A New Postharvest Fruit Rot in Apple and Pear Caused by *Phacidium lacerum*

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Abstract

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During surveys for postharvest diseases of apple and pear, an unknown postharvest fruit rot was observed in Washington State. The disease appeared to originate from infection of the stem and calyx tissue of the fruit or wounds on the fruit. An unknown pycnidial fungus was consistently isolated from the decayed fruit. Isolates from apple and pear were characterized and identified by molecular phylogenetic analysis and morphology. Pathogenicity of representative isolates on apple and pear fruit was tested under laboratory or field conditions. A BLAST search in GenBank showed that isolates differed from *Phacidium lacerum* and its synonym, *Ceuthospora pinastri*, by only 0 to 4 bp in sequences within part of the combined large ribosomal subunit + internal transcribed spacer + small ribosomal subunit regions. The phylogenetic analysis confirmed the taxonomic placement of the unknown fungus in the genus *Phacidium*, with the highest match being *C. pinastri* (formerly anamorphic *P. lacerum*) and with closely related taxa from GenBank forming congeneric clades. The fungus grew at 0 to 30°C and formed unilocular to multilocular pycnidial conidiomata on artificial media after approximately 5 to 7 days at room temperature. On potato dextrose agar incubated for a 12-h photoperiod, semi-immersed globose to subglobose pycnidial conidiomata were 250 to 1,000 µm in diameter (mean = 350), with 1 to 3

nonpapillate to slightly papillate ostioles and a buff conidial matrix. Conidia produced on phialides were 8 to 13 by 1.5 to 2.5 µm, hyaline, aseptate, cylindrical, with an abruptly tapered, typically slightly protuberant base, 2 to 3 guttules, and sometimes with a mucilaginous, flexuous, unbranched appendage which is attached to the apex of the conidium and disappears with age. Conidiogenous cells were flask shaped and 6 to 15 × 1.5 to 3 µm. Colony characteristics included felt-like aerial white mycelium, gray olivaceous at the center becoming greenish to colorless toward the margin, in concentric rings, with pycnidia forming in 5 to 7 days originating from the center of the plate. Morphological characteristics of the fungus had the greatest conformity with the description for *C. pinastri*. Based on molecular and morphological data, the fungus is identified as *P. lacerum*. ‘Fuji’ apple fruit and ‘d’Anjou’ pear fruit that were wounded, inoculated with representative isolates, and incubated at 0°C yielded the same symptoms as seen on decayed fruit collected from commercial fruit packinghouses. Stem-end rot, calyx-end rot, and wound-associated rot developed on fruit inoculated in the orchard after 3 months of cold storage. The fungus was reisolated from the diseased fruit. This is the first report of a fruit rot in apple and pear caused by *P. lacerum*. We propose *Phacidium* rot as the name of this disease.

Washington State leads apple and winter pear production in the United States. In 2011, apple and pear production in Washington grossed \$1.83 billion and \$186 million, respectively (National Agricultural Statistics Service 2012). Apple and pear fruit are harvested from August to early November, depending on the variety. At harvest, fruit are placed into either wooden or plastic bins and then transported immediately to storage and packing facilities. In Washington State, prior to cold storage, apple fruit are commonly drenched with a fungicide solution alone or in combination with the antioxidant diphenylamine for control of postharvest fruit rots and superficial scald (browning of apple fruit skin) (Xiao and Kim 2010). ‘D’Anjou’ pear is a major winter pear variety grown in the U.S. Pacific Northwest. D’Anjou pear fruit are either drenched prior to storage with a mixture of fungicide and the antioxidant ethoxyquin for decay control and scald prevention, or packed shortly after harvest, wrapped in paper impregnated with copper sulfate to prevent spread of decay, and stored in cardboard boxes (Kupferman 1998; Xiao and Boal 2004).

In storage, fruit are stored at –0.5 to 4°C in air or under controlled atmosphere conditions (1 to 2% O₂ and 0.5% CO₂), and may be stored for up to 8 months for d’Anjou pear and 12 months for apple, depending on the variety and volume of the fruit that need to be marketed (Meheriuk 1993). Because fruit may be stored for an extended period, postharvest diseases caused by various fungal pathogens can be a limiting factor to the long-term storage of fruit.

During surveys for postharvest diseases of d’Anjou pear during 2001 to 2002 and apple during 2003 to 2005, an unknown postharvest fruit rot was observed on apple and pear in Washington State (Kim and Xiao 2008; Xiao and Boal 2004). The disease appeared to originate from infection of the stem and calyx tissue of the fruit or wounds on the fruit. Lesions were light brown to brown and decayed tissues were spongy to firm. An unknown pycnidial fungus was consistently isolated from the decayed apple and pear fruit with symptoms described above. The objectives of this study were to (i) describe the symptoms and occurrence of this disease in storage, (ii) identify the cause of this disease, and (iii) test pathogenicity of the unknown fungus on apple and pear fruit.

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Materials and Methods

Symptoms and occurrence of the disease in storage. The unknown disease was initially observed during surveys of postharvest diseases on d’Anjou pear conducted in Washington State during fruit-packing operations during November 2001 and January 2002; details of the surveys have been previously described (Xiao and Boal 2004). Later, the same disease also was observed during surveys of postharvest diseases on apple conducted in Washington State during apple storage from June to August 2003 and from March to August 2004 and 2005; details of the surveys have been previously described

(Kim and Xiao 2008). In the pear survey, decayed d'Anjou pear fruit were sampled from 33 grower lots (each grower lot comprising fruit harvested from a given orchard) (Xiao and Boal 2004). In the apple survey study, decayed apple fruit were sampled from 26, 72, and 81 grower lots of 'Red Delicious', 'Golden Delicious', or 'Fuji' apple, respectively (each grower lot representing fruit harvested from a given orchard) (Kim and Xiao 2008). Symptoms and infection sites on decayed fruit were recorded, and isolations of fungi associated with the decayed fruit were conducted as previously described (Kim and Xiao 2008; Xiao and Boal 2004).

Fungal isolates. Ten isolates of the fungus were used throughout this study (Table 1). Isolates CLX1009 and CLX1010 were recovered from decayed d'Anjou pear fruit collected during surveys for postharvest diseases of d'Anjou pear in 2001 (Xiao and Boal 2004). The remaining isolates were recovered from decayed apple fruit collected during surveys for postharvest diseases of apple in 2003 to 2005 (Kim and Xiao 2008). All isolates were stored as mycelial plugs in 15% glycerol at -80°C . To obtain single-spore cultures of individual isolates, all isolates were revived on Boerema oat agar (BOA; Boerema et al. 2004) at room temperature (approximately 21°C) and under a 12-h photoperiod with a mixed spectrum of near-ultraviolet (NUV) and cool white fluorescent (CWF) light until the conidiomata had matured, indicated by cirrus oozing from the apex (approximately 3 to 5 weeks). Cirri from several pycnidia (approximately five to seven) were scraped from the medium with a sterile spatula and diluted in approximately 100 μl of sterile water. The diluted conidial suspensions were streaked onto potato dextrose agar (PDA; Difco Laboratories, Detroit) plates and incubated for 24 to 48 h at room temperature and under a 12-h photoperiod with a mixed spectrum of NUV and CWF light. Single-colony isolates were transferred onto fresh PDA and grown for approximately 3 to 5 weeks until the pycnidia were mature. Conidia were rinsed off with sterile water, centrifuged, rinsed twice with sterile water, and stored in 15% glycerol at -80°C .

Morphological characterization and culture studies. *Morphological characteristics.* To compare isolates with those from available literature, morphological traits and dimensions were observed and recorded on BOA according to Boerema et al. (2004). Mycelial plugs (1 cm in diameter) were removed from the margin of 7-day-old PDA cultures grown on PDA at 21°C under a 12-h photoperiod with a mixed spectrum of NUV and CWF light and plated onto BOA, malt agar (MA; Difco Laboratories), and PDA. All plates were then incubated at 21°C in the dark for 7 days, after which morphological, cultural, and color characteristics from the plates were recorded. Observations were once again recorded on day 14. Dimensions were measured at $\times 400$ and $\times 1,000$ using an Olympus BH2 microscope. Characteristics were compared with keys and descriptions in Nag Raj (1993). We also compared morphological characteristics of our isolates with those documented for all species in the clade encompassing our 10 isolates (CLX) in Crous et al. (2014).

Temperature studies. Mycelial growth at different temperatures of isolates CLX2095 and CLX3538 was evaluated on PDA. Two

replicates, each of three plates for each isolate, were inoculated with approximately 4-mm mycelial plugs removed from the margins of 10-day-old PDA plates. Inoculated plates were then sealed with Parafilm, wrapped in foil, and incubated in the dark at 0, 20, 30, or 37°C . Colony diameter on each plate was measured using an electronic caliper at 7, 9, 14, and 28 days, depending on how quickly an isolate reached the edge of the plate at a given temperature. Mycelial growth rates were calculated. This experiment was repeated.

Germination of conidia. Mature conidia from the conidiomata of a 5-week-old culture of isolate CLX3538 were scraped onto a sterile glass slide with a small amount of sterile water. A sterile cover slip was then placed over the conidia and the slide was placed into a petri dish sealed with Parafilm and stored at room temperature under a 12-h photoperiod. Conidia were checked for germination every 12 h.

Molecular phylogenetic analysis. *Production of mycelia and extraction of DNA.* Isolates were grown in yeast extract broth (Difco Laboratories) in deep-dish plates (8.0 cm deep and 10 cm in diameter; Pyrex) plates at 20°C under a 12-h photoperiod with a mixed spectrum of CWF and NUV light. After 3 weeks, the mycelium was harvested, rinsed three times with sterile distilled H_2O (dH_2O), centrifuged, and placed in a -80°C freezer for a minimum of 24 h. For DNA extraction, the mycelium was removed from the -80°C freezer and lyophilized for 48 h. Genomic DNA was extracted from the lyophilized mycelium using FastDNA SPIN Kit and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA). DNA was visualized under UV light in 0.8% agarose gels containing ethidium bromide at 0.5 $\mu\text{g}/\text{ml}$. Genomic DNA was diluted by 10 $\times$ with sterile dH_2O and stored at -20°C .

Polymerase chain reaction amplification conditions and phylogenetic analyses. DNA sequencing was performed for all isolates listed in Table 1. The nuclear ribosomal internal transcribed spacer (ITS) region (ITS ribosomal DNA, including ITS1, 5.8S, and ITS2 regions) was amplified using the primers ITS5 (5'-GGAAAGTAAAA GTCGTAACAAGG3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC3') (White et al. 1990). The small ribosomal subunit (SSU) region was amplified using the primers NS1 (5'-GTAGTCATATGCTT GTCTC3') and NS4 (5'-CTTCCGTCGAATTCCTTAAG3') (White et al. 1990). The large ribosomal subunit (LSU) region was amplified using primers LR0R (5'-ACCCGCTGAACCTTAAGC3') and LR5 (5'-TCCTGAGGGAAACTTCG3') (Vilgalys and Hester 1990). polymerase chain reaction (PCR) was performed with a final volume of 20 μl containing 14 μl of PCR Taq master mix (Qiagen Inc., Valencia, CA), 3 μl of sterile dH_2O , 1 μl of 10 ng of genomic DNA, and 1 μl of each primer using the Applied Biosystems Veriti Thermal Cycler under the conditions described in the literature (Vilgalys and Hester 1990; White et al. 1990). Amplicons were visualized under UV light in 1% agarose gels containing ethidium bromide at 0.5 $\mu\text{g}/\text{ml}$. PCR products were purified with QIAquick PCR purification columns (Qiagen Inc.) and then sequenced bidirectionally using the Sanger method (Eurofins MWG Operon, Huntsville, AL) with internal primers ITS2 for the ITS region, LR16 and LR3R for the LSU region, and NS3 for the SSU region (De Hoog et al.

Table 1. Details of isolates of *Phacidium lacerum* used in this study

Culture ^b	Collection date	Geographic origin	Host (fruit)	GenBank accession number ^a		
				ITS	LSU	SSU
CLX1009	11 December 2001	Peshastin, WA	D'Anjou Pear	KF493735	KF493745	KF493755
CLX1010	11 December 2001	Peshastin, WA	D'Anjou Pear	KF493736	KF493746	KF493756
CLX2095	14 July 2003	Chelan, WA	Red Delicious Apple	KF493737	KF493747	KF493757
CLX2357	3 July 2003	Chelan, WA	Red Delicious Apple	KF493738	KF493748	KF493758
CLX2362	5 August 2003	Chelan, WA	Red Delicious Apple	KF493739	KF493749	KF493759
CLX2379	3 July 2003	Wenatchee, WA	Red Delicious Apple	KF493740	KF493750	KF493760
CLX2406	5 August 2003	Manson, WA	Fuji Apple	KF493741	KF493751	KF493761
CLX2710	18 April 2004	Manson, WA	Red Delicious Apple	KF493742	KF493752	KF493762
CLX3538	3 November 2004	Manson, WA	Red Delicious Apple	KF493743	KF493753	KF493763
CLX3539	3 November 2004	Manson, WA	Red Delicious Apple	KF493744	KF493754	KF493764

^a ITS = internal transcribed spacer, LSU = large ribosomal subunit, and SSU = small ribosomal subunit regions.

^b Culture number. All isolates are maintained at the Tree Fruit Research and Extension Center, Washington State University, Wenatchee and at the United States Department of Agriculture–Agricultural Research Service Plant Introduction, Washington State University, Pullman, WA.

2005; Moncalvo et al. 2000; White et al. 1990). Sequences were edited using Sequencher sequence analysis software (version 5.1; Gene Codes Corporation, Ann Arbor, MI), and aligned using Clustal Omega (EMBL-EBI), and Mesquite, version 2.75 (Maddison and Maddison 2001). Sequences were deposited in GenBank under the accession numbers listed in Table 1.

Bayesian phylogenetic analyses were done using MrBayes v.3.0b4 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). Additional taxa used for the phylogenetic analyses were obtained from GenBank and chosen based on a reputable expert identification and high percent identity match (>87%) (Table 2). The best-fit model of sequence evolution for the dataset was selected and model parameter estimates obtained using jModeltest 2.1.3. The TIM2ef+G model, with equal base frequencies and three substitution rates, was deemed the best-fit model (Posada 2008) and was used for the Bayesian analysis.

Pathogenicity tests. Three separate experiments with different inoculation methods were conducted to test pathogenicity of the fungus on apple or pear fruit. Different isolates were used for some experiments due to the availability of the inoculum of the isolate.

Inoculation with mycelial plugs. Two isolates (CLX2379 from apple and CLX1010 from pear) were tested for pathogenicity on apple or pear fruit. Fruit of organic d'Anjou pear and Red Delicious apple were surface disinfested for 5 min in 0.5% NaOCl, rinsed three times, air dried, and then wounded with a sterile 4-mm-diameter nail head. A 4-mm-diameter mycelial plug from the leading edge of a 3-day-old PDA culture of each isolate or plain PDA (control) was placed in the wound of each of 10 replicate fruit of both apple and pear for each isolate or control (only the pear isolate was tested on pear fruit). Fruit were tray packed with polyethylene liners and stored in cardboard boxes in air at 0°C, and decay was evaluated 3 weeks after inoculation. Five decayed fruit from each treatment were selected for reisolation of the causal agent, as described above. The experiment was conducted twice.

Inoculation with conidial suspensions. The isolates of CLX3538 or CLX2095 recovered from decayed apple fruit (Table 1) were tested on both apple and pear fruit. Organically grown Fuji apple and D'Anjou pear were obtained from Tonnemaker Farms, Royal City, WA. Fruit were washed with a soap solution, gently rubbed, rinsed in sterile water, surface disinfested in a 0.8% NaOCl solution for 2 min, and independently rinsed two more times with sterile water. All fruit were stored at approximately 4°C prior to inoculation. Pycnidial conidiomata of the fungus produced on 10-day-old PDA cultures and incubated at 20°C under a 12-h photoperiod were used to make the conidial suspension inoculum. The final concentration of

conidial suspensions was adjusted with a hemacytometer to 5×10^5 conidia/ml in 0.2% semiliquid water agar. Surface-disinfested fruit were inoculated by placing 35 µl of the conidial suspension into each wound (0.25 mm in depth and 1 mm in diameter). The fruit treated with 0.2% water agar only served as controls. Fruit were incubated in individual plastic containers, placed in a larger container with a water reservoir separate from the fruit, sealed off with plastic wrap and tape, and stored in a cold storage room at 0 to 2°C for 8 to 11 weeks, at which time lesion diameter on each fruit was measured. If lesions formed around the inoculation site, reisolation of the causal agent was attempted. Fruit were sprayed twice in 70% ethanol with a squirt bottle, allowed to dry for approximately 30 min in a biological safety cabinet, and then rinsed with sterile deionized water. Tissues from the margin of the lesions were aseptically excised and plated onto PDA or acidified PDA (4 ml of 25% lactic acid solution per liter of PDA). The presence of the fungus was assessed at 4 to 7 days based on its colony morphology, and mycelium from selected isolation plates was harvested for sequencing of the ITS region to confirm the identity.

Field inoculation with conidial suspensions. Pathogenicity tests also were conducted on nonwounded apple fruit in the orchard to determine whether the fungus is able to infect sound fruit in the orchard, leading to stem-end rot and calyx-end rot. The isolate CLX3538 was used, and conidial suspensions of the fungus used for fruit inoculation were prepared as stated above. The experiment was conducted in an organic Red Delicious apple research orchard located at the Tree Fruit Research and Extension Center, Washington State University, near Wenatchee. Three replicate trees, each with 40 fruit, were selected for inoculation at each experiment time. The fruit were sprayed until runoff with the fungal inoculum, or with sterile deionized water as controls. The fruit were then covered with moistened plastic bags to maintain high humidity during the incubation period. Temperature and relative humidity inside the plastic bag during the incubation were recorded using a Watchdog data logger (Watchdog 450; Spectrum Technologies, Inc., Plainfield, IL). The experiments were conducted in fall 2012, 2 weeks before harvest, and repeated 1 week before harvest. All fruit were harvested, placed on sterile fiber foam apple trays wrapped in perforated polyethylene bags, and stored in cardboard boxes at 0°C in air. Decay development, infection sites (stem, calyx, or wounds) on the fruit, and percentage of the fruit with symptoms were recorded monthly starting 3 months after harvest for up to 6 months. Isolations were made from all decayed fruit to confirm the causal agent following the procedure described above.

Table 2. Fungal isolates used in *Phacidium lacerum* (*Ceuthospora pinastri*) phylogenetic study: hosts of isolates, source, collector, and GenBank accession numbers^a

Species	Strain or isolate number	Substrate	Source	Collector	ITS GenBank accession number
<i>Allantophomopsis lycopodina</i>	IFO 32908	<i>Rhododendron</i> sp.	—	—	AB041243.1
<i>Ceuthospora pinastri</i>	ZK205/08	<i>Picea abies</i>	Bohemia, Czech Republic	Z. Kolarova	FR717225.1
<i>C. pinastri</i>	Z58	Leaf litter	China	—	JN628108.1
<i>C. pinastri</i>	Z59	Leaf litter	China	—	JN628109.1
<i>Coleophoma empetri</i>	4	<i>Vaccinium macrocarpon</i>	New Jersey	—	FJ480107.1
<i>C. eucalyptorum</i>	CBS 131314	<i>Eucalyptus piperita</i> leaves	New South Wales, Australia	B. A. Summerell	JQ044430.1
<i>Lambertella corni-maris</i> ^b	CLX4050	Fuji apple fruit	Chelan, WA	C. L. Xiao	KC958561.1
<i>L. corni-maris</i>	CLX4075	Red apple fruit	Chelan, WA	C. L. Xiao	KC958562.1
<i>Neofabraea alba</i>	UASWS0534	—	—	—	HQ166308.1
<i>N. alba</i>	CBS 452.64	—	—	—	AF141190.1
<i>N. malicorticis</i> ^b	—	—	—	—	AF141189.1
<i>N. malicorticis</i>	CBS 141.22	—	—	—	AF141161.1
<i>Phacidiopeyrenis washingtonensis</i>	CLX2528	<i>Pyrus communis</i> twig	Wenatchee, WA	C. L. Xiao	AY608648.1
<i>Phacidium lacerum</i> ^b	ZK20/08	<i>Picea abies</i> needles	Bohemia, Czech Republic	Z. Kolarova	FR837911.1
<i>Potebniamyces pyri</i> ^b	AFTOL-ID 744	—	—	—	DQ491510.1
<i>Sphaeropsis pyriputrescens</i>	Sph 1962	—	British Columbia, Canada	—	EU156039.1
<i>S. pyriputrescens</i>	Sph 1409	—	British Columbia, Canada	—	EU156038.1
<i>S. pyriputrescens</i>	Sph 1206	—	British Columbia, Canada	—	EU156037.1

^a ITS = internal transcribed spacer and — indicates not available.

^b Type species of the genus.

Results

Symptoms and incidence of the disease. On d'Anjou pear fruit, the disease associated with the unknown fungus appeared to originate from infection in wounds on the fruit surface. As the disease advanced, the central part of the lesion usually turned black and, sometimes, black pycnidia of the fungus formed in the aged decayed area on d'Anjou fruit (Fig. 1A and B). On Red Delicious apple, common symptoms were stem-end rot and calyx-end rot, which presumably originated from infections of the stem and calyx of the fruit, respectively (Fig. 1C and D). Decayed areas appeared brown, with concentric, alternating rings of light brown and dark brown. Decayed tissue was spongy to firm. The internal decayed flesh appeared brown.

The disease associated with the unknown fungus was observed in 6 of 33 grower lots of d'Anjou pear collected from November 2001 to January 2002, with the incidence in the decayed fruit ranging from 2 to 4%. On apple (Red Delicious, Fuji, and Golden Delicious), the disease was observed in 4 of 26 grower lots sampled in 2003, accounting for 2 to 12% of the decayed fruit in the infected lots; 11 of 72 grower lots sampled in 2004, accounting for 2 to 4% of the decayed fruit in the infected lots; and 2 of 81 grower lots sampled in 2005, accounting for 2 to 3% of the decayed fruit in the infected lots.

Molecular phylogenetic analysis. Using jModeltest 2.1.3, we examined 88 candidate models with 11 different substitution schemes and determined the TIM2ef+G model to be the best fit based on the lowest Bayesian information criterion score. Among the taxa included in this study (Table 2), there were 660 bases spanning the ITS and part of the LSU regions. The results of the phylogenetic analysis revealed a well-supported clade of isolates bearing the name *Ceuthospora pinastri* (Fr.) Höhn., representative isolate CLX3539 of the unknown fungus, and isolates named *Phacidium lacerum* Fr. (current name, syn. = *C. pinastri*) (Fig. 2). The other clades successfully grouped according to their congeneric statuses. Only ITS blast identities of 87% and higher were used in the phylogenetic analysis. Members of the *Ceuthospora/Phacidium* clade shared 99% identity with the representative isolate CLX3539. Blasting other loci yielded high percentage matches with both *Ceuthospora* spp. and *Phacidium* spp. (Table 3).

From Crous et al. (2014), we discounted as candidate names *Potebniamyces pyri* (Berk. & Broome) Dennis, *Allantophomopsiella pseudotsugae* (M. Wilson) Crous, *Pseudophacidium ledi* (Alb. & Schwein.) P. Karst, and *Allantophomopsis cytisporae* (Fr.) Petr. because of insufficient morphological resemblance of their anamorphic states to characteristics of our isolates (i.e., their conidial states were not *Ceuthospora*-like). Also in the pertinent clade of Crous et al. (2014) was *Phacidium lauri* (Sowerby) Crous & D. Hawksw., whose *C. lauri* Grev. anamorph in Nag Raj (1993) did not match our isolate. More problematic was the new species *P. trichophoria* Crous & Quaedvlieg; however, conidia of that fungus tend toward lower length/width ratios than those of our isolates and the new species *P. pseudophacidiioides* Crous (whose anamorph, *C. phacidiioides* Grev., was regarded as a synonym of *C. lauri* by Nag Raj [1993]), whose conidia lack the lower size range and whose conidiophores are distinctly larger than those of our isolates. Also problematic is *P. fennicum* Butin, for which no *Ceuthospora* state is documented. Also in the clade of Crous et al. (2014) is *P. vaccinii* Fr., the "presumptive holomorph" of *C. anomala* Nag Raj (Nag Raj 1993), one of the species to which our isolates failed to key in Nag Raj (1993).

Taxonomy. Based on size of conidia, characteristics of the conidium base, and size of conidiogenous cells, our isolates conform to *C. pinastri* (Fr.) Höhn. in Nag Raj (1993). None of the taxa with species names other than *P. lacerum* in the pertinent clade of Crous et al. (2014) possess anamorphic morphological characteristics congruent with those of our isolates. Based on the above considerations and the presented morphological and molecular data, the fungus was identified as a *Ceuthospora* sp., for which the formerly anamorphic name *C. pinastri* (Fr.) Höhn. is the most descriptive.

***Ceuthospora pinastri* (Fr.) Höhn., Mitt. bot. Inst. tech. Hochsch. Wien 2(4): 104 (1925).** Mycelium immersed, branched, septate, pale brown to hyaline. Semi-immersed pycnidial conidiomata, globose to subglobose with 1 to 3 nonpapillate to slightly papillate ostioles.

Conidiomata uniloculate or multiloculate, 250 to 1,000 μ m in diameter (mean = 350). Conidial matrix pale buff. Conidia hyaline, aseptate, cylindrical, 8 to 13 by 1.5 to 2.5 μ m, base abruptly tapered, occasionally slightly protuberant, 2 to 3 guttules, sometimes with a mucilaginous, flexuous, unbranched appendage that attaches to the conidiogenous cell and disappears in age. Produced by phialidic conidiogenesis from a flask shaped conidiogenous cell 6 to 15 by 1.5 to 3 μ m. Conidiophores simple or branched (Fig. 3).

Colonies on OA approximately 6.5 to 7.0 cm in diameter after 7 days (reaching margin of plate after 14 days >8 cm), regular to irregular with felt-like aerial white mycelium. Gray olivaceous at the center becoming a greenish to colorless at margin. Reverse also gray olivaceous at center toward greenish to colorless at margin (Fig. 3). The colonies on PDA first appeared felty and white then olivaceous-gray after 2 to 3 days. Alternating rings of light olivaceous-gray and dark olivaceous-gray occur on the margin appearing greenish to colorless (Fig. 4). Black pycnidia formed on 5- to 7-day-old cultures starting from the center of the plate. Pale buff cirrhi from the pycnidia commonly appeared on 1.5- to 2.5-week-old cultures stored under a 12-h photoperiod at 20°C.

Specimens examined. United States, Washington State: Manson, Chelan County, on a decayed fruit of Red Delicious apple (*Malus domestica*), 4 November 2004, C. L. Xiao 3538 (WSP 72415; a dried agar culture bearing pycnidia also is included); Mattawa, Grant County, on a decayed fruit of Red Delicious apple (*M. domestica*), 4 November 2004, C. L. Xiao 3539 (WSP 72414; a dried agar culture bearing pycnidia also is included); Pullman, Whitman County, on an inoculated d'Anjou pear (*Pyrus communis*), original isolate from Manson, Chelan County, on a decayed fruit of Red Delicious apple (*M. domestica*), 7 February 2003, C. L. Xiao 3538 (WSP 72438);

Fig. 1. Symptoms on apple and pear fruit caused by *Phacidium lacerum* collected from commercial fruit packinghouses. **A** and **B**, Decay originating from infection of wounds on d'Anjou pear fruit showing concentric rings and formation of black pycnidia of the fungus on advanced decayed area (**B**); **C**, stem-end rot and **D**, calyx-end rot on Red Delicious apple showing alternating rings of light brown and dark brown on the decayed area.

Chelan, Chelan County, on a decayed fruit of Red Delicious apple (*M. domestica*), 3 July 2003, C. L. Xiao 2095 (WSP 72439). Because of the abolition of dual nomenclature (one name for anamorph, another for teleomorph or holomorph) in the International Code of Botanical Nomenclature (now the International Code of Nomenclature for algae, fungi, and plants; McNeill et al. 2012), the name with nomenclatural priority is *Phacidium lacerum* Fr.

Attempts to induce the teleomorph using a variety of artificial media and environmental conditions, plus pairing of single-conidium isolates in various combinations, were unsuccessful (data not shown).

Temperature studies. Both isolates of the fungus grew at temperatures of 0 to 30°C. Radial growth increased from 0 to 20°C, then decreased from 20 to 30°C. Growth ceased at 37°C. The average radial growth for CLX2095 was 0.55 (± 0.26), 7.38 (± 0.36), and 0.77 (± 0.09) mm/day at 0, 20, and 30°C, respectively. The average radial growth for CLX3538 was 0.51 (± 0.06), 6.75 (± 0.10), and 0.84 (± 1.39) mm/day at 0, 20, and 30°C, respectively. When mycelial plugs on plates with no active growth at 37°C were incubated at room temperature, no visible growth was observed after 4 weeks and, thus, the fungus was presumably dead at 37°C. Optimal radial growth appeared to occur around 20°C. Germination of viable conidia occurred between 24 to 48 h at approximately 21°C.

Pathogenicity tests. All apple and pear fruit inoculated with mycelial plugs of the fungus developed symptoms of decay 3 weeks after storage at 0°C. Both apple and pear isolates were pathogenic to apple fruit, and the pear isolate was pathogenic to pear fruit. The same fungus was reisolated from decayed fruit.

All apple and pear fruit inoculated with conidial suspensions of the fungus in the laboratory developed decay from the inoculation sites 12 weeks after inoculation. Isolation from lesions consistently displayed growth and morphological characteristics identical to those previously described. ITS sequencing from both isolates confirmed the identity of the casual agents.

During incubation after fruit inoculation in the orchard, Watchdog data loggers recorded near 100% relative humidity inside the bags and a temperature range from 8.6 to 24.8°C, with 14.5°C being the mean. These temperatures are favorable for conidial germination, as determined above. Symptoms on decayed fruit during cold storage from the field study were primarily stem-end rot and calyx-end rot. Infection also occurred in preexisting field wounds on the fruit. The decayed area appeared light brown to brown on the skin of the apple, often coupled with alternating light and dark concentric rings. The flesh of infected apple fruit was firm to spongy. The fungus

Table 3. Results of GenBank BLAST search with the representative isolate CLX3538 of the unknown fungus

BLAST ^a	Taxa	GenBank number	Match	Identity (%)
SSU	<i>Phacidium coniferarum</i>	AF203467.1	1,032/1,032	100
	<i>P. infestans</i>	GQ266410.1	470/472	99
	<i>P. infestans</i>	GQ266408.1	499/501	99
	<i>P. infestans</i>	GQ266406.1	484/486	99
	<i>P. infestans</i>	GQ266409.1	474/476	99
	<i>P. infestans</i>	GQ266407.1	479/481	99
	<i>P. infestans</i>	AF203466.A	1,021/1,032	99
	<i>P. lacerum</i>	DQ471028.1	1,069/1,069	100
LSU	<i>P. lacerum</i>	DQ470976	611/613	99
ITS	<i>Allantophomopsis lycopodina</i>	AB041243.1	547/554	99
	<i>Ceuthospora pinastri</i>	JN628108.1	432/436	99
	<i>C. pinastri</i>	JN628109.1	416/417	99
	<i>C. pinastri</i>	FR717225.1	528/530	99
	<i>P. lacerum</i>	FR837911.1	503/506	99

^a SSU = small ribosomal subunit, LSU = large ribosomal subunit, and ITS = internal transcribed spacer regions.

Fig. 2. Phylogenetic analysis of the internal transcribed spacer (ITS)1, 5.8S ribosomal RNA (rRNA), ITS2, and partial 28S rRNA sequences by Bayesian methods. The user-specified tree topology was analyzed by using the Bayesian methods based on the TIMe2+g model as determined by jModeltest. Numbers represent Bayesian posterior probabilities that were sampled from 3,864 trees generated from 10,000 Markov Chain Monte Carlo chains. Representative isolate CLX3539 consistently grouped into the *Phacidium/Ceuthospora* spp. clade. The tree was rooted using the *Lambertella corni-maris* clade as an outgroup.

formed pycnidia on the surface of the fruit after the fruit had been stored for an extended period. When infections arose from preexisting wounds, an amber-colored exudate appeared over the location of the wound. Pycnidial formation usually started from infection sites. While immature, the pycnidial conidiomata appeared white and then changed to an olivaceous-gray black upon maturity. Pycnidia were superficial or partially immersed.

At 9 months after harvest, 56.1 and 55.1% of the apple fruit inoculated in the orchard at 1 week and 2 weeks before harvest, respectively, developed decay symptoms. *P. lacerum* was isolated from 90.6 and 70.4% of the decayed fruit inoculated in the orchard at 1 week and 2 weeks before harvest, respectively. No decay resulting from *P. lacerum* was observed on noninoculated fruit. *P. lacerum* was consistently reisolated from decayed apple fruit with the symptoms described above. Stem, calyx, and wound infections accounted for 47.2, 40.5, and 12.3% of decay on the fruit inoculated 1 week prior to harvest, respectively. Stem, calyx, and wound infections accounted for 42.1, 52.6, and 5.3% of decay on the fruit inoculated 2 weeks prior to harvest, respectively. Morphological observations of isolates of anamorphic *P. lacerum* from decayed apple were consistent with our morphological observations of anamorphic *P. lacerum*.

Discussion

This is the first report of fruit rot in stored apple and pear fruit caused by a *Phacidium* or *Ceuthospora* sp., although Nag Raj (1993) indicates an old record from Austria on dried pear and there

are miscellaneous records on *Prunus*, *Rosa*, and some other species in the family Rosaceae (Farr and Rossman 2014). In the literature, *Ceuthospora* spp. that occur on members of Rosaceae are rare. Only *C. brevispora* Cooke & Harkn., *C. concava* Desm. (current name = *Pilidium concavum* (Desm.) Höhn.), *C. cookei* Thüm., *C. foliicola* (Lib.) Cooke, *C. lauri* Grev. (current name = *Phacidium lauri* (Sowerby) Crous & D. Hawksw.), *C. laurocerasi* Grove, *C. phacidioides* Grev. (= *P. lauri*), *C. photiniae* Bubák, and *C. rubi* Bubák ex Petr. (current name = *Pilidium concavum* (Desm.) Höhn.) have been documented on rosaceous hosts (Cooke 1875; Farr and Rossman 2014; Grove 1923; Nag Raj 1993; Saccardo 1884). Of these species, none were found to occur on either *Malus* or *Pyrus* spp. hosts.

Many investigators have made correlations between the asexual genus *Ceuthospora* and sexual genus *Phacidium* (Crous et al. 2014; DiCosmo et al. 1984; Höehnel 1924; Nag Raj 1993). *Phacidium* spp., *Phacidium denticulatum* (nom. inval.), *P. heteromelis* W. Phillips & Harkn. (= *Ceuthospora* sp.), *P. pusillum* Lib. (= *Hysteropezizella pusilla* (Lib.) Nannf., with an *Apostrasseria* anamorph.), and *P. rubi* have been documented on rosaceous hosts—either *Rubus* spp. or *Heteromeles arbutifolia* (Lindl.) M. Roem., toyon (DiCosmo et al. 1984; Farr and Rossman 2014; Nag Raj 1993; Saccardo 1884). Recently, Crous et al. (2014) drew attention to records of *P. lauri* on *Prunus* spp. Regardless, the *Ceuthospora* sp. associated with *P. heteromelis* has conidia that are much larger than the unknown fungus (*P. heteromelis* conidia 20 by 3 μ m; DiCosmo et al. 1984), *P. pusillum* is associated with an

Fig. 3. Photomicrographs of anamorphic *Phacidium lacerum* (isolate CLX3538). **A**, Vertical section illustrating a multiloculate pycnidial conidiomata with arrows pointing to the ostiole (O) and the brown textura globulosa (TG) surrounding the ostiole; **B**, conidium with mucilaginous appendage (MA); **C**, scanning electron microscopy of a conidium, with a mucilaginous appendage (MA), attached to a hyphae; **D**, Calcofluor image of the flask shaped phialide; **E**, Nomarsky depiction of a biguttulate conidium; **F**, pale buff cirrus oozing out of conidiomata on potato dextrose agar; **G**, vertical section of a superficial pycnidium on apple; **H**, vertical section illustrating conidiogenesis with collarettes indicated by the red circles. Scale bars: B and C = 5 μ m; D, E, and H = 10 μ m; G = 125 μ m.

Apostrasseria sp. anamorph with lunate conidia, and *P. rubi* has no documented anamorph (DiCosmo et al. 1984; Nag Raj 1993). To date, the only appendaged coelomycetous anamorph found on a *Malus* host is *Aphanofalx mali* B. Sutton, which bears conidia that are fusiform, curved, and much larger than our fungus's conidia (26 to 33 by 2 to 2.5 μm ; Nag Raj 1993).

Examining fungi with a maximum identity from BLAST searches (Table 3), *Allantophomopsis lycopodina* (Höhn.) Carris and *C. pinastri* are the only fungi to share overlapping characteristics with our fungus. *P. infestans* P. Karst (now *Gremmenia infestans* (P. Karst.) Crous) cannot be accurately compared with our fungus because there are no descriptions of an asexual state in the current literature (Crous et al. 2014; DiCosmo et al. 1984; Minter and Miller 1980). Furthermore, *P. coniferarum* (G. G. Hahn) DiCosmo, Nag Raj & W. B. Kendr. has an *Apostrasseria* sp. anamorph that produces kidney-shaped conidia (DiCosmo et al. 1984). *A. lycopodina* differs from the unknown fungus by its lunate-shaped conidia as compared with the unknown fungus's cylindrical conidia (Carris 1990). Moreover, the ITS sequences of the unknown fungus and *A. lycopodina* differ by 7 bp (GenBank accession number AB041243.1). In comparison, both *C. pinastri* and the unknown fungus share similar conidia, conidiophore, and conidiomata morphology and dimensions (DiCosmo et al. 1984). In addition, both fungi reproduce by phialidic enteroblastic conidiogenesis with conidiophores that line the interior of their pycnidial conidiomata. Nonetheless, *C. pinastri* and the unknown fungus differ on their recognized hosts, conidiomata dimensions, and observed sexual stage (or lack thereof). The two fungi are very similar molecularly and morphologically. Notably, the neotype of *P. lacerum* clustered with two other isolates of that name but at a slight distance from our CLX isolates and three other isolates of *P. lacerum* on the basis of the LSU phylogeny in Crous et al. (2014); therefore, further genetic analysis is warranted. Unfortunately, ITS and ribosomal RNA sequences seem insufficiently variable to separate *Phacidium* or *Phacidium*-like taxa at the species level, and all appear with little or no separation in Crous et al. (2014), thereby forcing reliance on morphological

characteristics. Further investigation into the presence or production of a sexual stage is also desirable.

To date, *C. pinastri* has only been documented on fallen needles and cone scales of conifers or on *Ilex* spp. (Crous et al. 2014; DiCosmo et al. 1984; Farr and Rossman 2014; Minter 1981; von Rack and Scheidemann 1987). The teleomorph of *C. pinastri*, *P. lacerum* (demonstrated by Gremmen [1959]), has also been found on fallen needles of conifers (Cannon et al. 1985; Dennis 1978, 1986; Farr and Rossman 2014; Gremmen 1959, 1977; Minter 1981; von Arx and Müller 1954). Furthermore, *P. lacerum* was also reported on *Arctostaphylos uva-ursi* in Idaho (Shaw 1973). No literature has been found indicating that either *P. lacerum* or *C. pinastri* are pathogens or have any rosaceous hosts (although there is literature indicating the presence of various *Phacidium* or *Ceuthospora* spp. on some of these hosts, as shown above). In the present study, we demonstrated that the fungus identified as *P. lacerum* on the above criteria was able to cause decay on apple fruit under both laboratory and field conditions and decay on wounded and inoculated pear fruit. It is unknown how this fungus is associated with apple and pear trees in the orchard: whether it survives as a saprophyte on the trees or is pathogenic to the trees. Future research needs to test the life cycle and epidemiology of this fungus on apple and pear trees under field conditions.

On apple fruit inoculated with *P. lacerum* in the orchard, stem-end rot and calyx-end rot were observed on those fruit during storage, indicating that the fungus is able to colonize or infect the stem and calyx tissue of the fruit in the orchard, leading to stem-end and calyx-end rots during storage. Stem-end rot and calyx-end rot also are the common symptoms of Sphaeropsis rot of apple and pear caused by *Sphaeropsis pyriputrescens* and speck rot of apple caused by *Phacidiopycnis washingtonensis*, two recently reported postharvest diseases of apple and pear (Kim and Xiao 2006; Xiao and Rogers 2004; Xiao et al. 2004). *S. pyriputrescens* and *P. washingtonensis* can infect apple fruit from after bloom to harvest, remain latent at harvest, and develop decay symptoms on the fruit during storage 2 to 3 months after harvest (Kim et al. 2014; Sikdar et al. 2014). In the

Fig. 4. Colony morphology of *Phacidium lacerum* (left to right; isolates CLX3539, CLX3538, and CLX1010). Top row, malt extract agar plates; middle row, potato dextrose agar plates; bottom row, Boerema oat agar plates. Picture taken 2 weeks after inoculation date. Plates were incubated in the dark at 20°C.

present study, fruit inoculation was conducted at 1 or 2 weeks before harvest. Whether the fungus can infect apple fruit during early fruit-growing season and remain latent before harvest, leading to stem-end rot and calyx-end rot during storage, remains to be determined.

How many years this fungus has been present in the region is unknown. The postharvest disease surveys of apple and pear indicated a low incidence of this disease under commercial storage conditions. It is likely that this disease, if present, is under control with the current postharvest handling practices because currently registered postharvest fungicides fludioxonil, pyrimethanil, and thiabendazole are all highly effective in controlling this disease when they are used as a postharvest drench treatment (C. L. Xiao, unpublished).

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