Lower Limb Dieback and Trunk and Scaffold Canker Diseases in California

Project No.: **15-PATH12-Trouillas**

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Objectives:

1. Survey almond orchards for trunk and scaffold canker diseases (TSCD), sample diseased trees and identify the main pathogens associated with TSCD
2. Determine the pathogenicity and aggressiveness on almond of the putative pathogens
3. Management of TSCD

Interpretive Summary:

Trunk and scaffold canker diseases (TSCD) of almond have become a major concern recently as they can affect young trees and are usually prevalent as orchards get older. Symptoms of TSCD include discoloration of vascular tissues, wood necrosis and extensive gumming. Dieback of scaffold branches may occur and eventually the whole tree can die. Canker diseases can also impact the longevity of trees in an orchard. Field diagnosis of TSCD has been challenging as symptom delineation among these diseases is not clear. Management strategies against TSCD rely for the most part on remedial surgery, cultural and prophylactic practices and no chemical or biological control practices are yet available to control TSCD.

Survey of orchards with TSCD were conducted in 2015 and 2016. The survey revealed a broad diversity of fungal pathogens associated with cankers including Botryosphaeriaceae spp., *Ceratocystis variospora*, *Eutypa lata*, *Cytospora* spp., *Collophora* spp., *Phomopsis/Diaporthe* spp., *Phytophthora* and Basidiomycete spp. A total of 292 isolates were isolated from cankers in almond and identified in this study. Botryosphaeriaceae spp.

accounted for the largest group of pathogens with *Spencermartinsia viticola, Neofusicoccum vitifusiforme, Neoscytalidium dimidiatum, Diplodia mutila* being reported for the first time in California almond. The main symptoms associated with Botryosphaeriaceae infections were cankers developing in the trunk or main scaffolds, and originating at pruning wounds made for the selection of primary and secondary scaffolds. Symptoms expressed in young (3-4 leaf) to mature trees and appeared as cankers and gummosis near pruning wounds. Ceratocystis infections were encountered throughout the state in orchards that suffered bark injury caused by mechanical shakers, suggesting that *Ceratocystis* is ubiquitous in California. Phylogenetic analysis of *Ceratocystis* isolates confirmed the occurrence of a single species in almond. Six new species of *Cytospora* were found and pathogenicity in almond of two species was shown *in vitro*. *Collophora hispanica* and *C. paarla* also were present in almond cankers in California. These pathogens have emerged as canker-causing pathogens on almond in Spain and Iran, and constitute first reports in California almond. The fungus *Eutypa lata* appeared to be rather common in cankers in the Sacramento Valley and Northern San Joaquin region and it is being reported for the first time in almond in California. *Phytophthora cinnamomi* was isolated for the first time in almond from aerial cankers in a young orchard in Kern County. Finally, we also report here common Basidiomycete fungi isolated from cankers, heart rot or wood decays. Pathogenicity tests of different isolates from all major pathogen groups are on-going in the field and green house. Results will determine the most aggressive fungal species to be included in our control studies. Fungicides and biocontrols will be screened in fall 2016 and winter 2017 to determine the most effective products for preventing infection of pruning wounds by these fungi. Detailed illustrations of the various canker diseases are provided in the present report.

Based on our results from the survey we conclude that of the TSCD present in California almonds Botryosphaeria canker, Ceratocystis canker, Eutypa dieback, Collophora canker and Cytospora canker seem to be the most prevalent. Detailed illustrations of these various diseases is provided in the present report. Further research is being conducted to investigate the biology of these pathogens and their control. On-going research on the pathogenicity of these isolates is being conducted and will provide answers about the potential aggressiveness of the different species. Results will determine fungal species to be included in our control studies. Fungicides and biocontrols will be screened in fall 2016 and winter 2017 to determine the most effective products for preventing infection of pruning wounds by these fungi.

Materials and Methods:

Surveys of trunk and scaffold canker diseases in California almond orchards and identification of pathogens. During 2015-2016, orchards with trees displaying symtoms of TSCD were identified. Portions of symptomatic branches, scaffolds or trunks were collected and taken to the laboratory for isolation on various media. Potato Dextrose Agar (PDA) was used for the isolation of true fungi, and a combination of selective media (PARP and V8 juice agar) for the isolation of *Phytophthora* spp. and *Ceratocystis* spp. Identification of pathogens associated with TSCD were carried out using cultural morphology and DNA sequence analyses. Amplification of the internal transcribed spacer (ITS) region of the rDNA was conducted using primers ITS1 and ITS4 for the identification of fungi. Species identifications were finalized using the nucleotide query algorithms BLAST in GenBank. Sequences of the ITS locus were aligned and phylogenetic analyses were run to represent a fungal genus or family. Reference sequences representing type specimens were included in the alignments and phylogenies were constructed using MEGA 7 software.

Pathogenicity of TSCD fungi on almond. *In vitro* pathogenicity tests have been initiated using 1-to-2-year old, lignified branches of almond. Branches were wounded using a cork borer and inoculated by placing a small agar plug with mycelium, ensuring contact of the fungal mycelium with the vascular tissues. Branches were incubated in moist chambers in the laboratory. After one-month incubation, vascular discoloration was measured and necrotic tissues were plated into PDA in attempt to re-isolate the inoculated fungi and complete Koch's postulates. The length of vascular discoloration and percent reisolation were used to determine the pathogenicity and aggressiveness of the various fungi. More pathogenicity tests are being set in the field.

Management of TSCD in vitro and in the field. Trials will be initiated in fall 2016 and winter 2017.

Results and Discussion:

The 2015-2016 survey of almond orchards for TSCD resulted in a total of 292 fungal isolates from cankers. The survey extended throughout the Central Valley region in 53 orchards across 13 counties (Colusa, Fresno, Glenn, Kern, Kings, Madera, Merced, Modesto, San Joaquin, Solano, Stanislaus, and Tulare). Fungi isolated from wood included: *Alternaria* spp. (4%), Botryosphaeriaceae spp. (22%), *Calosphaeria pulchella* (0.6%), *Ceratocystis variospora* (14%), *Chaetomium* spp. (1.7%), *Collophora* spp. (10%), *Cytospora* spp. (10%), *Phomopsis/Diaporthe* spp. (5%), Diatrypaceae spp. (3%), *Fusarium* spp. (5%), Basidiomycete spp. (6%), *Paecilomyces* spp. (2%), *Petriella sordida* (3%), *Phaeoacremonium* spp. (0.7%), *Phoma* spp. (2%), *Phytophthora* spp. (7%), and *Verticillium dahliae* (4%). *Verticillium dahliae* is not considered a canker-causing fungus, however the fungus does infect the wood and was included in this study to revisit the species diversity associated with Verticillium wilt. Based on previous research on canker diseases and the incidence of these fungi we believe that the most important and potentially threatening pathogens of almond include Botryosphaeriaceae spp., *Ceratocystis variospora*, *Collophora* spp., *Cytospora* spp., *Phomopsis/Diaporthe* spp., *Eutypa lata*, Basidiomycete spp. and *Phytophthora* spp.

Botryosphaeriaceae spp. constituted the majority of the pathogens isolated from cankers in this survey (60 isolates from 8 counties). Species included *Botryosphaeria dothidea, Neofusicoccum mediterraneum, N. vitifusiforme, N. arbuti, N. parvum, Diplodia seriata, D. mutila, Dothiorella iberica, Macrophomina phaseolina*, *Spencermartinsia viticola* and *Neoscytalidium dimidiatum*. Botryosphaeriaceae were found in Colusa, Solano, Merced, Fresno, Glenn, San Joaquin, Madera and Kern counties (total of 14 orchards). The main symptoms (**Figure 1**) associated with Botryosphaeriaceae infections were cankers in the trunk originating at pruning wounds made for the selection of the tree primary and secondary scaffolds. Symptoms expressed in young (3-4 leaf) to mature trees as gummosis near pruning wounds. Botryosphaeriaceae spp. have been reported as the causal agents of band canker which forms a band or ring of necrotic cambial tissue near growth cracks on the trunk or main scaffolds branches of vigorous almond cultivars. Symptoms of band canker are very distinct as gumballs form in a ring around the infected area. However, most of the Botryosphaeriaceae-associated diseases encountered during our 2015-2016 surveys did not resemble the classic symptoms of band canker. In contrast, infections with Botryosphaeriaceae appeared as perennial cankers characterized by discoloration of the wood of trunks and scaffold branches. This suggests a broader diversity of canker diseases resulting from infections by these fungi. Additionally, there was a high frequency of *Neoscytalidium* spp. isolated from cankers in young trees. *Neoscytalidium* is a recently emerging pathogen of table grapes, citrus, English walnut and figs in California. A phylogeny was constructed of the ITS locus for the Botryosphaeriaceae spp. (**Figure 2**). This phylogeny confirmed the large diversity of Botryosphaeriaceae species infecting almond (Inderbitzin et al. 2010). Of all the species found during this survey, *Neofusicoccum vitifusiforme, N. arbuti, Diplodia mutila,* and *Neoscytilidium dimidiatum* constitutes new reports for almond in California. Botryosphaeriaceae species are known to infect and cause disease on many other agricultural hosts in California including grapes, walnut, pistachio, olive, stone fruits and many ornamentals.

Ceratocystis variospora (formerly *Ceratocystis fimbriata*) was associated mainly with trunk cankers. Nearly all orchards visited, where trees had suffered bark injury caused by mechanical shakers, expressed symptoms of Ceratocystis canker. A total of 40 isolates were collected from Colusa, Merced, Fresno and Kern counties in 12 orchards. Symptoms consistent with Ceratocystis canker (**Figure 3**) included cankers on trunks near shaker damage. Occasionally, some infections were found in the scaffolds of trees near a pruning wound or cracked bark in poorly trained scaffolds. Gumming (gummosis) also accompanied infections; gum balls were found at the margin of the canker, which is diagnostic of Ceratocystis infections. Ceratocystis canker has long been known to occur in California almond orchards. Nevertheless, the great challenge of isolating this fungus in culture from symptomatic trees has rendered unsuccessful previous attempts to characterize the pathogen biology and associated disease. A re-investigation of this disease and its causal agent using modern molecular techniques is underway to understand the spread and population genetic diversity of *Ceratocystis variospora* in almond in California. One of the first steps in doing so is improving diagnostic and isolation capabilities of this pathogen from infected wood. A major outcome of our research is the development of a successful protocol for isolating *Ceratocystis* by using an extended disinfection period prior to incubation of diseased bark and cambium tissues in humid chambers. This method has made isolation and culturing much more efficient and has allowed us to accurately diagnose this disease. This improvement allowed us to conduct further studies on the pathogen biology and genetic diversity. Preliminary phylogenetic analyses of the ITS locus (**Figure 4**) indicate that all the isolates of Ceratocystis collected from almond in California are genetically uniform and consist of a single species (*Ceratocystis variospora*).

Collophora species represented 28 of the total isolates found in this survey. Isolates were obtained from 6 orchards in Kings, Fresno and San Joaquin counties. Symptoms appeared as dieback of twigs and branches as well as cankers originating from pruning wounds (**Figure 5**). *Collophora* species have become a fungal group of interest within the last several years as they have emerged as canker-causing pathogens and recently been reported on almond in Spain (Gramaje et al. 2012) and Iran (Arzanlou et al. 2016). The results of the phylogenetic analyses of the ITS locus (**Figure 6**) suggest that *Collophora hispanica* and *C. paarla* are present on almond in California. These constitute first reports in California almond.

Cytospora species represented 29 of the isolates collected from Modesto, Fresno, Stanislaus, and San Joaquin counties in 8 orchards. Symptoms of Cytospora canker (**Figure 7**) included longitudinal cankers in branches and scaffolds, often associated with pruning wound, vascular discoloration of the wood and moderate gumming. *Cytospora* species have traditionally been considered secondary to stress or injury in stone fruits, however their prevalence in cankers on almond suggest that this group constitutes virulent pathogens. Phylogenetic analysis of the ITS locus (**Figure 8**) revealed 6 new species of *Cytospora* on almond in California. Results of preliminary pathogenicity tests show that several isolates of *Cytospora* are highly virulent on almond. The same isolates were able to produce asexual fruiting bodies shortly (one month) after infection of twigs. This result suggests an abundance of inoculum of *Cytospora* spp. within almond orchards. Nevertheless, additional research is being carried out to determine the host range and possible inoculum sources of *Cytospora* species as these appear to emerge as severe new pathogens of almond.

Diatrypaceae species represented 10 isolates collected in the survey. Isolates were obtained from 5 orchards in Merced, San Joaquin, Fresno and Colusa counties. Symptoms associated with infection by Diatrypaceous fungi were cankers on trunks, branches or scaffolds, and moderate gumming surrounding a pruning wound or cracks at the tree crotch (**Figure 9**). Phylogenetic analysis of the ITS locus (**Figure 10**) identified *Eutypa lata*, *Diatrype stigma* and *D. oregonensis*. *Eutypa lata* is the main causal agent of Eutypa dieback of grapevine, apricot and sweet cherry, and diatrypaceous fungi have been well studied in California grapevines as canker pathogens (Trouillas et al. 2010; Trouillas and Gubler, 2010). *Eutypa lata* seems to have been overlooked in the past in almond in California and was found rather commonly in cankers in the Sacramento Valley and Northern San Joaquin region and it is reported for the first time in almond in California.

Phytophthora species represented 18 isolates collected in the survey. Isolates were obtained from 2 orchards in Merced and Kern counties. Symptoms of aerial Phytophthora are illustrated in **Figure 11**. Phylogenetic analysis of the ITS locus (**Figure 12**) identified two species, *Phytophthora cinnamomi* and *P. cactorum*. This is the first time *P. cinnamomi* is being reported on almond. *Phytophthora cactorum* was associated with symptoms typical of the aerial Phytophthora and included cankers in the trunk and scaffolds initiating at the tree crotch, often accompanied by profuse gumming (**Figure 11A-B**). *Phytophthora cinnamomi* was isolated for the first time in a young orchard in Kern County. Symptoms associated with this species consisted of a trunk canker initiating at the soil line and numerous amber-colored gumballs (**Figure 11C-D**). Both species have been reported on walnut in California, and result in a severe root and crown rot, trunk cankers and dieback (Mircetich and Matheron, 1983).

Basidiomycete species represented 18 isolates collected in the survey. Isolates were obtained from 5 orchards in Kings, Fresno, Colusa and Tulare counties. Symptoms associated with Basidiomycetes included heart rot and wood decay (*Ganoderma adspersum*) (**Figure 13**), cankers on tree trunks or scaffold branches (*Schizophyllum commune, Stereum hirsutum, Trametes versicolor*). *Chondrostereum purpureum* was associated with Silver leaf symptoms. Species phylogeny and diversity are illustrated in **Figure 14**.

Diaporthe (*Phomopsis*) species represented 14 isolates collected in the survey. Isolates were obtained from 8 orchards in Modesto, Kern, Colusa, Stanislaus and San Joaquin counties.

Isolates were found mainly in branches expressing symptoms of lower limb dieback (LLDB). This suggests that infection of the wood by these fungi may be secondary to a physiological or water stress thought to be responsible for LLDB. Cankers associated with *Phomopsis* were usually undefined, light in color and occurred in lower twigs or branches. *Diaporthe* species are known to cause *Phomopsis* dieback in grape, and similar species have emerged recently as pathogen within the walnut canker disease complex. Other species have been reported from cankers in other fruit and nut crops (Lawrence et al. 2015) but their ability to infect almond wood and cause cankers remains unclear. Phylogenetic analysis of the ITS locus (**Figure 15**) revealed that *Diaporthe eres*, *D. chamaeropsis* and a putative new species of *Diaporthe* constitute new reports in California almond.

Verticillium dahliae represented 11 isolates in this survey. Isolates were obtained from 2 orchards in Fresno and Madera counties. Verticillium wilt is not considered a canker disease on almond, however we have included it in this report as it is encountered in the wood and causes distinct vascular discoloration in branches and wilting of the leaves. Phylogenetic analysis of the ITS locus (**Figure 16**) found that all isolates were identical and consist of a single species (*Verticillium dahliae*).

Based on our results from the survey we conclude that of the TSCD present in California almonds Botryosphaeria canker, Ceratocystis canker, Eutypa dieback, Collophora canker and Cytospora canker seem to be the most prevelant. Detailed illustrations of these various diseases is provided in the present report. Further research is being conducted to investigate the biology of these pathogens and their control. On-going research on the pathogenicity of these isolates is being conducted and will provide answers about the potential aggressiveness of the different species. Results will determine fungal species to be included in our control studies. Fungicides and biocontrols will be screened in fall 2016 and winter 2017 to determine the most effective products for preventing infection of pruning wounds by these fungi.

Research Effort Recent Publications:

No publication at this time.

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Trouillas F.P., Urbez-Torres J.R. and Gubler W.D. 2010. Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. *Mycologia*, 102:319-336.

Figure 1: Symptoms of Botryosphaeria canker in almond

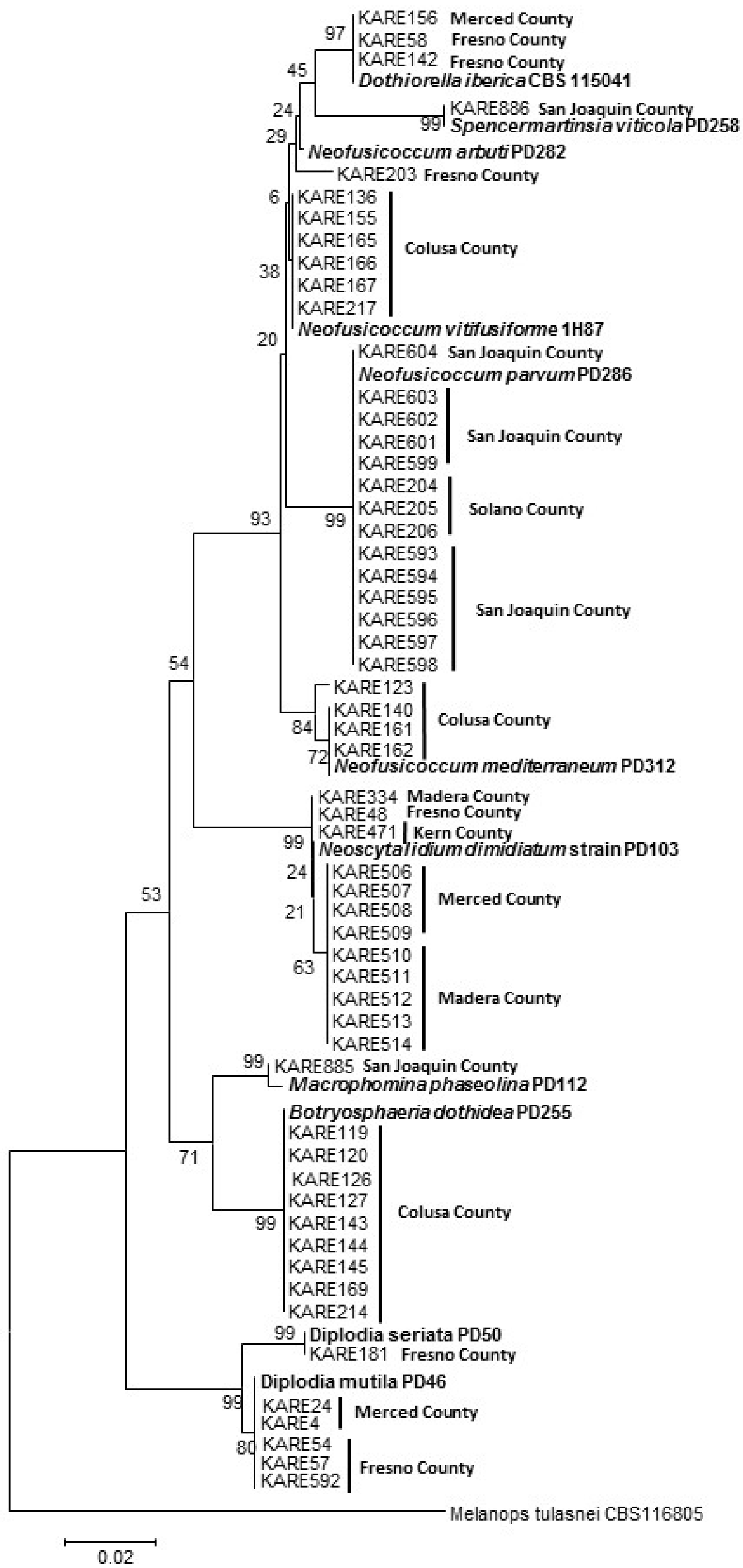

Figure 2: Phylogenetic diversity of Botryosphaeriaceae spp. associated with almond cankers

Figure 3: Symptoms of Ceratocystis canker in almond

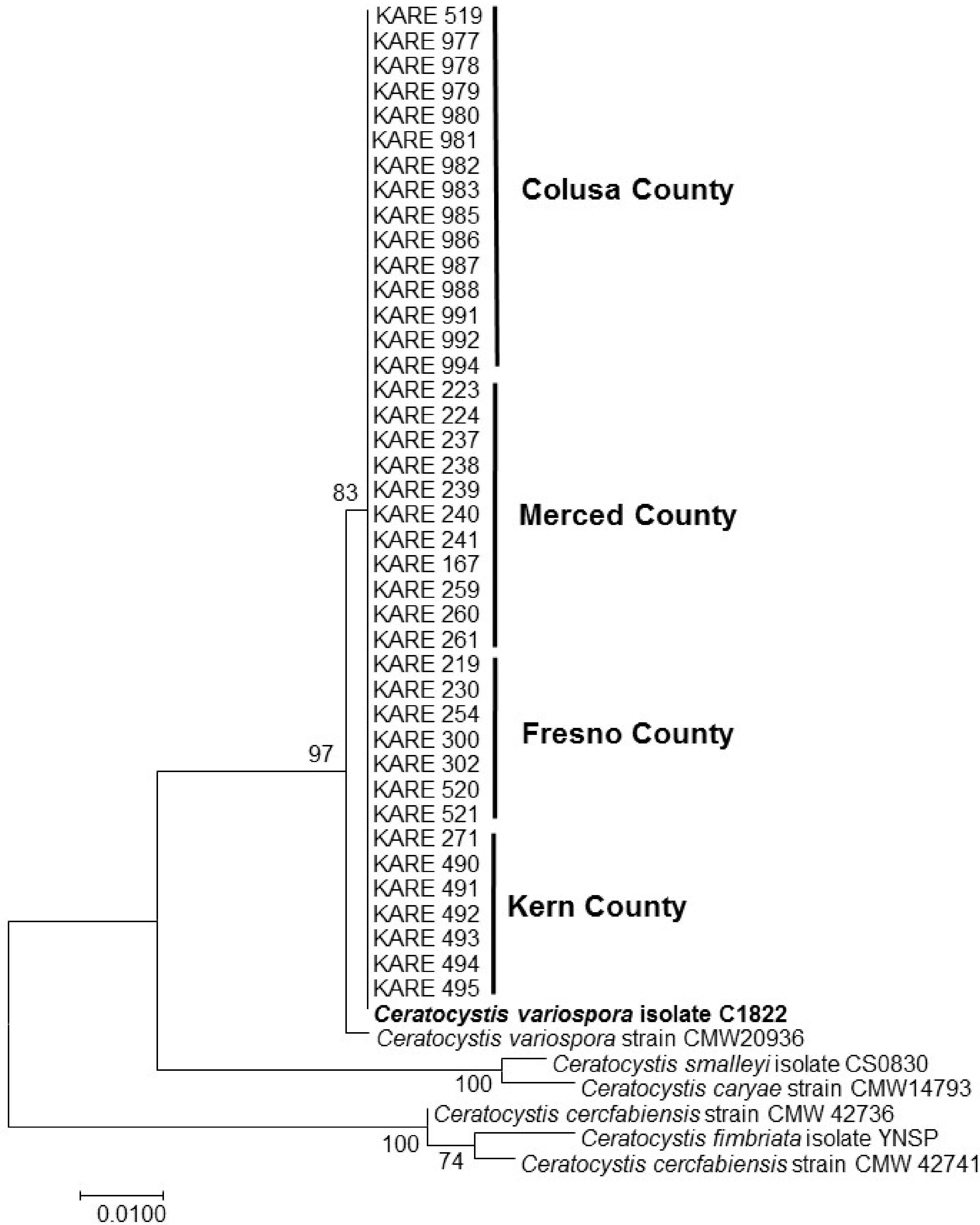

Figure 4: Phylogenetic diversity of *Ceratocystis* species associated with almond cankers

Figure 5: Symptoms of Collophora canker in almond

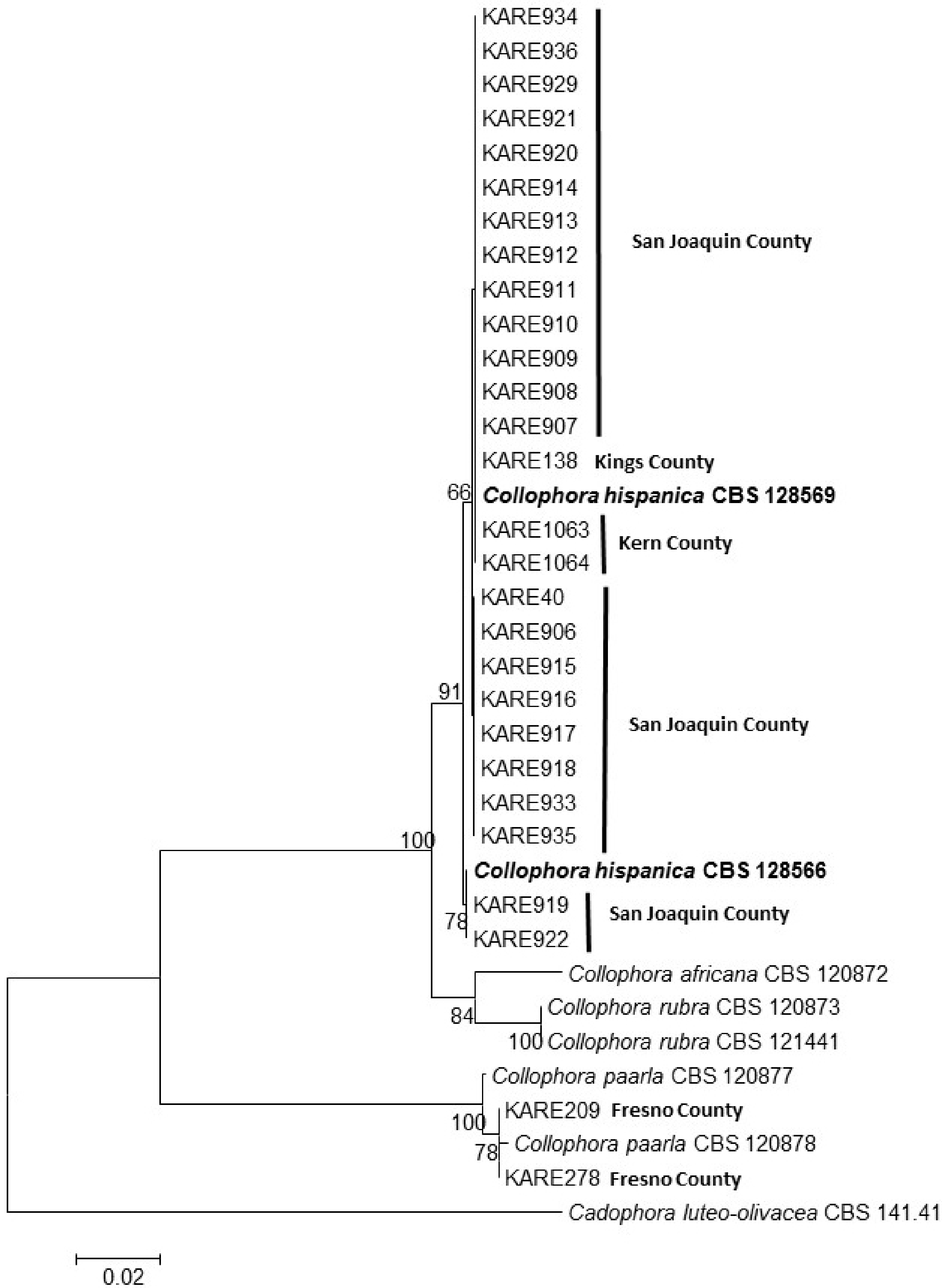

Figure 6: Phylogenetic diversity of *Collophora* spp. associated with almond cankers

Figure 7: Symptoms of Cytospora canker in almond

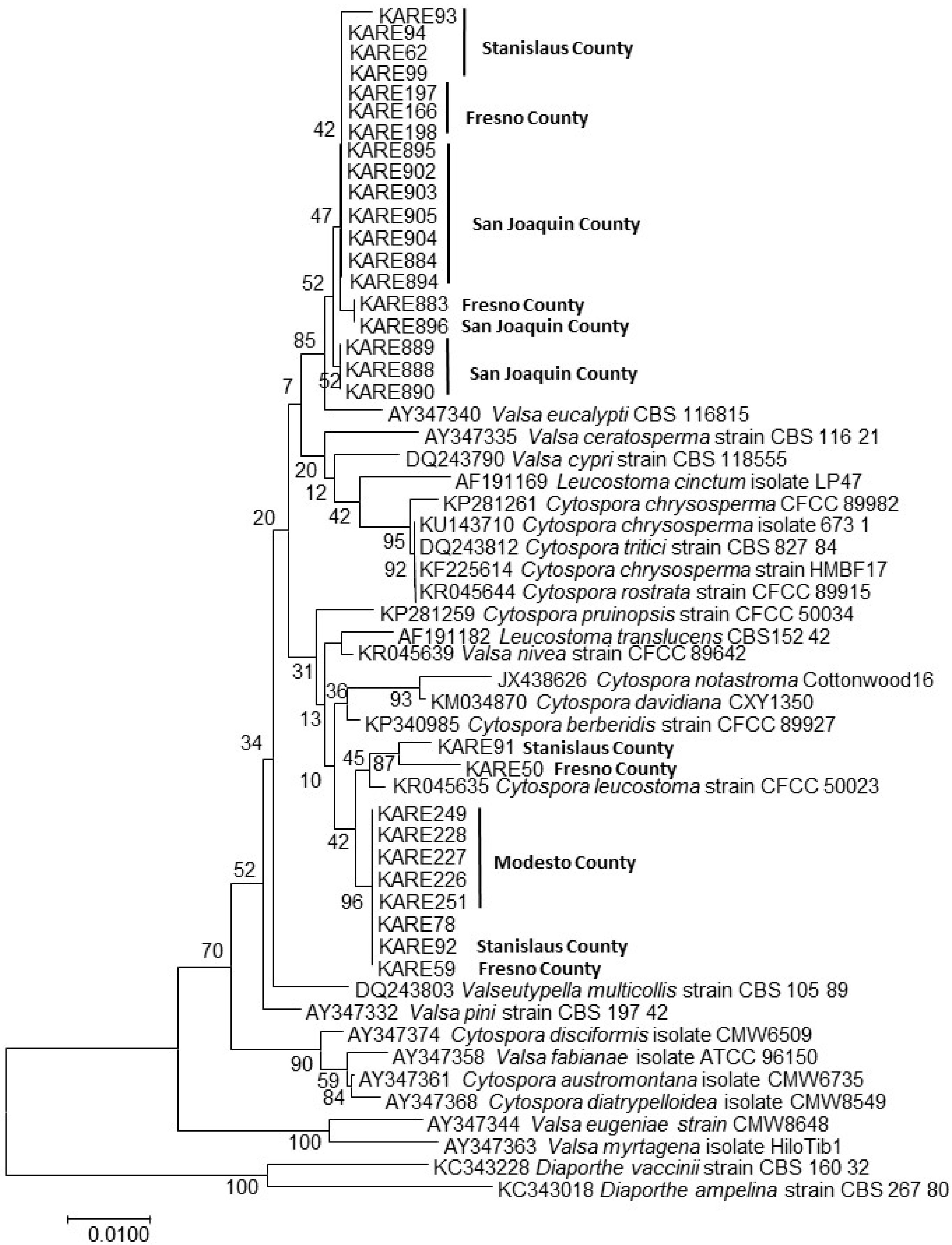

Figure 8: Phylogenetic diversity of *Cytospora* spp. associated with almond cankers

Figure 9: Symptoms of Eutypa dieback (a Diatrypaceous fungi) in almond

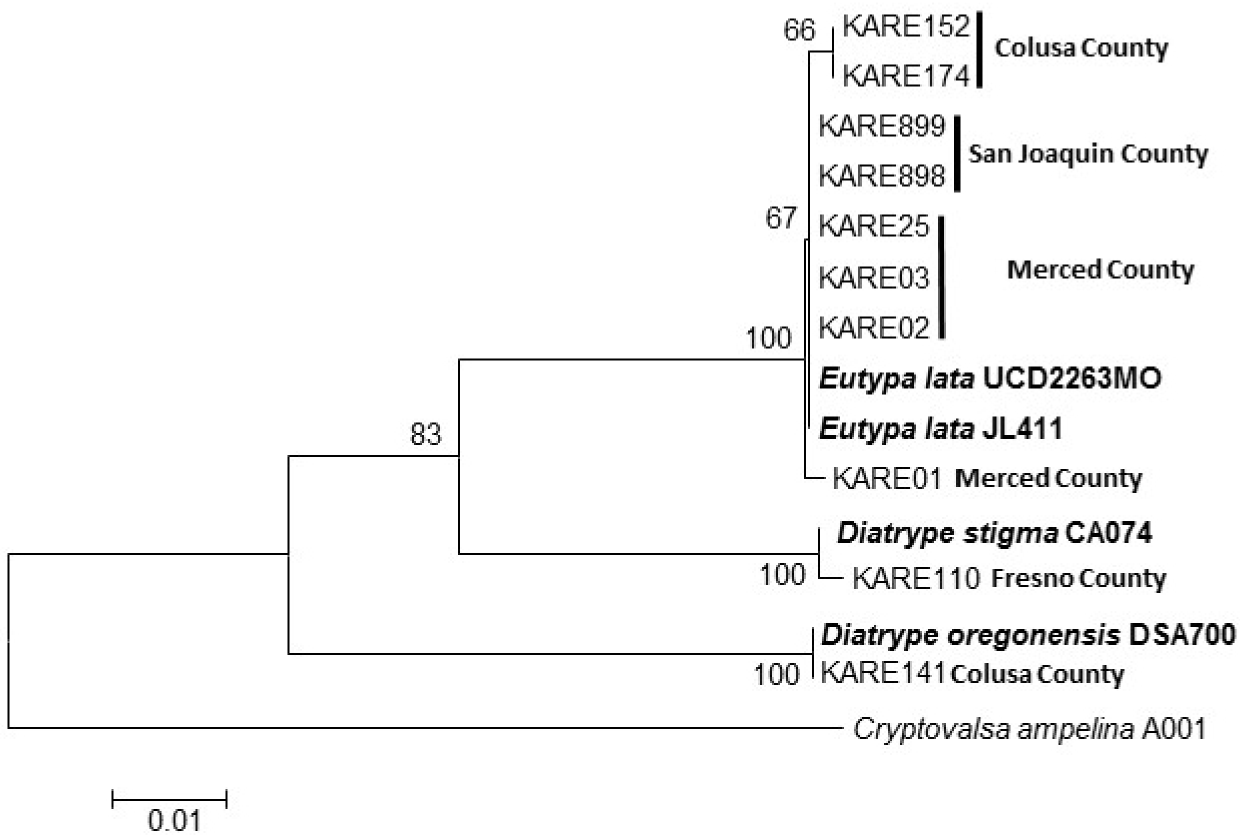

Figure 10: Phylogenetic diversity of Diatrypaceae spp. associated with almond cankers

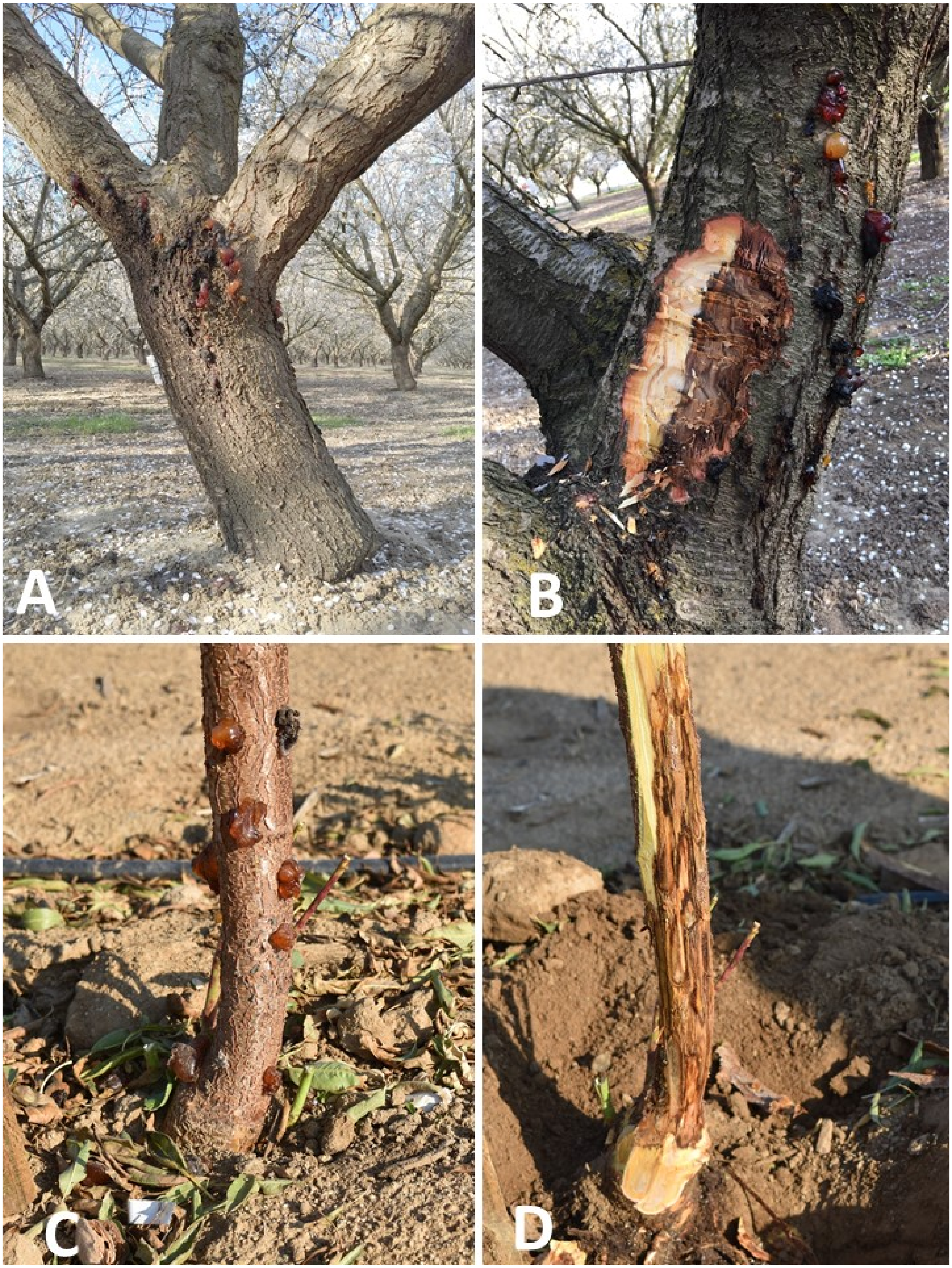

Figure 11: Symptoms of aerial Phytophthora in almond. **A-B:** Symptoms associated with *P. cactorum.* **C-D:** Symptoms associated with *P. cinnamomi*

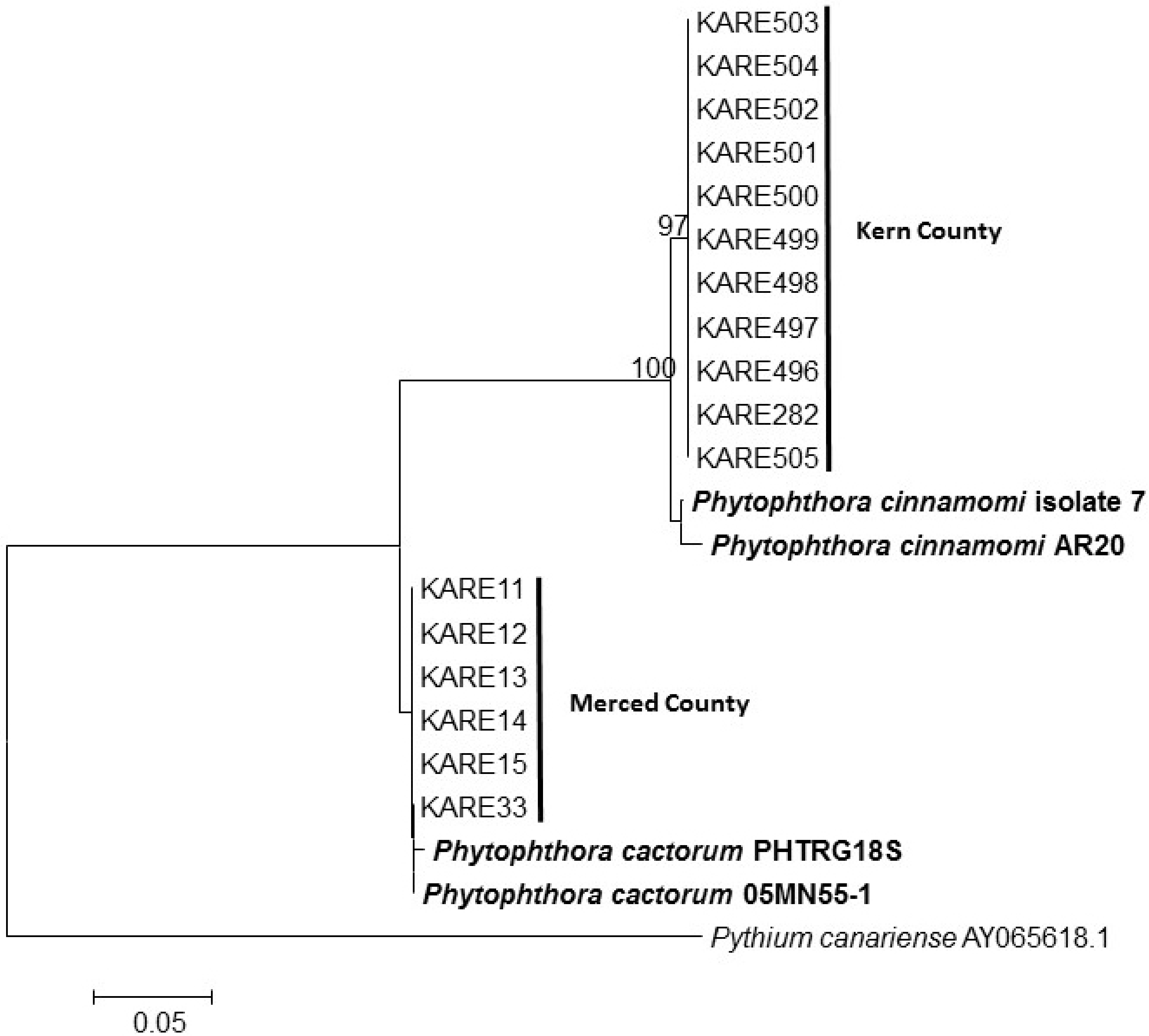

Figure 12: Phylogenetic diversity of *Phytophthora* spp. associated with aerial Phytophthora

Figure 13: Symptoms and signs of heart rot and wood decay in almond caused by *Ganoderma adspersum*

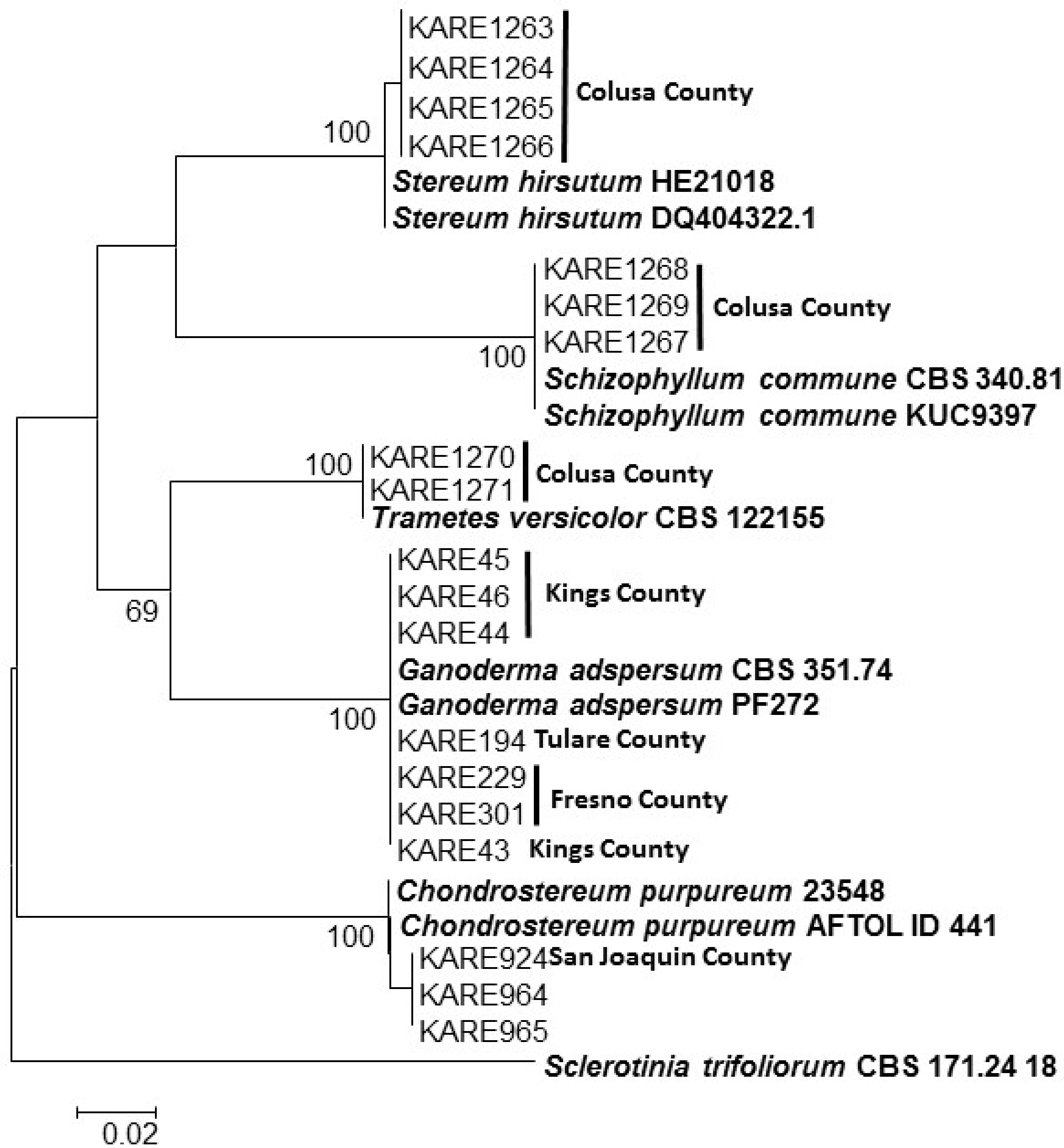

Figure 14: Phylogenetic diversity of Basidiomycete spp. associated with wood diseases in almond

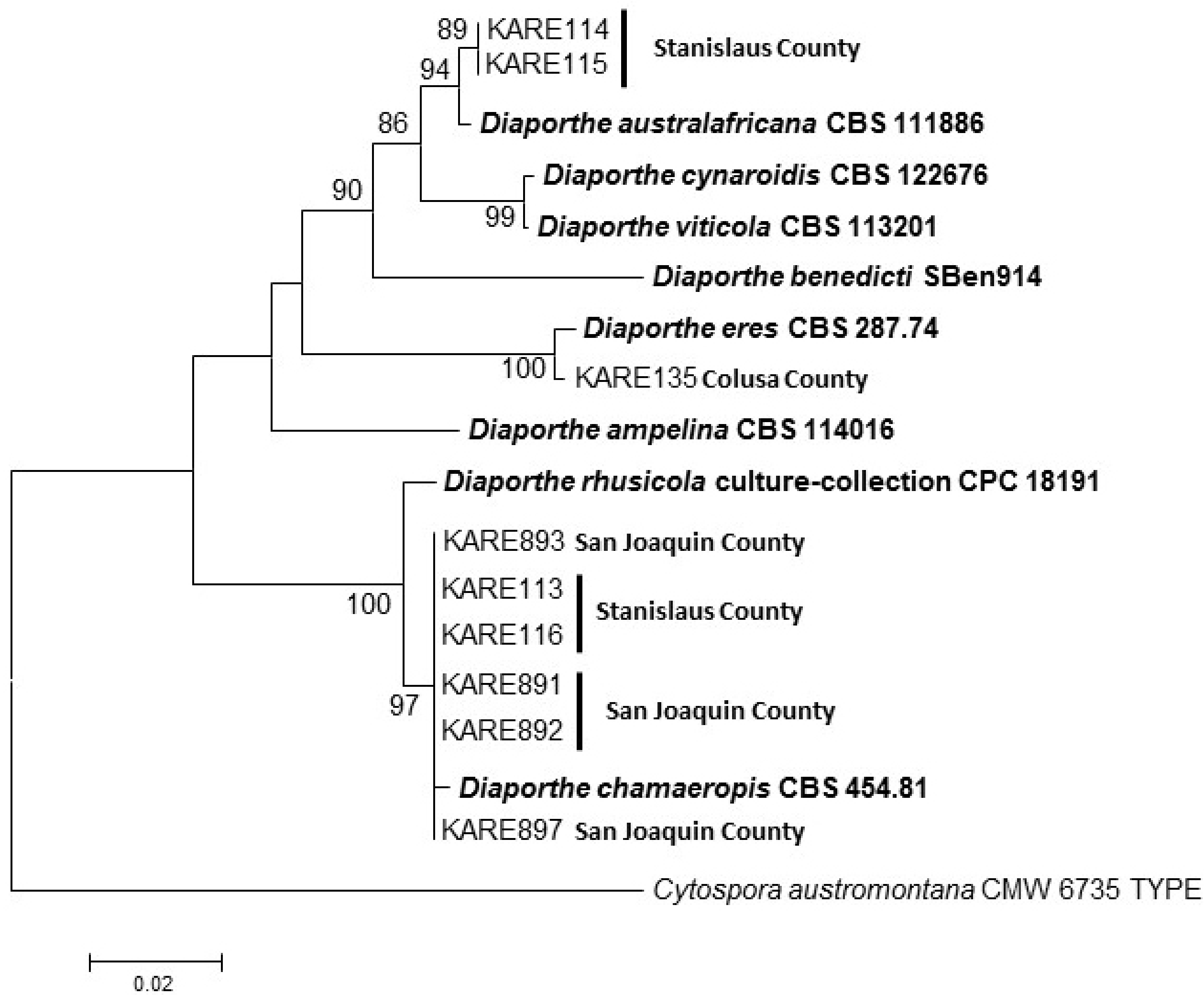

Figure 15: Phylogenetic diversity of *Phomopsis/Diaporthe* spp. associated with almond cankers

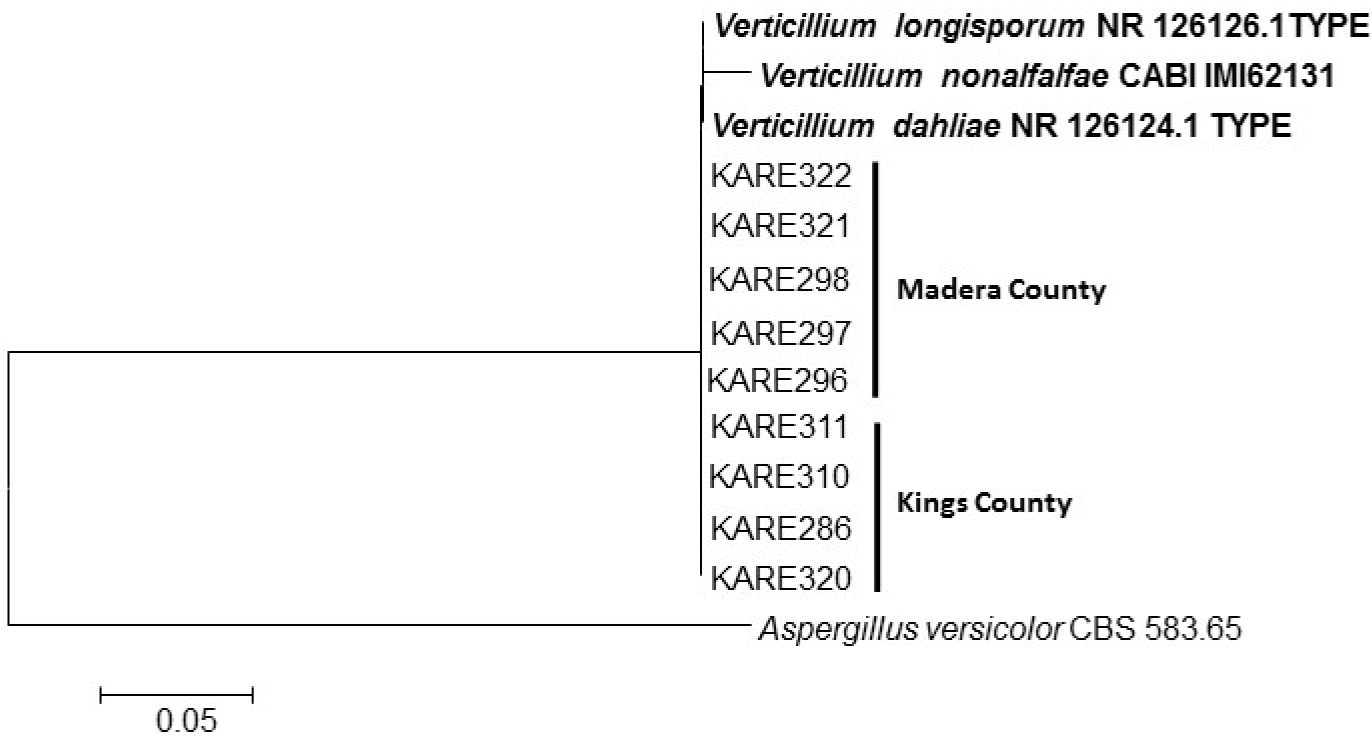

Figure 16: Phylogenetic diversity of *Verticillium* species associated with Verticillium wilt