Calosphaeria Canker of Sweet Cherry Caused by *Calosphaeria pulchella* in California and South Australia

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Abstract

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California is the second largest sweet cherry producer in the United States with annual revenues up to \$200 million. The South Australian cherry industry generates about 10% of Australia's overall production with approximately 1,500 metric tons of cherries produced yearly. In California, perennial canker diseases and subsequent branch dieback are responsible for extensive damage throughout sweet cherry orchards, reducing annual yields and tree longevity. Surveys of cherry orchards and isolation work were conducted in California to identify the main canker-causing agents. *Calosphaeria pulchella* was the main fungus isolated from cankers, followed by *Eutypa lata* and *Leucostoma persoonii*, respectively. Preliminary surveys in cherry orchards in

South Australia documented *C. pulchella* and *L. persoonii* in cankers. The pathogenicity of *C. pulchella* in sweet cherry was confirmed following field inoculations of 2- to 3-year-old branches. *C. pulchella* was able to infect healthy wood and produce cankers with as much virulence as *E. lata* or *L. persoonii*. Spore trapping studies were conducted in two sweet cherry orchards in California to investigate the seasonal abundance of *C. pulchella* spores. Experiments showed that rain and sprinkler irrigation were important factors for aerial dissemination. Finally, this study illustrates the symptoms and signs of the new disease *Calosphaeria* canker.

The California cherry industry currently produces about 25% of all sweet cherry (*Prunus avium*) grown in the United States, with 80,000 metric tons of cherries produced each year on approximately 12,800 ha (24,29,30). California produces the earliest sweet cherry crop in the United States as well as approximately 40 varieties of cherries. The California cherry industry is mostly represented by family-owned businesses, and current trends are for acreage increases and planting of new varieties.

The Australian cherry industry is a relatively small farming industry with approximately 485 growers distributed throughout the southern half of the country (12). The industry is concentrated in New South Wales, Victoria, Tasmania, and South Australia and has smaller production areas in Western Australia and Queensland. In 2010, the industry generated 15,243 metric tons of cherries on 2,845 ha according to the Cherry Growers of Australia Inc. (12). The gross value of the cherry industry for 2008–2009 was AU\$126 million (Australian Bureau of Statistics). The South Australian cherry industry counts approximately 120 growers and produces 10% of Australia's production (12). Most cherry plantings occur in the Adelaide Hills and Riverland areas.

In recent years, the incidence of dieback and canker diseases has increased significantly in the main cherry producing regions of California. Canker diseases generally constitute major threats to productivity by reducing tree health, yield, and longevity. Canker

diseases commonly develop in tree branches as necroses of vascular tissues (23). Water, as well as phloem movement, becomes increasingly diminished as functional conductive pathways are lost (3). Characteristic dieback symptoms appear when water demand exceeds the conductive capacity of the narrowing pathways (3). Despite the common occurrence of dieback in cherry trees in California, associated fungi have not been fully investigated. *Eutypa lata* (Pers.:Fr.) Tul. & C. Tul. (Syn: *E. armeniacae* Hansford & Carter) is known to be responsible for *Eutypa* dieback of sweet cherry (16), and since the first report of *E. lata* on sweet cherry in California, this fungus has been thought responsible for most dieback symptoms observed in cherry orchards. Nevertheless, the actual importance of *E. lata* and its contribution to the numerous wood cankers and branch dieback symptoms observed in California has not been determined. *Leucostoma* canker (=Cytospora or Valsa canker) caused by *Leucostoma persoonii* (Nitschke) Höhn. also is known to occur in stone fruits in California, but it is believed to be of relatively minor importance on sweet cherry (6). Other known pathogens of stone fruits in California include wood rotting and wood decay fungi, primarily in the Basidiomycetes (2).

In 2010, we first reported *Calosphaeria pulchella* (Pers.:Fr.) J. Schröt (anamorph: *Calosphaeriophora pulchella* Réblová, L. Mostert, W. Gams & Crous) to be associated with dieback of sweet cherry in California (27). However, much work remains to characterize the disease, incidence, distribution, and aggressiveness of *C. pulchella* in relation to other fungal pathogens of sweet cherry in California. *C. pulchella* has also been reported from decaying scaffold branches of peach trees (*Prunus persica*) in South Carolina; however, the type of decay produced by this fungus remained undetermined (1). The taxonomy of *C. pulchella* has been investigated using morphological and/or phylogenetic analyses (4,7,9,15,19,20). These studies have positioned *C. pulchella* in the order Calosphaeriales and have provided detailed descriptions and illustrations of its anamorphic and teleomorphic states.

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*The e-Xtra logo stands for "electronic extra" and indicates that Figures 1, 2, and 3 appear in color online.

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During the course of surveys for *Eutypa lata* and allied species in Australia (28), perithecia resembling those of *C. pulchella* were detected from old sweet cherry trees in the Adelaide Hills region of South Australia. The occurrence of *Calosphaeria* canker of sweet cherry in Australia has not yet been investigated, and overall, little is known about canker diseases of sweet cherry. In South Australia, *Cytospora leucostoma* (= *L. persoonii*) was reported to cause canker in *P. avium* (8), and there are no known reports of any other canker causing pathogens.

The objectives of this study were to (i) determine the incidence and geographic distribution of *C. pulchella* in sweet cherry cankers in California and evaluate the relative importance of *E. lata* and *L. persoonii*; (ii) ascertain if *Calosphaeria* canker also occurs in South Australia; (iii) verify the pathogenicity of *C. pulchella* in sweet cherry and compare its virulence to that of *E. lata* and *L. persoonii*; (iv) identify the main environmental conditions allowing spore dissemination and determine the seasonal quantity of *C. pulchella* spores in sweet cherry orchards in California; and (v) characterize and illustrate the disease caused by *C. pulchella* in sweet cherry.

Materials and Methods

Surveys and fungal isolates. A survey of Californian sweet cherry orchards began in San Benito County in April 2006 to identify the main pathogens associated with cankers. Subsequent sampling was conducted in Stanislaus County (May 2006), San Joaquin County (May to July 2006), Contra Costa County (September 2006), and Yolo County (August 2006 and July 2007). Two hundred samples were taken from 20 orchards (10 cankers per orchard) from all major sweet cherry growing regions in California. In December 2008, approximately five cankers per orchard were collected from three commercial sweet cherry orchards in the Adelaide Hills region of South Australia. Trees in a 25-year-old experimental cherry orchard of the South Australian Research and Development Institute (S.A.R.D.I.) were examined to detect the perithecia of *C. pulchella*.

In both locations, sampling consisted of sweet cherry branches (~15 to 150 mm diameter) exhibiting typical dieback symptoms (death, premature leaf senescence, and desiccation), external lesions, and cankers. Trees showing dieback were also inspected for the presence of perithecia of *C. pulchella*. Diseased branches with cankers were collected and were taken to the laboratory to proceed to isolations. Symptom characteristics including dieback and shape of cankers were recorded together with isolation results in order to recognize symptoms attributed to *Calosphaeria* canker.

For isolation work, the bark was removed and wood samples were surface sterilized by spraying them with 95% ethanol and briefly flaming. Six to eight fragments (~8 × 8 mm) from the margin between healthy and necrotic tissue were taken from each sample and placed onto petri dishes filled with potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 100 ppm tetracycline (PDA-tet) for isolation of fungi. Pure cultures of each fungal isolate were obtained by transferring single hyphal tips onto PDA-tet. Cultures were maintained in the laboratory and incubated at ambient laboratory light and temperature conditions.

Additional isolates of *C. pulchella* were isolated from perithecia on sweet cherry and/or nectarine trees in California, Australia, Italy, and France to be added to the phylogenetic analysis. Strains were obtained by placing 5 to 10 perithecia from a single stroma in a 1-ml Eppendorf tube containing 200 µl of sterile water. Perithecia were then crushed using a sterile pestle, and tubes were vortexed at maximum speed for 1 min. Subsequently, 200 µl of the resulting suspension was spread onto the surface of 85-mm PDA-tet plates and incubated at 24°C. Colonies typical of *C. pulchella* (20) were transferred as hyphal tips onto fresh PDA plates to obtain pure cultures for use in morphological and phylogenetic analyses.

Strains from California used in this study are maintained in our laboratory's culture collection in the Department of Plant Pathology of the University of California, Davis. Strains from South Australia are maintained at the South Australian Research and De-

velopment Institute. Isolates collected for this study are presented in Table 1.

Morphological characterization. *C. pulchella* isolates from California and South Australia were tentatively identified in accordance with earlier treatments (9,20). For the anamorph, colony morphology was observed 2 weeks following incubation at 24°C on PDA agar petri dishes maintained in the dark. The size, color, and shape of conidiophores, phialides, mycelia, conidia, and colony color were used for identification. For the teleomorph, morphology, stromata, and perithecia were examined using a Leica MZ95 stereo microscope. Slides were prepared to inspect microscopic characteristics using a Leica DMLB light microscope. All measurements were conducted in water at ×1,000 magnification with a 200-division eyepiece reticule. Photographs were taken using a Leica DFC480 digital camera.

DNA extraction, amplification, and phylogenetic analyses. Identification of the various isolates collected for this study was completed using polymerase chain reaction (PCR), amplification of the internal transcribed spacer region (ITS) of the rDNA, and comparison with reference sequences from GenBank. Total genomic DNA was obtained from mycelium of 2-week-old cultures using a DNeasy Plant Mini kit (Qiagen Sciences, MD) and following manufacturer's instructions. DNA amplification of the ITS region was conducted using primers ITS5 and ITS4 (32). PCR reactions were performed in a thermal cycler (PTC-100; MJ Research, Watertown, MA) as follows: 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min. Amplicons were purified using QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Sequencing (both strands) was done using a ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, CT) at the Division of Biological Sciences (DBS) sequencing facility at University of California, Davis.

Sequencing results were edited and assembled using Sequencher version 3.1.1. Sequences were aligned using ClustalW multiple alignment program (26). Phylogenetic analyses were performed with PAUP version 4.0b10 (25) using maximum parsimony (MP) with a heuristic search and 1,000 random addition sequence replicates. Alignment gaps were treated as missing data. Tree length, consistency index (CI), retention index (RI), and rescaled consistency index (RC) were estimated. Bootstrap support (BS) was evaluated using 500 replicates to test branch strengths. Reference ITS sequences of *C. pulchella* isolates from California were deposited previously into GenBank (HM237297 to HM237300) (27).

Pathogenicity tests. The first experiment was performed in December 2006 on mature sweet cherry trees (*P. avium* 'Bing') located at the research station of the plant pathology department of the University of California, Davis. Using three trees as replicates, 2- to 3-year-old branches were inoculated using nine isolates and one control per tree. Inoculated branches received a single isolate or control. Treatments consisted of three isolates of *C. pulchella* (LM06, DC04, SS07), three isolates of *L. persoonii* (DC06, IV01, LM07), and three isolates of *E. lata* (LM03D, DS09, IV09). Holes (5 mm diameter and 5 mm deep) were made in branches using a cork borer, and plugs of developing mycelium were inserted mycelium-side-down, sealed with petroleum jelly, and wrapped with Parafilm. Sterile blank plugs of PDA-tet were used as controls. Inoculated branches were harvested 14 months later and taken to the laboratory to be examined. The bark was removed and canker development was assessed by measuring the length of vascular discoloration extending from the point of inoculation, both acropetally and basipetally. Branches were then surface sterilized by spraying with 95% ethanol and flaming. Isolations from the edge of discolored tissue were conducted as described above to fulfill Koch's postulates. Statistical analyses of the extent of vascular discoloration were performed using analysis of variance (ANOVA) in JMP (Version 8.0; SAS Institute Inc., Cary, NC). Log transformations were performed when necessary to reduce heteroscedasticity. Tukey-Kramer HSD test was used to assess significant differences in the extent of vascular discoloration between the various treatment group means.

A second experiment was conducted in December 2008 at the Lenswood Research Station of S.A.R.D.I. located in the Adelaide Hills. In this experiment, we used 2- to 3-year-old branches (10 to 15 mm diameter) on mature sweet cherry trees (*P. avium* 'Vega').

Treatments included five isolates of *C. pulchella* (ACH02, ACP01, ACH05, ACP100, ACH01A) as well as three isolates of *E. lata* (ADSC300, LNI, LI). One branch on each of six trees (replicates) was inoculated with one isolate as described above. Control

Table 1. Isolates collected for this study and used either in the morphological, phylogenetic, or pathogenicity studies

Fungal species	Collection number	Host	Country	Collector / isolator
<i>Calosphaeria pulchella</i>	ACH01	<i>Prunus mahaleb</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	ACH02	<i>Prunus mahaleb</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	ACH05	<i>Prunus mahaleb</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	ACP10	<i>Prunus avium</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	CHG1	<i>Prunus avium</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	CHG2	<i>Prunus avium</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	CHG4	<i>Prunus avium</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	CHG6	<i>Prunus avium</i>	Adelaide Hills, South Australia	Florent Trouillas / Mark Sosnowski
<i>C. pulchella</i>	TOB5B	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	TOB7C	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	TOB9B	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	TOB12	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	2RE	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	3E	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	6E	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	8E	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	9E	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	12E	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	14E	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DS02	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DS03	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DS04	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DS05	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DS07	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DS11	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	RS01	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	RS02	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	RS03	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	RS07	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	RS010	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	SM04	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	GRP3	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	GRP4	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	SM05	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	SS02	<i>Prunus avium</i>	Stanislaus Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	SS07	<i>Prunus avium</i>	Stanislaus Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	WF02	<i>Prunus avium</i>	Stanislaus Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	WF03	<i>Prunus avium</i>	Stanislaus Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	WF05	<i>Prunus avium</i>	Stanislaus Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	LM01	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	LM03	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	LM04	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	LM05	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	LM06	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	LM07	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	LM08	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	MS04	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	MS05	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	MS06	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DC01	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DC03	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DC04	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DC12	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DC14	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	DC16	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	SS02	<i>Prunus avium</i>	Stanislaus Co., California, USA	Florent Trouillas / Jake Lorber
<i>C. pulchella</i>	NEC03	<i>Prunus persica</i>	San Joaquin Co., California, USA	Florent Trouillas
<i>C. pulchella</i>	NEC06	<i>Prunus persica</i>	San Joaquin Co., California, USA	Florent Trouillas
<i>C. pulchella</i>	NEC07	<i>Prunus persica</i>	San Joaquin Co., California, USA	Florent Trouillas
<i>C. pulchella</i>	NEC08	<i>Prunus persica</i>	San Joaquin Co., California, USA	Florent Trouillas
<i>C. pulchella</i>	CFSA100	<i>Prunus avium</i>	Adèche, France	Florent Trouillas
<i>C. pulchella</i>	CFMAL100	<i>Prunus avium</i>	Adèche, France	Florent Trouillas
<i>C. pulchella</i>	CIT100	<i>Prunus persica</i>	Tuscany, Italy	Florent Trouillas
<i>Leucostoma persoonii</i>	DC06	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>L. persoonii</i>	LM07B	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>L. persoonii</i>	IV01	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>Eutypa lata</i>	LM03B	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>E. lata</i>	IV09	<i>Prunus avium</i>	San Benito Co., California, USA	Florent Trouillas / Jake Lorber
<i>E. lata</i>	DS09	<i>Prunus avium</i>	San Joaquin Co., California, USA	Florent Trouillas / Jake Lorber

branches were inoculated with blank agar plugs. After 12 months, inoculated branches were removed from trees and returned to the laboratory for assessment of canker length and extent of fungal colonization. The bark of twigs was peeled off and the total length of vascular discoloration was measured and cumulated for acropetal and basipetal distances of necroses developing from the point of inoculation. Each branch was then surface sterilized in 2.5% sodium hypochlorite for 10 min and rinsed in sterile distilled water. Wood fragments (5 to 8 mm discs) were taken at every 10-mm interval up to 100 mm in both directions from the inoculation point and transferred to PDA plates. Following 7 to 10 days of incubation at 25°C under fluorescent light (Phillips TLD 36W/865 cool daylight) for 12 h each day, cultures were identified as *C. pulchella* and *E. lata* based on colony morphology. The furthest distance from the inoculation point that *C. pulchella* and *E. lata* was isolated, acropetal and basipetal, was recorded. All data were subjected to analysis of variance (Statistix for Windows v. 4.1, Tallahassee, FL). Differences among means for length of staining and extent of mycelium colonization were compared for all isolates using Tukey's test.

Spore trapping. Spore trapping studies were conducted to investigate the seasonal sporulation pattern and spore abundance of *C. pulchella* in California. Spore traps were placed in two separate sweet cherry orchards. The first orchard was located at the research station of the plant pathology department of the University of California, Davis (Yolo County) and was furrow-irrigated. The second orchard was located near Lodi (San Joaquin County) and was sprinkler-irrigated weekly during the growing season.

Experiments were conducted using microscope slides covered on both sides with a thin layer of petroleum jelly as described by Eskalen and Gubler (10). Ten spore traps were placed in each orchard. Two to three spore traps per sweet cherry tree were positioned in areas where perithecia occurred, usually near the old scaffold branches and upper trunk area. Spore traps were collected and changed weekly from October 2008 through August 2010. The number of spores per 10 slides was estimated weekly for both orchards as follows. Slides were collected in sterile 50-ml screw-cap tubes (Sarstedt, Inc., Newton, NC) and brought to the laboratory. Spores were detached from slides by adding 10 ml of distilled water into the screw-cap tube and shaking manually for 60 s. Consequently, 200 µl per spore trap of the resulting spore suspension were placed in a petri dish containing PDA-tet. The solution was then spread evenly over the surface of the agar using a sterile bent glass rod, and petri dishes were left open for 10 min in a laminar flow hood to dry the surface. Cultures were then incubated at ambient laboratory light and temperature conditions. The number of *C. pulchella* colonies was recorded after 14 days. Weekly records of precipitation averages were obtained from local weather stations (Adcon Telemetry, Metos, IPM).

Results

Field survey. *C. pulchella* was isolated from 95% of the orchards ($n = 20$) that were visited in California. Other pathogens commonly isolated from sweet cherry in California included *E. lata* and *L. persoonii*. Overall, *C. pulchella* was the most common fungal species isolated from cankers in nearly all counties and was recovered up to 100% ($n = 30$) from cankers collected in Contra Costa, 93% ($n = 15$) in Yolo, 57% ($n = 46$) in San Benito, 53% ($n = 70$) in San Joaquin, 37% ($n = 46$) in Stanislaus. *E. lata* was only more abundant in Santa Benito County and was recovered at 0, 27, 61, 29, and 24%, respectively, from the various counties. *L. persoonii* accounted for 3, 13, 26, 11, and 17% of all isolations from the respective counties. Additional putative pathogens isolated in California for this study included fungi in the Botryosphaeriaceae, *Collophora* spp., *Cryptovalsa ampelina* (Nitschke) Fuckel, *Phaeoacremonium* spp., and a few basidiomycetous fungi, mostly *Schizophyllum commune* Fries and *Trametes versicolor* (L.:Fries) Pilát. However, incidences of these fungi were too limited to be further investigated in the present study.

In the Adelaide Hills region of South Australia, *C. pulchella* was isolated from cankers in two out of the three commercial sweet cherry orchards that were visited. Of all cankers collected ($n = 15$), seven were infected with *C. pulchella* only, four with *L. persoonii* only, two with *Trametes versicolor* only. The last two cankers generated unidentified species of *Chaetomium* and *Chondrostereum*, respectively. *E. lata* was not isolated from any of the cankers collected in South Australia. Perithecia of *C. pulchella* were detected in the 25-year-old experimental orchard in Lenswood on diseased *P. avium* and *P. mahaleb* trees.

Symptoms and signs. Symptoms attributed to *Calosphaeria* canker included branch and main scaffold dieback (Fig. 1a and b) as well as internal cankers and vascular necroses. Cankers from which *C. pulchella* was isolated generally initiated around the pith and progressively invaded the xylem, cambium, phloem, and cortical tissues (Fig. 1c). In older scaffold branches, cankers usually developed gradually from the heartwood into the sapwood. *C. pulchella* also was isolated from wedge-shaped to irregularly shaped cankers. External symptoms of *Calosphaeria* canker were habitually less visible during the early stages of infection, particularly in large diameter branches, whereas older infections caused wilting of leaves. Signs of the disease consisted of perithecia of *C. pulchella* beneath the periderm of infected branches, scaffold branches, and trunks (Fig. 1d).

Morphological characterization. At 7 days, colonies of *C. pulchella* on PDA were pink to red in the center with white margins. Twenty-eight day old colonies became dark pink to dark red with moderate aerial mycelium (Fig. 2a and b). One isolate, identified as *C. pulchella* based upon 100% ITS sequence similarity with GenBank reference EU367451 (20), appeared light yellow in the center with a white margin (Fig. 2c). Overall, *C. pulchella* colonies appeared quite distinctive and could easily be separated from *L. persoonii* (Fig. 2d), *Phaeoacremonium* spp. (Fig. 2e and f), and *E. lata* also occurring in cankers. Hyphae of *C. pulchella* showed red pigmentation when examined with the light microscope (Fig. 3a). Conidiogenesis was typically phialidic, producing round, slimy conidial masses usually aggregated at the tip of the phialides (Fig. 3b and c). Conidia were hyaline, allantoid to oblong-ellipsoidal, (3–) 4–6 (–9) $\times$ 1.5–2 (–2.5) µm (Fig. 3d). Perithecia were non-stromatic, black, flask-shaped, with elongated necks and arranged in dense circinate groups beneath the bark (Fig. 3e). Ostioles were radially arranged and converged together toward a crack or stomatal opening in the periderm. Asci were unitunicate, club-shaped, 45–55 $\times$ 5–5.5 µm (Fig. 3f). Ascospores were allantoid to suballantoid, hyaline, 5–6 $\times$ 1 µm (Fig. 3f).

Phylogeny. The final alignment included 61 isolates of *C. pulchella* obtained from California, Australia, and Europe, nine sequences of allied species added from GenBank as well as *C. pulchella* isolate CBS 115999 (EU367451) (9,20). The ITS analysis contained 644 total characters, with 401 being constant, 93 parsimony-uninformative, and 147 parsimony-informative. The heuristic search produced one most parsimonious tree with 436 steps each (CI = 0.8165, RI = 0.7004, RC = 0.5719, and HI = 0.2492). The most parsimonious tree is shown in Figure 4. The analysis confirmed the identification of *C. pulchella* from California and Australia. All sequences of *C. pulchella* isolates from California, South Australia, and Europe shared 100% similarity with GenBank sequence of *C. pulchella* isolate CBS 115999 (ex-epitype) (9,20). Isolates of *C. pulchella* from *P. persica* also shared 100% similarity with isolates from *P. avium*. The clade consisting of *C. pulchella* isolates was strongly supported with 100% bootstrap value and clearly separated from the related species *Calosphaeria africana* Damm & Crous occurring in *Prunus armeniaca* and *P. salicina* in South Africa (9).

Pathogenicity tests. In the experiments in California, all three species tested were able to cause cankers and dark vascular streaking in sweet cherry branches. Shapes of cankers were quite similar among the various pathogens and varied from wedge-shaped to round-shaped in cross-sections. Most isolates tested were recovered at a significant distance from the inoculation points, and per-

centages of fungal recovery were highest for *C. pulchella* (100%), followed by *L. personii* (56%) and *E. lata* (33%). After 14 months, average vascular discoloration was 98 mm in branches inoculated with *L. personii* isolates, 59 mm in branches inocu-

lated with *C. pulchella* isolates, 50 mm in branches inoculated with *E. lata* isolates, and 20 mm in branches inoculated with agar-only control (Fig. 5). ANOVA revealed highly significant differences among the various treatment means ($P < 0.0001$). The mean of the

Fig. 1. Symptoms and signs of *Calosphaeria* canker. **a and b**, Typical branch dieback and leaf desiccation symptoms associated with *Calosphaeria pulchella* and visible during summer months. **c**, Longitudinal and transversal view of an active *Calosphaeria* canker likely to originate from infection of a pruning wound. Canker developed from the heartwood and later spread into the sapwood. **d**, Circinate groups of *C. pulchella* perithecia beneath the periderm of sweet cherry scaffold branch.

extent of vascular discoloration in branches inoculated with *L. persoonii* isolate DC06 was significantly higher than all the other treatments. Lesions produced by the isolates of *L. persoonii* (IV01, LM07), *C. pulchella* (DC04, LM06, SS07), and *E. lata* (LM03) were comparable in length and were significantly longer than those produced in the control according to Tukey-Kramer HSD (Fig. 5). Lesions caused by *E. lata* isolates (DS09, IV09) did not differ significantly from those of the agar-only inoculated control (Fig. 5).

In the South Australia experiment, all isolates of *E. lata* and *C. pulchella* were found to be pathogenic based on observations and mean comparisons. Branches had developed cankers as well as dark streaking 12 months following inoculation with *E. lata* and *C. pulchella*. Cankers produced by the two pathogens varied inconsistently from wedge-shaped to less regularly shaped when observed from cross-sections. Average lesion length in twigs inoculated with *E. lata* isolates (72 mm) and those inoculated with *C. pulchella* isolates (53 mm) were significantly higher than the average lesion length in branches inoculated with the control (11 mm) ($P = 0.0003$) (Fig. 6A). Average lesion length in branches inoculated with *E. lata* isolates LI was significantly the highest in this experiment. All the other *C. pulchella* and *E. lata* isolates produced lesions that were statistically similar in length and that distinguished from the control (Fig. 6A). Percentage of reisolations for *E. lata* isolates varied from 67 to 83% and was 100% for all *C. pulchella* isolates. Average distances of reisolation varied between 48 and 96 mm for *E. lata* isolates and between 65 and 83 mm for *C. pulchella* isolates (Fig. 6B). Maximum distances of reisolation were significantly longer than the respective length of vascular discoloration for all *C. pulchella* isolates, whereas they were somewhat equivalent for all the *E. lata* isolates (ADSC300, LI, and LNI).

Spore trapping studies. In the Davis orchard, *C. pulchella* spores were trapped from mid-fall to early spring, coinciding with

rain events (Fig. 7A). In year one, most spores were trapped during 24 to 31 October 2008 and during 7 to 14 January 2009. In year two, most spores were trapped during 30 November to 7 December 2009 and during April and May 2010.

In the Lodi orchard, *C. pulchella* spores were trapped during rain events from early fall and throughout the spring months (Fig. 7B). In this experiment, large amounts of *C. pulchella* spores were also captured during the summer months (early June through early September) and following overhead irrigation.

Discussion

This study identifies the fungus *C. pulchella* as a widespread pathogen of sweet cherry in California and reports its occurrence in South Australia. *C. pulchella* was the most common fungal pathogen isolated from sweet cherry cankers in nearly all counties surveyed in California, and *E. lata* appeared less common than previously believed. In the past, vascular cankers and branch dieback in sweet cherry in California have been mostly attributed to *E. lata* as the causal agent of Eutypa dieback (16), and occasionally to *L. persoonii* as the causal agent of Leucostoma canker (6). Leucostoma or Cytospora canker was found to be fairly common in sweet cherry orchards in California even though previous authors have suggested minor importance to this host (6). Additional fungi isolated in this study included *C. ampelina*, fungi in the Botryosphaeriaceae, *Collophora*, *Phaeoacremonium*, and a few Basidiomycetes species. However, the overall incidence of these fungal pathogens in active cankers was low, and they did not appear to constitute a major threat to the Californian cherry industry. Nevertheless, further studies are under way to investigate the taxonomy and phylogeny of these putative pathogens of sweet cherry in California.

Fig. 2. Colony morphology of different fungi isolated from cankers of sweet cherry on 85 mm potato dextrose agar dishes after 28 days incubation at 25°C in the dark. **a** and **b**, Typical colonies of *Calosphaeria pulchella*. **c**, Unusual colony of *C. pulchella*. **d**, Colony of *Leucostoma persoonii*. **e** and **f**, Colonies of *Phaeoacremonium* spp.

Fig. 3. Morphology of the anamorph and teleomorph of *Calosphaeria pulchella*. **a**, Red pigmentation of the hyphae. **b**, Conidiophores and phialides. **c**, Round conidial masses aggregated at the tip of the phialides. **d**, Hyaline, allantoid to oblong-ellipsoidal conidia. **e**, Nonstromatic, black, flask-shaped perithecia with elongated necks, arranged in dense circinate groups beneath the bark. **f**, Unitunicate, club-shaped asci with hyaline, allantoid to suballantoid ascospores. Scale bar in **a**, **b**, **c**, **d**, and **f** = 5 µm; in **e** = 500 µm.

Fig. 4. Most parsimonious tree with bootstrap values based on the complete sequence of the internal transcribed spacer (ITS) of the rDNA (436 steps, CI = 0.8165, RI = 0.7004, RC = 0.5719, and HI = 0.2492).

Pathogenicity of *C. pulchella* in sweet cherry was verified after inoculating branches with a plug of mycelium into an artificial hole. The fungus was capable of colonizing healthy wood tissues and subsequently produced cankers. Recovery rates and size of lesions associated with *C. pulchella* 12 to 14 months following artificial inoculations of branches were comparable with those of *E. lata* and *L. personii*. Dieback symptoms were not reproduced in our experiments, but the ability of *C. pulchella* to cause branch dieback was strongly suspected by field observations and subsequent isolation work. Extended incubation periods may be required in future experiments to better characterize expression of dieback symptoms. Similarly, the influence of environmental factors such as water stress on symptom expression and disease severity should also be investigated in future research.

Examinations of diseased sweet cherry trees during surveys revealed a common occurrence and high incidence of perithecia of *C. pulchella* beneath the bark of branches and trunks. On the other hand, asexual fruiting structures on trees were not identified. These observations led us to believe that ascospores may constitute the primary inoculum for this disease and that inoculum originates principally from within orchards. Inspections of cankered branches and isolation work also let us postulate that *C. pulchella* could initiate infection through pruning wounds and sunburn lesions as well as scars left by the abscission of leaves near the buds. Further research involving infection studies will be necessary to test these hypotheses.

In California, rainfall occurs typically during mid-fall (mid-October) throughout winter and early spring (March–April), whereas weather conditions usually remain dry during the summer season (from early June through September). The present study demonstrated the occurrence in the air of high spore concentrations of *C. pulchella* throughout the California rainy season. The aerial concentration of *C. pulchella* spores also was high during sprinkler irrigation events in the spring and summer months in Lodi. Perithecial fungi are known to release their ascospores in response to wetting caused by rain or irrigation, which are dispersed by wind or rain splashing (13). Spores of the closely related species *Togninia minima* (Tul. & C. Tul.) Berl. are forcibly discharged into the air in Esca-infected vineyards of California, mostly during rain periods in the winter and spring (22). In California, *E. lata* typically releases its ascospores following rainfall events from late fall to late

winter (17,18). Release and dispersal of spores of *L. personii* in California occur during conditions of rain and wind during all seasons (5). Other studies have shown the role of irrigation water in disseminating conidia of *Leucostoma cinctum* (Fr.) Höhn. in peach and cherry orchards (11). Overhead sprinkler irrigation also has been associated previously with spore release of various grapevine fungal pathogens in the Botryosphaeriaceae (31). Under the environmental conditions of the California Central Valley (low rainfall and hot summers), sweet cherry orchards are typically irrigated weekly throughout the spring and summer months. Sprinkler irrigation increases risks of wetting tree trunks and scaffolds, contributing significantly to the release of ascospores of *C. pulchella*. Drip irrigation or furrow irrigation are less likely to cause spread of ascospores. Alternatively, low-angle sprinkler heads and splitters could be used to avoid wetting the trunk and lower branches. If using a drip system, emitters should be placed at least a foot away from the trunk. Also, wounding (pruning) of woody crops during rains or during irrigation events generally should be avoided, as it usually increases the risks of infection with fungal pathogens (5,18,21,31).

Intensive cultural practices that have contributed to high productivity of the California cherry industry include high-density plantings, high-analysis fertilizers, and chemical control of diseases (2). The implementation of systematic pruning and repeated sprinkler irrigation also has constituted major changes in the traditional cultivation practices of sweet cherry in California (14). It has been suggested that this new intensive production system is responsible for the emergence of new disease, previously of little importance in California (2). Overall, the intensification of agricultural practices as well as plant stress provoked by excessive sprinkler irrigation and extensive pruning in sweet cherry may have contributed to the expression of Calosphaeria canker.

In the past, diagnosis for canker diseases has been particularly challenging due to the lack of knowledge on fungal pathogens associated with sweet cherry and the possible confusion of Calosphaeria canker with Eutypa dieback. Poor understanding of canker diseases has also hindered attempts to control them. As with grapevine canker diseases, most products were initially developed and field tested for control of *E. lata*, and recent research is aiming to protect grapevines against a broad range of taxonomically unrelated pathogens (21). Future research on the control of sweet

Fig. 5. Mean lesion length caused by *Leucostoma personii*, *Calosphaeria pulchella* and *Eutypa lata* isolates 14 months following inoculations into 2- to 3-year old twigs of sweet cherry 'Bing' located in the field at the research station of the plant pathology department of the University of California, Davis. Mean not represented with the same letter differ significantly according to Tukey's test ($P < 0.05$). Bars represent standard error of the mean.

Fig. 6. Results of the pathogenicity study performed between December 2008 and December 2009 using *Calosphaeria pulchella* and *Eutypa lata* isolates inoculated into 2- to 3-year old twigs of sweet cherry 'Vega' located at the Lenswood Research Station of the South Australian Research and Development Institute in the Adelaide Hills, South Australia. **A.** Mean lesion length 1 year after inoculation of various *C. pulchella* and *E. lata* isolates. Means not represented with the same letter differ significantly according to Tukey's test ($P < 0.05$). Bars represent standard error of the mean. **B.** Cumulated means (acropetal and basipetal) of distance of re-isolation from inoculation point 1 year after inoculation ($P < 0.0001$). Means not represented with the same letter differ significantly according to Tukey's test ($P < 0.05$). Bars represent standard error of the mean.

cherry canker diseases will need to incorporate all the various pathogens to develop appropriate control strategies and avoid unnecessary applications.

This study proved the occurrence of *C. pulchella* on *P. avium* in California and South Australia, but also identified this fungus in Italy and France. *C. pulchella* was also isolated from perithecia and cankers in nectarine trees (*P. persica*). The occurrence of *Calosphaeria* canker in other cherry producing countries as well as in additional *Prunus* spp. should be explored in future research in order to extend our knowledge on the distribution, epidemiology, and control options for this disease.

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Fig. 7. Number of *Calosphaeria pulchella* spores trapped weekly according to rainfall and/or sprinkler irrigation events in sweet cherry orchards located **A**, in Davis, Yolo County, CA, and **B**, in Lodi, San Joaquin County, CA.

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