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Fungicide Resistance Genetics of Apple Scab fungus *Venturia inaequalis*

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Honors Thesis

Dr. Kirk Broders

Abstract

Apple scab, caused by the ascomycete fungus *Venturia inaequalis*, is considered the most devastating disease on domestic apple crops. Apples are the most important cultivated crop in temperate regions and the United States produced about 4.6 million tons of apples in 2010. Traditional methods to control fungal plant diseases like apple scab are based on the use of chemical compounds that may produce serious negative effects, mainly related with environmental pollution and the development of fungicide resistance. Identifying genes and mechanisms of fungicide resistance in *V. inaequalis* is imperative to developing new and more effective defenses against the spread of resistance.

Introduction

Apples are the most important and most widely cultivated fruit crop in temperate regions around the world (Gladieux *et al.*, 2010). Apples are the second most important non-citrus fruit crop in the United States following only grapes. The United States produced approximately 4.6 million tons of apples worth an estimated \$2.2 billion in 2010 (USDA & NASS 2011). However, this price does not reflect profits from apple production, as it does not include the amount of money spent on fungicides and their application for the management of apple scab, as well as other input costs. Apple scab, caused by the fungus *Venturia inaequalis*, is the most important disease of apples and has invaded all apple-growing regions around the world (Gladieux *et al.*, 2008). Apple scab causes deformation in affected fruits, enhances susceptibility of the tree to chilling and freezing injuries, and causes premature leaf and fruit fall (Thakur *et al.*, 2013). Apple scab can lead to severe crop losses if it is not controlled properly (Gusberti *et al.*, 2012), and has negative effects on the economy due to the impact of yield losses, the use of fungicide

inputs, and with the corresponding environmental and health hazards (Gladieux *et al.*, 2008). Knowledge of the pathogenicity and virulence factors needed for fungal infection is highly important because it represents the targets that will allow researchers to identify and deploy resistance genes against these microorganisms (Acero *et al.*, 2011).

Venturia inaequalis is an ascomycete fungus that reproduces both sexually and asexually and is responsible for the most prominent disease in apple production commonly known as apple scab (Gladieux *et al.*, 2008). As *V. inaequalis* reproduces sexually and asexually, it is able to rapidly evolve resistance against fungicides and overcome host resistance genes. No part of the plant structure is exempt from fungal infections. This capacity is supported by a complex fungal life and infection cycle (Acero *et al.*, 2011). *Venturia inaequalis* overwinters primarily as pseudothecia that develop on the orchard floor. Infection is initiated in spring by ascospores that are released by rainfall from pseudothecia (Bowen *et al.*, 2011). The release of ascospores is timed to coincide with host budburst and leaf unfurling, increasing the probability of host infection. Germ tubes arise from ascospores and penetrate through the cuticle of the host to form multilayered pseudoparenchymatous structures, called stromata (Bowen *et al.*, 2011). The stromata, and the conidia they produce, form the characteristic lesions of the disease. Conidia are disseminated by wind and rain and allow for secondary infections throughout the fruit development period (Bowen *et al.*, 2011).

Genes that Confer Fungicide Resistance

The *cytochrome b* (*cyt b*) genes are the targets of the Quinone outside inhibitor (QoI) fungicides. Cytochrome b is a membrane protein that forms the center of the mitochondrial bcI complex in the respiratory chain of eukaryotes (Luo *et al.*, 2013). The *cyt b* gene is located in the

mitochondrial genome. The QoI fungicide binds to the Qo site on Cytochrome b, which blocks the electron transfer through the respiration pathway, which leads to energy deficiency and eventual cell death by preventing the production of ATP. In many cases, QoI resistance is conferred by a single amino acid change in the *cyt b* gene. It is known that in *V. inaequalis*, resistance is conferred by an amino acid substitution at position 143 from glycine to alanine (G143A) (Luo *et al.*, 2013). The G143A mutation is most likely the most potent of all *cyt b* mutations. The presence of an intron just downstream of position 143 has been shown to prevent the G143A mutation. *Monilia fucticola* has a group 1 intron directly downstream of *cyt b* indicating that it is unlikely that a G143A mutation will develop in this species (Luo *et al.*, 2013).

The fungicide Benomyl binds to the β -tubulin protein and prevents cellular processes that are dependent on the cytoskeleton, including chromosome transport and cell division (Deising *et al.*, 2008). This disruption in the cytoskeleton leads to reduced cell growth and eventual cell death. In several plant pathogenic fungi, specific amino acid mutations resulted in increased resistance to the fungicidal compound intracellularly derived from benomyl, carbendazium (Deising *et al.*, 2008). As the amino acids involved in fungicide binding are highly conservative, it would follow that resistance mutations involve the same amino acids in various fungi belonging to different taxa.

In fungi, efflux systems have evolved to expel naturally occurring toxic substances and thus confer resistance to these substances (Deising *et al.*, 2008). These transporter mechanisms also confer fungicide resistance and because they export substrates belonging to different chemical classes, it is thought that they are crucial to the adaptation of pathogenic fungi to various fungicides (Deising *et al.*, 2008). There are two main efflux systems known to be

involved in the transport of toxins in pathogenic fungi: the ATP-Binding Cassette (ABC) transporter and the Major Facilitator Superfamily (MFS) transporters. The ABC transporter uses energy from the hydrolysis of ATP to transport substances against the concentration gradient. ABC transporters are part of a rapidly growing efflux pump superfamily and they contain a highly conserved ABC module. Although not their primary role, some MFS transporters have been shown to protect pathogenic fungi from fungicides (Deising *et al.*, 2008).

Azole fungicides inhibit the synthesis of ergosterol in fungi through the non-competitive, direct binding to 14 α -demethylase (CYP51). Point mutations in the target enzyme gene *CYP51* can confer resistance to the Azole classification of fungicides (Parker *et al.*, 2014). Resistance to azoles is increasing due to the selective pressures of azole, particularly triazole, treatments. CYP51 is located in the outer membrane of the endoplasmic reticulum. Azole binding leads the accumulation of 14-methylated sterols, which slow fungal growth by disrupting the cell membrane (Parker *et al.*, 2014). Triazoles have become the most widely used class of fungicide in agriculture, accounting for 20% of fungicide use (Parker *et al.*, 2014).

Bioinformatic Approach

The Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information identifies regions of local similarity between sequences. Nucleotide sequences are compared to the NCBI sequence database and the statistical significance is calculated for each match. BLAST can be used to identify functional relationships. In BLAST results, cover represents the percentage of query covered by alignment to the database sequence (Fassler, 2011). Identity represents the extent to which two sequences have the same residues at the same positions in an alignment (Fassler, 2011.). The E-value represents the number of

different alignments with scores equivalent to or better than the raw score that is expected to occur in a database by chance (Fassler, 2011). Neighbor-joining trees are accomplished by sequentially identifying neighbors that minimize the total length of the tree (Graur and Li, 2000). The bootstrap is a technique that estimates the confidence level of the phylogenetic hypothesis by resampling data from the original sample set with replacement (Graur and Li, 2000). Bootstrap values are expressed in percentages and can be interpreted as confidence levels for clades (Graur and Li, 2000).

Hypothesis

Similarities in function of genes may be elucidated from similarities in their structure. Similarities in structure between *Venturia inaequalis* and other organisms may lead to information on the spread of fungicide resistance and possible techniques for control of this spread.

Materials and Methods

Accession numbers of 155 experimentally validated nucleotide sequences of *V. inaequalis* were obtained from Thakur *et al.* 2013 and the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information was utilized to find similar sequences. Standard nucleotide blasts, optimized for highly similar sequences (megablast), against nucleotide collection on NCBI (nr/nt) were completed for each experimentally validated nucleotide sequence of *V. inaequalis*. Only results with query covers over 40% and an E-values smaller than 1E-5 were considered significant. If no significant results were found, the alignment was repeated using BLAST optimized for somewhat similar sequences. Again, only results with

query covers over 40% and an E-values smaller than 1E-5 were considered significant. The most significant result for each experimentally validated sequence that was not from a variety of *V. inaequalis* or either of its anamorph *Spilocaea pomi* and *Fusicladium pomi* were considered the most significant alignment.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013). The following neighbor-joining trees were generated under standard, pre-set conditions. Bootstrap consensus was conducted under standard, pre-set conditions with 500 pseudosamples. Bootstrap values were considered confident if they were greater than 50%. Sequences and alignments from the *V. inaequalis* genes with any of the following: 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA, were exported to the program MEGA, aligned with MUSCLE, and a neighbor-joining tree with bootstrap consensus was generated. Sequences and alignments for the *V. inaequalis actin* genes were exported to the program MEGA and aligned with MUSCLE. A neighbor-joining tree could not be generated for *V. inaequalis actin* genes and alignments because at least four sequences are needed. Sequences and alignments for the *V. inaequalis cyt b* genes were exported to the program MEGA and aligned with MUSCLE. Sequences and alignments for the *V. inaequalis β -tubulin* genes were exported to MEGA and a neighbor-joining tree was generated with bootstrap consensus. The *β -tubulin* genes and alignments could not be aligned by MUSCLE. Sequences and alignments from the *translation elongation factor 1-alpha* genes were exported to the program MEGA, aligned with MUSCLE, and a neighbor-joining tree with bootstrap consensus was created based on the alignment. Sequences and alignments from the *V. inaequalis GAPDH* genes were exported to the program MEGA, aligned with MUSCLE, and a neighbor-joining tree with bootstrap consensus was generated based on the alignment.

Sequences and alignments from *V. inaequalis ABC transporter* genes were exported to the program MEGA, aligned with MUSCLE, and a neighbor-joining tree with bootstrap consensus was generated. Sequences and alignments from *V. inaequalis CYP51* genes were exported to the program MEGA, aligned with MUSCLE, and a neighbor-joining tree with bootstrap consensus was generated. The *V. inaequalis* genes *Cin1*, *RNA Polymerase largest subunit*, *Cin1L2*, *alternative oxidase*, *small subunit rRNA*, and *Group 1 introns* were exported to the program MEGA and aligned with MUSCLE.

Results

No hits, or no significant hits, were found for: *V. inaequalis cellophane-induced protein 1-like 1 (Cin1L1)* gene, complete cds (JN159972.1); *V. inaequalis* isolate MNH 135 *cellophane-induced protein 3 (CIN3)* gene, partial cds (EU189193.1); *V. inaequalis* strain Ent7 *14 alpha-demethylase (CYP51A1)* gene, partial cds (AF227918.1); *V. inaequalis* strain Ent23 *14 alpha-demethylase (CYP51A1)* gene, partial cds (AF227916.1); *V. inaequalis* strain UZ4 *14 alpha-demethylase (CYP51A1)* gene, partial cds (AF227919.1); *V. inaequalis* strain Ent2 *14 alpha-demethylase (CYP51A1)* gene, partial cds (AF227917.1); *V. inaequalis* candidate effector 15 mRNA, complete cds (FJ621513.1); *V. inaequalis* candidate effector 13 mRNA, complete cds (FJ621511.1); *V. inaequalis* candidate effector 11 mRNA, complete cds (FJ621509.1); *V. inaequalis* candidate effector 5 mRNA, complete cds (FJ621507.1); *V. inaequalis* candidate effector 14 mRNA, complete cds (FJ621512.1); *V. inaequalis* candidate effector 12 mRNA, complete cds (FJ621510.1); *V. inaequalis* candidate effector 10 mRNA, complete cds (FJ621508.1); *V. inaequalis* microsatellite TG9/129 sequence (AY502077.1); *V. inaequalis* microsatellite AACs10 sequence (AY491494.1); *V. inaequalis* microsatellite CA9/X sequence

(AY491492.1); *V. inaequalis* microsatellite GA7/116 sequence (AY491490.1); *V. inaequalis* microsatellite TC2/16 sequence (AY491488.1); *V. inaequalis* microsatellite CACG8/42 sequence (AY491486.1); *V. inaequalis* microsatellite CA9/134 sequence (AY491484.1); *V. inaequalis* microsatellite TCCA7/P sequence (AY491482.1); *V. inaequalis* microsatellite AGGT8/I sequence (AY491480.1); *V. inaequalis* microsatellite CT1/130 sequence (AY491478.1); *V. inaequalis* microsatellite TC1/2 sequence (AY491476.1); *V. inaequalis* microsatellite TG10/epsilon sequence (AY491495.1); *V. inaequalis* microsatellite 10/154 sequence (AY491493.1); *V. inaequalis* microsatellite TG9/99 sequence (AY491491.1); *V. inaequalis* microsatellite GA3/Z sequence (AY491489.1); *V. inaequalis* microsatellite GT8/146 sequence (AY491487.1); *V. inaequalis* (AY491485.1); *V. inaequalis* microsatellite TG11/70 sequence (AY491483.1); *V. inaequalis* microsatellite CA9/152 sequence (AY491481.1); *V. inaequalis* microsatellite TC2/D sequence (AY491479.1); *V. inaequalis* microsatellite TC1/82 sequence (AY491477.1); *ViECP6* nucleotide sequence.

The neighbor-joining tree with bootstrap consensus for *V. inaequalis* sequences and alignments for the genes with any of the following: *18S ribosomal RNA*, *internal transcribed spacer 1*, *5.8S ribosomal RNA*, *internal transcribed spacer 2*, and *28S ribosomal RNA*, is represented in Figure 1.

Venturia inaequalis actin (act1) gene, partial cds (AY748993.1) has 99% cover, 88% identity, and E-value of 0.0 with *Phoma dennisii* strain CBS 631.68 *actin (act1)* gene, partial cds (AY748974.1). *Venturia inaequalis actin gene*, partial cds (AF269254.1) has 100% cover, 87% identity, and E-value of 7E-136 with *P. dennisii* strain CBS 631.68 *actin (act1)* gene, partial cds (AY748974.1). The three genes are aligned consistently from site number 373 to site number

826. *Phoma dennisii* strain CBS 631.68 *actin (act1)* gene and *V. inaequalis actin (act1)* gene, partial cds (AY748993.1) are aligned consistently for all 904 sites.

Venturia inaequalis cytochrome b (cytb) mRNA, mitochondrial gene encoding mitochondrial protein, complete cds (AF047029.1) has 90% cover, 83% identity, and an E-value of 0.0 with *Monilinia laxa* isolate MbhMF08-14B *cytochrome B (cytb) mRNA*, complete cds; mitochondrial (GQ423060.1). *Venturia inaequalis* RT-like protein and *cytochrome b (cytb)* genes, mitochondrial genes encoding mitochondrial proteins, complete cds (AF004559.1) has 44% cover, 76% identity, and E-value of 0.0 with *Fusarium graminearum* strain CBS 139513 mitochondrion, complete genome (KP966561.1).

The neighbor-joining tree with bootstrap consensus for *V. inaequalis β-tubulin* genes and alignments is represented in Figure 2.

The neighbor-joining tree with bootstrap consensus for *V. inaequalis translation elongation factor 1 alpha* genes and alignments is represented in Figure 3.

The neighbor-joining tree with bootstrap consensus for *V. inaequalis GAPDH* genes and alignments is represented in Figure 4.

The neighbor-joining tree with bootstrap consensus for *V. inaequalis ATP-binding cassette transporter* genes and alignments is represented in Figure 5.

The neighbor-joining tree with bootstrap consensus for *V. inaequalis CYP51* genes and alignments is represented in Figure 6.

Venturia inaequalis isolate MNH 135 *cellophane-induced protein 1 precursor (CIN1)* gene, complete cds (EU189192.1) has 42% cover, 72% identity, and E-value of 5E-27 with *Venturia pirina cellophane-induced protein 1 (Cin1)* gene, complete cds (JN159974.1). *Venturia inaequalis* strain CBS 815.69 *RNA polymerase II largest subunit* gene, partial sequence

(GU357756.1) has 96% cover, 92% identity, and E-value of 0.0 with *Venturia populina* strain CBS 256.38 *RNA polymerase II largest subunit* gene, partial sequence (GU357769.1). *Venturia inaequalis* strain CBS 594.70 *RNA polymerase II largest subunit* gene, partial sequence (GU357757.1) has 98% cover, 91% identity, and E-value of 0.0 with *V. populina* strain CBS 256.38 *RNA polymerase II largest subunit* gene, partial sequence (GU357769.1). *Venturia inaequalis cellophane-induced protein 1-like 2 (Cin1L2)* gene, complete cds (JN159973.1) has 99% cover, 67% identity, and E-value of 1E-21 with *V. pirina cellophane-induced protein 1-like 2 (Cin1L2)* gene, complete cds (JN159976.1). *Venturia inaequalis cellophane-induced protein 1 (Cin1)* gene, complete cds (JN159971.1) has 42% cover, 72% identity, and E-value of 4E-28 with *V. pirina cellophane-induced protein 1 (Cin1)* gene, complete cds (JN159974.1). *Venturia inaequalis alternative oxidase* gene, complete cds (AF363785.1) has 41% cover, 66% identity, and E-value of 3E-61 with *Pyrenophora tritici-repentis* Pt-1C-BFP *alternative oxidase, mitochondrial precursor, mRNA* (XM_001941903.1). *Venturia inaequalis alternative oxidase (AOX1)* gene, AOX1-A1 allele, complete cds (AF279690.1) has 49% cover, 69% identity, and E-value of 9E-66 with *Pseudocercospora fijiensis* CIRAD86 hypothetical protein mRNA (XM_007930318.1). *Venturia inaequalis candidate effector 16 mRNA*, complete cds (FJ621514.1) has 55% cover, 67% identity, and E-value of 1E-30 with *Oryza sativa Japonica Group cDNA clone:002-151-G12*, full insert sequence (AK108837.1). *Venturia inaequalis ribosomal protein L12 mRNA*, complete cds (EU853840.1) has 64% cover, 85% identity, and E-value of 5E-139 with *Arthrobotrys oligospora* ATCC 24927 hypothetical protein mRNA (XM_011125321.1). *Venturia inaequalis small subunit ribosomal RNA gene, mitochondrial gene encoding mitochondrial RNA*, partial sequence (AF051644.1) has 60% cover, 97% identity, and

E-value of 0.0 with *V. chlorospora* voucher Kruys 502 (UPS) *small subunit ribosomal RNA gene*, partial sequence; mitochondrial (DQ384084.1).

Venturia inaequalis ribosomal RNA gene, group I intron (U63628.1) has 100% cover, 99% identity, and E-value of 0.0 with Uncultured fungus clone OTU_2778_62_32857 *18S ribosomal RNA gene*, partial sequence; *internal transcribed spacer 1*, complete sequence; and *5.8S ribosomal RNA gene*, partial sequence (KF221914.1). *Venturia inaequalis ribosomal RNA gene*, group I intron (U63626.1) has 100% cover, 97% identity, and E-value of 0.0 with Uncultured fungus clone OTU_2778_62_32857 *18S ribosomal RNA gene*, partial sequence; *internal transcribed spacer 1*, complete sequence; and *5.8S ribosomal RNA gene*, partial sequence (KF221914.1). *Venturia inaequalis ribosomal RNA gene*, group I intron (U63624.1) has 100% cover, 98% identity, and E-value of 0.0 with Uncultured fungus clone OTU_2778_62_32857 *18S ribosomal RNA gene*, partial sequence; *internal transcribed spacer 1*, complete sequence; and *5.8S ribosomal RNA gene*, partial sequence (KF221914.1). *Venturia inaequalis ribosomal RNA gene*, group I intron (U63627.1) has 100% cover, 97% identity, and E-value of 0.0 with Uncultured fungus clone OTU_2778_62_32857 *18S ribosomal RNA gene*, partial sequence; *internal transcribed spacer 1*, complete sequence; and *5.8S ribosomal RNA gene*, partial sequence (KF221914.1). *Venturia inaequalis ribosomal RNA gene*, group I intron (U63625.1) has 100% cover, 99% identity, and E-value of 0.0 with Uncultured fungus clone OTU_2778_62_32857 *18S ribosomal RNA gene*, partial sequence; *internal transcribed spacer 1*, complete sequence; and *5.8S ribosomal RNA gene*, partial sequence (KF221914.1).

Discussion

No hits and no significant hits for *V. inaequalis* sequences indicate that no alignments were found for these sequences under the set parameters of significance. No hits and no significant hits may indicate highly evolving or unique *V. inaequalis* genes.

Sequences and alignments from the *V. inaequalis* genes with any of the following: *18S ribosomal RNA*, *internal transcribed spacer 1*, *5.8S ribosomal RNA*, *internal transcribed spacer 2*, and *28S ribosomal RNA* are represented in the neighbor-joining tree in Figure 1. Ribosomal genes (18S, ITS 1, 5.8S, ITS 2, 28S) can be targets used for identification of fungal species (Dannaoui, 2009). A high number of bootstrap values, 31 out of a total of 68, were considered below the confidence interval, indicating that the neighbor-joining tree configuration is not definitive. According to this tree configuration and the BLAST results *V. inaequalis* genes in this category are related to ten different species: *Fusicladium eriobotryae*, uncultured fungus, *Fusicladium peltigericola*, uncultured pezizomycotina, uncultured Ascomycota, *Fusicladium mandshuricum*, *Venturia tremulae*, *Proventuria barriar*, *Patellaria atrata*, and *Lentinula lateritia*.

Venturia inaequalis actin (act1) gene, partial cds, *P. dennisii* strain CBS 631.68 *actin (act1)* gene, and *V. inaequalis actin* gene, partial cds (AF269254.1) align consistently from site number 373 to site number 826 of 904 with gaps. *P. dennisii* strain CBS 631.68 *actin (act1)* gene and *V. inaequalis actin (act1)* gene, partial cds (AY748993.1) are aligned consistently, with few misalignments, for all 904 sites with gaps. Genes coding for actin can be used for phylogenetic species recognition (Dannaoui, 2009). The alignments in MEGA indicate that the *V. inaequalis actin* genes are highly related to the *P. dennisii actin* gene. *Venturia inaequalis actin* gene with accession number AY748993.1 aligns better to the *P. dennisii actin* gene, indicating that it is more closely related.

Genes encoding for Cytochrome b can be used as targets for phylogenetic species recognition (Dannaoui, 2009). *Venturia inaequalis cytochrome b (cytb)* mRNA, mitochondrial gene encoding mitochondrial protein, complete cds (AF047029.1) and *Monilinia laxa* isolate MbhMF08-14B *cytochrome b (cytb)* mRNA, complete cds; mitochondrial (GQ423060.1) are close to fully aligned throughout all three genes except a portion at the beginning, a portion at the end, and a few scattered amino acids throughout the genes. In *V. inaequalis* resistance is conferred by a single point mutation in the *cyt b* gene, leading to an amino acid substitution at position 143 from glycine to alanine (Yin *et al.*, 2012). The high query cover, high identity, and low E-value indicate that *M. laxa* may also have an amino acid substitution at position 143 from glycine to alanine. This indicates that *M. laxa* may be resistant to, or will develop resistance to, QoI fungicides. Due to the high similarity between the *cyt b* gene of *V. inaequalis* and *M. laxa*, the later should be monitored for QoI resistance. *Venturia inaequalis RT-like protein* and *cytochrome b (cytb)* genes, mitochondrial genes encoding mitochondrial proteins, complete cds aligns to *Fusarium graminearum* strain CBS 139513 mitochondrion, complete genome with a high number of gaps, as the *F. graminearum* gene is much larger than the *V. inaequalis RT-like protein* and *cytochrome b* genes. The alignment does not indicate a strong relationship between the two genes. The BLAST results and the neighbor-joining tree indicate that *V. inaequalis*, *M. laxa*, and *F. graminearum* may react similarly to QoI fungicides.

Venturia inaequalis beta-tubulin genes and alignments are represented in Figure 2. The neighbor-joining tree contains only one bootstrap value of the four that is below the confidence level at 33%. The bootstrap values indicate that this neighbor-joining tree is rather stable and can be used with relative confidence to identify evolutionary relationships between the genes it contains. *Venturia inaequalis* isolate HIR-2 *beta-tubulin* gene, partial cds and *V. inaequalis*

isolate Ibaraki 1 *beta-tubulin* gene, partial cds are in the same clade. This clade is in a larger clade as *Botryotinia fuckeliana* T4 SuperContig_34_1 genomic supercontig. This indicates that these three sequences are highly similar. *Venturia inaequalis beta tubulin mRNA*, complete cds and *Cochliobolus sativus* ND90Pr hypothetical protein mRNA are in the same clade. This indicates that these two sequences are highly similar. VENBETATUB *V. inaequalis beta-tubulin* gene, complete cds and *Venturia tremulae* var. *tremulae* culture-collection CBS:693.85 *beta-tubulin* gene, partial cds are in the same clade. The BLAST results and neighbor-joining tree indicate that *V. inaequalis*, *B. fuckeliana*, *C. sativus*, and *V. tremulae* may interact similarly to Benomyl fungicides.

Venturia inaequalis translation elongation factor 1-alpha genes and alignments are represented in Figure 3. The neighbor-joining tree contains no bootstrap values of the six below the confidence level indicating that this phylogenetic tree is rather stable and can be used with confidence to identify phylogenetic relationships between the genes it contains. Genes encoding for translation elongation factor 1-alpha can be targets for fungal species recognition (Dannaoui, 2009). *Venturia inaequalis* isolate Ibaraki 1 *translation elongation factor 1 alpha* and *V. inaequalis* isolate HIR-2 *translation elongation factor 1 alpha (EF 1-a)* gene, partial cds share a clade. *Venturia minuta* culture-collection CBS:479.61 *translation elongation factor 1-alpha* gene, partial cds diverged to form a separate clade more recently and *F. catenosporem* culture-collection CBS:447.91 *translation elongation factor 1-alpha gene*, partial cds diverged to form a separate clade more distantly. *Venturia inaequalis* strain CBS 476.61 *translation elongation factor-1 alpha (TEF1)* gene, partial cds, *V. inaequalis* strain CBS 594.70 *translation elongation factor 1 alpha* gene, partial sequence, and *V. inaequalis* strain CBS 815.69 *translation elongation factor 1 alpha* gene, partial sequence form a clade the diverged more distantly. *Spilocaea pomi*

strain CBS 176.42 *translation elongation factor 1 alpha* gene, partial sequence and *G. conferta* strain CBS 191.53 *translation elongation factor 1 alpha* gene, partial sequence form a clade together that diverged most distantly.

Venturia inaequalis GAPDH genes and alignments are represented in Figure 4. Of the 15 bootstrap values, only three fall below the set confidence level. *F. eriobotryae* CAC31 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744568.1), *F. eriobotryae* CAC61 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744570.1), *F. eriobotryae* ST1 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744566.1), and *F. eriobotryae* E5 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744574.1) share a clade. This clade shares a larger clade with *V. inaequalis* CBS 813.69 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744579.1), *V. inaequalis* CBS 476.61 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744577.1), and *V. inaequalis* CBS 593.70 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744578.1). This indicates that these seven genes are very similar and the *V. inaequalis* GAPDH gene is similar to the *F. eriobotryae* GAPDH gene.

Spilocaea pomi CBS 180.47 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744581.1) and *C. sativus* ND90Pr hypothetical protein mRNA (XM_007706671.1) diver from the large original clade indicating that they are more distantly related.

Fusicladium eriobotryae E6 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744575.1 and *V. inaequalis* CBS 595.70 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, partial sequence (EU744576.1) share a clade with a confident

bootstrap of 100. This clade shares a larger clade with *V. inaequalis glyceraldehyde-3-phosphate dehydrogenase (gapdh)* mRNA, complete cds (EU873167.1) and this clade is more distantly related to the original clade.

The neighbor-joining tree with bootstrap consensus of *V. inaequalis ATP-binding cassette transporter* genes and alignments is represented in Figure 5. Of the five bootstrap values, only one falls below the set confidence level. This indicates that the neighbor-joining tree is rather reliable and can be used to identify phylogenetic species relationships with confidence. *Botrytis cinerea* B05.10 chromosome 1 and *A. oryzae* RIB40 DNA, SC010 share a clade. *Venturia inaequalis ATP-binding cassette transporter ABC3* gene and *V. inaequalis ATP-binding cassette transporter ABC1* gene share a larger clade with *B. cinerea* chromosome 1 and *A. oryzae* RIB40 DNA. This indicates that *V. inaequalis ABC1* and *ABC3* genes are evolutionarily similar to *B. cinerea* chromosome 1 and *A. oryzae* RIB40 DNA. *Neofusicoccum parvum* UCRNP2 putative abc transporter protein mRNA and *N. parvum* UCRNP2 putative abc multidrug transporter *mdr1* protein mRNA share a clade. *Venturia inaequalis ATP-binding cassette transporter ABC2* gene and *V. inaequalis ATP-binding cassette transporter ABC4* gene share a larger clade with the *N. parvum* genes. This indicates that *V. inaequalis ABC2* and *ABC4* genes are evolutionarily similar to *N. parvum ABC* genes. The BLAST results and neighbor-joining tree indicate that *V. inaequalis*, *B. cinerea*, *A. oryzae*, and *N. parvum* may develop new fungicide resistances in a similar manner.

The neighbor-joining tree with bootstrap consensus of *V. inaequalis CYP51* genes and alignments are represented in Figure 6. *Venturia nashicola* partial CYP51 gene for euburicol 14 alpha-demethylase, exons 1-3 (AJ314649.1) is in a clade that diverges from the *V. inaequalis*

CYP51 genes. The BLAST results and neighbor-joining tree indicate that *V. inaequalis* and *V. nashicola* may develop Azole fungicide resistance similarly.

Venturia inaequalis isolate MNH 135 *cellophane-induced protein 1 precursor (CIN1)* gene, complete cds and *V. pirina cellophane-induced protein 1 (Cin1)* gene, complete cds (JN159974.1) align with 158 consensus sites out of 677 total sites, this indicates that the two sequences are not highly similar.

Venturia inaequalis strain CBS 815.69 *RNA polymerase II largest subunit* gene, partial sequence (GU357756.1) and *V. populina* strain CBS 256.38 *RNA polymerase II largest subunit* gene, partial sequence (GU357769.1) align with 685 consensus sites out of 769 total sites, indicating that the two sequences are highly similar.

Venturia inaequalis strain CBS 594.70 *RNA polymerase II largest subunit* gene, partial sequence and *V. populina* strain CBS 256.38 *RNA polymerase II largest subunit* gene, partial sequence (GU357769.1) align with 19 consensus sites out of 254 total sites indicating that this *V. inaequalis* strain *RNA polymerase II largest subunit* gene is not highly similar to the *V. populina* *RNA polymerase II largest subunit* gene.

Venturia inaequalis cellophane-induced protein 1-like 2 (Cin1L2) gene, complete cds and *V. pirina cellophane-induced protein 1-like 2 (Cin1L2)* gene, complete cds (JN159976.1) align with 42 consensus sites out of 148 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis cellophane-induced protein 1 (Cin1) gene, complete cds and *V. pirina cellophane-induced protein 1 (Cin1)* gene, complete cds (JN159974.1) align with 78 consensus sites out of 598 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis alternative oxidase gene, complete cds and *P. tritici-repentis* Pt-1C-BFP alternative oxidase, mitochondrial precursor, mRNA (XM_001941903.1) align with 80 consensus sites out of 699 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis alternative oxidase (AOX1) gene, AOX1-A1 allele, complete cds and *P. fijiensis* CIRAD86 hypothetical protein mRNA (XM_007930318.1) align with 57 consensus sites out of 474 total sites, indicating that the two gene sequences are not highly similar.

Sequence 28329 from Patent EP2199304 and *V. nashicola* partial *CYP51* gene for eburicol 14 alpha-demethylase, exons 1-3 (AJ314649.1) align with 122 consensus sites out of 603 total sites, indicating that the two gene sequences are not highly similar.

Sequence 28329 from Patent EP2096177 and *V. nashicola* partial *CYP51* gene for eburicol 14 alpha-demethylase, exons 1-3 (AJ314649.1) align with 122 consensus sites out of 603 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis candidate effector 16 mRNA, complete cds and *O. sativa* Japonica Group cDNA clone:002-151-G12, full insert sequence (AK108837.1) align with 50 consensus sites out of 340 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis ribosomal protein L12 mRNA, complete cds and *A. oligospora* ATCC 24927 hypothetical protein mRNA (XM_011125321.1) align with 46 consensus sites out of 260 total sites, indicating that the two genes are not highly similar.

Venturia inaequalis small subunit ribosomal RNA gene, mitochondrial gene encoding mitochondrial RNA, partial sequence and *V. chlorospora* voucher Kruys 502 (UPS) small subunit ribosomal RNA gene, partial sequence; mitochondrial (DQ384084.1) align with 43 consensus sites out of 345 total sites, indicating that the two genes are not highly similar.

Venturia inaequalis ribosomal RNA gene, group I intron (U63628.1) and Uncultured fungus clone OTU_2778_62_32857 18S ribosomal RNA gene, partial sequence; *internal transcribed spacer 1*, complete sequence; and 5.8S ribosomal RNA gene, partial sequence (KF221914.1) align with 46 consensus sites out of 161 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis ribosomal RNA gene, group I intron (U63626.1) and Uncultured fungus clone OTU_2778_62_32857 18S ribosomal RNA gene, partial sequence; *internal transcribed spacer 1*, complete sequence; and 5.8S ribosomal RNA gene, partial sequence (KF221914.1) align with 102 consensus sites out of 155 total sites, indicating that the two gene sequences are somewhat similar.

Venturia inaequalis ribosomal RNA gene, group I intron (U63624.1) and fungus clone OTU_2778_62_32857 18S ribosomal RNA gene, partial sequence; *internal transcribed spacer 1*, complete sequence; and 5.8S ribosomal RNA gene, partial sequence (KF221914.1) align with 44 consensus sites out of 161 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis ribosomal RNA gene, group I intron (U63627.1) and Uncultured fungus clone OTU_2778_62_32857 18S ribosomal RNA gene, partial sequence; *internal transcribed spacer 1*, complete sequence; and 5.8S ribosomal RNA gene, partial sequence (KF221914.1) align with 60 consensus sites out of 163 total sites, indicating that the two gene sequences are not highly similar.

Venturia inaequalis ribosomal RNA gene, group I intron (U63625.1) and Uncultured fungus clone OTU_2778_62_32857 18S ribosomal RNA gene, partial sequence; *internal transcribed spacer 1*, complete sequence; and 5.8S ribosomal RNA gene, partial sequence

(KF221914.1) align with 45 consensus sites out of 163 total sites, indicating that the two gene sequences are not highly similar.

Conclusion

The classical approach to the control of pathogenic fungi includes use of chemical compounds that produce negative side effects like environmental pollution and development of fungicide resistance. *Venturia inaequalis*, the causal agent of apple scab, rapidly develops fungicide resistance and is known to be resistant to a high number of fungicides. The research in this paper identifies similar sequences to the 155 experimentally validated nucleotide sequences of *V. inaequalis* obtained from Thakur *et al.* 2013. Neighbor-joining trees were generated for six phylogenetically important genes, including four that are known to confer fungicide resistance.

Further Research

To further research the mechanisms of fungicide resistance in *V. inaequalis*, the full genome should be sequenced. The genes known to confer fungicide resistance should be monitored for change to elucidate the movement of fungicide resistance.

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Figures

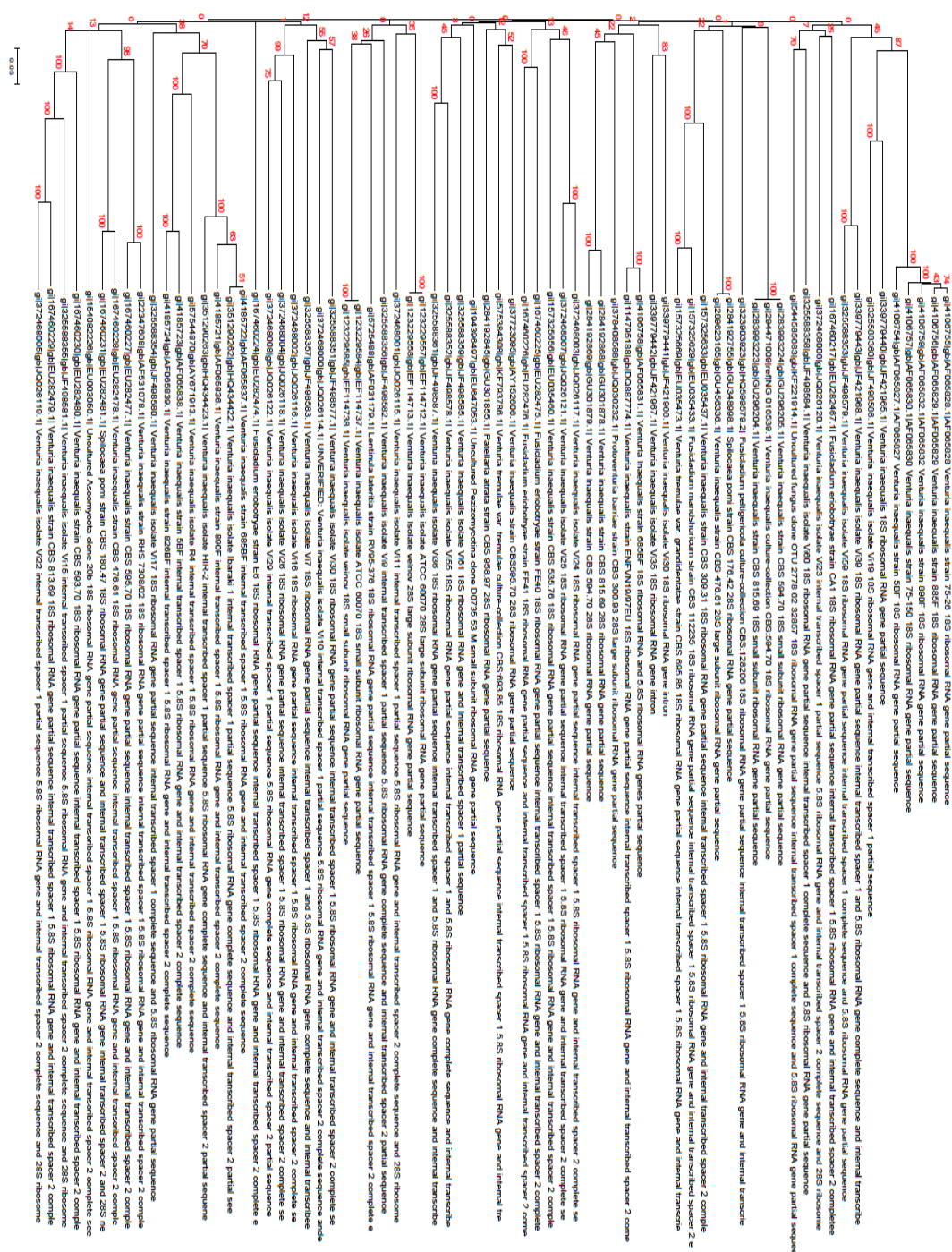


Figure 1. Neighbor-joining tree with bootstrap consensus of *V. inequalis* gene region 18S–28S with BLAST alignments.

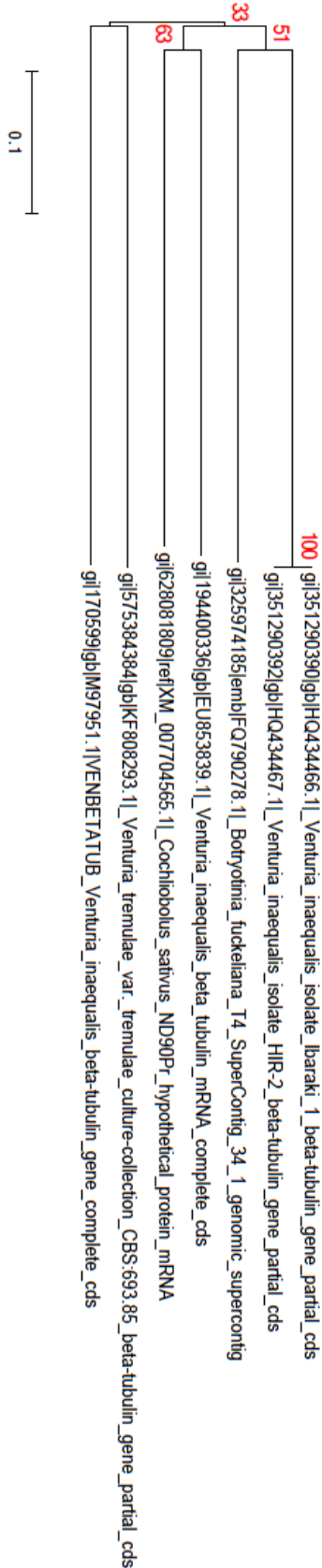


Figure 2. Neighbor-joining tree with bootstrap consensus of *V. inaequalis* beta-tubulin genes and BLAST alignments.

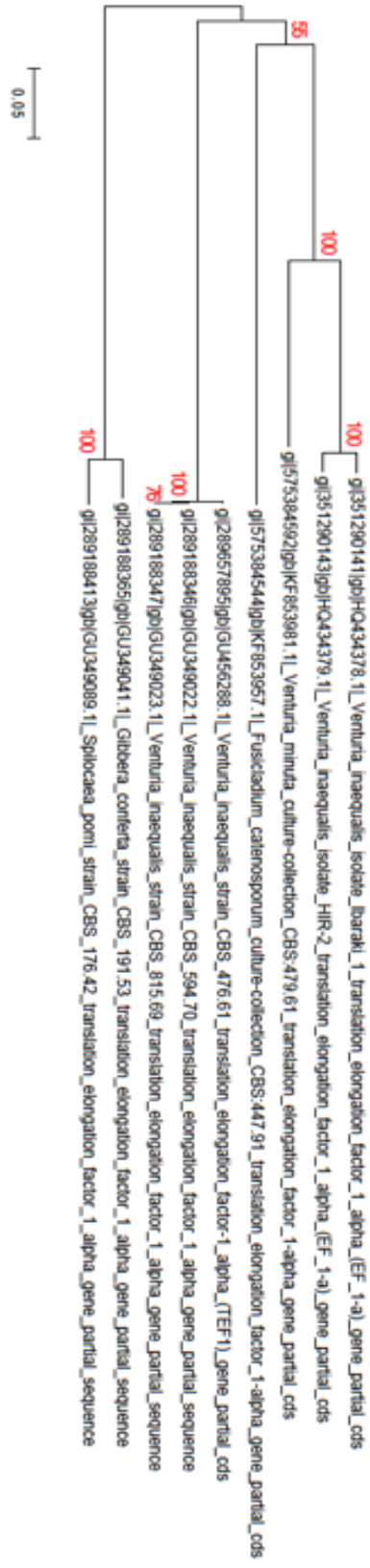
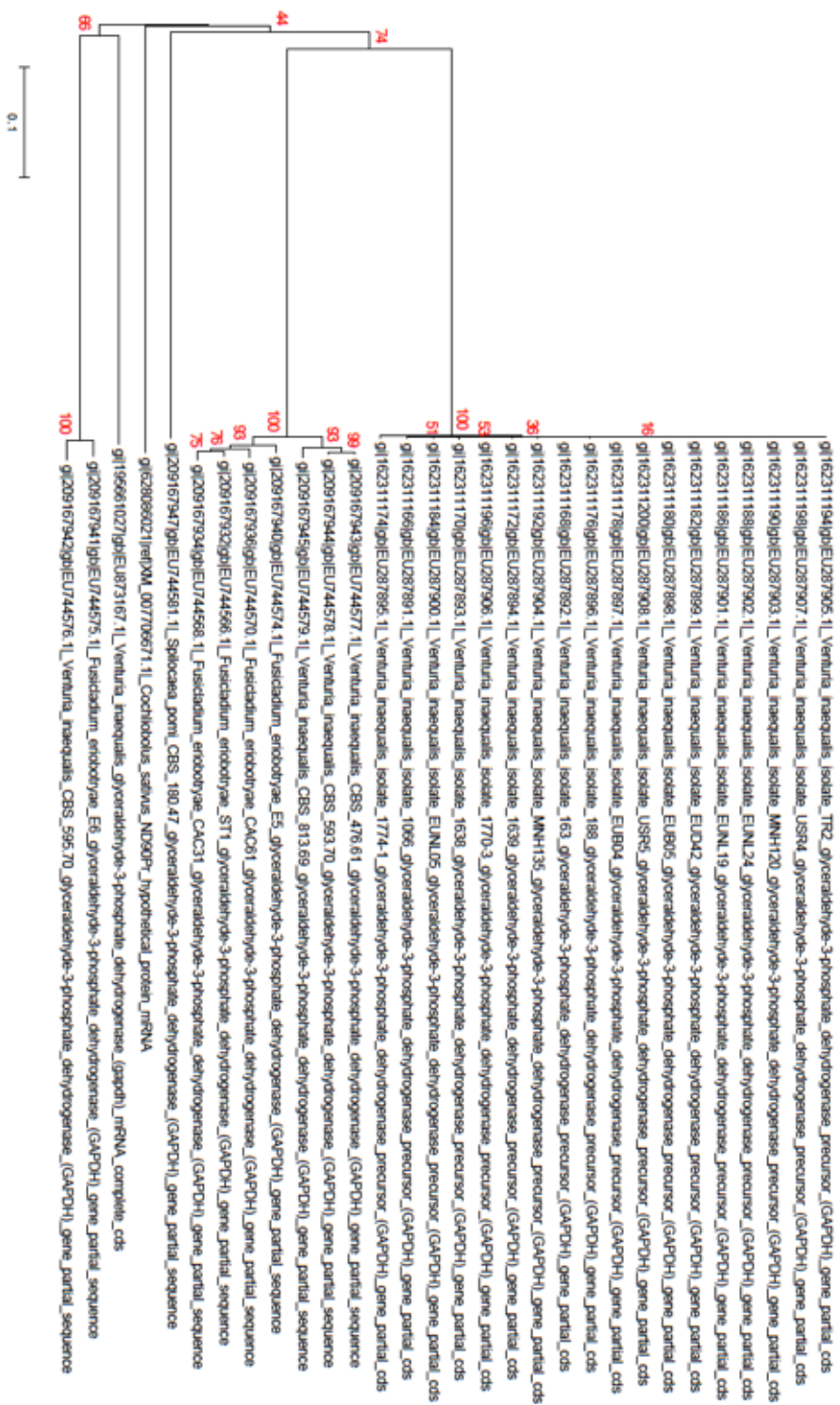


Figure 3. Neighbor-joining tree with bootstrap consensus of *V. inaequalis* translation elongation factor 1- α genes and BLAST alignments.



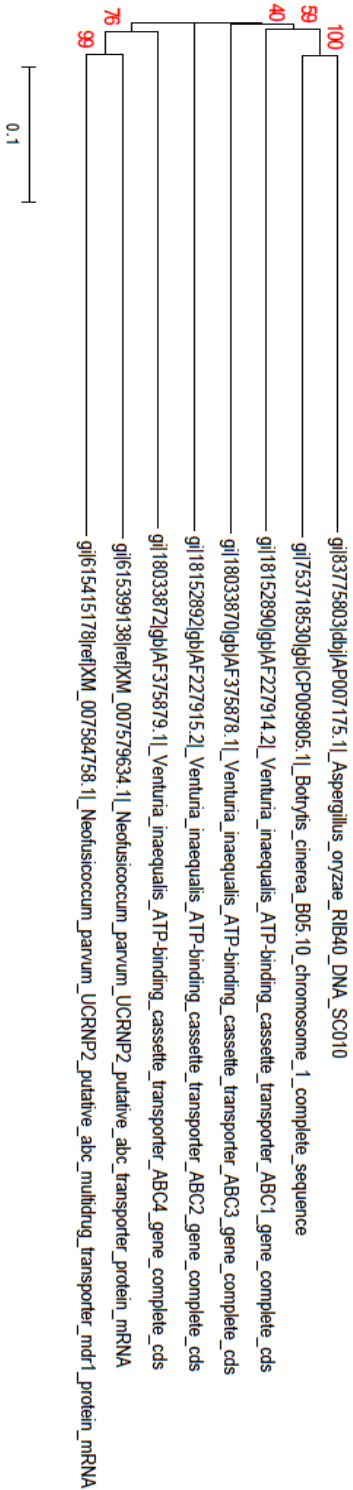


Figure 5. Neighbor-joining tree with bootstrap consensus of *V. inaequalis* ABC transporter genes and BLAST alignments.

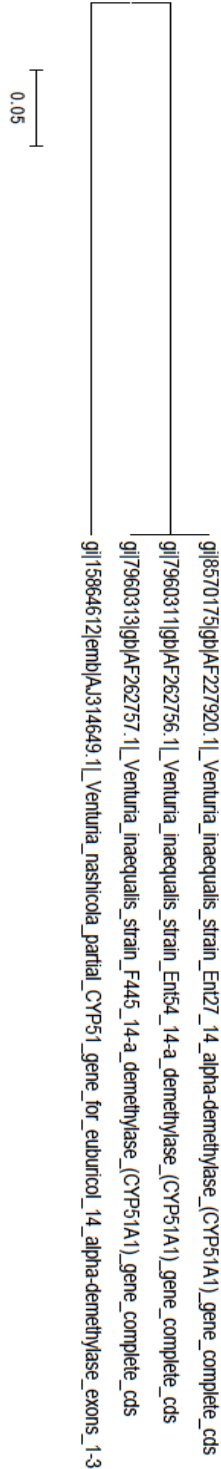


Figure 6. Neighbor-joining tree with bootstrap consensus of *V. inaequalis* CYP51 genes and BLAST alignments.