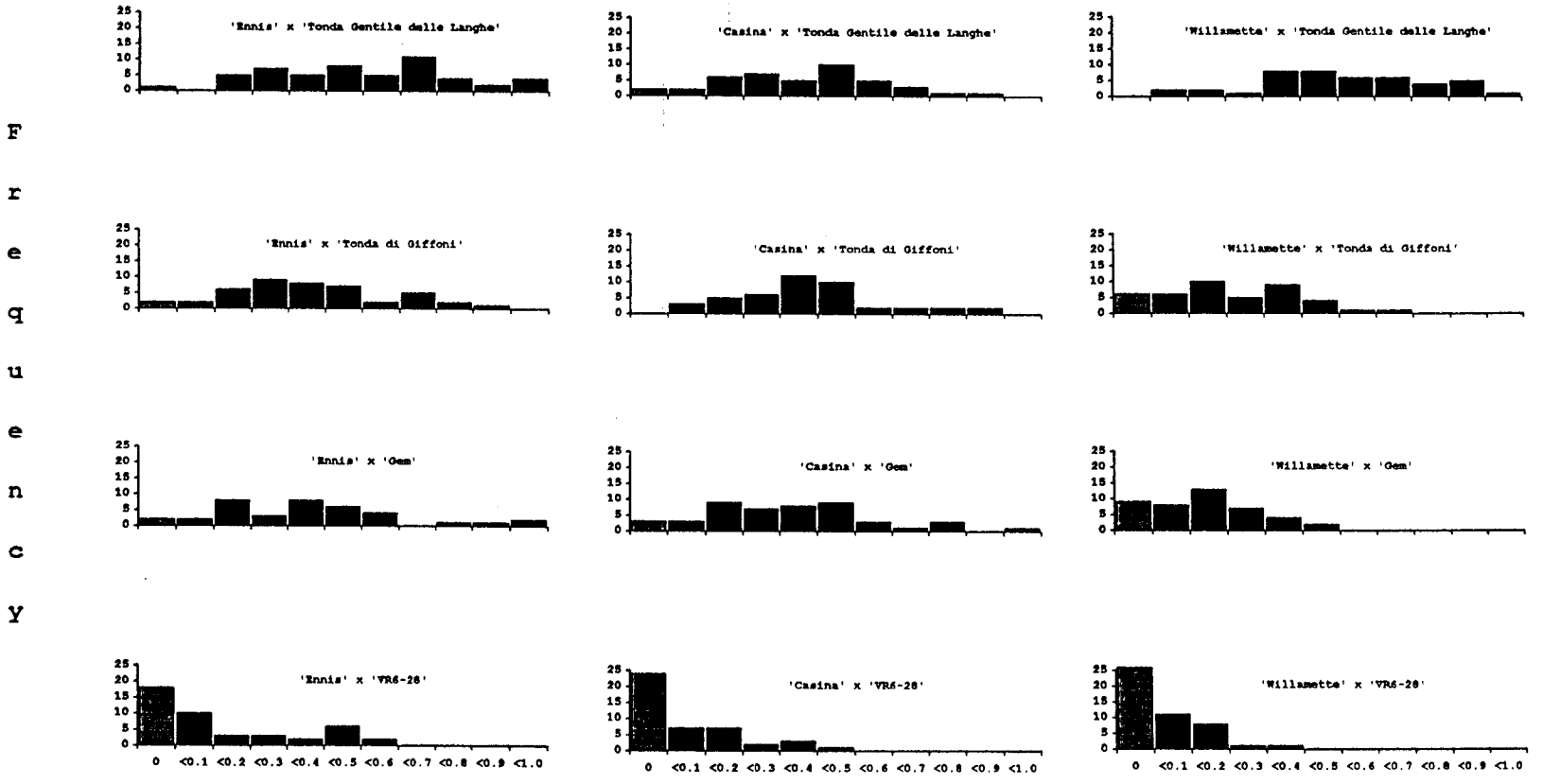
	Controlled Cross	Number of progeny		Expected ratio	χ^2		
		Non-diseased	Diseased		Value	df	*P* - value
GH[a]	'Ennis' x 'VR6-28'	22	11	1:1	3.03	1	0.10 - 0.05
	'Will.' x 'VR6-28'	24	25	1:1	0.00	1	0.99 - 0.95
	'Casina' x 'VR6-28'	25	15	1:1	2.03	1	0.25 - 0.10
	Pooled[b]	71	51		2.96	1	0.25 - 0.10
	Homogeneity[c]				2.10	2	0.50 - 0.25
FY 1	'Ennis' x 'VR6-28'	18	27	1:1	1.42	1	0.25 - 0.10
	'Will.' x 'VR6-28'	26	19	1:1	0.80	1	0.50 - 0.25
	'Casina' x 'VR6-28'	24	20	1:1	0.20	1	0.75 - 0.50
	Pooled	68	66		0.01	1	0.95 - 0.90
	Homogeneity				2.41	2	0.50 - 0.25
FY 2	'Ennis' x 'VR6-28'	19	24	1:1	0.37	1	0.75 - 0.50
	'Will.' x VR6-28'	23	21	1:1	0.02	1	0.90 - 0.75
	'Casina' x VR6-28'	28	13	1:1	4.78	1	0.05 - 0.01
	Pooled	70	58		0.95	1	0.75 - 0.50
	Homogeneity				4.22	2	0.25 - 0.10

[a] Disease incidence ratios for experiments in the greenhouse (GH), the first year of the field study (FY1), and the second year of the field study (FY2).
[b] Total of non-diseased and diseased plants for all three crosses combined.
[c] The homogeneity of ratio for the pooled results.

dominant gene for complete resistance in one-half of the progeny. The disease response number of cankers per tree showed frequency distributions similar to those in Figure 2.1 for all crosses.

Combining ability analysis was performed on the parents whose progeny exhibited a continuous range of responses to infection by *A. anomala*. These progeny were examined for disease incidence, mean

Figure 2.1. Histograms showing the frequency distribution for proportion of wood diseased with eastern filbert blight in the progeny of three seed parental cultivars and four pollen parental cultivars of European hazelnut crossed in all combinations. Results presented are from data collected from the progeny after the first year of exposure in the field.



number of cankers, and mean proportion of wood diseased (Table 2.1), and general combining abilities for the parents were calculated for each disease response. Analysis of variance was performed on the disease responses observed in the progeny to determine the significance of general and specific combining ability in these parents (Table 2.4). General combining ability was not significant ($P > 0.05$) for any of the disease responses examined in the greenhouse, whereas specific combining ability was significant ($P \leq 0.05$) for disease incidence (Table 2.4). In contrast, both general and specific combining ability were significant ($P \leq 0.05$) for all disease responses measured in the first year of the field study (Table 2.4). For each disease response, general combining ability was nearly twice the magnitude of specific combining ability. Specific combining ability was significant for both disease incidence ($P \leq 0.05$) and mean proportion of wood diseased ($P \leq 0.01$) in field year two, while general combining ability was significant only for mean proportion of wood diseased ($P \leq 0.01$)(Table 2.4).

In the first year of the field study, progeny of the pollen parents 'Tonda di Giffoni' (GCA = -0.33) and 'Gem' (GCA = -3.93) had lower levels of disease incidence than the grand mean for all crosses as did progeny of the seed parent 'Willamette' (GCA = -4.63). The fewest cankers were observed on progeny of 'Gem' (GCA = -1.08) and on progeny of 'Willamette' (GCA = -1.05). Proportions of wood diseased that were lower than the grand mean were observed on progeny of 'Gem', 'Tonda di Giffoni', and 'Willamette' (Table 2.5)(14). The general ranking of 'Gem', 'Tonda di Giffoni', and 'Willamette' as parents capable of transmitting high levels of partial disease resistance to

Table 2.4. Analysis of variance of combining ability in *Corylus avellana* for the three disease responses to eastern filbert blight collected in the greenhouse study (GH), field year one (FY1), and field year two (FY2).

	Source	df	Mean Squares		
			Disease Incidence	Mean # of Cankers	Mean Prop. of Wood Diseased
Green House Study	Experiment	1	47.68	0.050	0.002
	Seed GCA	2	94.66	0.001	0.007
	Pollen GCA	2	10.53	0.051	0.006
	Seed x Pollen SCA	4	169.53*[a]	0.075	0.014
	Error[b]	4	24.37	0.021	0.005
Field Year One	Block	2	77.93	19.086**	0.008
	Seed GCA	2	146.18**	6.296**	0.028*
	Pollen GCA	2	149.20**	10.415**	0.099**
	Seed x Pollen SCA	4	99.22*	4.007*	0.030**
	Error	16	23.39	0.965	0.005
Field Year Two	Block	2	19.52	NA[c]	0.003
	Seed GCA	2	48.64	NA	0.014**
	Pollen GCA	2	40.62	NA	0.086**
	Seed x Pollen SCA	4	55.34*	NA	0.014**
	Error	16	17.53	NA	0.001

[a] *, ** Significant at the $P = 0.05$ and $P = 0.01$ probability level, respectively.
[b] The error term accounts for environmental interaction (e.g. block x cross).
[c] No values were available for analysis.

their progeny did not change from the first year to the second in the field study (Table 2.5).

Specific combining ability, as measured by the difference between an observed mean disease response for a cross and an expected value based on the estimated general combining ability values of the parents, was important in a few crosses, such as 'Willamette' x 'Gem' and 'Willamette' x 'Tonda di Giffoni' (Figure 2.2). In other crosses (e.g. 'Ennis' x 'Tonda Gentile delle Langhe'), it was of little importance.

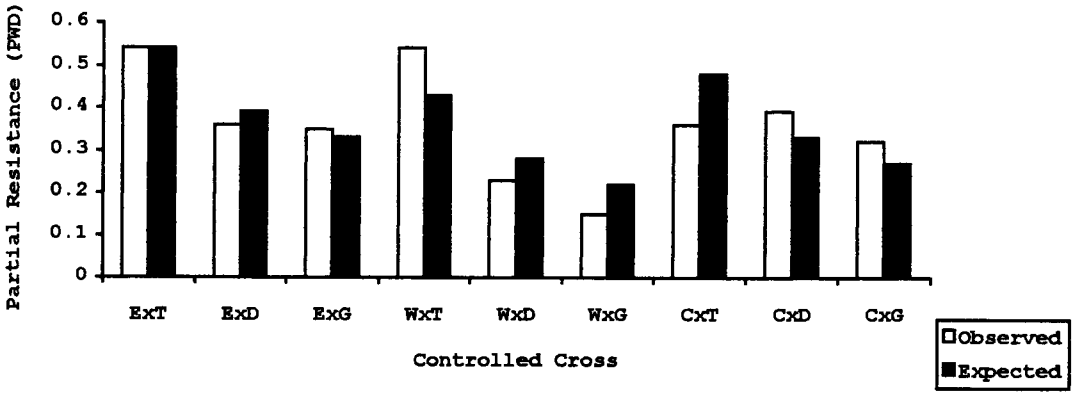
Table 2.5. General combining ability (GCA) values for six European hazelnut cultivars based on eastern filbert blight incidence and severity in progeny populations inoculated with *Anisogramma anomala* in the greenhouse and for two consecutive years in the field.

	Disease Incidence			Mean Number of Cankers				Mean Proportion of Wood Diseased			
	Pollen Parent	GCA	Seed Parent	GCA	Pollen Parent	GCA	Seed Parent	GCA	Pollen Parent	GCA	Seed Parent	GCA
Greenhouse:												
	TDG[a]	-0.67[b]	Casina	-5.17	TDG	-0.04	Casina	0.00	Gem	-0.02	Casina	-0.02
	TGDL[c]	+0.17	Will.[d]	+0.87	Gem	-0.04	Ennis	0.00	TDG	-0.01	Will.	-0.01
	Gem	+0.40	Ennis	+4.20	TGDL	+0.10	Will.	+0.01	TGDL	+0.03	Ennis	+0.03
Field Year One:												
	Gem	-3.93	Will.	-4.63	Gem	-1.08	Will.	-1.05	Gem	-0.09	Will.	-0.05
	TDG	-0.33	Ennis	+1.83	TDG	+0.02	Ennis	+0.45	TDG	-0.03	Casina	0.00
	TGDL	+4.20	Casina	+2.73	TGDL	+1.06	Casina	+0.61	TGDL	+0.12	Ennis	+0.06
Field Year Two:												
	TDG	-2.33	Will.	-2.60	NA[e]	NA	NA	NA	Gem	-0.08	Will.	-0.04
	Gem	+0.70	Casina	+0.97	NA	NA	NA	NA	TDG	-0.03	Casina	+0.01
	TGDL	+1.77	Ennis	+1.77	NA	NA	NA	NA	TGDL	+0.11	Ennis	+0.04

Table 2.5. Continued.

[a] Pollen parent 'Tonda di Giffoni'.
[b] A negative GCA value indicates a mean disease response lower than the grand mean for all crosses was detected in the progeny of this parent.
[c] Pollen parent 'Tonda Gentile delle Langhe'.
[d] Seed parent 'Willamette'.
[e] No values were available for analysis.

Figure 2.2. Observed mean proportion of wood diseased with eastern filbert blight in progeny populations from nine crosses of European hazelnut cultivars compared to an expected proportion of wood diseased based on the estimated general combining abilities of each parent in a cross. The difference between observed and expected values is the measure of specific combining ability. Standard deviations for observed values are reported in Table 2.1. Parentage of the crosses are indicated as follows: E = 'Ennis', W = 'Willamette', C = 'Casina', T = 'Tonda Gentile delle Langhe', D = 'Tonda di Giffoni', and G = 'Gem'.



The estimate of heritability for number of cankers per tree was 0.39 for cultivars Ennis, Willamette, Casina, Tonda Gentile delle Langhe, Tonda di Giffoni, and Gem. The estimate of heritability for proportion of wood diseased was 0.47 and for disease incidence was 0.21 for these same cultivars. The standard errors of these estimates are 0.05 for disease incidence, 0.02 for number of cankers, and 0.04 for proportion of wood diseased.

Discussion

The results from this study show that partial resistance to eastern filbert blight is a moderately heritable trait in *C. avellana*, the European hazelnut. The heritability levels calculated for disease incidence, number of cankers per tree, and proportion of wood diseased are consistent with those calculated for other traits observed in *C.*

avellana (11). The progeny of cultivars Gem and Willamette and, to a lesser extent, Tonda di Giffoni, showed relatively high levels of partial resistance to eastern filbert blight. The significance and magnitude of general combining ability relative to specific combining ability suggest this partial resistance to eastern filbert blight is controlled mainly by additive gene action.

The selection VR6-28, a pollen parent examined in this study, is an offspring of Gasaway, a cultivar that carries a single dominant gene for complete resistance to eastern filbert blight (13). Progeny from all crosses with 'VR6-28' segregated 1:1 for complete resistance to this disease in both the greenhouse and in the field. These results show that progeny of 'VR6-28' inherited the 'Gasaway' resistance gene from 'VR6-28' in a single, dominant fashion. In 1995, Coyne test crossed resistant offspring of 'Gasaway' with 'Gasaway' and obtained a 3:1 ratio of resistant to susceptible seedlings (3). Her results combined with the results reported here support the conclusion that the 'Gasaway' resistance gene is inherited in a single, dominant fashion (13). Thus, European hazelnut possesses both qualitative (major gene) and quantitative resistance to eastern filbert blight.

Disease responses measured on progeny seedlings inoculated with *A. anomala* in the greenhouse differed from those measured on 1- to 2-yr-old progeny inoculated in the field. For example, progeny of the seed parent 'Casina' had low values for disease incidence, number of cankers, and proportion of wood diseased in the greenhouse (Table 2.1). However, in the field, the progeny of this cultivar were much more susceptible to eastern filbert blight (Table 2.1). As a result, the general combining ability values for each disease response in cultivar Casina changed from the greenhouse to the field (Table 2.5). This phenomenon occured in cultivars Willamette and Gem, as well. These results suggest the greenhouse inoculation method is not suitable for

identifying parents that transmit high levels of partial disease resistance to their progeny. A similar problem was encountered in a study of the heritability of fire blight resistance in crosses of pear cultivars (22). The results of the study showed that disease responses were weakly correlated between plants inoculated in the greenhouse and plants inoculated in the field (22). Fortunately, the heritability estimates for number of cankers per tree and proportion of wood diseased after one year of exposure in the field were moderately high in European hazelnut. This suggests that for the purposes of applied breeding, the phenotype of the parent is an adequate predictor of the parent's general combining ability. Progeny testing in the field need only be done when more accurate measures of combining ability are required.

The level of disease detected in several parental cultivars was similar to that reported previously (19). Cultivars Ennis and Tonda Gentile delle Langhe were highly susceptible to eastern filbert blight, cultivars Willamette and Tonda di Giffoni were moderately susceptible, and selection VR6-28 was completely resistant. However, the level of disease observed in two cultivars, Gem and Casina, differed from previous reports (3,19). Casina has been reported to be a moderately susceptible cultivar (19), but was considered highly susceptible to eastern filbert blight in this study. No disease was detected on 'Gem' trees in the previous report (19), but 30% of the trees included in this study became infected with eastern filbert blight. Our results match observations that have been made in an orchard of mixed cultivars in which eastern filbert blight has been allowed to develop naturally. In this orchard, eastern filbert blight was observed on 100% of the 'Casina' trees and on 11% of the 'Gem' trees (S. A. Mehlenbacher and D. Smith, unpublished data).

In other pathosystems, partial disease resistance in a host may be controlled by one to several genes (1,16,17,29). For example, resistance to fire blight in pears is apparently quantitative (22). When a frequency distribution of progeny disease responses in pear progenies was constructed, a continuous range of responses was observed (22). Like the disease responses in pear progenies, the frequency distribution for proportion of wood diseased for European hazelnut progenies was mostly continuous over the range of responses observed (Figure 2.1). Only disease responses in progeny of 'VR6-28' were dramatically skewed towards one end of the range, in this case towards no disease. This skewing pattern suggests the presence of a dominant gene for resistance, whereas the continuous pattern suggests multiple genes are involved. In a recent paper by Pinkerton et al, the response of *C. avellana* to infection by *A. anomala* was examined at the microscopic level (21). Their conclusions state that successful infection by the pathogen is dependent on several factors, including structural characteristics of the epidermis and cuticle, cell vacuolation, and accumulation of fungitoxic substances. These factors are the result of plant growth and development, traits normally under multigenic control (23). This would be consistent with our observations that partial resistance is inherited quantitatively and is controlled primarily by additive gene action. For example, certain cultivars may carry genes that allow for faster maturation of new shoots, whereas other cultivars may carry genes that control host defense responses such as the production of fungitoxic substances. A more extensive study must be done on the interaction of host and parasite in this system to better elucidate the mechanisms of partial disease resistance in *C. avellana*.

Gasaway, the cultivar from which the single dominant gene for complete resistance was identified, can become diseased with eastern

filbert blight when *A. anomala* cankers grow across a graft union with a disease scion cultivar (21). These results suggest this single dominant gene plays a role in preventing initial infection by ascospores of the pathogen. Specific combining ability, a measure of non-additive gene action (dominance and epistasis), was significant ($P \leq 0.05$) for all disease responses measured in this study (Table 2.4). This suggests dominant genes other than the 'Gasaway' gene exist that determine how the host, European hazelnut, reacts to infection by *A. anomala*. However, the results of this study show the contribution of these dominant genes to host defense responses is of less importance than the contribution of multiple genes controlled quantitatively. Specific combining ability was of lesser magnitude than general combining ability (Table 2.4) and disease responses in the progeny were distributed continuously (Figure 2.1).

In the field, mean disease incidence for crosses with the parent 'VR6-28' changed from one year to the next for some crosses (Table 2.1). For example, the increase in disease incidence in cross 'Willamette' x 'VR6-28' from one year to the next may be a result of plants escaping inoculation during the first exposure period. In the remaining two crosses, decreases in disease incidence were observed. These decreases may have been due to experimental error or, as in cross 'Casina' x 'VR6-28', missing trees. A third explanation is that some infected plants successfully recovered from infection. The conclusions of Pinkerton et al (21), as well as personal observations by the authors, support the latter hypothesis. Pinkerton et al found that European hazelnut reacts to infection by ascospores of *A. anomala* with a hypersensitive-like response (21). In some plants, this reaction may have successfully limited mycelial growth to dead or dying tissues; this would eventually kill the pathogen, an obligately biotrophic parasite (27). Small cankers observed on an offspring of 'Gasaway',

selection VR24-16, were missing the following spring (S. A. Mehlenbacher, pers. obs.). A similar observation was made on offspring of 'Casina' x 'VR6-28' in this study; cankers observed on four trees in June 1994 were absent the following year. This supports the conclusion that a few infected plants did recover from infections by the pathogen. The report by Pinkerton et al (21) suggests a mechanism by which this could be accomplished.

Combining partial resistance with resistance controlled by a single dominant gene is considered a good strategy for increasing the longevity of dominant resistance genes in the field (9). Such resistance combinations should prevent severe disease outbreaks should the dominant resistance gene be rendered ineffective by a virulence shift in the pathogen (9). Only one dominant gene for complete resistance to *A. anomala* has been identified in European hazelnut (13). Results from this study show that the high levels of partial resistance, as expressed by fewer and smaller cankers, observed in some cultivars is heritable. VR6-28, a selection that carries the dominant gene for complete resistance, may be a potential source of high levels of partial resistance for hazelnut breeders, as well. Diseased progeny of 'VR6-28' tended to have fewer, smaller cankers than the diseased progeny of other parents (Figure 2.1). In conclusion, it should be possible to breed new cultivars with increased levels of partial resistance to eastern filbert blight. Growing these new, partially resistant cultivars with cultivars carrying the single dominant gene for complete resistance may present a durable strategy for combating this devastating disease.

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CHAPTER THREE
VARIATION IN THE INTERNAL TRANSCRIBED SPACER REGION OF *ANISOGRAMMA ANOMALA*, THE EASTERN FILBERT BLIGHT FUNGUS

N. K. Osterbauer, A. Liston, and K. B. Johnson

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Lawrence, KS

Abstract

Sixty-seven isolates of *Anisogramma anomala* (Pyrenomycetes, Diaporthales), an introduced pathogen of European hazelnuts grown in Oregon and Washington, were collected from five geographically separated infection centers. DNA was extracted from ascospores of these isolates and genetic variation in the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA of these isolates was examined using the polymerase chain reaction (PCR) and restriction enzyme digestion. The entire ITS region was amplified. Sixty-six isolates yielded a PCR product approximately 649 base pairs (bp) in length. Double digestions were used to map restriction sites in the 649 bp PCR product. A total of 14 restriction sites were found, three of which were polymorphic. The ITS I regions of 17 isolates, two to four from each infection center, were sequenced and the sequences subjected to analysis. Little sequence divergence was observed between isolates both within (mean sequence divergence = 2%) and among (mean sequence divergence = 2%) infection centers, although four isolates featured relatively numerous mutations. Mutations in the ITS I region were mostly transversions, however, one insertion mutation was detected in a single isolate. A second region of the nuclear DNA, the β-tubulin locus, also was investigated for variation using PCR followed by restriction enzyme digestion of the PCR product. No restriction site polymorphisms were observed at this locus. The phylogenetic relationship of *A. anomala* to other Ascomycetes was determined by comparing the DNA sequences of the ITS I regions of seven randomly selected isolates of *A. anomala* to those of 22 other fungi, including 14 members of the Diaporthales. *Anisogramma anomala* was most closely related to *Phomopsis* spp., *Cryphonectria* spp. and *Endothia gyrosa*, all

diaporthaceous pathogens of woody plants; however, all genera of the Diaporthales examined appeared to be relatively different from one another at the ITS I locus.

Introduction

Anisogramma anomala (Peck.) Muller, a member of the family Gnomoniaceae in the order Diaporthales (subclass Pyrenomycetes)(2), is responsible for a devastating canker disease on European hazelnut (*Corylus avellana* L.) grown commercially in Oregon and Washington (4,5,17). This fungus is indigenous to the eastern coast of North America where it exists as an obligately biotrophic parasite on *C. americana* Marshall, the American hazel (9,16,24). The fungus was introduced to southern Washington in the early 1970s (5), apparently in a single event, and has since become firmly established in the northern one-third of Oregon's Willamette Valley, where 98% of United States hazelnut production occurs (4,17).

Anisogramma anomala is one of several members of the order Diaporthales that cause serious plant diseases on a variety of hosts (11,14,19,22,23,26). Other well-known plant pathogens within this order include *Cryphonectria parasitica* (Murill) M. E. Barr, the cause of chestnut blight (2), and *Gaeumannomyces graminis* (Sacc.) Art ex Oliver, the cause of take-all disease on wheat, barley, and oats (3). These genera and species have been classified in the order Diaporthales based on their morphological and physiological characteristics (2,3,11,26). Over the last 20 yr, new physiological characters have been introduced (11) and previous morphological characters reexamined (2) resulting in the shifting of several species between genera and

some genera between families. Recently, efforts have been made to use DNA sequencing data to better clarify the classification of fungi within the Diaporthales (1,3).

The morphological characteristics of *A. anomala* place it within the Diaporthales (2,8). However, *A. anomala* has physiological characteristics that are relatively unique among the phytopathogenic genera and species of this order. For example, the infection biology of *A. anomala* is similar to that of obligately biotrophic rusts and mildews (18,25), it has a limited host range (8,16,24), and it has no known anamorph (8,25). *Anisogramma anomala* also is considered to be homothallic (8,25), although this observation has not been rigorously verified. Therefore, reexamining the relationship of this fungus to other members of the Diaporthales is of interest.

In another study (15), genetic variation in *A. anomala* was detected using the random amplified polymorphic DNA (RAPD) technique. The RAPD technique detects differences between individual isolates using arbitrary sequences of DNA. For this study, *A. anomala* was investigated for intraspecific variation at two specific genetic loci, the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) and the β-tubulin locus of the nuclear DNA, using the polymerase chain reaction (PCR), restriction enzyme digestions, and DNA sequencing. The DNA sequences were used to evaluate the phylogenetic relationship of *A. anomala* to other phytopathogenic members of the Diaporthales and to other members of the Ascomycetes.

Materials And Methods

Isolate collection: Isolates were collected from five geographically separated *A. anomala* infection centers in northwest Oregon and

southwest Washington (Figure 3.1). In the field, individual cankers approximately 15 to 30 cm long with mature stromata present were collected by pruning the branches bearing the cankers from living trees. Each canker was considered an isolate. The isolates were labeled, sealed in plastic bags, then placed in a cooler for transport to the laboratory. Isolates were stored at -20C in the laboratory until needed. A listing of the isolates examined in this study is presented in Table 3.1.

DNA extraction: Ascospores of *A. anomala* were collected by removing individual perithecia from a single stromata under a dissection microscope. The perithecia were washed in 95% ethanol for 30 sec, then rinsed in sterile distilled water for 1 min. Ascospores were forcibly removed from the perithecia and placed in a sterile 1.5 mL microcentrifuge tube. DNA was extracted from the ascospores using a rapid, high yield mini-preparation method for isolation of total genomic DNA from fungi (10). The procedure was stopped after the first DNA precipitation. Total genomic DNA was extracted from each isolate at least twice and the concentration of DNA adjusted to 25 to 50 ng prior to amplification.

PCR amplification: Reactions were performed in volumes of 25 µL containing 50 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 10 µM each dATP, dCTP, dTTP, and dGTP (Promega), 0.05% glycerol (v:v)(Sigma), 5 nM each of the primers, 25 to 50 ng DNA, and 0.1 units of *Tfl* polymerase (Epicentre). Amplification was performed in a Perkin Elmer-Cetus DNA Thermal Cycler programmed for 1 min at 94C, 35 cycles of 1 min at 94C, 1 min at 54C, and 3 min at 72C, with a final extension step of 7 min at 72C. Products were held at 4C at the conclusion of the

Figure 3.1. Map of the commercial hazelnut production area in Oregon's Willamette Valley (modified from Pinkerton et al, 1992). Letters represent the sites isolates were collected from: Sandy, OR (J); Troutdale, OR (Z); Vancouver, WA (V); Bald Peak, OR (B); and Damascus, OR (D). The hazelnut production region (▨), areas affected by EFB (▩), and sites of initial detection in Washington (⊛) and Oregon (⊠) are shown.

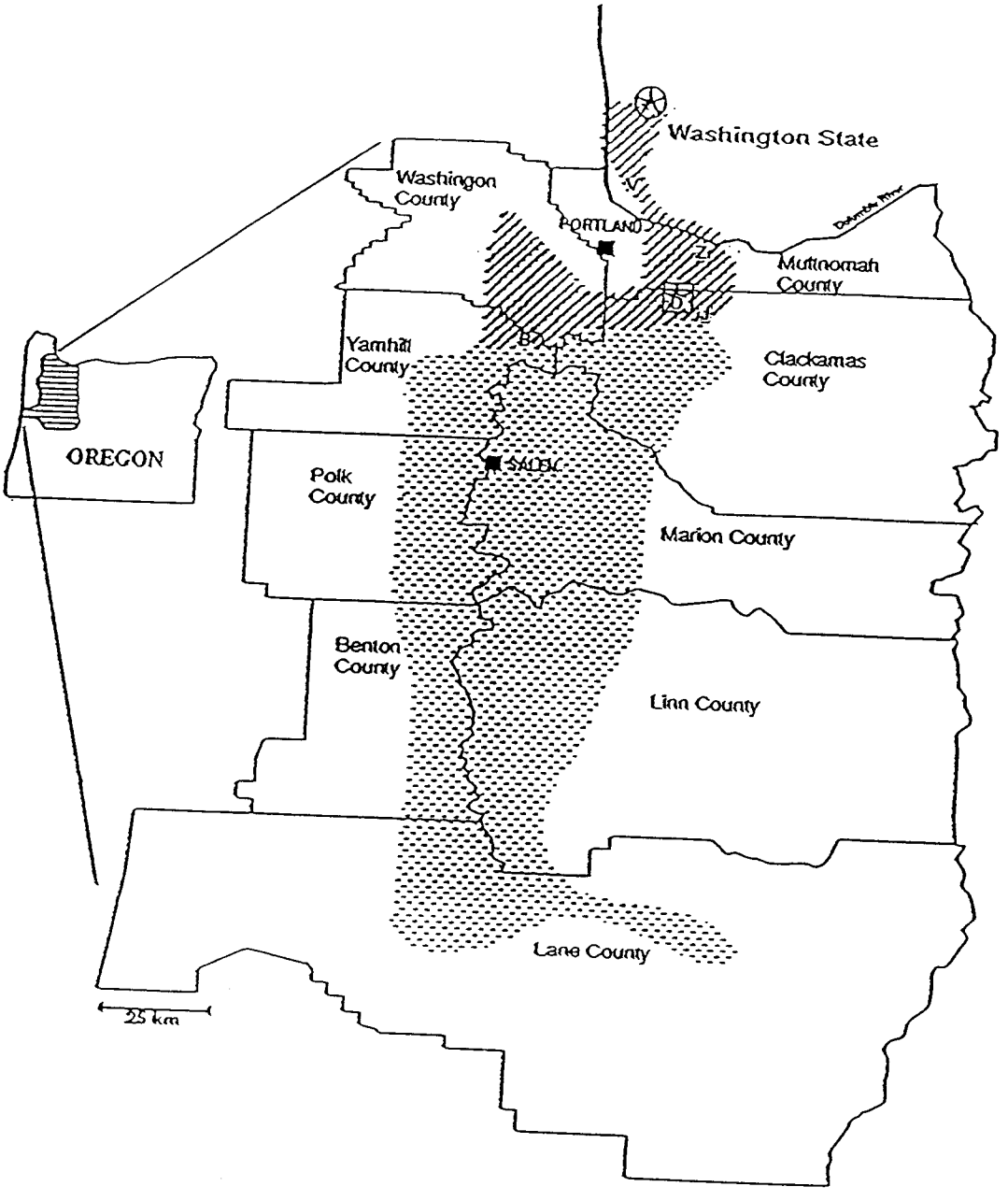


Table 3.1. Isolates of *Anisogramma anomala* examined for variation in the internal transcribed spacer region of the nuclear ribosomal DNA.

Isolate Number	State	Location	Host[1]
AAN6011B[2]	Oregon	Bald Peak	Royal
AAN6021B[2]	Oregon	Bald Peak	Royal
AAN6031B	Oregon	Bald Peak	Royal
AAN6011D	Oregon	Damascus	Unknown
AAN6012D	Oregon	Damascus	Unknown
AAN6013D	Oregon	Damascus	Unknown
AAN6014D[2]	Oregon	Damascus	Unknown
AAN6015D	Oregon	Damascus	Unknown
AAN6021D	Oregon	Damascus	Unknown
AAN6022D	Oregon	Damascus	Unknown
AAN6023D[2]	Oregon	Damascus	Unknown
AAN6024D	Oregon	Damascus	Unknown
AAN6025D[2]	Oregon	Damascus	Unknown
AAN6031D[2]	Oregon	Damascus	Unknown
AAN6032D[2]	Oregon	Damascus	Unknown
AAN6033D	Oregon	Damascus	Unknown
AAN6034D	Oregon	Damascus	Unknown
AAN6035D	Oregon	Damascus	Unknown
AAN6011J	Oregon	Sandy	Gem
AAN6012J	Oregon	Sandy	Gem
AAN6021J	Oregon	Sandy	J-14
AAN6022J	Oregon	Sandy	J-14
AAN6031J	Oregon	Sandy	J-14
AAN6041J	Oregon	Sandy	Ennis
AAN6042J	Oregon	Sandy	Ennis
AAN6043J	Oregon	Sandy	Ennis
AAN6044J[2]	Oregon	Sandy	Ennis
AAN6051J	Oregon	Sandy	Ennis
AAN6052J	Oregon	Sandy	Ennis
AAN6053J	Oregon	Sandy	Ennis
AAN6054J	Oregon	Sandy	Ennis
AAN6061J[2]	Oregon	Sandy	Nottingham
AAN6062J	Oregon	Sandy	Nottingham
AAN6063J[2]	Oregon	Sandy	Nottingham
AAN6064J	Oregon	Sandy	Nottingham
AAN6011V	Washington	Vancouver	Unknown
AAN6012V	Washington	Vancouver	Unknown
AAN6013V	Washington	Vancouver	Unknown
AAN6021V[2]	Washington	Vancouver	Unknown
AAN6022V	Washington	Vancouver	Unknown
AAN6023V	Washington	Vancouver	Unknown
AAN6031V[2]	Washington	Vancouver	Daviana
AAN6032V	Washington	Vancouver	Daviana
AAN6033V[2]	Washington	Vancouver	Daviana
AAN6041V[2]	Washington	Vancouver	USOR 8-73
AAN6042V	Washington	Vancouver	USOR 8-73
AAN6043V	Washington	Vancouver	USOR 8-73
AAN6011Z	Oregon	Troutdale	Barcelona
AAN6012Z	Oregon	Troutdale	Barcelona
AAN6013Z	Oregon	Troutdale	Barcelona
AAN6014Z[2]	Oregon	Troutdale	Barcelona

Table 3.1. Continued.

Isolate	State	Location	Host
AAN6021Z	Oregon	Troutdale	Barcelona
AAN6022Z	Oregon	Troutdale	Barcelona
AAN6023Z	Oregon	Troutdale	Barcelona
AAN6024Z	Oregon	Troutdale	Barcelona
AAN6031Z	Oregon	Troutdale	Barcelona
AAN6032Z[2]	Oregon	Troutdale	Barcelona
AAN6033Z[2]	Oregon	Troutdale	Barcelona
AAN6034Z	Oregon	Troutdale	Barcelona
AAN6041Z	Oregon	Troutdale	Daviana
AAN6042Z	Oregon	Troutdale	Daviana
AAN6043Z	Oregon	Troutdale	Daviana
AAN6044Z	Oregon	Troutdale	Daviana
AAN6051Z	Oregon	Troutdale	Daviana
AAN6052Z[2]	Oregon	Troutdale	Daviana
AAN6053Z	Oregon	Troutdale	Daviana
AAN6054Z	Oregon	Troutdale	Daviana

[1] Cultivar or hazelnut selection from which the isolate was collected.
[2] The ITS I region of the nrDNA of these isolates were sequenced.

amplification. Products were analyzed by electrophoresis in 2% agarose (Gibco-BRL) gels, detected by staining with ethidium bromide (1 µg/mL), then photographed after visualization under UV light. Amplification reactions were repeated at least twice for each DNA extraction. A negative control consisting of the reaction mixture minus fungal DNA was included with each reaction. DNA from *Fusarium graminearum* and from European hazelnut were included as positive controls. Isolates from which multiple products were obtained (three isolates) were not considered for restriction enzyme digestion or for DNA sequencing because of possible contamination by other DNA.

A forward primer homologous to the 3′ end of the small subunit (SSU) nrDNA and a reverse primer homologous to the 5′ end of the large subunit (LSU) nrDNA were used to amplify the entire ITS region. Sequences of the primers were as follows: 5′ GGA AGT AAA AGT CGT AAC

AAG G for the forward primer, ITS5, and 5′ TCC TCC GCT TAT TGA TAT GC for the reverse primer, ITS4 (27). The internal spacer region between the fourth and fifth exons of the β-tubulin locus was amplified using the forward primer T12 (5′ AAC AAC TGG GCC AAG GGT CAC) and reverse primer T22 (5′ TCT GGA TGT TGT TGG GAA TCC)(K. O'Donnell, unpublished data, USDA, Peoria, IL 61604).

Restriction digestions: All restriction enzymes were purchased from New England Biolabs, Inc., and digestions carried out according to manufacturer's specifications. The digested DNA fragments were separated on a 2% agarose gel, stained and photographed as described above. The ITS region of isolates randomly selected from each infection center (e.g. AAN6011B, AAN6031D, AAN6054J, AAN6013V, AAN6034Z) were digested with the enzymes *Csp*6I, *Dpn*II, *Eco*RI, *Eco*RV, *Hha*I, *Hin*fI, and *Taq*αI. The restriction sites were mapped using double digestions.

The PCR products corresponding to the β-tubulin regions of seven randomly selected isolates (AAN6021B, AAN6041J, AAN6061J, AAN6021V, AAN6041V, AAN6021Z, and AAN6032Z) were digested with the enzymes *Dpn*II, *Hha*I, *Hin*fI, and *Taq*αI. The restriction sites were mapped using double digestions.

DNA sequencing and analysis: The PCR products from 17 randomly selected isolates, two to four from each infection center, were purified for sequencing from the reaction mixture using the Prep-A-Gene Purification System (BIORAD, DNA MiniPrep Kit #732-6017). Isolates used for DNA sequencing are indicated in Table 3.1. DNA sequencing was performed by the Center for Gene Research and Biotechnology Central Services Laboratory located at Oregon State University using the automated fluorescence sequencer ABI 373. A search of the GenBank database using the default parameters for the Genetics Computer Group

program BLAST (7) and the ITS I sequence of isolate AAN6031D confirmed fungal DNA had been sequenced. Sequences were aligned using the Genetics Computer Group programs PILEUP and LINEUP (7). Alignment was performed using two sets of weight parameters: A) gap weight = 1.0 and gap length weight = 0.2, B) gap weight = 1.0 and gap length weight = 0.5. The former set of weight parameters favors insertion/deletion events in the alignment whereas the second favors single nucleotide mutations. For each *A. anomala* isolate, the sequence of the entire ITS I region and a partial sequence of the 5.8S nrDNA were used to determine the sequence divergence between isolates. The sequence divergences were calculated using the program PAUP (Phylogenetic Analysis Using Parsimony) v. 3.0s+4. This same program was utilized to determine the phylogenetic relationship of *A. anomala* to other Ascomycetes by comparing the DNA sequences of the ITS I regions from seven of the *A. anomala* isolates to published DNA sequences. Twenty-two fungi, including 14 members of the Diaporthales, were used (Table 3.2). These fungi were selected because they represent a wide range of Pyrenomycetes, which includes the order Diaporthales. *Talaromyces flavus*, a member of the Eurotiales (Subclass Plectomycetes) was used as an outgroup. A phylogenetic analysis of the DNA sequences was performed using the heuristic search algorithm with a maximum of 2000 trees saved and 100 bootstrap replications. Branches with a maximum length of zero were collapsed to show polytomies.

Results

An approximately 649 bp PCR product corresponding to the entire ITS region of the nrDNA of *A. anomala* was observed in 66 of the 67 isolates examined. This result was consistent between DNA extractions

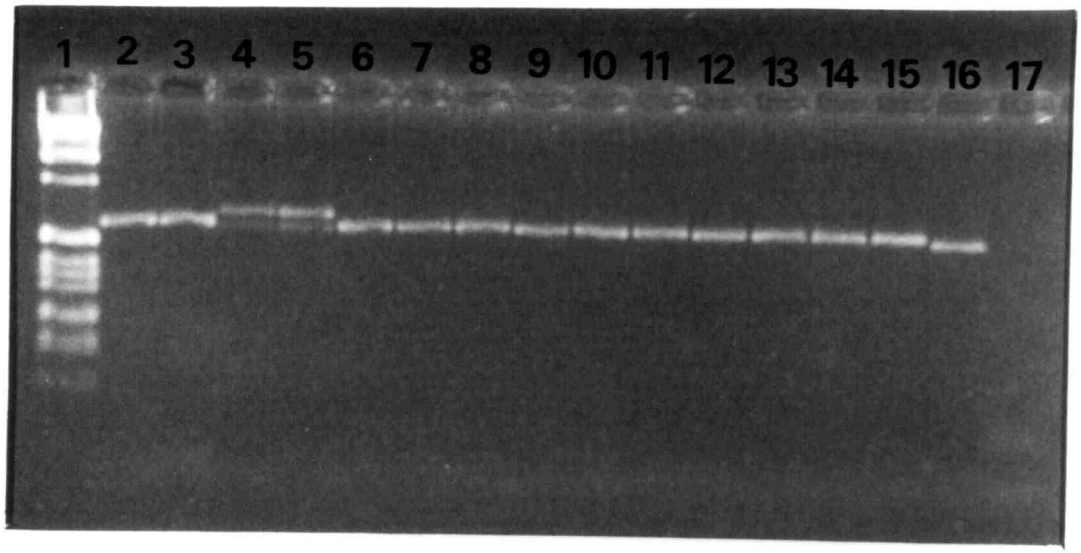
Table 3.2. Fungi examined for relatedness to *Anisogramma anomala* based on their sequences for the ITS I region of the nuclear ribosomal DNA.

Fungus	Order	GenBank accession number
Colletotrichim destructivum	Phyllachorales	Z32939
Cryphonectria cubensis	Diaporthales	L42444
Cr. havanensis	Diaporthales	L42445
Cr. parasitica	Diaporthales	L42446
Cr. radicalis	Diaporthales	L42447
Endothia gyrosa	Diaporthales	L42448
Cylindrocarpon heteronema	Hypocreales[1]	X73805
Fusarium oxysporum	Hypocreales[1]	U28161
F. sambucinum	Hypocreales[1]	X65477
Gauemannomyces cylindrosporus	Diaporthales	U17211
G. graminis var. *avenae*	Diaporthales	U17207
G. graminis var. *graminis*	Diaporthales	U17212
G. graminis var. *tritici*	Diaporthales	U17222
G. incrustans	Diaporthales	U17214
Magnaporthe grisea	Phyllachorales	U17328
Nectria haematococca	Hypocreales	L36616
Phialophora sp.	Diaporthales[2]	U17216
Phomopsis sp.	Diaporthales[2]	U11315
Phomopsis sp.	Diaporthales[2]	U11316
Phomopsis sp.	Diaporthales[2]	U11314
Talaromyces flavus	Eurotiales	U18354
Trichoderma harzianum	Hypocreales	Z48812

[1] Reported as anamorphs for members of the Hypocreales.
[2] Reported as anamorphs for members of the Diaporthales.

and amplification reactions. Isolates from Vancouver, WA, and Bald Peak, OR, all exhibited the 649 bp product. All 17 isolates collected from Sandy, OR, showed the 649 bp product. Two of these isolates, AAN6012J and AAN6043J, had an additional 750 bp PCR product. This product corresponded in size to the ITS region of the nrDNA of European hazelnut suggesting contamination by hazelnut DNA (Figure 3.2). Of the 20 isolates from Troutdale, OR, only one exhibited the additional larger product corresponding in size to the hazelnut ITS region. The remaining 19 isolates showed the 649 bp product.

Figure 3.2. The PCR products resulting from amplification of the ITS region of the nrDNA for isolates of *Anisogramma anomala* collected from Sandy, OR. Lane 1, 1 kb DNA size marker; lanes 2 and 3, isolate AAN6042J; lanes 4 and 5, isolate AAN6043J; lanes 6 and 7, isolate AAN6044J; lanes 8 and 9, isolate AAN6022J; lanes 10 and 11, isolate AAN6061J; lanes 12 and 13, isolate AAN6062J; lanes 14 and 15, isolate AAN6064J; lane 16, *Fusarium* sp. (positive control); and lane 17, reaction mixture minus DNA (negative control). Note the dual products produced by isolate AAN6043J indicating contamination by hazelnut DNA. pair changes were observed at several positions within the ITS I region.



Fourteen of 15 isolates collected from Damascus, OR, exhibited the 649 bp product. A single PCR product approximately 555 bp in length was observed in isolate AAN6025D. Numerous mutations were detected in the 555 bp product when it was digested with restriction enzymes. In addition, the DNA sequence of the ITS I region of AAN6025D was >30% divergent from all other isolates examined. Since these results suggested the 555 bp product was the result of inadvertant amplification of an unidentified fungal contaminant, isolate AAN6025D was excluded from further analyses.

The results from amplification of the β-tubulin region with primers T12 and T22 were consistent across extractions and amplification reactions for each isolate examined. All isolates had products approximately 810 bp in length. No additional PCR products were detected.

Restriction digestions: A restriction site map of the 649 bp PCR products was constructed using double digestions (Figure 3.3). A total of 14 restriction sites were detected in this product (Figure 3.3) and the map was oriented based on the locations of the *Eco*RI and *Eco*RV restriction sites. This orientation was later confirmed by DNA sequence analysis. Three restriction sites, the *Csp*6I site and both *Hha*I sites, were polymorphic (Figure 3.3) in the isolates examined.

Double digestions were used to construct a restriction site map of the β-tubulin locus (Figure 3.4). Nine restriction sites were detected using four restriction enzymes. The enzymes *Dpn*II and *Taq*αI had three restriction sites apiece, *Hha*I had two sites and *Hin*fI had one site. No nucleotide polymorphisms were observed in the isolates analyzed using restriction enzyme digestion.

ITS I region of *A. anomala*: The DNA sequences of the ITS I regions of the 17 isolates of *A. anomala* examined are shown in Figure 3.5. Base

Figure 3.3. Restriction site map of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA of *Anisogramma anomala*. The key for restriction sites and corresponding enzymes is as follows: C = *Csp*6I, D = *Dpn*II, I = *Eco*RI, V = *Eco*RV, H = *Hha*I, F = *Hin*fI, and T = *Taq*αI. Lower case letters indicate sites where nucleotide polymorphisms were detected. Locations of the PCR primer binding sites are indicated.

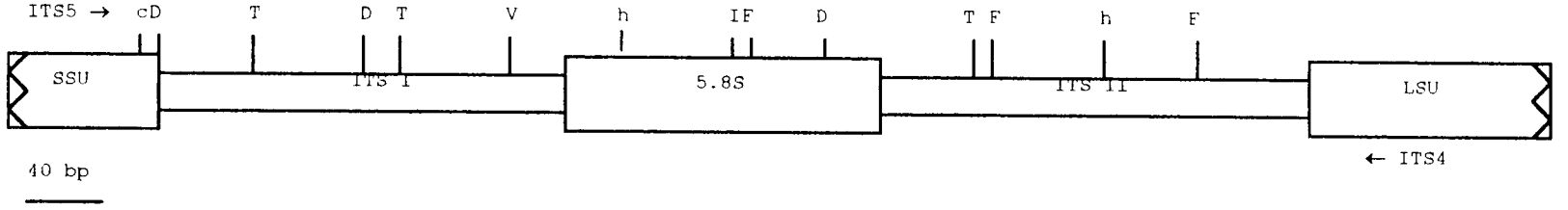


Figure 3.4. Restriction site map of the β-tubulin locus of the nuclear DNA of *Anisogramma anomala*. The key for the restriction sites and corresponding enzymes are as follows: D = *Dpn*II, H = *Hha*I, F = *Hin*fI, and T = *Taq*αI. Locations of the PCR primer binding sites are indicated. Orientation of the map is approximate.

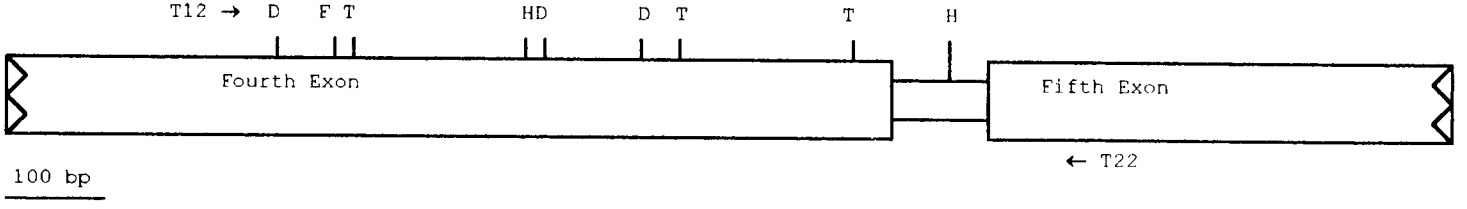


Figure 3.5. The DNA sequences of the ITS I regions of 17 isolates of *Anisogramma anomala*. Partial sequences of the SSU nrDNA and of the 5.8S nrDNA, as indicated by bold type, are included for each isolate. Gaps are indicated by a minus sign (-) and identical bases by an asterisk (*). Nucleotides that differ from those in the reference sequence are indicated as follows: G = guanine, C = Cytosine, A = adenine, and T = Thymine.

Position								SSU nrDNA									1										11
AAN6031D	**C**	**A**	**G**	**C**	**G**	**G**	**A**	**G**	**G**	**G**	**A**	**T**	**C**	**A**	**T**	**T**	**G**	C	T	G	G	A	G	C	A	A	A	C
AAN6063J	*	*	*	*	*	**A**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6061J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6041V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6031V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6023D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*
AAN6021B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
AAN6044J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*
AAN6011B	*	*	*	**T**	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*
AAN6052Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Position	12																										39
AAN6031D	G	C	C	C	C	C	C	C	C	T	T	C	C	C	C	G	G	G	G	G	G	G	G	G	G	C	T	A
AAN6063J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6061J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6041V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6014D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*
AAN6033V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6032D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6031V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6032Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6023D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6021B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
AAN6033Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*
AAN6014Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*
AAN6044J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6011B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6052Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*

Figure 4.5. Continued.

Position	40																										68
AAN6031D	C	C	C	A	G	A	A	A	C	C	C	T	T	T	G	T	G	A	A	T	C	T	T	C	T	C	C	G
AAN6063J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021V	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6061J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6041V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6031V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6023D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6044J	*	*	*	*	*	*	G	G	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6011B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6052Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Position	68																										95
AAN6031D	T	-	T	G	C	C	T	C	G	G	C	C	T	G	G	T	T	G	C	C	C	C	C	G	A	A	C	G
AAN6063J	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021V	*	-	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6061J	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6041V	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014D	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033V	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032D	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6031V	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032Z	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6023D	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021B	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033Z	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014Z	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6044J	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6011B	*	-	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6052Z	*	C	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Figure 4.5. Continued.

Position	96																										123
AAN6031D	G	G	G	G	C	C	C	C	T	C	C	T	C	C	T	C	C	G	G	G	A	G	G	G	A	G	C	T
AAN6063J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6061J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6041V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6031V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6023D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6044J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6011B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6052Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Position	124																										151
AAN6031D	G	A	C	C	G	G	C	C	G	G	C	G	G	C	C	C	C	A	T	A	A	A	C	A	C	T	G	C
AAN6063J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021V	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	T
AAN6061J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6041V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A
AAN6014D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6031V	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6023D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014Z	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	A
AAN6044J	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T
AAN6011B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6052Z	*	*	*	*	*	*	*	*	*	*	T	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Figure 4.5. Continued.

Position	152																											179
AAN6031D	T	C	C	T	G	T	T	A	C	G	A	T	A	T	C	T	G	A	G	C	C	T	T	T	G	A	C	A
AAN6063J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6061J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6041V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6031V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6032Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6023D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6021B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6033Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6014Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*
AAN6044J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAN6011B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*
AAN6052Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*

Position	180										190			5.8S nrDNA					
AAN6031D	G	A	A	A	T	G	A	A	T	C	A	**A**	**A**	**A**	**C**	**T**	**T**	_	**T**
AAN6063J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	*
AAN6021V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	*
AAN6061J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	C
AAN6041V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	C
AAN6014D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	C
AAN6033V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	*
AAN6032D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	C
AAN6031V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	*
AAN6032Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	C
AAN6023D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	C
AAN6021B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	C
AAN6033Z	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	*
AAN6014Z	*	*	*	*	*	*	*	*	*	*	*	*	**T**	*	**A**	*	*	_	*
AAN6044J	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	*
AAN6011B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	_	*
AAN6052Z	*	*	*	*	*	A	*	*	*	*	*	**T**	**C**	**T**	*	*	*	**G**	**C**

Both transitions and transversions occurred at these sites, with transversions predominating 2.5:1. The most common transversion mutations were G→C or C→G and the most common transition mutations T→C or C→T. Transversions at position 35 and at position 137 created new restriction sites for the enzymes *Hha*I and *Hae*III, respectively, in the ITS I regions of some of the isolates examined using DNA sequencing (Figure 3.5). For example, 18% of the isolates sequenced had a *Hae*III site in the ITS I region. Seven isolates, at least one from each infection center, had identical ITS I sequences (Figure 3.5). Four isolates, one each from Sandy, OR, and from Bald Peak, OR, and two from Troutdale, OR, showed a large number of mutations (Figure 3.5) with isolate AAN6052Z from Troutdale, OR, having the most mutations. This isolate had a single nucleotide insertion at position 69 in the ITS I region in addition to several transversion mutations (Figure 3.5).

The sequence divergences between the individual isolates based on the sequences of their ITS I regions were determined (Table 3.3). The range of divergence between isolates within an infection center was lowest for isolates from Damascus, OR, and for isolates from Vancouver, WA (range = 0.00 to 0.01 for both locations). One of the older infection centers, the Troutdale, OR site, featured relatively large divergences among isolates (range = 0.02 to 0.09) as did a young infection center, the Bald Peak, OR site (sequence divergence between isolates = 0.03). Sequence divergences between isolates among infection centers ranged from 0.00 to 0.06.

Phylogenetic analysis of *A. anomala* and other Ascomycetes: The ITS I region of the nrDNA was chosen to determine the relationship of *A. anomala* to other Diaporthales and Ascomycetes because of the availability of published sequence data. A total of 870 most parsimonious trees for Alignment A (gap weight = 1.0, gap length weight

Table 3.3. Sequence divergence between 17 isolates of *Anisogramma anomala* collected from Oregon and Washington based on their ITS I sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. AAN6011B	–	.03	.04	.04	.03	.04	.04	.03	.02	.03	.03	.04	.04	.06	.04	.03	.06
2. AAN6021B		–	.00	.00	.01	.00	.03	.00	.01	.00	.01	.01	.01	.04	.00	.01	.05
3. AAN6014D			–	.01	.01	.00	.03	.00	.01	.00	.01	.01	.01	.05	.00	.02	.05
4. AAN6023D				–	.01	.01	.03	.01	.01	.00	.01	.01	.01	.05	.01	.02	.05
5. AAN6031D					–	.01	.03	.01	.00	.01	.01	.01	.01	.04	.01	.01	.05
6. AAN6032D						–	.03	.00	.01	.00	.01	.01	.01	.05	.00	.02	.05
7. AAN6044J							–	.03	.02	.03	.03	.03	.02	.06	.03	.03	.06
8. AAN6061J								–	.01	.00	.01	.01	.01	.05	.00	.01	.05
9. AAN6063J									–	.01	.01	.01	.01	.04	.01	.01	.04
10. AAN6031V										–	.00	.01	.01	.04	.00	.01	.04
11. AAN6033V											–	.01	.01	.04	.01	.01	.05
12. AAN6041V												–	.01	.03	.01	.01	.05
13. AAN6021V													–	.04	.01	.02	.05
14. AAN6014Z														–	.05	.03	.09
15. AAN6032Z															–	.02	.05
16. AAN6033Z																–	.06
17. AAN6052Z																	–

= 0.2) and 68 most parsimonious trees for Alignment B (gap weight = 1.0, gap length weight = 0.5) were saved from the phylogenetic analyses of these alignments using the heuristic search algorithm of PAUP v.3.0s+4. Fifty percent majority rule consensus trees for Alignments A and B generated from 100 bootstrap replicates of the heuristic search algorithm of PAUP v.3.0s+4 are illustrated in Figures 3.6 and 3.7, respectively.

When gaps were favored in the sequence alignment (Alignment A), the seven *A. anomala* isolates included in the analysis formed a weakly supported (61% bootstrap support) clade with *Phomopsis* spp., *Cryphonectria* spp., and *E. gyrosa* (Figure 3.6). *Gaeumannomyces* spp., *Phialophora* sp., and members of the Hypocreales and Phyllachorales formed a separate, more strongly supported (85% bootstrap support) clade (Figure 3.6). When single nucleotide mutations were favored by the sequence alignment (Alignment B), the seven isolates of *A. anomala* formed a distinct clade from all other members of the Diaporthales (100% bootstrap support)(Figure 3.7). *Phomopsis* spp., *Cryphonectria* spp., *and E. gyrosa*, formed another distinct clade of strictly diaporthaceous fungi (96% bootstrap support) whereas *Phialophora* sp. and *Gaeumannomyces* spp. formed a clade with members of the Phyllachorales and Hypocreales (99% bootstrap support)(Figure 3.7).

Discussion

A low level of intraspecific variation was detected within the Oregon and Washington population of the hazelnut pathogen, *A. anomala*, at one genetic locus, the ITS region of the nrDNA. Despite this low level of intraspecific variation, nucleotide polymorphisms were detected in the ITS regions of 23 *A. anomala* isolates examined with

Figure 3.6. A 50% majority-rule consensus tree generated from 100 bootstrap replications of the heuristic search algorithm of PAUP v.3.0+s4. The tree is based on the aligned nucleotides (gap weight = 1.0, gap length weight = 0.2) of the ITS I regions of 29 Ascomycetes. Branches having a maximum length of zero were collapsed to reveal polytomies. The percentages are the frequencies with which a given branch appeared in 100 bootstrap replications. The tree was rooted by making *Ta. flavus* an outgroup.

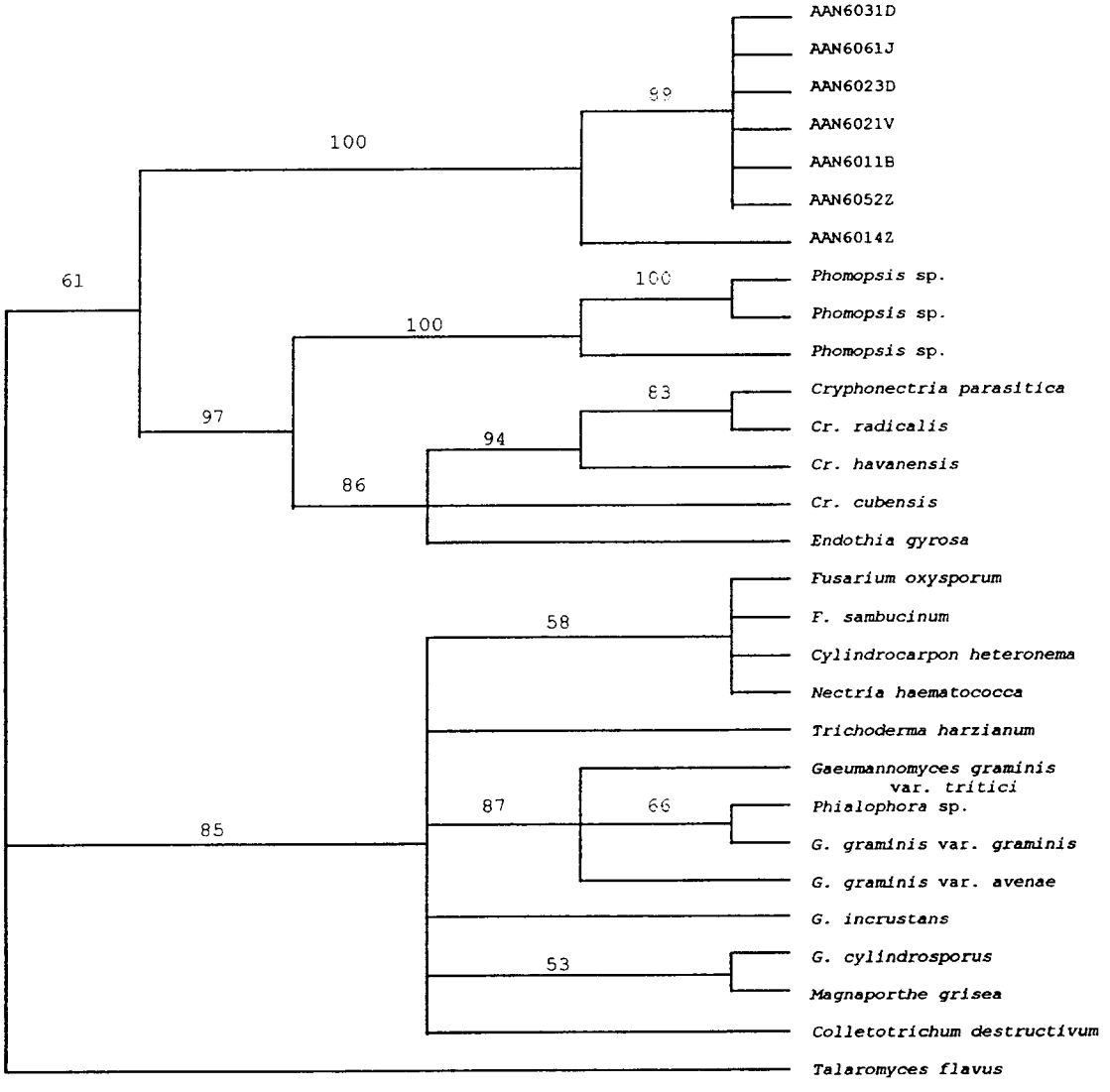
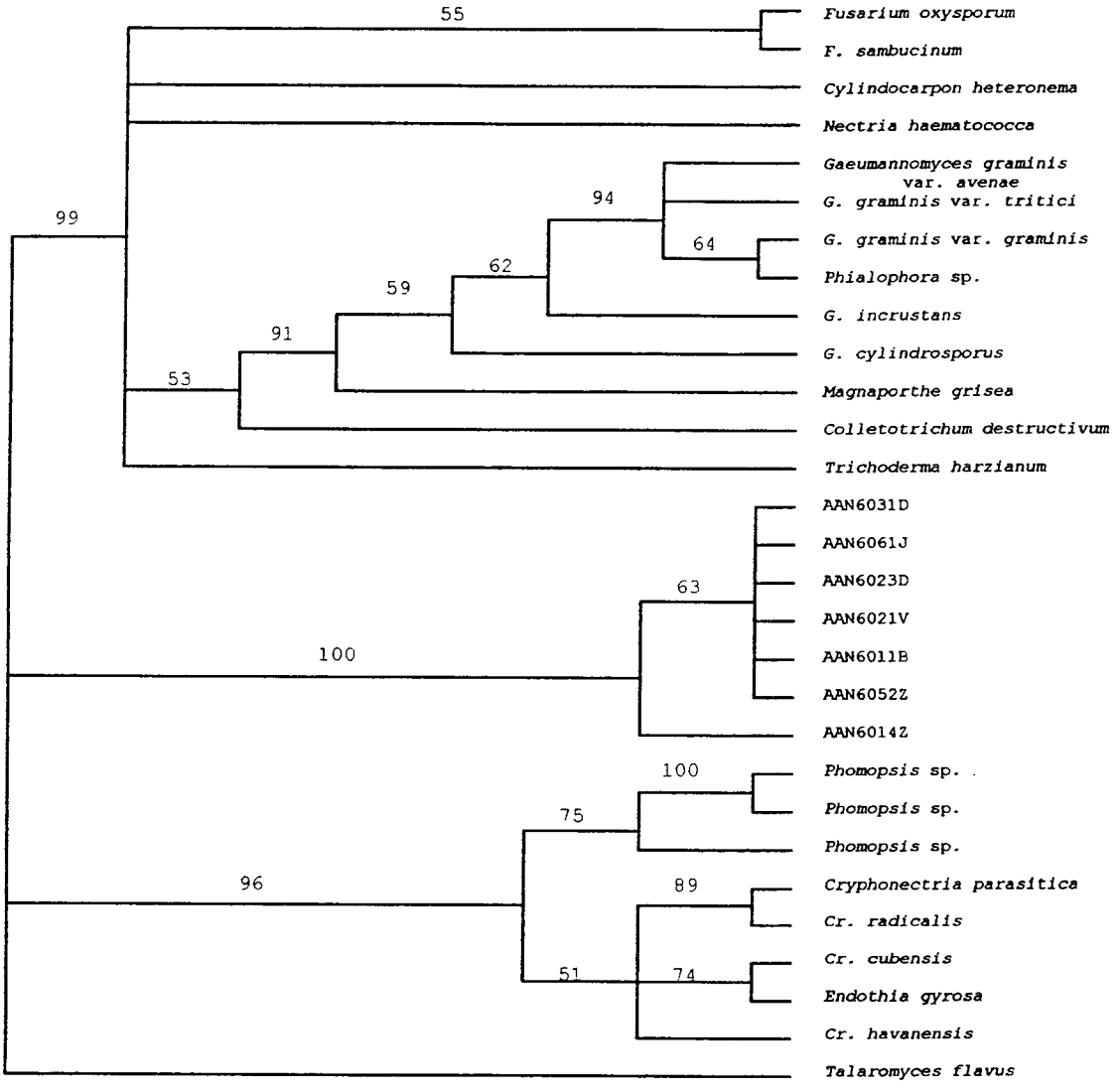


Figure 3.7. A 50% majority rule consensus tree generated from 100 bootstrap replicates of the heuristic search algorithm in PAUP V.3.0s+4. The tree is based on the aligned nucleotides (gap weight = 1.0, gap length weight = 0.5) of the ITS I regions of 29 Ascomycetes. Branches having a maximum length of zero were collapsed to reveal polytomies. The percentages are the frequencies with which a given branch appeared in 100 bootstrap replications. The tree was rooted by making *Ta. flavus* an outgroup.



restriction enzyme digestions and/or DNA sequencing. A phylogenetic analysis of the ITS I sequences of seven *A. anomala* isolates and of 22 other Ascomycetes, including 14 members of the Diaporthales, suggested the relationships between the diaporthaceous genera examined could not be resolved using ITS I sequence data, although there was weak support (61% bootstrap support) for a relationship between *A. anomala* and members of the Diaporthales pathogenic on woody plants.

Overall, little sequence divergence was detected between the *A. anomala* isolates among infection centers (mean percent sequence divergence = 2%). While a few isolates were more divergent than others (e.g. AAN6052Z, mean percent sequence divergence = 5%), the divergences observed between isolates both within and among infection centers are consistent with those observed in other largely homogeneous fungal populations (3,13). For example, Gardes et al found that within *Laccaria* spp. Berk. & Br., intraspecific sequence divergence was relatively low (1 to 5%)(6). The fact that several *A. anomala* isolates, at least one from each infection center, have identical DNA sequences for the ITS I region supports the conclusion that the Oregon and Washington population of *A. anomala* is relatively homogeneous. The lack of nucleotide polymorphisms in the β-tubulin locus of the nuclear DNA is consistent with this finding.

The presence of such a large number of transversions within the ITS I region of *A. anomala* was somewhat surprising. Previous reports cite transitions as the predominant mutations occuring within the ITS I region of the nrDNA of Ascomycetes. O'Donnell discovered a bias towards transition mutations in *Fusarium sambucinum* Fckl. (3) and a similar bias was observed in *Gaeumannomyces* spp. by Bryan et al (3).

At least two of the transversions in *A. anomala* resulted in the formation of new restriction sites within the ITS regions of some isolates. As these new restriction sites occured relatively frequently within different isolates of *A. anomala* (e.g. 56% of the isolates examined had an additional *Hha*I site), these sites may prove useful for population genetics studies performed on this fungus. Nucleotide polymorphisms in the ITS region have been used in other studies to distinguish between populations of individuals (12,21,28). O'Donnell identified three distinct populations of *F. sambucinum* using restriction enzyme digestions (13).

Two sets of weight parameters, one favoring gaps and the other favoring single nucleotide mutations, were used to align the sequences of the ITS I regions of 29 Ascomycetes, including seven isolates of *A. anomala*. Phylogenetic analysis of the alignment favoring gaps resulted in weak support (61% bootstrap support) for a clade including the seven *A. anomala* isolates, *Phomopsis* spp., *Cryphonectria* spp., and *E. gyrosa* (Figure 3.6). This result is supported somewhat by morphological data which places *Anisogramma* in the same family (Gnomoniaceae) as *Phomopsis* and *Endothia* (2,8). Phylogenetic analysis of the alignment favoring single nucleotide mutations suggests the relationships between the genera of the Diaporthales examined in this study could not be resolved; the genera examined were found in three separate clades (Figure 3.7). Because the most current morphological data are consistent with the alignment favoring gaps, we prefer this alignment to the other. Assuming this tautology to be correct, this suggests that evolution within the ITS I region of diaporthaceous fungi occurs primarily by insertion/deletion events. Because these genera were so

different at this locus, additional genera should be examined for divergence in the ITS I region before this hypothesis is accepted.

Two relatively unusual mutations in the SSU nrDNA and in the ITS I region of *A. anomala* were observed in this study. An *Eco*RV restriction site was detected in the ITS region using restriction enzyme digestions (Figure 3.3). In plants, a highly conserved *Eco*RV restriction site is found within the 5.8S nrDNA (M. Hershkovitz and L. Lewis, unpublished data, Smithsonian Institute, Washington, DC). This feature is sometimes used by plant systematists to confirm the successful amplification of plant ITS. The existence of an *Eco*RV restriction site in *A. anomala*'s ITS region suggests plant systematists should be careful about using the presence of an *Eco*RV site to verify the identity of plant ITS. The presence of a guanine in the final position of the SSU nrDNA (Figure 3.5) is the second relatively unusual trait of *A. anomala*. A search of the GenBank database suggests this trait is generally one of plants (A. Liston, unpublished data), however, in this study *Phomopsis* spp., *Cryphonectria* spp., and *E. gyrosa* (see Table 3.2 for GenBank accession numbers) shared this trait with *A. anomala*. All of the remaining fungi examined, including the diaporthaceous fungi *Gaeumannomyces* spp. and *Phialophora* sp., had an adenine at this position. This might suggest common ancestry between *Anisogramma*, *Phomopsis*, *Cryphonectria*, and *Endothia*, although further analysis of the SSU nrDNA should be done before accepting this hypothesis.

Some care must be taken to prevent overinterpretation of the results of the phylogenetic analysis performed in this study. A small genetic locus with relatively few positions was examined and a limited

sample size was used. Also, the alignment of the ITS I regions of the 29 Ascomycetes affected the results of the phylogenetic analysis of these fungi. Thus, we recommend a more highly conserved locus such as the SSU nrDNA or LSU nrDNA be examined before any firm conclusions about the phylogenetic relationships between these diaporthaceous genera are drawn.

This limited study suggests some intraspecific variation does exist in *A. anomala* in the ITS region of the nrDNA. This variation could be used, i) to examine the population genetics of this fungus and ii) to develop species-specific PCR primers for *A. anomala*. Such primers have proven useful for the detection of other fungal phytopathogens in vivo and in vitro (12,21,28). Finally, our observations of high levels of divergence between members of the Diaporthales in the ITS I region indicate an extensive analysis of the order Diaporthales at a more highly conserved locus such as the SSU nrDNA would be appropriate.

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CHAPTER FOUR
EVALUATION OF RELATIVE PATHOGENIC AND GENETIC VARIATION IN ISOLATES OF *ANISOGRAMMA ANOMALA* FROM VARIOUS GEOGRAPHIC REGIONS

N. K. Osterbauer, K. B. Johnson, and S. A. Mehlenbacher

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Abstract

In the greenhouse, a total of 40 *Anisogramma anomala* isolates collected from Illinois, Michigan, Minnesota, New York, Ontario, Virginia, and Oregon/Washington were examined for their ability to infect two European hazelnut clones: VR6-28, a selection that carries a single dominant gene for complete resistance to eastern filbert blight, and Barcelona, a moderately susceptible cultivar grown commercially in Oregon and Washington. Two isolates, one from Ontario and one from Minnesota, caused small sunken lesions on 'VR6-28', raising concern about the durability of the resistance gene in this selection. Isolates from all seven regions infected 'Barcelona'. Based on disease incidence and canker length data measured, the virulence of isolates on 'Barcelona' did not differ significantly among geographic regions ($P > 0.05$) with the exception that isolates from Ontario had a significantly ($P < 0.05$) higher incidence of infection (18.3%) than did isolates from Illinois (9.7%) or Minnesota (5.0%). Seventy RAPD primers were evaluated for polymorphic amplification of *A. anomala* DNA. Of those tested, four were used to compare the relative genetic similarity of 33 isolates both within and among regions. Cluster analysis of the RAPD data showed some clustering of isolates by region. Isolates from Oregon/Washington, New York, and Ontario tended to group together, whereas isolates from the remaining four regions did not. Isolates from Oregon/Washington formed a discrete cluster with the highest similarity (> 88%) among isolates. Clustering of isolates among regions (e.g. > 74% similarity between Illinois isolates AAN1021 and AAN1083 and Minnesota isolate AAN3101) also was evident. With respect to cultivar Barcelona, the results from this study indicate that a relatively low level of pathogenic variation exists in the *A. anomala*

populations sampled in this study. Based on RAPD data, however, some genetic variation attributable to region of origin was apparent in the collected isolates. This observation, coupled with the fact that two isolates from different indigenous populations infected the resistant hazelnut selection VR6-28, suggests the quarantine laws prohibiting the importation of hazelnut plant material from eastern North America to Oregon and Washington should be maintained.

Introduction

Eastern filbert blight is an endemic disease on American hazel (*Corylus americana* Marsh.) in northeastern North America. The causal pathogen, *Anisogramma anomala* (Peck) E. Muller, an obligately biotrophic parasite, was first described in 1876 (21,30). In the late 1800s, orchards of the more commercially desirable European hazelnut (*C. avellana* L.) planted in eastern North America proved highly susceptible to eastern filbert blight (2,15). The presence of *A. anomala* prevented the establishment of a hazelnut industry in this region (2,31). Commercial European hazelnut orchards were established, however, in western North America, outside of the natural range of *A. anomala* (15). At present, all commercial hazelnut production in North America occurs in Oregon, Washington, and British Columbia; the U.S. ranks fourth in production worldwide (5,31).

Quarantine laws were established in 1921 to prevent the importation of diseased *C. americana* and *C. avellana* from eastern North America into Oregon, Washington, and British Columbia (2). Nonetheless, eastern filbert blight was found in a commercial orchard of *C. avellana* located near Woodland, WA, in 1973 (6). This disease

has spread from Washington to Oregon and is now firmly established in the northern one-third of Oregon's Willamette Valley, where 98% of U.S. hazelnut production occurs (4,22).

In Oregon and Washington, ascospores of *A. anomala* are disseminated during periods of rain from November to May (30). Ascospores infect young growing shoots in early spring (10,24,30). Hyphae of the fungus invade the cambial layer of the plant, eventually forming a perennial canker 1 to 2 yr following initial infection (24,30). Perennial expansion of these cankers results in girdling of limbs and death of the tree within a few years (24,30).

Several control methods for eastern filbert blight have been investigated (5,9,18,19,23). Cultural methods, such as the removal of infected limbs, vary in the level of control provided (9,30). Chemical methods, such as fungicide applications, are effective but costly for growers because of the perennial nature of the disease (5,9). Based on 20 yr of observation, resistance to eastern filbert blight in hazelnut selections appears to be a promising strategy for long-term control of this disease (5,14,17,18,19,23).

There is concern that the introduced *A. anomala* population in Oregon and Washington may be not be similar to *A. anomala* populations from within the pathogen's indigenous range, and that this introduced population may be able to shift its virulence to overcome the resistance that will be deployed in new hazelnut cultivars (5,16,17,19). A single dominant gene for complete resistance to eastern filbert blight was identified in an old cultivar named Gasaway (18). This gene has since been bred into new, more commercially acceptable hazelnut selections (17). Development and deployment of a

commercially acceptable hazelnut cultivar with the 'Gasaway'-type resistance to eastern filbert blight is expected to require 20 to 30 yr (31). The breakdown of resistance in this new cultivar would represent a major setback for the hazelnut industry and for breeding efforts. Similar problems have been encountered in attempts to breed resistance to the obligate heteroecious rust *Cronartium quercuum* f. sp. *fusiforme* into loblolly pine (*Pinus taeda* L.)(8). Because loblolly pines are grown for 25 yr before harvest, the form of host resistance employed must be durable for an extended period of time. Previously, pine breeders found it necessary to characterize rust populations using pathogenicity surveys. Recently, Hamelin et al (8) found that genetic variability in *C. quercuum* f. sp. *fusiforme*, as measured by random amplified polymorphic DNA (RAPD)(35) markers, was highly correlated to pathogenic variability in this rust. Thus, populations of *C. quercuum* f. sp. *fusiforme* can be rapidly and reliably screened for pathogenic variation using the RAPD technique. The RAPD technique has proven valuable for identifying genetic diversity in many fungi (1,3,7,8,13,28,32,33). It has also been used to identify the origins of introduced pest populations (e.g. 34).

The purpose of this research was to examine pathogenic variation in *A. anomala* on two hazelnut clones with isolates from indigenous eastern North American populations of this pathogen and within the introduced Oregon/Washington population of *A. anomala*. In addition, the random amplified polymorphic DNA technique (35) was used to assess relative genetic variation within the same set of *A. anomala* isolates examined in the pathogenicity study.

Materials and Methods

Plant material. Two-yr-old trees of `Barcelona' and of 'VR6-28', a selection carrying a single dominant gene for resistance (19), were obtained from commercial nurseries in January 1994. 'Barcelona' tress had been propagated by layering whereas trees of 'VR6-28' had been grafted on roots of hazelnut seedlings at one year of age. Trees of both cultivars were planted in a mixture of peat, sand, and pumice (1:1:2, v:v:v) in 8 L pots and treated with a slow-release fertilizer (Nutricote 16-10-10, N:P:K). All trees were maintained in a temperature-controlled greenhouse (day temperature 18C, night temperature 13C) until November, 1994. At this time, the trees were removed to an enclosed, unheated greenhouse for a period of 4 mo to induce cold dormancy.

Fungal isolates. A total of 40 isolates of *Anisogramma anomala* were collected from seven geographically separated regions (Table 4.1). Individual cankers 15 to 30 cm long with mature stromata present were collected by pruning the branches bearing the cankers from living trees. Each canker was considered an isolate (20). The isolates were numbered, sealed in plastic bags, then placed in a cooler for shipping to the laboratory. Once in the laboratory, isolates were stored at -20C until needed. The isolates were collected by several individuals (Table 4.1).

Pathogenicity Study. Inoculum for this study was prepared as follows (10,19,23,30). For each isolate examined, perithecia were ground in a mortar containing sterile distilled water. Concentration of the ascospores in suspension was determined with a hemacytometer and adjusted to 4 x 10^5 spores/mL. This concentration matched the highest concentration available from one of the isolates examined, but was

Table 4.1. Isolates of *Anisogramma anomala* examined for pathogenicity and virulence on two European hazelnut clones.

Isolate	State	Location	Host
AAN1021	Illinois	Galesburgh	*Corylus* sp.
AAN1042	Illinois	Galesburgh	*Corylus* sp.
AAN1051	Illinois	Galesburgh	*Corylus* sp.
AAN1083	Illinois	Galesburgh	*Corylus* sp.
AAN1102	Illinois	Galesburgh	*Corylus* sp.
AAN2022	Michigan	East Lansing	*C. avellana* x *C. colurna*
AAN2041	Michigan	East Lansing	*Corylus* sp.
AAN3021	Minnesota	Canton	*C. americana* x *C. avellana*
AAN3083	Minnesota	Canton	*C. americana* x *C. avellana*
AAN3093	Minnesota	Canton	*C. americana* x *C. avellana*
AAN3101	Minnesota	Canton	*C. americana* x *C. avellana*
AAN3113	Minnesota	Canton	*C. americana* x *C. avellana*
AAN4011	New York	Amherst	*Corylus* sp.
AAN4021	New York	Amherst	*Corylus* sp.
AAN4031	New York	Amherst	*Corylus* sp.
AAN4041	New York	Amherst	*Corylus* sp.
AAN4051	New York	Amherst	*Corylus* sp.
AAN4091	New York	Amherst	*Corylus* sp.
AAN4121	New York	Amherst	*Corylus* sp.
AAN4201	New York	Amherst	*Corylus* sp.
AAN4251	New York	Amherst	*Corylus* sp.
AAN4271	New York	Amherst	*Corylus* sp.
AAN5061	Ontario	Niagara on the Lake	*Corylus* sp.
AAN5071	Ontario	Niagara on the Lake	*Corylus* sp.
AAN5151	Ontario	Niagara on the Lake	*Corylus* sp.
AAN5221	Ontario	Niagara on the Lake	*Corylus* sp.
AAN5241	Ontario	Niagara on the Lake	*Corylus* sp.
AAN6015D	Oregon	Damascus	*C. avellana*
AAN6025D	Oregon	Damascus	*C. avellana*
AAN6031D	Oregon	Damascus	*C. avellana*
AAN6063J	Oregon	Sandy	*C. avellana*
AAN6021V	Washington	Vancouver	*C. avellana*
AAN6033V	Washington	Vancouver	*C. avellana*
AAN6014Z	Oregon	Troutdale	*C. avellana*
AAN6021Z	Oregon	Troutdale	*C. avellana*

Table 4.1. Continued.

Isolate	State	Location	Host
AAN6033Z	Oregon	Troutdale	*C. avellana*
AAN6041Z	Oregon	Troutdale	*C. avellana*
AAN6052Z	Oregon	Troutdale	*C. avellana*
AAN7021	Virginia	Blackridge	*C. heterophylla*
AAN7031	Virginia	Blackridge	*C. heterophylla*

slightly lower than the concentration routinely used for inoculation studies (10,19,23). Ascospores were removed only from stromata that were apparently in good condition.

For each isolate, the new shoots on two trees of each cultivar were inoculated with a suspension of *A. anomala* ascospores. The shoots on a single branch of each tree were left uninoculated to serve as a control. Shoots were inoculated by brushing the spore suspension onto the tissues near the apical bud with a paintbrush (10). Plastic bags were placed over the inoculated shoots for a period of 5 days to provide conditions of high humidity (5,23). The new shoots were inoculated twice over a 2 wk period beginning in March 1994. Approximately three shoots per 'VR6-28' tree and 11 shoots per 'Barcelona' tree were inoculated. In November 1994, the trees were allowed to cycle through a natural period of cold dormancy which is required for development of cankers of eastern filbert blight (30). Data on disease development were collected in June 1995 after cankers had formed. The data collected included incidence of disease, number of cankers, and total canker length. Absence of infection in symptomless trees was confirmed by microscopic examination of hand sections taken from inoculated segments of stem tissues (5,10,19,23, 30).

Prior to statistical analysis, the percentage of inoculated shoots infected for each isolate was determined by dividing number of

cankers by number of shoots inoculated. The mean canker length for each isolate also was determined. The percentage of inoculated shoots infected and the mean canker length were considered measures of virulence for each isolate. Isolates that caused symptoms on 'Barcelona' were used to examine the relative virulence of *A. anomala* among regions. This was done by subjecting percent shoots infected and mean canker length to analysis of variance (SAS-PROC GLM)(27). Fischer's protected least significance difference (27) was used to compare disease responses as affected by geographic region.

Relative genetic variation. Because the number of ascospores and stromata available for some isolates was limited, only 34 of the 40 *A. anomala* isolates examined for pathogenic variation were examined for genetic variation using the RAPD technique (35). Perithecia and ascospores were removed from the stromata of individual isolates and DNA extracted from the ascospores of these isolates using a rapid, high yield mini-preparation method for isolation of total genomic DNA from fungi (12). The extraction procedure was halted after the first DNA precipitation. Total genomic DNA was extracted from each isolate at least twice and the concentration of DNA adjusted to 25 ng prior to amplification. The internal transcribed spacer region of the nuclear ribosomal DNA was amplified for each isolate as described previously (20); this was done to insure that the DNA extracted from each isolate was that of *A. anomala*. A product of the expected length (649 bp) was amplified from all 34 isolates except one, the Oregon/Washington isolate AAN6025D. A smaller product was amplified from this isolate indicating possible contamination by another organism. Thus, AAN6025D was excluded from any further analyses.

Amplification reactions were performed in volumes of 25 µL containing 50 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 10 µM

each of dATP, dCTP, dGTP, and dTTP (Promega), 0.05% glycerol (v:v) (Sigma), 100 nM primer, 25 ng DNA, and 1.0 units of *Tfl* polymerase (Epicentre). Amplification was performed in a Perkin Elmer-Cetus DNA Thermal Cycler programmed for two cycles of 30 s at 94C, 30 s at 36C, and 2 min at 72C, 36 cycles of 20 s at 94C, 15 s at 36C, 15 s at 45C, and 90 s at 72C, and a final extension of 10 min at 72C (7). Products were held at 4C at the conclusion of amplification. Products were analyzed by electrophoresis in 2% agarose (Gibco-BRL) gels, detected by staining with ethidium bromide (1 µg/mL), then photographed after visualization under UV light (7,35). Amplification reactions were repeated at least three times for each DNA extraction. A negative control consisting of the reaction mixture minus DNA was included with each amplification reaction.

A total of 70 decamer oligonucleotide primers were screened against *A. anomala* DNA. Of the 41 primers that successfully amplified *A. anomala* DNA, four amplified clear and consistent polymorphic products. Two of the four primers were obtained from the University of British Columbia Nucleic Acid - Protein Service Unit and two primers were obtained from Operon Technologies, Inc. The primers and their sequences are as follows: UBC759, 5′ CCCACCACAC; UBC771, 5′ CCCTCCTCCC; OPE06, 5′ AGGACCCCTC; and OPE07, 5′ AGATGCAGCC.

To evaluate the genetic similarity of these geographic isolates, the results were analyzed by creating a data matrix indicating the presence or absence of specific RAPD primer products for each isolate (26,28,32,34). Products of the same length were assumed to be identical. Only those products between 300 and 1500 bp in length and those products that appeared in all three amplification reactions were used to create the matrix. Genetic similarity scores were calculated using Jaccard's coefficient in the SimQual option of the program NTSYS-

pc v.1.8 (26,32,34). An unweighted-pair-group method with arithmetic means (UPGMA) dendrogram was generated from the similarity scores (26). The goodness of fit of the cluster analysis was determined (26).

Results

Pathogenicity study. Twenty-two of the 40 isolates examined caused symptoms on one of the two European hazelnut clones inoculated in this study (Tables 4.2 and 4.3). Twenty-one of the 22 isolates caused disease on 'Barcelona' (Table 4.2), while two produced lesions on 'VR6-28' (Table 4.3). The mean length of cankers observed on symptomatic 'Barcelona' trees was 19.0 cm. The lesions produced on 'VR6-28' (mean canker length = 6.0 cm) were significantly smaller than those produced on 'Barcelona' ($P < 0.01$).

On symptomatic 'Barcelona' trees, 11.0% of the inoculated shoots on these trees were infected by *A. anomala*. The percentage of infected shoots on diseased trees ranged from a low of 4.0% (isolates AAN6033Z and AAN3093) to a high of 20% (isolate AAN5151)(Table 4.2). Eight isolates produced cankers over 20.0 cm in length (ANN6025D, AAN1083, AAN1102, AAN3093, AAN4021, AAN4041, AAN4051, AAN4251)(Table 4.2). Four of these isolates were from New York, two were from Illinois, one was from Minnesota, and one was from Oregon/Washington. Isolates AAN1083 and AAN1102 from Illinois and isolate AAN4021 from New York produced multiple, large cankers. Twelve isolates produced medium-sized cankers (10.0 to 19.9 cm in length) and a single isolate, AAN6033Z, produced one, small canker (< 9.9 cm in length). Fungal stromata were present in all cankers produced on 'Barcelona'.

Two isolates produced sunken lesions on 'VR6-28'; AAN5221 from Ontario and AAN3093 from Minnesota. Ascospores of AAN5221 infected 43% of the shoots inoculated and produced multiple, small cankers (Table

Table 4.2. Disease responses collected from the European hazelnut cultivar Barcelona inoculated with geographic isolates of *Anisogramma anomala*.

Isolate	Geographic Origin	# Shoots Inf./# Shoots Inoc.	% Shoots Infected	Mean Canker Length (cm)
AAN6015D	Oregon	1/11	9	13.0
AAN6025D	Oregon	1/11	9	36.0
AAN6031D	Oregon	0/23	0	0.0
AAN6063J	Oregon	0/16	0	0.0
AAN6033V	Washington	0/34	0	0.0
AAN6021V	Washington	3/17	18	19.0
AAN6014Z	Oregon	0/43	0	0.0
AAN6021Z	Oregon	0/33	0	0.0
AAN6033Z	Oregon	1/25	4	4.0
AAN6041Z	Oregon	0/20	0	0.0
AAN6052Z	Oregon	0/14	0	0.0
AAN1021	Illinois	0/18	0	0.0
AAN1042	Illinois	1/9	11	11.0
AAN1051	Illinois	0/17	0	0.0
AAN1083	Illinois	2/24	8	35.0
AAN1102	Illinois	2/20	10	23.5
AAN2022	Michigan	3/19	16	17.6
AAN2041	Michigan	3/27	11	16.0
AAN3021	Minnesota	0/13	0	0.0
AAN3083	Minnesota	0/21	0	0.0
AAN3093	Minnesota	1/25	4	22.0
AAN3101	Minnesota	1/16	6	11.0
AAN3113	Minnesota	0/33	0	0.0
AAN4011	New York	2/27	7	16.0
AAN4021	New York	3/16	19	28.3
AAN4031	New York	0/40	0	0.0
AAN4041	New York	1/12	8	24.0
AAN4051	New York	1/17	6	31.0
AAN4091	New York	0/10	0	0.0
AAN4121	New York	0/14	0	0.0
AAN4201	New York	0/10	0	0.0
AAN4251	New York	1/9	11	20.0
AAN4271	New York	1/8	13	16.0
AAN5061	Ontario	2/11	18	15.0
AAN5071	Ontario	1/6	17	19.0
AAN5151	Ontario	2/10	20	11.0
AAN5221	Ontario	0/12	0	0.0
AAN5241	Ontario	0/8	0	0.0
AAN7021	Virginia	1/8	13	10.0
AAN7031	Virginia	0/9	0	0.0

Table 4.3. Disease responses collected from the European hazelnut selection VR6-28 inoculated with geographic isolates of *Anisogramma anomala*.

Isolate	Geographic Origin	# Shoots Inf./# Shoots Inoc.	% Shoots infected	Mean Canker Length (cm)
AAN6015D	Oregon	0/3	0	0.0
AAN6025D	Oregon	0/5	0	0.0
AAN6031D	Oregon	0/6	0	0.0
AAN6063J	Oregon	0/6	0	0.0
AAN6014Z	Oregon	0/5	0	0.0
AAN6021Z	Oregon	0/10	0	0.0
AAN6033Z	Oregon	0/7	0	0.0
AAN6041Z	Oregon	0/8	0	0.0
AAN6052Z	Oregon	0/6	0	0.0
AAN6033V	Washington	0/4	0	0.0
AAN6021V	Washington	0/5	0	0.0
AAN1021	Illinois	0/4	0	0.0
AAN1042	Illinois	0/4	0	0.0
AAN1051	Illinois	0/8	0	0.0
AAN1083	Illinois	0/8	0	0.0
AAN1102	Illinois	0/4	0	0.0
AAN2022	Michigan	0/5	0	0.0
AAN2041	Michigan	0/7	0	0.0
AAN3021	Minnesota	0/5	0	0.0
AAN3083	Minnesota	0/5	0	0.0
AAN3093	Minnesota	1/6	17	9.0
AAN3101	Minnesota	0/3	0	0.0
AAN3113	Minnesota	0/6	0	0.0
AAN4011	New York	0/5	0	0.0
AAN4021	New York	0/6	0	0.0
AAN4031	New York	0/5	0	0.0
AAN4041	New York	0/8	0	0.0
AAN4051	New York	0/4	0	0.0
AAN4091	New York	0/7	0	0.0
AAN4121	New York	0/6	0	0.0
AAN4201	New York	0/5	0	0.0
AAN4251	New York	0/9	0	0.0
AAN4271	New York	0/6	0	0.0
AAN5061	Ontario	0/4	0	0.0
AAN5071	Ontario	0/5	0	0.0
AAN5151	Ontario	0/5	0	0.0
AAN5221	Ontario	3/7	43	5.0
AAN5241	Ontario	0/7	0	0.0
AAN7021	Virginia	0/3	0	0.0
AAN7031	Virginia	0/7	0	0.0

4.3)(Figure 4.1). AAN3093 infected 17% of the inoculated 'VR6-28' shoots and produced a single, small lesion (Table 4.3). Isolate AAN3093 caused symptoms on 'Barcelona' (Table 4.2), whereas isolate AAN5221 did not.

Based on data collected from the 'Barcelona' inoculations, Ontario isolates infected the most shoots (18.3%) whereas Minnesota isolates infected the least (5.0%)(Table 4.4). Isolates from the

Table 4.4. Disease responses by region of geographic origin for the isolates of *Anisogramma anomala* pathogenic on the European hazelnut cultivar Barcelona.

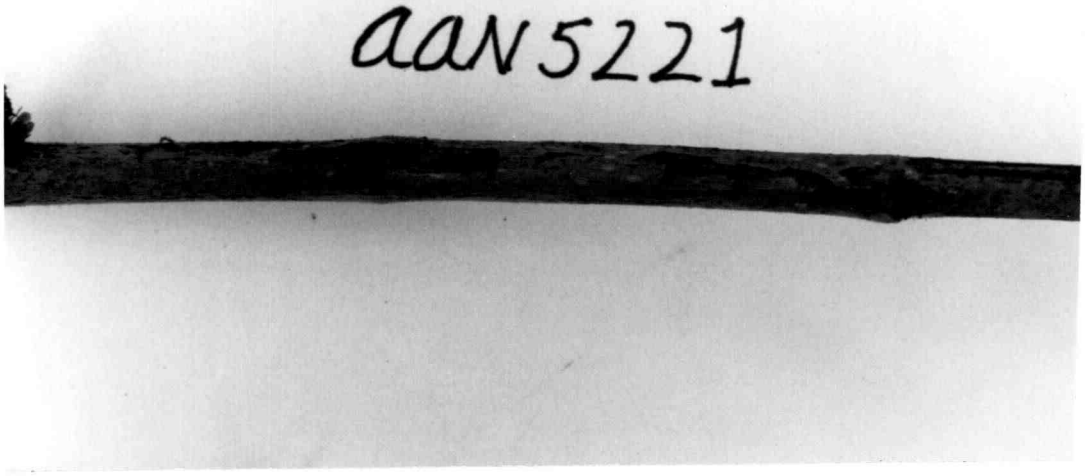
Geographic Origin	% Shoots Infected	Mean Canker Length
Illinois	9.6 bc[1]	23.2 a
Michigan	13.5 ab	16.8 a
Minnesota	5.0 c	16.5 a
New York	10.7 abc	22.6 a
Ontario	18.3 a	15.0 a
Oregon/Washington	10.0 abc	18.0 a
Virginia	13.0 abc	10.0 a

[1] Values with the same letter are not significantly different at the $P = 0.05$ level according to Fischer's protected least significant difference (27).

remaining regions were ranked as follows for percentage of shoots infected: Michigan, 13.5%; Virginia, 13.0%; New York, 10.7%; Oregon/Washington, 10.0%; and Illinois, 9.7%. Ontario differed significantly from Illinois and Minnesota ($P < 0.05$) for percentage of shoots infected. Illinois and New York isolates produced the longest cankers (mean canker length equaled 23.2 cm and 22.6 cm, respectively). However, these cankers were not significantly longer than those produced by isolates from other regions (Table 4.4).

Relative genetic variation. The DNA of 33 isolates was amplified using four RAPD decamer primers. A total of 23 RAPD primer products between 300 and 2000 bp in length were used to produce the data matrix; 22 of

Figure 4.1. A sunken lesion formed by an isolate of *Anisogramma anomala*, AAN5221, on the European hazelnut selection VR6-28. Selection VR6-28 carries a single dominant gene for complete resistance to this pathogen (20).



these products were polymorphic (e.g. Figure 4.2)(Table 4.5). Cluster analysis of the data matrix resulted in the UPGMA dendrogram shown in Figure 4.3. The goodness of fit for this cluster analysis was 0.87 on a 0.00 to 1.00 scale (1.00 = perfect correlation).

Clustering of isolates by region was evident. Isolates from New York, Oregon/Washington, and Ontario tended to cluster together, although some individual isolates within these regions were dissimilar (e.g. New York isolate AAN4031)(Figure 4.3). The isolates from Oregon/Washington formed a small, discrete cluster with the highest similarity between isolates (> 88% similar). Isolates from Illinois, Michigan, Minnesota, and Virginia did not form discrete clusters (Figure 4.3). Similarity between isolates within these regions was relatively low (e.g. > 48% similarity between Minnesota isolates). Some clustering of isolates between regions was evident (e.g. > 90% similar between Illinois isolates AAN1042 and AAN1102 and Minnesota isolate AAN3093). Isolates from the Oregon/Washington population were most similar to a single isolate from Ontario (AAN5241, > 80% similar) and several isolates from New York (> 76% similar).

Discussion

Isolates of *A. anomala* from all seven geographic regions that were sampled are capable of infecting Barcelona, the principal European hazelnut cultivar grown commercially in Oregon and Washington (5,15). According to two measures of disease, percentage of shoots infected and mean canker length, the relative virulence observed in *A. anomala* isolates from Oregon/Washington was similar to that observed in isolates from all other geographic regions examined (Table 4.4). The only isolates that differed significantly ($P < 0.05$) were those from

Figure 4.2. Agarose gel (ethidium bromide stain) electrophoresis showing randomly amplified polymorphic DNA products from six geographic isolates of *Anisogramma anomala* using the decamer primer UBC771. Isolates shown include: lane 1, AAN4041; lane 2, AAN4051; lane 4, AAN3083; lane 5, AAN3101; lane 6, AAN3093; lane 8, AAN3113. Lane 3 shows a 1 kb DNA size marker (Gibco-BRL) and lane 7 the negative control. Products used to create the data matrix are indicated (–).

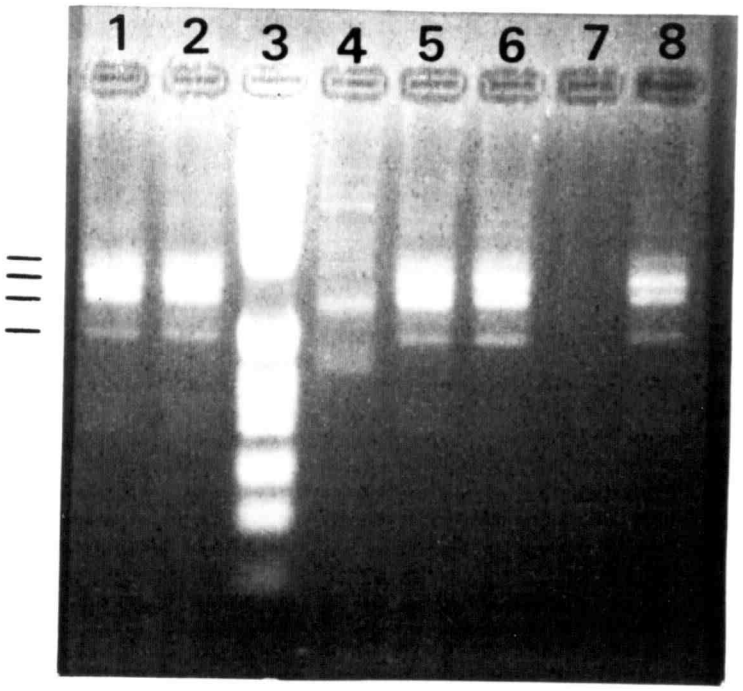


Table 4.5. Data matrix for 33 isolates of *Anisogramma anomala* examined using the RAPD technique (35).

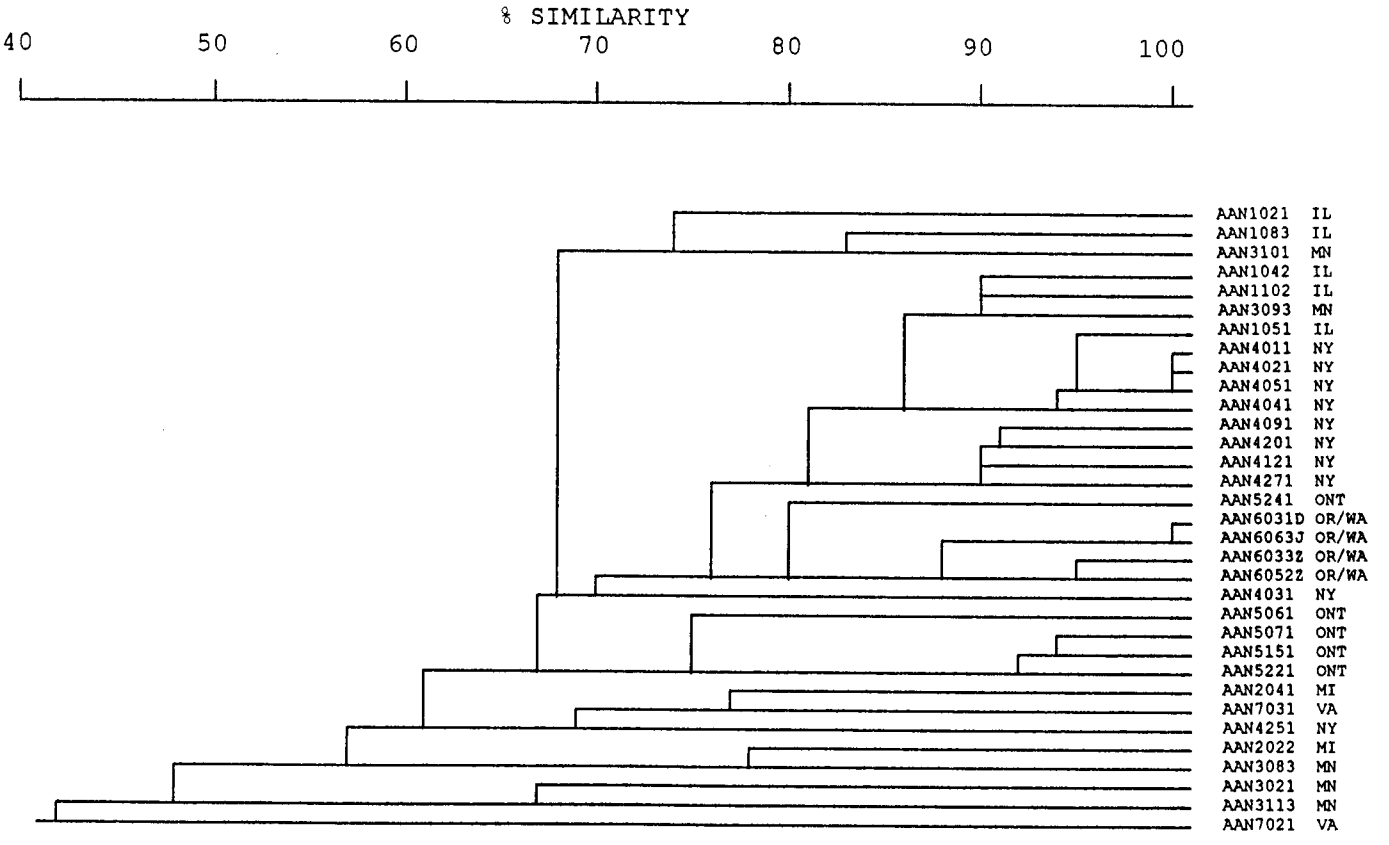
		Primer																							
		OPE06						OPE07								UBC759					UBC771			
		350[1]	400	650	850	900	1500	350	400	500	600	650	700	850	1000	425	475	550	550	800	500	750	800	900
Isolate	Origin																								
AAN1021	IL	1[2]		1			1					1		1		1	1	1	1	1	1	1	1	1
AAN1042	IL	1		1	1	1	1		1	1	1		1	1		1	1	1	1	1	1	1	1	1
AAN1051	IL	1	1	1	1	1	1		1	1	1	1	1	1		1	1	1	1	1	1	1	1	1
AAN1083	IL	1		1	1	1	1					1	1	1		1	1	1	1		1	1	1	1
AAN1102	IL	1		1	1	1	1		1	1	1	1	1	1		1	1	1	1		1	1	1	1
AAN2022	MI	1	1		1			1	1			1	1	1	1		1		1	1		1	1	
AAN2041	MI	1	1		1	1	1	1		1			1						1	1	1	1	1	1
AAN3021	MN		1			1						1	1		1			1	1	1	1	1	1	1
AAN3083	MN			1		1	1			1	1		1		1	1	1	1	1	1		1		1
AAN3093	MN	1		1	1	1	1			1	1	1	1	1		1	1	1	1	1	1	1	1	1
AAN3101	MN	1		1	1	1	1			1		1	1	1		1	1	1		1	1	1	1	1
AAN3113	MN		1			1							1	1	1	1	1	1	1		1	1	1	1
AAN4011	NY	1	1	1	1	1	1		1	1	1	1	1	1			1	1	1	1	1	1	1	1
AAN4021	NY	1	1	1	1	1	1		1	1	1	1	1	1			1	1	1	1	1	1	1	1
AAN4031	NY	1		1			1	1	1	1	1	1	1		1		1	1	1	1	1	1	1	1
AAN4041	NY	1	1		1	1	1		1	1	1	1	1	1			1	1	1	1	1	1	1	1
AAN4051	NY	1	1	1	1	1	1		1	1	1	1	1	1			1	1	1	1	1	1	1	1
AAN4091	NY	1	1	1	1	1	1		1	1	1		1	1	1		1	1	1	1	1	1	1	1
AAN4121	NY	1	1	1	1	1	1		1	1	1		1	1			1	1	1	1		1	1	1
AAN4201	NY	1	1	1	1	1	1	1	1	1	1		1	1	1		1	1	1	1		1	1	1
AAN4251	NY	1	1		1	1	1	1	1	1	1			1	1			1	1	1	1	1	1	1
AAN4271	NY	1	1	1	1	1	1		1	1	1		1	1	1			1	1	1		1	1	1
AAN5061	ONT	1	1	1	1		1		1	1						1	1	1		1		1	1	
AAN5071	ONT	1	1	1	1	1	1		1	1				1		1	1	1		1	1	1	1	1
AAN5151	ONT	1	1	1	1	1	1		1	1				1	1	1	1	1		1	1	1	1	1
AAN5221	ONT	1	1	1	1	1	1		1	1				1	1	1	1	1		1	1	1	1	
AAN5241	ONT	1	1	1	1		1		1	1		1	1	1	1	1	1	1		1		1	1	1
AAN6031D	OR	1		1	1	1	1		1	1		1	1	1	1	1	1	1	1	1	1	1	1	1

Table 4.5. Continued.

		Primer																						
		OPE06						OPE07								UBC759					UBC771			
		3 5 0	4 0 0	6 5 0	8 5 0	9 0 0	1 5 0 0	3 5 0	4 0 0	5 0 0	6 0 0	6 5 0	7 0 0	8 5 0	1 0 0 0	4 2 5	4 7 5	5 5 0	6 0 0	8 0 0	5 0 0	7 5 0	8 0 0	9 0 0
Isolate	**Origin**																							
AAN6063J	OR	1		1	1	1	1		1	1		1	1	1	1	1	1	1	1	1	1	1	1	1
AAN6033Z	OR	1		1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1		1	1	1
AAN6052Z	OR	1		1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1		1	1	
AAN7021	VA	1	1			1		1		1							1	1	1			1	1	
AAN7031	VA	1	1		1	1	1	1		1		1	1				1	1	1		1	1	1	1

[1] Approximate size in bp of the RAPD products amplified by this primer.
[2] A one (1) indicates the RAPD product was present.

Figure 4.3. A dendogram constructed from RAPD marker data indicating the relationships among *Anisogramma anomala* isolates. A similarity matrix was calculated using the Jaccard coefficient and an UPGMA dendogram generated from the similarity matrix (26). Origin of isolates by region is indicated.



Ontario compared to those from Illinois and Minnesota; the Ontario isolates successfully infected more shoots than those from Illinois and Minnesota (Table 4.4). Thus, based on 'Barcelona', *A. anomala* isolates from eastern North America do not appear to represent a significantly greater threat to hazelnut production in Oregon and Washington than the isolates that are already present in this region.

The ability of two isolates of *A. anomala*, one from Ontario and one from Minnesota, to infect VR6-28, a selection carrying a single dominant gene for resistance, may be cause for greater concern. Only extremely rarely have cankers been observed on trees of this selection grown in the field (19) and in each case, the canker was apparent for only one season. Similar observations were made on an *A. anomala*-inoculated tree of VR24-16, a selection that carries the same single dominant gene for complete resistance as 'VR6-28' (S. A. Mehlenbacher, pers. comm.). Thus, the lesions we observed on 'VR6-28' may not indicate an actual breakdown of the single dominant resistance gene. Pinkerton et al (24) reported that eastern filbert blight cankers grew from a diseased scion wood into the mainstem of Gasaway, the cultivar in which this resistance gene was first observed (18). In that same report, Pinkerton et al stated that even susceptible cultivars frequently produce a hypersensitive reaction in response to infection by *A. anomala* (24). For cankers that formed on 'VR6-28' in this study, their size was limited compared to canker size on susceptible 'Barcelona'. In addition, cankers did not continue to expand from the spring of 1995 to the spring of 1996, indicating that further hyphal growth was stopped by host defense responses. These results suggest temporal factors may influence host defense responses controlled by this single dominant gene. Therefore, our results do not allow us to

draw a strong conclusion on the significance of lesions or cankers on 'VR6-28' to the durability of this single dominant resistance gene.

Examination of the isolates using the RAPD technique indicated some intraregional dissimilarity exists between isolates of *A. anomala*. This was indicated by the lack of clustering of isolates from within four of the regions sampled, Illinois, Michigan, Minnesota, and Virginia (Figure 4.3). Isolates from the remaining regions, Ontario, New York, and Oregon/Washington, formed fairly discrete clusters (Figure 4.3). High levels of interregional similarity among some isolates were observed (e.g. AAN1042, AAN1102, and AAN3093, all 90% similar). Such variation has been observed in other large, relatively homogeneous pathogen populations that experience occasional influxes of new genetic material (3,13,32). For example, *C. quercumm* f. sp. *fusiforme*, the cause of oak-pine rust, is a highly heterogeneous pathogen for which isolates cannot be differentiated based on geographic origin (8). The isolates of *A. anomala* from eastern North America analyzed in this study were collected by members of the Northern Nut Growers Association. Because the exchange of plant material between Association members is common (N. K. Osterbauer, pers. obs. and K. B. Johnson and S. A. Mehlenbacher, pers. comm.), the high levels of interregional similarity observed between some isolates may be due to the exchange of infected plant material.

The Oregon/Washington population of *A. anomla* was apparently introduced in 1968 (2,6) and there is no evidence of additional introductions. A secondary goal of this research was to identify possible origins of the Oregon/Washington population. Plants that have been exposed to a pathogen population for an extended period of time often develop resistance genes unique to that pathogen population (25). Identifying possible origins of the introduced population would

identify regions from which wild hazelnut germplasm should be collected. Unfortunately, the results of this study were inconclusive. Based on virulence on cultivar Barcelona, isolates from Oregon/Washington were statistically similar to isolates from all other regions (Table 4.4), yet the results of the RAPD analysis indicated the isolates from Oregon/Washington were most similar to isolates from Ontario and New York (> 76% similarity). A more extensive sampling of isolates from isolated wild American hazelnut populations located throughout the indigenous range of this pathogen may be necessary to better understand regional specialization by *A. anomala*. Also, additional RAPD primers that produce consistent, clear polymorphisms like the four used in this study may be found. The use of additional RAPD primers may further clarify similarities among isolates and help identify the origins of the introduced population of *A. anomala* in Oregon and Washington.

The number of stromata within a canker (isolate) was limited for some isolates (e.g. AAN7021, from Virginia), therefore, the concentration of the ascospore suspension used to inoculate trees was restricted to the highest concentration available for all isolates examined. This concentration, 4×10^5 spores/mL, was slightly lower than the concentration normally used in greenhouse inoculation studies (10,19,23,30). Previous studies have shown that the concentration and condition of *A. anomala* ascospores affects their ability to germinate and infect *C. avellana* (10,24). Care was taken to use ascospores only from those stromata that appeared in good condition; however, some of the variability in inoculation success observed among isolates (Table 4.4) may have been caused by unknown quality differences as well as by the relatively low inoculum dose.

The results of this study indicate that some genetic variation exists in indigenous (eastern North American) populations of *A. anomala*, but this variation was not apparent in our evaluation of virulence of isolates from these populations on 'Barcelona'. This study does raise concerns about the durability of the single dominant resistance gene in 'VR6-28'. Virulence shifts leading to the breakdown of resistance genes have been observed in several other host-parasite pathosystems, including those of obligately biotrophic parasites (e.g. 8,11,13,28). The ability of two isolates from eastern North America to infect selection VR6-28 suggests *A. anomala* may be capable of such a virulence shift. For this reason, present quarantine laws prohibiting the importation of plant material from east of the Rocky Mountains should be maintained and additional studies on the virulence of indigenous *A. anomala* populations on 'VR6-28' should be performed.

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CHAPTER FIVE
SUMMARY

The perennial nature of the host, European hazelnut, and parasite, *Anisogramma anomala*, in the eastern filbert blight pathosystem makes eastern filbert blight a difficult disease to control culturally or chemically. Growing resistant cultivars is considered the most durable strategy for controlling this disease. As a species, European hazelnut is considered susceptible to eastern filbert blight, however, a wide range of disease responses have been observed in European hazelnut clones. High levels of partial resistance have been observed in some European hazelnut cultivars and a single dominant gene for complete resistance was found in the commercially unacceptable pollenizer 'Gasaway'. In this study, the heritability of partial disease resistance in six European hazelnut cultivars was determined and the presence of the 'Gasaway' resistance gene in 'VR6-28' confirmed. The progeny from crosses made between all seven cultivars, the pollen parents Tonda Gentile delle Langhe, Tonda di Giffoni, Gem, and VR6-28, and the seed parents Ennis, Casina, and Willamette, were exposed to *A. anomala* ascospores as potted trees in the field under conditions designed to simulate natural inoculation. Trees of the parents also were included. The disease responses incidence of disease, number of cankers, and proportion of wood diseased, were collected and mean disease responses calculated. Disease incidence in the progeny of crosses made between 'VR6-28' and the three susceptible seed cultivars was subjected to χ^2 analysis to determine if the progeny segregated 1:1 for complete resistance to susceptibility. The offspring of 'VR6-28' did not deviate significantly ($P > 0.05$) from

this 1:1 ratio, indicating that the 'Gasaway' resistance gene was inherited in a single, dominant fashion from 'VR6-28'. Histograms showing the frequency distribution of disease responses in the progeny of the remaining six parents showed continuous distributions for all crosses examined. Disease responses in the progeny of these parents were subjected to combining ability analysis. Analysis of variance of combining ability values showed that both general and specific combining ability were significant in the field ($P < 0.05$), with general combining ability having twice the magnitude of specific combining ability. This suggests that the partial disease resistance in these six European hazelnut cultivars is controlled quantitatively. Based on general combining ability values, high levels of partial resistance were transmitted by the seed parent 'Willamette' and the pollen parents 'Gem' and 'Tonda di Giffoni'. Fewer and smaller cankers were observed on the progeny of these cultivars. The mean disease responses for each cross were regressed on the midparent disease responses. The regression coefficients were considered the estimates of heritability for the disease responses. Heritabilities of disease incidence, number of cankers per tree, and proportion of wood diseased per tree were estimated to be 0.21, 0.39, and 0.47, respectively, suggesting it will be possible to use partially resistant parents to breed for hazelnuts with fewer and smaller cankers.

Anisogramma anomala (Order Diaporthales, Family Gnomoniaceae), a fungus with a reportedly homothallic mating system, was introduced relatively recently into Oregon and Washington. Because of this, the Oregon and Washington population of *A. anomala* is believed to be genetically homogeneous. To evaluate the validity of this hypothesis, 67 isolates of *A. anomala* were collected from five geographically

separated infection centers and DNA extracted from ascospores of these isolates. Variation in the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA of these isolates was examined using the polymerase chain reaction (PCR) and restriction enzyme digestion. An approximately 649 bp PCR product corresponding to the entire ITS region was amplified from 66 of the isolates examined. The DNA of one isolate was apparently contaminated, which resulted in amplification of a smaller PCR product. Transversion mutations within the ITS regions of these isolates resulted in polymorphic restriction sites for three enzymes, *Csp*6I, *Hha*I, and *Hae*III. In addition, an *Eco*RV site was discovered in the ITS I region; this is considered an unusual restriction site for a fungus. The ITS I regions of 17 isolates, two to four from each infection center, were sequenced and the sequences subjected to analysis. Sequence divergences between isolates both within (~2%) and among (~2%) infection centers were low, suggesting the Oregon and Washington population of *A. anomala* is relatively homogeneous. The sequences of the ITS I regions of seven *A. anomala* isolates were compared to those of 22 other Ascomycetes. Results of a phylogenetic analysis suggest *A. anomala* is most closely related to *Phomopsis* spp., *Cryphonectria* spp., and *Endothia gyrosa*, all diaporthaceous pathogens of woody plants, although the ITS I sequences of these organisms were relatively divergent.

The durability of the 'Gasaway' gene for complete resistance to eastern filbert blight is of major concern to hazelnut breeders. There is concern that *A. anomala* may be able to shift its virulence to overcome this resistance gene. Breakdown of this gene would represent a major setback for breeders as the 'Gasaway' gene is being bred into new hazelnut selections. To address this concern, pathogenic and

genetic variation in *A. anomala* was evaluated. Forty isolates of *A. anomala* collected from seven geographical regions, Illinois, Michigan, Minnesota, New York, Ontario, Oregon/Washington, and Virginia, were examined for their ability to infect two European hazelnut clones: VR6-28, a selection carrying the 'Gasaway' gene, and 'Barcelona', a moderately susceptible cultivar. New shoots on trees of each clone were inoculated with a 4 x 10^5 spore suspension of *A. anomala* ascospores and the disease responses subjected to analysis of variance and Fischer's Protected least significant difference. Two isolates, one from Ontario and one from Minnesota, caused small sunken lesions on 'VR6-28', raising concerns about the durability of the 'Gasaway' gene. These lesions apparently stopped growing after one season, however. Isolates from all seven regions were capable of infecting 'Barcelona', although there were no significant ($P > 0.05$) differences in isolate virulence among regions. Genetic variation in 33 of the 40 geographic isolates was determined using the randomly amplified polymorphic DNA (RAPD) technique. Cluster analysis of the RAPD data revealed that isolates from New York, Ontario, and Oregon/Washington tended to cluster together whereas isolates from the remaining regions did not. This indicates at least some regional adaptation exists within three of the indigenous populations examined, since little dissimilarity was observed in two of the indigenous populations and in the introduced Oregon/Washington population (>88% similar). This high level of similarity in the Oregon/Washington population of *A. anomala* suggests this population is relatively homogeneous. Although the results of the RAPD analysis indicated some genetic variation exists within the populations of *A. anomala* sampled from different regions, this

variation was not apparent in the evaluation of virulence on 'Barcelona'. The ability of two geographic isolates to infect 'VR6-28', which carries the 'Gasaway' resistance gene, may be cause for greater concern. This, coupled with the genetic variation observed in the indigenous populations of *A. anomala*, suggests the quarantine law preventing the importation of European hazelnut and American hazelnut plant material from east of the Rocky Mountains should be maintained.

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APPENDICES

APPENDIX A
ISOLATES OF *ANISOGRAMMA ANOMALA*

Table A.1. A listing of all isolates of *Anisogramma anomala* collected from Oregon, Washington, and the eastern coast of North America.

Isolate Number	State or Province	Location	Host	Collector
AAN1011	Illinois	Galesburgh	*Corylus americana* x *C. avellana*	V. Luvall
AAN1021	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1031	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1032	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1033	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1041	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1042	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1051	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1061	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
ANN1062	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
ANN1063	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1071	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1072	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1081	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1082	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1083	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1091	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1101	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1102	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1111	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1121	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1131	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1132	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1133	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1141	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1142	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1143	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN1144	Illinois	Galesburgh	*C. americana* x *C. avellana*	V. Luvall
AAN2011	Michigan	East Lansing	*C. avellana*	C. Farris & S. Grinnell

Table A.1. Continued.

Isolate Number	State or Province	Location	Host	Collector
AAN2012	Michigan	East Lansing	*C. avellana*	C. Farris & S. Grinnell
AAN2021	Michigan	East Lansing	*C. avellana* x *C. colurna*	C. Farris & S. Grinnell
AAN2022	Michigan	East Lansing	*C. avellana* x *C. colurna*	C. Farris & S. Grinnell
AAN2031	Michigan	East Lansing	*C. avellana*	C. Farris & S. Grinnell
AAN2041	Michigan	East Lansing	*Corylus* sp.	C. Farris & S. Grinnell
AAN2042	Michigan	East Lansing	*Corylus* sp.	C. Farris & S. Grinnell
AAN2043	Michigan	East Lansing	*Corylus* sp.	C. Farris & S. Grinnell
AAN2044	Michigan	East Lansing	*Corylus* sp.	C. Farris & S. Grinnell
AAN2051	Michigan	East Lansing	*C. avellana* x *C. colurna*	C. Farris & S. Grinnell
AAN2052	Michigan	East Lansing	*C. avellana* x *C. colurna*	C. Farris & S. Grinnell
AAN2053	Michigan	East Lansing	*C. avellana* x *C. colurna*	C. Farris & S. Grinnell
AAN2054	Michigan	East Lansing	*C. avellana* x *C. colurna*	C. Farris & S. Grinnell
AAN2055	Michigan	East Lansing	*C. avellana* x *C. colurna*	C. Farris & S. Grinnell
AAN2061	Michigan	East Lansing	*C. colurna*	C. Farris & S. Grinnell
AAN2071	Michigan	East Lansing	*C. colurna*	C. Farris & S. Grinnell
AAN2072	Michigan	East Lansing	*C. colurna*	C. Farris & S. Grinnell
AAN3011	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3012	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3013	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3021	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3022	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3023	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3024	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3025	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3031	Minnesota	Canton	*C. americana*	N. Osterbauer
AAN3041	Minnesota	Canton	*C. americana*	N. Osterbauer
AAN3051	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3052	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3053	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3061	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3062	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3063	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer

Table A.1. Continued.

Isolate	State	Location	Host	Collector
AAN3071	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3072	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3081	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3082	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3083	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3084	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3085	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3091	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3092	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3093	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3101	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3102	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3111	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3112	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3113	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3114	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN3115	Minnesota	Canton	*C. americana* x *C. avellana*	N. Osterbauer
AAN4011	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4021	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4031	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4041	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4051	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4061	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4071	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4072	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4081	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4082	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4091	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4101	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4102	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4111	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4121	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4131	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4141	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4151	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4161	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4171	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4181	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4191	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4201	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4211	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4221	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4231	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4241	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4251	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4261	New York	Amherst	*Corylus* sp.	J. Gordon

Table A.1. Continued.

Isolate	State	Location	Host	Collector
AAN4271	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4281	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4291	New York	Amherst	*Corylus* sp.	J. Gordon
AAN4301	New York	Amherst	*Corylus* sp.	J. Gordon
AAN5011	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5021	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5031	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5041	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5051	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5061	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5071	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5081	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5091	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5101	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5111	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5121	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5131	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5141	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5151	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5161	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5171	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5181	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5191	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5201	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5211	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5221	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5231	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5241	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5251	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5261	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5271	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5281	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5291	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell
AAN5301	Ontario	Niagara on the Lake	*Corylus* sp.	R. Campbell

Table A.1. Continuted.

Isolate	State	Location	Host	Collector
AAN6011B	Oregon	Bald Peak	*C. avellana*	N. Osterbauer & K. Johnson
AAN6021B	Oregon	Bald Peak	*C. avellana*	N. Osterbauer & K. Johnson
AAN6031B	Oregon	Bald Peak	*C. avellana*	N. Osterbauer & K. Johnson
AAN6011D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6012D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6013D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6014D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6015D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6021D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6022D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6023D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6024D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6025D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6031D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6032D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6033D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6034D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6035D	Oregon	Damascus	*C. avellana*	N. Osterbauer
AAN6011J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6012J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6021J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6022J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6031J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6041J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6042J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6043J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6044J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6051J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6052J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6053J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6054J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6061J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6062J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6063J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6064J	Oregon	Sandy	*C. avellana*	N. Osterbauer & K. Johnson
AAN6011Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6012Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6013Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6014Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson

Table A.1. Continued.

Isolate Number	State	Location	Host	Collector
AAN6021Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6022Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6023Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6024Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6031Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6032Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6033Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6034Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6041Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6042Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6043Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6044Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6051Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6052Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6053Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6054Z	Oregon	Troutdale	*C. avellana*	N. Osterbauer & K. Johnson
AAN6011V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6012V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6013V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6021V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6022V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6023V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6031V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6032V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6033V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6041V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6042V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN6043V	Washington	Vancouver	*C. avellana*	N. Osterbauer & K. Johnson
AAN7011	Virginia	Blackridge	*C. heterophylla*	M. Katszarab
AAN7021	Virginia	Blackridge	*C. heterophylla*	M. Katszarab
AAN7031	Virginia	Blackridge	*C. heterophylla*	M. Katszarab
AAN7041	Virginia	Blackridge	*C. heterophylla*	M. Katszarab
AAN7051	Virginia	Blackridge	*C. heterophylla*	M. Katszarab

APPENDIX B
HISTOGRAMS OF DISEASE RESPONSES

Figure B.1. Histograms showing the frequency distribution for number of cankers observed on the progeny of 12 hazelnut crosses after 1 yr of exposure to eastern filbert blight in the field.

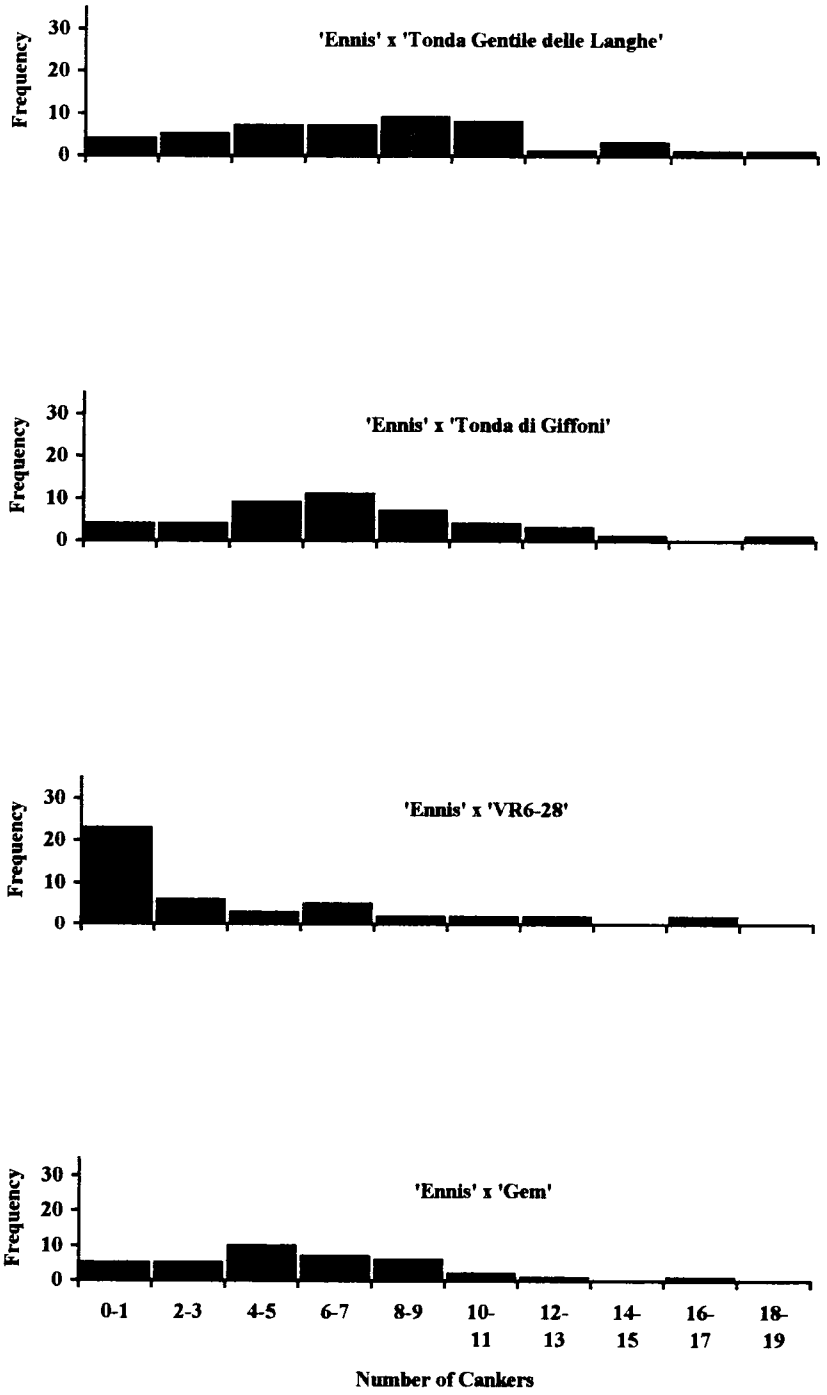


Figure B.1. Continued.

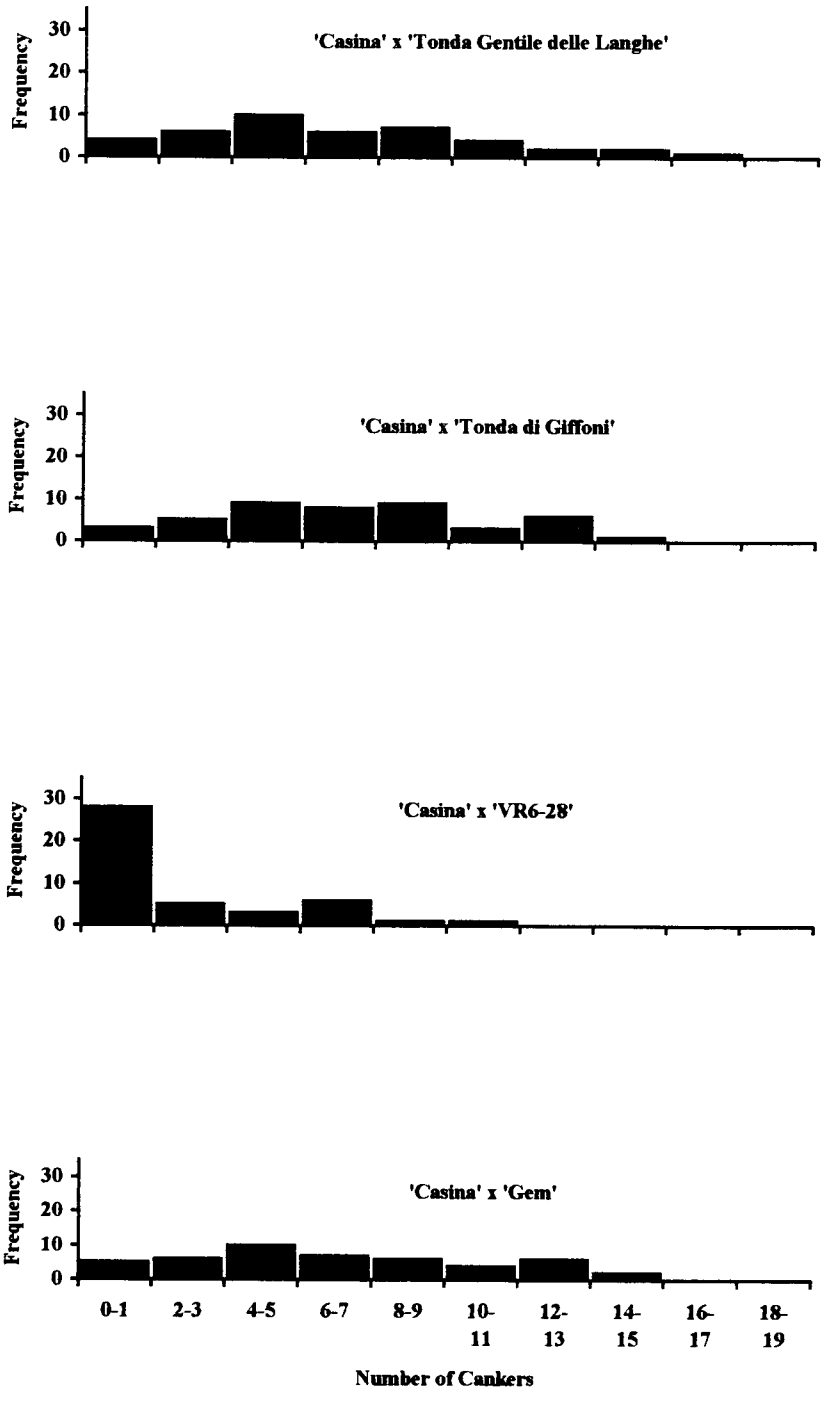
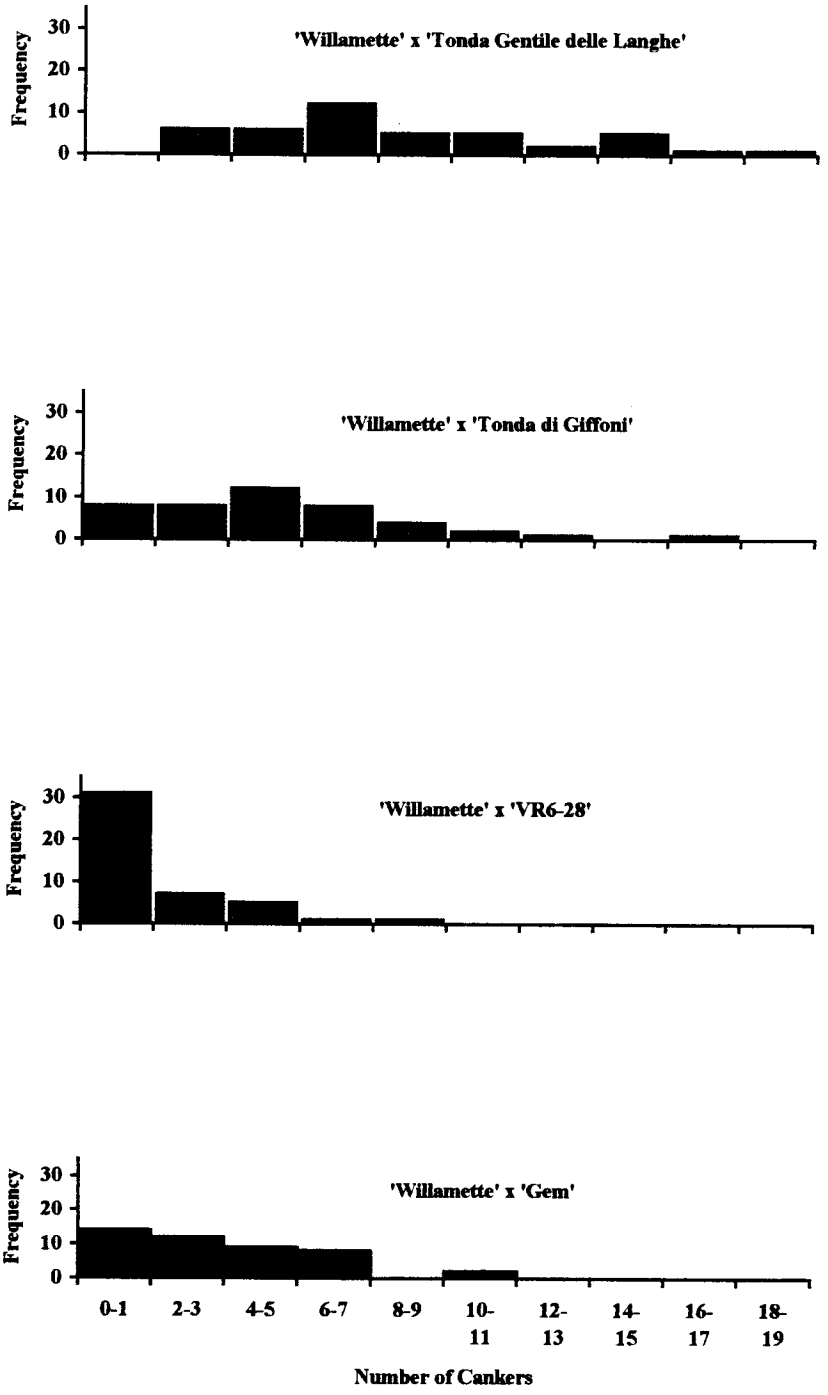


Figure B.1. Continued.



APPENDIX C
ITS I SEQUENCE ALIGNMENTS

Figure C.1. Alignment of the ITS I sequences of 29 Ascomycetes using a gap weight of 1.0 and gap length weight of 0.2.

```
                                                    1                                        40
Anisogramma anomala AAN6031D (1)                    NTGGAGCAAA CGCCCCCCCC TTCCCCGGGG GGGGGGCTAC
A. anomala AAN6061J (2)                             CTGGAGCNAA CGCCCCCCCC TTCCCCGGGG GGGGNGCTAC
A. anomala AAN6023D (3)                             ATGGAGCAAA CGCCCCCCCC TTCCCCGGGG GGGGCGCTAC
A. anomala AAN6021V (4)                             NTGGAGCAAA CGCCCCCCCC TTCCCCGGGG GGGGNGCTAC
A. anomala AAN6011B (5)                             NTGGAGTAAA CGNCCTCNCC TTCNCCGGGG GGGGGGCTAC
A. anomala AAN6014Z (6)                             TTNGAGCAAA CGCNCCCCCC TTCCCCGGCG GGGGGGGTAC
A. anomala AAN6052Z (7)                             CTGGAGCAAA CGCCCCCCCC TTCCCCGGGG GGGGCGCTAC
Phomopsis sp. (8)                                   .........C TGGAAC...G CGCCCCAGCG ......AGGC
Phomopsis sp. (9)                                   .......... ..GGAC...G .GCCCCAGCG ......A.GC
Phomopsis sp. (10)                                  .........C TGGAAC...G CGCTTCGGCG .......CAC
Cryphonectria parasitica (11)                       .........C TGGAACTGTG .GCCCTCACG GGCGC.AACC
Cr. radicalis (12)                                  .........C TGGAACTGTG .GCCCTCACG GGCGC.AACC
Cr. cubensis (13)                                   .........C TGGAACTGCG CCCCCCCACG GGCGCAAACC
Endothia gyrosa (14)                                .........C TGGAACTGTG ..CCCTCGCG GGCAC.AACC
Cr. havanensis (15)                                 .........C TGGAATTTGT TGCCCTCACG GGCAC.AACC
Fusarium oxysporum (16)                             .......... .......... .....CCGAG TTTACAA.CT
F. sambucinum (17)                                  .......... .......... .....CCGAG TTTACAA.CT
Cylindrocarpon heteronema (18)                      .......... .......... .....CCGAG TTTACAA.CT
Nectria haematococca (19)                           .......... .......... .....CCGAG TTTTCAA.CT
Trichoderma harzianum (20)                          .......... .......... .....CCGAG TTTACAA.CT
Gaeumannomyces graminis var. tritici (21)           .......... .......... .....CAGAG TTGAAAAACT
Phialophora sp. (22)                                .......... .......... .....CAGAG TTGAAAAACT
G. graminis var. avenae (23)                        .......... .......... .....CAGAG TTGAAAAACT
G. graminis var. graminis (24)                      .......... .......... .....CAGAG TTGAAAAACT
G. incrustans (25)                                  .......... .......... .....AAGAG TTGCAAAACT
G. cylindrosporus (26)                              .......... .......... .....CAGAG TTGAAAAACT
Magnaporthe grisea (27)                             .......... .......... .....CTGAG TTGAAAAACT
Colletotrichum destructivum (28)                    .......... .......... .....CTGAG TTACCGCTCT
Talaromyces flavus (29)                             .......... .......... .....CCGAG TGCGGGCCCT

          41                                                                          110
1         C.CAGA.AAC CCTTTGTGAA .TCTTCTC.C GT........ .TGNCTCGGC .......CTG GTTGC.....
2         C.CAGA.AAC CCTTTGTGAA .TCTTCTC.C GT........ .TGCCTCGGC .......CTG GTTGC.....
3         C.CAGA.AAC CCTTTGTGAA .TCTTCTC.C GT........ .TGCCTCGGC .......CTG GTTGC.....
4         C.CAGA.NTC CCTTTGTGAA .TCTTCTC.C GT........ .TGNCTCGGC .......CTG GTTGN.....
5         C.CAGA.NAC CCTTTGTGAA .TCTTCTC.C GT........ .TGNCTCGGN .......CTG GTTGC.....
6         C.CAGA.NAC CCTTTGTGAA .TCTTCTC.N GC........ .TGCCTCGGC .......CTG GTTGC.....
7         C.CAGA.AAC CCTTTGTGAN .TCTTCTC.C GTC....... .TGGCTCGGC .......CTG GTTGC.....
8         C.CAGA.AAC CCTTTGTGAA CTTAT.AC.C TT....ACTG TTGCCTCGGC GCTA..C... ...GTGG...
9         C.CAGA.AAC CCTTTGTGAA CTTAT.AC.C TT....ACTG TTGCCTCGGC GCTA..C... ...GTGG...
10        C.CAGA.AAC CCTTTGTGAA CTTAT.AC.C .T....ATTG TTGCCTCGGC G.TAGGC... ...GCGC...
11        C.CAGATA.C CCTTTGTGAA CTTATAAC.C ATTTT.ATCG TTGCCTCGGC GCTGAGCCCG GGGGGGGGTT
12        C.CAGATA.C CCTTTGTGAA CTTATAAC.C ATTTT.ATCG TTGCCTCGGC GCTGAGCCCG GGGGGGGGTT
13        C.CAGATA.C CCTTTGTGAA CTTAT.AC.C TTTTT.ATCG TTGCCTCGGC GCCGAGCC.. ...GGGAG..
14        C.CAGATA.C CCTTTGTGAA CTTAT.AC.C ATTTT.ATCG TTGCCTCGGC GCTGAGCT.. ...GGGGG..
15        C.CAGATACC CCTATGTGAA CTTATTCC.T TTTTTAATCG TAGGCTCGGC GCTGAGCCC. ..GGGGGGAA
16        C.C..CAAAC CC.CTGTGAA ..CAT.AC.C A.C...T.TG TTGCCTCGGC .........G GATCA.G...
17        C.C..CAAAC CC.CTGTGAA ..CAT.AC.C T.C...TATG TTGCCTCGGC .........G GATCA.G...
18        C.C..CAAAC CC.CTGTGAA ..CAT.AC.C ..C...ATCG TTGCCTCGGC .........G G...T.G...
19        C.A..CCAAC CC.CTGTGAA ..CTT.TT.T ACC...AACG TTGCTTCGGC G........G GAACA.G...
20        C.C..CAAAC CCAATGTGAA C.CAT.AC.C A.A...ACTG TTGCCTCGGC G........G GGTCACG...
21        C.C....AAC CC.CTGTGAA C..AT.AC.C TTT...ACTG TTGCTTCGGC .........G GACGATG...
22        C.C....AAC CC.CTGTGAA C..CT.AC.C TTT...ACTG TTGCTTCGGC .........G GACGATG...
23        C.C....AAC CC.CTGTGAA C..AT.AC.C TTT...ACTG TTGCTTCGGC .........G GACGATG...
24        C.C....AAC CC.CTGTGAA C..CT.AC.C TTT...ACTG TTGCTTCGGC .........G GACGATG...
25        C.C....AAC CC.CTGTGAA C.TTT.AC.C TTT...ACTG TTGCTTCGGC .........G GTTGGCG...
26        C.C....AAC CC.CTGTGAA C.CTT.AC.C TCT....TTG TTGCTTCGGC GTGCCCTCTG GAGGCCG...
27        C.C....AAC CC.CTGTGAA C.ATA.AC.C TCT...GTCG TTGCTTCGGC .........G G.........
28        A.C....AAC CCTTTGTGAA C..AT.AC.C TTA...ACTG TTGCTTCGGC G.GGCAGGAG GACAACC...
29        CGCGGCCCAA CCTC...CCA CCCTTGTCTC TATACACCTG TTGCTTTGGC G........G G.........
```

Figure C.1. (cont.)

```
       111                                                                                                  180
1      ......... ......... .....CCCCG AACGGGGGCC CCTCCTCCTC CGGGA..... .......GGG
2      ......... ......... .....CCCCG AACGGGGGCC CCTCCTCCTC CGGGA..... .......GGG
3      ......... ......... .....CCCCG AACGGGGGCC CCTCCTCCTC CGGGA..... .......GGG
4      ......... ......... .....CCCCG AACGGGGGNC CCTCCTCCTC CGGGA..... .......GGG
5      ......... ......... .....CCCCG AACGGGGGCC CCTCCTCCTC NGGGA..... .......GGG
6      ......... ......... .....CCCCG AACGGNGGCC CCTCCTCCTC CGGGA..... .......GGG
7      ......... ......... .....CCCCG AACGGGGGCC CCTCCTCCTC CNGGA..... .......GGG
8      ......... ......... ...TCCTTC. ..GGGGCCCC ......TCAC CCT.....CG GGTGTT...G
9      ......... ......... ...TCCTTC. ..GGGGCCCC ......TCAC CCT.....CG GGTGTT...G
10     ......... ......... ...CTCTTCA CTGAGGCCCC ......CTGG AAA.....CA GG.......G
11     GGCGAAGGAA GATTTTCTTC CTTCTCCCCT CCCTCCCCCC CCTCTTCCAC CGTGAAAACG GTTGTTGGGG
12     GGCGAAGGTA GATTTTCTTC CTTCTCCCCT CCCTCCCCCC CCTCTTCCAC CGTCAAAACG GTTGTTGGGG
13     ......... ......... .TGCTCTTCT ..GTGCTCCC ......CCAC CGCGCAAGCA GT......GG
14     ......... ......... .CACTCTCCT ..GTGCCCCC ......CCAC CGTGCAAGCG GT......GG
15     GAGAAAGCTT GAT..... ....TTCCCT CCCTCACCC. ..TTCACGGG GGTGAAAG.G CCAGGGTTGG
16     ......... ......... .......CCC ...GCTCCCG GTAA.AACG. ......... ......GGAC
17     ......... ......... .......CCC ...GTTCC.. ...T.CACG. ......... ......GAAC
18     ......... ......... .......CCC ...GCTC... .......CG. ......... ......G..C
19     ......... ......... .......ATG ...G..CCCT GTAG.AACG. ......... ......GGCC
20     ......... ......... .......CCC CGGGTGCGTC GCAGCCCCG. ......... ......GAAC
21     ......... ......... ......... ...G.CCC.. ......CCCC CGGG..... .GGC.CGGA.
22     ......... ......... ......... ...G.CCC.. ......CC.. ...G..... .GGC.CGGA.
23     ......... ......... ......... ...GTTCC.. ......CCCC GGGG..... .GGC.CGGA.
24     ......... ......... ......... ...GCCCC.. ......CC.. ..GG..... .GTC.CGGA.
25     ......... ......... .......C.. ..CGGTGC.. ......CCAG ATGG..... .GCC.TGGAG
26     ......... ......... .......C.. ..AGGTCCGC CCCTCACCGG GTGGCCGCGG CGCC.CGGCG
27     ......... ......... ......... ......... ......... ......... .GCA.CG...
28     ......... ......... .......CCC CTCGGGGGGC GGTCCCCCTC CCGGCCGCGC CCTCACGGGC
29     ......... ......... ......CCCA CTGGGGGCCC CCTGGTCGCC GGGGGACATC CGTCCCCGGG


       181                                                                                                  250
1      AGCTGACCGG CCGGCGGCCC CA..TAAACA C.TGCTCCTG T......... TACGATA.TC TGAGCCTTTG
2      AGCTGACCGG CCGGCGGCCC CA..TAAACA C.TGCTCCTG T......... TACGATA.TC TGAGCCTTTG
3      AGCTGACCGG CCGGCGGCCC CA..TAAACA C.TGCTCCTG T......... TACGATA.TC TGAGCCTTTG
4      AGCTGACCGG CCGGCGGNCC CA..TAAACA C.TGTTCCTG T......... TACGATA.TC TGAGCCTTTG
5      AGTTGACCGG CCGGCGGCCC CA..TAAACA C.TGCTCNTG T......... TACGATA.TC TGAGCCTTCG
6      AGCTGACCGG NCGGCGGNCC CA..TAAACA C.TGATCCTG T......... TACGATA.TC TGNGTCTTTG
7      AGTTNACCNN CCNGTGNGCC CA..TAANCA C.TGCTCCTG T......... TAAGATN.TC TNAGCNTTTG
8      AGACAGCCCG CCGGCGG..C CAACCCAACT C.TTGTT.TT TACA.CTGAA AC......TC TGAGA..AAT
9      AGACAGCCCG CCGGCGG..C CAACCCAACT C.TTCTT.TT TACA.CTGAA AC......TC TGAG...AAT
10     AG.CAGCCCG CCGGCGG..C CAACCAAACT C.TTGTT.TC TACA.GTGAA TC......TC TGAGT..AA.
11     AACAGGCCCG CCGGCGG..C CCACTAAACT CTTTGTT.TT TATAA.CCTA TC...TCTTC TGAGT.ACAT
12     AGCAGGACCA CCGGCGG..C CCACTAAACT CCTTGTT.TT AATAA.CCTA TC...TCTTC TGAGT.ACAT
13     AGCAGGCCCG CCGGCGG..C CCACCAAACT CTTTGTT.TT TAGAA.CGTA TC...TCTTC TGAGT.GTTT
14     AGCAGGCCCG CCGGCGG..C CCACCAAACT CTTTGTT.TT TAGAC.CGTA TC...TCCTC TGAGT.GTTT
15     AGCAGGTCCG CCCGCGG..C CCAGTTACGA TCTAGTTATT AATAACCGTA TC...TCTTC TGAGTGGCAT
16     ....GGCCCG CCAGAGGACC C..CTAAACT C.TG.TT.TC TA......TA .TGTAACTTC TGAGT..AAA
17     ....GGCCCG CCGCAGGA.C C..CTAAACT C.TG.TT.TT TA.......G .TGGAACTTC TGAGT..AAA
18     ....GGCCCG CCAGAGGACC C..CCAAACT C.TT.GT.TT TA......TA CAGCATCTTC TGAGT..AAC
19     ...ACCCCCG CCAGAGGACC C..CCAAACA C.CC.TG.TA TC......TC TAGT.TTTTC TGAGT..AA.
20     CAGGCGCCCG CCGGAGGGAC CAACCAAACT C.TT.TTCTG TAGTCCCCTC GCGGACGTTA TTTCT..TAC
21     ..CG...CCG CCGGAGGTTA C....AAACC C.TGAATTT. TA.......G T.GTATC.TC TGAGT..AT.
22     ..CG...CCG CCGGAGGTTA C....AAACC C.TGAATTT. TA.......G T.GTATC.TC TGAGT..AC.
23     ..CG...CCG CCGGAGGTTA C....AAACC C.TGAATTT. TA.......G T.GTATC.TC TGAGT..AC.
24     ..CG...CCG CCGGAGGTTA C....AAACC C.TGAATTT. TA.......G T.GTATC.TC TGAGT..AC.
25     GTCG...CCG CCGGAGGTTC G....AAACC C.TGAATTC. TA.......G T.GTGTC.TC TGAGA..AA.
26     GTCG.GCACG CCGGAGGTTC C....AAACT C.TGAATTTA TA.......G T.GTATC.TC TGAGA..TT.
27     ......CCCG CCGGAGGTTC A....AAACT C.TTATTTTT TC.......C A.GTATC.TC TGAGC..CTG
28     GTGGCGCCCG CCGGAGGATA C..CAAAACT C.T.ATTTTA AC.......G ACGTTTCTTC TGAGT..GGC
29     CCCGCGCCCG CCGAAGCGCT C.TGTGAACC C.TGATGAAG .........A TGGGCTG.TC TGAGTATTAT
```

Figure C.1. (cont.)

```
        251                 270
1       AC......AG AAATGAATC.
2       AC......AG AAATGAATCA
3       AC......AG AAATGAATCA
4       AC......AG AAATGAATCA
5       AC......AG AAATGAATCA
6       AC......AG AAATGAATCA
7       AT......AG AAATNAATCA
8       AAAAC...AT AAATGAATCA
9       AAAAC...AT AAATGAATCA
10      AAAAC...AT AAATGAATCA
11      AAACCAAAAA AAATGAATTA
12      AAACCAAAAT AAATGAATCA
13      ATAAC.AAAC AAATGAATCA
14      ACAA..AAAC AAATGAATCA
15      AATC....AA AAGTTAATCA
16      A....CCA.T AAATAAATCA
17      A....AAA.C AAATAAATCA
18      A....CGATT AAATAAATCA
19      A....CAAGC AAATAAATC.
20      AGCTCTGAGC AAA.AATTCA
21      A....AAACC AAATAA.TTA
22      A....AAACC AAATAA.TTA
23      A....AAACC AAATAA.TTA
24      A....AAACC AAATAA.TTA
25      A....GAATA AAACAA.TCA
26      A....AAAAC AAATAA.TCA
27      A....AAGAC AAATAA.TCA
28      A....CAAGC AAATAA.TTA
29      .........G AAAATTGTCA
```

Figure C.2. Alignment of the ITS I sequences of 29 Ascomycetes using a gap weight of 1.0 and gap length weight of 0.5.

```
                                                  1                                                  40
Fusarium oxysporum (1)                            ..... ..... ..... .....
F. sambucinum (2)                                 ..... ..... ..... .....
Cylindrocarpon heteronema (3)                     ..... ..... ..... .....
Nectria haematococca (4)                          ..... ..... ..... .....
Gaeumannomyces graminis var. avenae (5)           ..... ..... ..... .....
G. graminis var. tritici (6)                      ..... ..... ..... .....
G. graminis var. graminis (7)                     ..... ..... ..... .....
Phialophora sp. (8)                               ..... ..... ..... .....
G. incrustans (9)                                 ..... ..... ..... .....
G. cylindrosporus (10)                            ..... ..... ..... .....
Magnaporthe grisea (11)                           ..... ..... ..... .....
Colletotrichum destructivum (12)                  ..... ..... ..... .....
Trichoderma harzianum (13)                        ..... ..... ..... .....
Anisogramma anomala AAN6031D (14)                 ..... ..... ..... .....
A. anomala AAN6061J (15)                          ..... ..... ..... .....
A. anomala AAN6023D (16)                          ..... ..... ..... .....
A. anomala AAN6021V (17)                          ..... ..... ..... .....
A. anomala AAN6011B (18)                          ..... ..... ..... .....
A. anomala AAN6014Z (19)                          ..... ..... ..... .....
A. anomala AAN6052Z (20)                          ..... ..... ..... .....
Phomopsis sp. (21)                                .....C TGGAACGCGC CCCAGCGAGG CCCAGA.AAC
Phomopsis sp. (22)                                ..... ..GGACG.GC CCCAGCGA.G CCCAGA.AAC
Phomopsis sp. (23)                                .....C TGGAACGCGC TTCGGCG.CA CCCAGA.AAC
Cryphonectria parasitica (24)                     CTGGAACTGT G.GCCCTCAC GGGCGC.AAC CCCAGATA.C
Cr. radicalis (25)                                CTGGAACTGT G.GCCCTCAC GGGCGC.AAC CCCAGATA.C
Cr. cubensis (26)                                 CTGGAACTGC GCCCCCCCAC GGGCGCAAAC CCCAGATA.C
Endothia gyrosa (27)                              CTGGAACTGT G..CCCTCGC GGGCAC.AAC CCCAGATA.C
Cr. havanensis (28)                               CTGGAATTTG TTGCCCTCAC GGGCAC.AAC CCCAGATACC
Talaromyces flavus (29)                           ..... ..... ..... .....

      41                                                                                         110
1     ..... ....CCGAGT TTACAACTCC CAAACCC.CT GTGAAC.ATA CCA.CT.TGT TGCCTCGGC.
2     ..... ....CCGAGT TTACAACTCC CAAACCC.CT GTGAAC.ATA CCT.CTATGT TGCCTCGGC.
3     ..... ....CCGAGT TTACAACTCC CAAACCC.CT GTGAAC.ATA CC..CATCGT TGCCTCGGC.
4     ..... ....CCGAGT TTTCAACTCA CCAACCC.CT GTGAAC.TTT TTACCAACGT TGCTTCGGCG
5     ..... ....CAGAGT TGA.AAAACT CCAACCC.CT GTGAAC.ATA CCTTTACTGT TGCTTCGGC.
6     ..... ....CAGAGT TGA.AAAACT CCAACCC.CT GTGAAC.ATA CCTTTACTGT TGCTTCGGC.
7     ..... ....CAGAGT TGA.AAAACT CCAACCC.CT GTGAAC.CTA CCTTTACTGT TGCTTCGGC.
8     ..... ....CAGAGT TGA.AAAACT CCAACCC.CT GTGAAC.CTA CCTTTACTGT TGCTTCGGC.
9     ..... ....AAGAGT TGC.AAAACT CCAACCC.CT GTGAACTTTA CCTTTACTGT TGCTTCGGC.
10    ..... ....CAGAGT TGA.AAAACT CCAACCC.CT GTGAACCTTA CCTCT.TTGT TGCTTCGGCG
11    ..... ....CTGAGT TGA.AAAACT CCAACCC.CT GTGAACATAA CCTCTGTCGT TGCTTCGGC.
12    ..... ....CTGAGT T.ACCGCTCT ACAACCCTTT GTGAAC.ATA CCTTAACTGT TGCTTCGGCG
13    ..... ....CCGAGT TTACAACTCC CAAACCCAAT GTGAACCATA CCA.AACTGT TGCCTCGGCG
14    ...NTG.GAG CAAACGCCCC .....CCCCT TCCCCGGGGG GGGGGCTACC CAGAAACCCT TTGTGAATCT
15    ...CTG.GAG CNAACGCCCC .....CCCCT TCCCCGGGGG GGGNGCTACC CAGAAACCCT TTGTGAATCT
16    ...ATG.GAG CAAACGCCCC .....CCCCT TCCCCGGGGG GGGCGCTACC CAGAAACCCT TTGTGAATCT
17    ...NTG.GAG CAAACGCCCC .....CCCCT TCCCCGGGGG GGGNGCTACC CAGANTCCCT TTGTGAATCT
18    ...NTG.GAG TAAACGNCCT .....CNCCT TCNCCGGGGG GGGGGCTACC CAGANACCCT TTGTGAATCT
19    ...TTN.GAG CAAACGCNCC .....CCCCT TCCCCGGCGG GGGGGGTACC CAGANACCCT TTGTGAATCT
20    ...CTG.GAG CAAACGCCCC .....CCCCT TCCCCGGGGG GGGCGCTACC CAGAAACCCT TTGTGANTCT
21    CCTTTGTGAA CTTAT.ACCT T....ACTGT TGCCTCGGCG CTACGT.... ..GGT..... ..........
22    CCTTTGTGAA CTTAT.ACCT T....ACTGT TGCCTCGGCG CTACGT.... ..GGT..... ..........
23    CCTTTGTGAA CTTAT.ACC. T....ATTGT TGCCTCGGCG .TAGGC.... ..GCG..... ..........
24    CCTTTGTGAA CTTATAACCA TTTT.ATCGT TGCCTCGGCG CTGAGCCCGG GGGGGGGTTG GCGAAGGAAG
25    CCTTTGTGAA CTTATAACCA TTTT.ATCGT TGCCTCGGCG CTGAGCCCGG GGGGGGGTTG GCGAAGGTAG
26    CCTTTGTGAA CTTAT.ACCT TTTT.ATCGT TGCCTCGGCG CCGAGCC... ..GGGAG... ..........
27    CCTTTGTGAA CTTAT.ACCA TTTT.ATCGT TGCCTCGGCG CTGAGCT... ..GGGGG... ..........
28    CCTATGTGAA CTTATTCCTT TTTTAATCGT AGGCTCGGCG CTGAGCCC.. .GGGGGGAAG AGAAAGCTTG
29    ..... ..... ..... ....CCGAGT GCGGGCCCTC GCGGCCCAAC CTCCCACCCT
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Figure C.2. (cont.)

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        111                                                                 180
1       ........GG ATCAGCCCGC T....C.CCG GTAAA..... ....ACGGGA C......GG. ......CCC.
2       ........GG ATCAGCCCGT T....C.C.. ...TC..... ....ACGGAA C......GG. ......CCC.
3       ........GG ...TGCCCGC T....C.... .......... .....CGG.. C......GG. ......CCC.
4       ........GG AACAGATGG. .....C.CCT GTAGA..... ....ACGGGC CA.....CC. ......CCC.
5       ........GG ACGATG..GT TC.C.C.CCC .......... ....GGGGGG CC.....GGA ...CG..CC.
6       ........GG ACGATG..G. CC.C.C.CCC .......... ....CGGGGG CC.....GGA ...CG..CC.
7       ........GG ACGATG..G. .....C.CCC .......... ....CCGGGT CC.....GGA ...CG..CC.
8       ........GG ACGATG..G. .....C.CCC .......... ....C..GGG CC.....GGA ...CG..CC.
9       ........GG TTGGCGCCGG TG.C.C.CAG .......... ....ATGGGC CT.....GGA GGTCG..CC.
10      TGCCCTCTGG AGGCCGCAGG TC.CGC.CCC TCACCGGGTG GCCGCGGCGC CC.....GGC GGTCGGCAC.
11      .......... .......... .......... .......... ......GGGC AC.....G.. ......CCC.
12      .GGCAGGAGG ACAACCCCCC TCGGGG.GGC GGTCCCCCTC CCGGCCGCGC CCTCACGGGC GTGGCGCCC.
13      GGGTCACGCC CCGGGTGCGT CGCAGC.CCC GGAACCAGGC GCCCGCCGGA GGGACCAACC AAACTCTTT.
14      TCTCCGT.TG NCTCGGCCTG GTTGCC.CCC GAACGGGGGC CCCTCCTCCT CC.GGGAGGG AGCTGACCG.
15      TCTCCGT.TG CCTCGGCCTG GTTGCC.CCC GAACGGGGGC CCCTCCTCCT CC.GGGAGGG AGCTGACCG.
16      TCTCCGT.TG CCTCGGCCTG GTTGCC.CCC GAACGGGGGC CCCTCCTCCT CC.GGGAGGG AGCTGACCG.
17      TCTCCGT.TG NCTCGGCCTG GTTGNC.CCC GAACGGGGGN CCCTCCTCCT CC.GGGAGGG AGCTGACCG.
18      TCTCCGT.TG NCTCGGNCTG GTTGCC.CCC GAACGGGGGC CCCTCCTCCT CN.GGGAGGG AGTTGACCG.
19      TCTCNGC.TG CCTCGGCCTG GTTGCC.CCC GAACGGNGGC CCCTCCTCCT CC.GGGAGGG AGCTGACCG.
20      TCTCCGTCTG GCTCGGCCTG GTTGCC.CCC GAACGGGGGC CCCTCCTCCT CC.NGGAGGG AGTTNACCN.
21      .......... ......CCTT CGGGGC.CCC ......TCAC CCT.....CG GGTGTT..GA GACA.GCCC.
22      .......... ......CCTT CGGGGC.CCC ......TCAC CCT.....CG GGTGTT..GA GACA.GCCC.
23      .......... ......CCTC TTCACT.GAG ......GCCC CCT.....GG AAACAG..GG AGCA.GCCC.
24      ATTTTCTTCC TTCTCCCCTC CCTCCC.CCC CCTCTTCCAC CGTGAAAACG GTTGTTGGGG AACAGGCCC.
25      ATTTTCTTCC TTCTCCCCTC CCTCCC.CCC CCTCTTCCAC CGTCAAAACG GTTGTTGGGG AGCAGGACC.
26      .......... TGCTCTTCT. .GTGCT.CCC ......CCAC CGCGCAAGCA GT......GG AGCAGGCCC.
27      .......... CACTCTCCT. .GTGCC.CCC ......CCAC CGTGCAAGCG GT......GG AGCAGGCCC.
28      AT........ ...TTCCCTC CCTCAC.CC. ..TTCACGGG GGTGAAAG.G CCAGGGTTGG AGCAGGTCC.
29      TGTCTCTATA CACCTGTTGC TTTGGCGGGC CCACTGGGGG CCCCCTGGTC GCCGGGGGAC ATCCGTCCCC


        181                                                                 250
1       ..GCCAGAGG ACCCCTAAAC TCTGTTTCTA TA.T...... GTAACTTCTG AGTAAAACCA .T..AAATAA
2       ..GCCGCAGG A.CCCTAAAC TCTGTTTTTA .G.T...... GGAACTTCTG AGTAAAAAAA .C..AAATAA
3       ..GCCAGAGG ACCCCCAAAC TCTTGTTTTA TACA...... GCATCTTCTG AGTAACACGA TT..AAATAA
4       ..GCCAGAGG ACCCCCAAAC ACCCTGTATC TCTA...... GT.TTTTCTG AGTAA.ACAA GC..AAATAA
5       ..GCCGGAGG TTAC..AAAC CCTGAATTT. TAGT...... GTATC.TCTG AGTAC.AAAA CC..AAAT.A
6       ..GCCGGAGG TTAC..AAAC CCTGAATTT. TAGT...... GTATC.TCTG AGTAT.AAAA CC..AAAT.A
7       ..GCCGGAGG TTAC..AAAC CCTGAATCT. TTGT...... GTATC.TCTG AGTAC.AAAA CC..AAAT.A
8       ..GCCGGAGG TTAC..AAAC CCTGAATTT. TAGT...... GTATC.TCTG AGTAC.AAAA CC..AAAT.A
9       ..GCCGGAGG TTCG..AAAC CCTGAATTC. TAGT...... GTGTC.TCTG AGAAA.AGAA TA..AAAC.A
10      ..GCCGGAGG TTCC..AAAC TCTGAATTTA TAGT...... GTATC.TCTG AGATT.AAAA AC..AAAT.A
11      ..GCCGGAGG TTCA..AAAC TCTTATTTTT TCCA...... GTATC.TCTG AGCCTGAAAG AC..AAAT.A
12      ..GCCGGAGG ATACCAAAAC TCTATTTTAA CGAC...... GTTTCTTCTG AGTGGCACAA GC..AAAT.A
13      ..TCTGTAGT CCCCTCGCGG ACGTTATTTC T..T...... ACAGC.TCTG AGCAA.AAAT TC..A.....
14      ..GCCGGCGG CCCCATAAAC AC..TGCTCC TGTTACG... ATA...TCTG AGC..CTTTG ACAGAAATGA
15      ..GCCGGCGG CCCCATAAAC AC..TGCTCC TGTTACG... ATA...TCTG AGC..CTTTG ACAGAAATGA
16      ..GCCGGCGG CCCCATAAAC AC..TGCTCC TGTTACG... ATA...TCTG AGC..CTTTG ACAGAAATGA
17      ..GCCGGCGG NCCCATAAAC AC..TGTTCC TGTTACG... ATA...TCTG AGC..CTTTG ACAGAAATGA
18      ..GCCGGCGG CCCCATAAAC AC..TGCTCN TGTTACG... ATA...TCTG AGC..CTTCG ACAGAAATGA
19      ..GNCGGCGG NCCCATAAAC AC..TGATCC TGTTACG... ATA...TCTG NGT..CTTTG ACAGAAATGA
20      ..NCCNGTGN GCCCATAANC AC..TGCTCC TGTTAAG... ATN...TCTN AGC..NTTTG ATAGAAATNA
21      ..GCCGGCGG CCAACCCAAC TC.TTGTT.T TTACA.CTGA AAC...TCTG AGAAATA.AA ACATAAATGA
22      ..GCCGGCGG CCAACCCAAC TC.TTCTT.T TTACA.CTGA AAC...TCTG AG.AATA.AA ACATAAATGA
23      ..GCCGGCGG CCAACCAAAC TC.TTGTT.T CTACA.GTGA ATC...TCTG AGTAA.A.AA ACATAAATGA
24      ..GCCGGCGG CCCACTAAAC TCTTTGTT.T TTATAA.CCT ATCTCTTCTG AGT.ACATAA ACCAAAAAAA
25      ..ACCGGCGG CCCACTAAAC TCCTTGTT.T TAATAA.CCT ATCTCTTCTG AGT.ACATAA ACCAAAATAA
26      ..GCCGGCGG CCCACCAAAC TCTTTGTT.T TTAGAA.CGT ATCTCTTCTG AGT.GTTTAT AAC.AAACAA
27      ..GCCGGCGG CCCACCAAAC TCTTTGTT.T TTAGAC.CGT ATCTCCTCTG AGT.GTTTAC AA..AAACAA
28      ..GCCCGCGG CCCAGTTACG ATCTAGTTAT TAATAACCGT ATCTCTTCTG AGTGGCATAA TCAAAAGTTA
29      GGGCCCGCGC CCGCCGAAGC GCTCTGTGAA CCCTGA.TGA AGATGGGCTG TCTGAGTATT ATGAAAATT.
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Figure C.2. (cont.)

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          251
1       ATCA....
2       ATCA....
3       ATCA....
4       ATC.....
5       ATTA....
6       ATTA....
7       ATTA....
8       ATTA....
9       ATCA....
10      ATCA....
11      ATCA....
12      ATTA....
13      ........
14      ATC.....
15      ATCA....
16      ATCA....
17      ATCA....
18      ATCA....
19      ATCA....
20      ATCA....
21      ATCA....
22      ATCA....
23      ATCA....
24      ATGAATTA
25      ATGAATCA
26      ATGAATCA
27      ATGAATCA
28      ATCA....
29      GTCA....
```