Etiology of Bull's Eye Rot of Pear Caused by *Neofabraea* spp. in Oregon, Washington, and California

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ABSTRACT

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A collection of *Neofabraea* isolates from pear fruit grown in Oregon, Washington, and California was screened with species-specific primers in a multiplex polymerase chain reaction (PCR). *Neofabraea alba* was identified most frequently in samples from Oregon and California, whereas *N. perennans* was found most frequently in samples from Washington. *N. alba* also was identified from tissue of small cankers and pruning stubs on pear trees using PCR. Bull's eye rot pathogens were isolated from fruit of nine different European pear cultivars, Asian pear, and quince. Overall, *N. alba* was the most prevalent species in 2001 whereas *N. perennans* was more prevalent in 2002. An undescribed species of *Neofabraea* was identified in samples from Medford, OR that corresponds to a species previously found by others using molecular methods. This information increases the known geographic distribution of this undescribed species.

Additional keywords: canker diseases, *Pyrus communis*

Bull's eye rot of apple and pear, caused by species of *Neofabraea*, is an important postharvest disease in the Pacific Northwest (PNW) of the United States, and also occurs in Europe and other growing areas. Fruit can be infected in the orchard at any time during the growing season, and spores or incipient infections remain latent until development of symptoms after several months of postharvest storage (5,12,25). On apple trees, bull's eye pathogens produce cankers on branches and twigs or develop saprophytically on dead wood, from which spores are produced and dispersed in splashing water to the fruit (3,14,17,18,21).

Four species of the genus *Neofabraea* are known to cause bull's eye rot of apple (8). *Neofabraea alba* is the main pathogen causing bull's eye rot in continental Europe (6,13), and also has been reported as a minor disease of apple in eastern North America (25). *N. malicorticis* causes anthracnose canker of apple trees and is found principally in the more humid areas of the PNW. It is an aggressive pathogen able to infect sound wood directly (20). *N. perennans* causes perennial canker of ap-

ple trees. The pathogen requires wounds to infect the wood, and the fungus survives in cankers by infecting injured tissue at the border of old cankers (7,20). *N. malicorticis* and *N. perennans* are considered a single species in Europe (6,13,19,24), whereas they have been regarded as distinct species in North America (9,10,17,20). A putative new species, yet to be described, recently has been reported in a phylogenetic study of *Neofabraea* spp. causing tree cankers and bull's eye rot of apple (8). It was represented by one isolate from Nova Scotia, Canada, and one from Portugal. The undescribed species also has been identified recently in a molecular study in Australia (J. Cunningham, *personal communication*).

There is no canker disease described on pear trees associated with the bull's eye rot pathogens, although pear has been mentioned as a host by some authors describing apple canker diseases (7). An association of bull's eye rot fungi with dead or injured pear bark also has been reported (15,25).

Morphological differences among bull's eye pathogens are subtle and often overlap, making specific identification difficult and undependable. Several comparative studies on the morphology have been performed, especially to differentiate *N. malicorticis* from *N. perennans* (9,10,20). Verkley (26), in a world monograph of the genus *Pezizula*, matched morphological characters with molecular data (restriction fragment length polymorphisms of nuclear rDNA) and revalidated the genus *Neofabraea*. Abeln et al. (1) conducted a phylogenetic study of *Pezizula*, *Dermea*, and *Neo-*

fabraea by partial sequencing of ribosomal RNA genes, and concluded that *Neofabraea* is a separate evolutionary lineage and should not be included among *Pezizula*. Later, de Jong et al. (8) identified four species of *Neofabraea* associated with apple tree cankers and bull's eye rot, including *N. alba*, *N. malicorticis*, *N. perennans*, and the putative new species. They found genetic differences between *N. malicorticis* and *N. perennans* that merited the recognition of these taxa as distinct species. Furthermore, they developed species-specific primers by sequencing the β -tubulin gene for the five known species of *Neofabraea*, including *N. krawtzevii*, a pathogen of poplar trees. Garipey et al. (16) established the protocol for a multiplex polymerase chain reaction (PCR) for the specific identification of bull's eye rot pathogens using mycelium from cultures or diseased tissue.

The objective of this study was to determine the species of *Neofabraea* causing bull's eye rot of pear grown in Oregon, Washington, and California using molecular and morphological techniques. Sources of inoculum on pear trees also were investigated in a PCR screening.

MATERIALS AND METHODS

Collection and storage of isolates. In 2001, 450 isolates of bull's eye rot fungi were accumulated for PCR identification. Pear fruit showing symptoms of bull's eye rot were collected from packinghouses in Medford and Hood River, OR (including the White Salmon area of Washington). Additional samples were obtained from the orchards of the Southern Oregon Research and Extension Center (SOREC), several commercial orchards in Medford, and from the Mid-Columbia Agricultural Research and Extension Center in Hood River. Cultures of fungi isolated from pear fruit with bull's eye rot from elsewhere in Washington and a small number of symptomatic pear fruit from Philo, CA were included. In addition, fruit from nine different pear cultivars from the orchards of SOREC were screened to evaluate possible differential cultivar susceptibility.

In 2002, 205 pear fruit with bull's eye rot obtained from four orchards in Medford and one orchard in Hood River were screened and the causal agents identified as *N. alba* or *N. perennans* based on the morphology of their macroconidia.

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Direct tissue isolations were performed from fruit that had been surface sterilized in 0.5% sodium hypochlorite. Alternatively, monospore cultures were obtained when sporulation was present. Monospore cultures were made by immersing an individual fruiting body, extracted from a fruit lesion, in 100 to 300 µl of sterile distilled water and subsequently spreading the suspension on water agar. The cultures then were incubated at approximately 4°C for 1 to 2 weeks. Individual germinating conidia then were transferred to acidified potato-dextrose agar (aPDA) in petri dishes. Morphology of the germinating conidia and presence or absence of microconidia were noted. Isolates were stored at 4°C until molecular screening.

Sampling of woody tissues. In all, 60 samples each of dead bark, pruning stubs, previous-season fruit spurs, and 72 samples of small superficial cankers were taken from a block of Bosc pear trees with a history of bull's eye rot in an orchard at SOREC. These woody tissues were screened using PCR and *Neofabraea* species-specific primers in order to detect the presence of bull's eye pathogens and to determine possible inoculum sources on pear trees.

DNA extraction. Total genomic DNA was extracted using the FastDNA Kit, following manufacturer's instructions (Qbiogene, Carlsbad, CA). Approximately 200 mg of mycelium from cultures growing on aPDA were transferred to FastPrep 2-ml tubes containing 800 µl of cell lysis buffer solution for plant samples (CLS-VF) and 200 µl of protein-precipitating solution to eliminate inhibitors that could be present in the culture media. Samples were processed in a FastPrep machine (FP 120) for 30 s at speed 4, incubated for 15 min at room temperature, and extracted following the protocol for CLS-VF buffer. DNA extraction from pear tree woody tissues was conducted as described above but at speed 5, incubated for 30 min at room temperature, and extracted according to the protocol for CLS-VF buffer. The extraction product containing total DNA then was subjected to a second extraction using CLS-Y buffer (cell lysis solution for yeasts, algae, and fungi) for the extraction of fungal DNA.

DNA amplification. Amplification of DNA was performed in a multiplex reaction, with the addition of four species-specific primers simultaneously, following the protocol developed by Garipey et al. (16). The species-specific primer sets use the same forward primer in combination with the specific reverse primer. PCR reactions were conducted in 200-µl tubes in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA). A total of 25 µl of reaction solution contained 0.2 µl of 10 mM dNTPs; 0.1 µl of 5 µM of the universal fungal primers UN-UP18S-42 and UN-LO28S-22 (2), which amplify a por-

tion of the internal transcribed spacer region and were used as positive control to demonstrate the presence of fungal DNA after the extraction; 0.4 µl of 20 µM of the forward primer; 0.4 µl of a 5-µM solution of each of four species-specific primers; 0.5 µl of *Taq* titanium DNA polymerase and 2.5 µl of 10× titanium *Taq* PCR buffer (Clontech Lab., Palo Alto, CA); and distilled water to the final volume. The PCR reaction was performed using four different annealing temperatures to achieve higher specificity. It consisted of an initial denaturation step of 3 min at 95°C followed by 40 cycles of 45 s at 95°C, 45 s at four different annealing temperatures, and 2 min at 72°C, with a final extension of 10 min at 72°C. The annealing temperatures started at 72°C for 5 cycles and decreased to 70 and 68°C for 5 cycles each and to 66°C for 25 cycles. Isolates where DNA could not be amplified in PCR reactions were identified using morphological characteristics.

Agarose gel electrophoresis and visualization of PCR products. PCR products were resolved on 1.5% agarose gels stained with ethidium bromide and visualized by UV transillumination at 302 nm.

Band sizes resulting from the PCR reaction were estimated based on a low mass DNA ladder (Gibco/Invitrogen, Carlsbad, CA).

RESULTS

Molecular identification of bull's eye rot isolates from pear fruit. A total of 450 isolates from pear fruit showing symptoms of bull's eye rot were screened with PCR. DNA fragment sizes of 358, 300, and 236 bp were obtained, consistent with those previously amplified for *N. alba*, *N. perennans*, and the undescribed *Neofabraea* sp. respectively (16; Fig. 1).

N. alba was the most common species isolated, with a total of 282 isolates identified in samples from Oregon and California (Table 1). *N. perennans* was the second most common species, and was identified from 95 isolates originating in Wenatchee, WA and Hood River and Medford, OR. Eighteen isolates of the undescribed *Neofabraea* sp. were found, obtained from 10 orchards in the Medford area. Fourteen isolates were identified as *Cylindrocarpum magnusianum* Wollenweb. based on morphological characters. The *Neofabraea*-specific primers did not amplify any fragment of DNA from these isolates, but uni-

Fig. 1. DNA fragments from polymerase chain reaction products of the *Neofabraea* spp. isolated from bull's eye rot lesions on pear fruit. Lane 1: a low-mass DNA ladder (Invitrogen, Inc. Rockville, MD); lanes 2–4: *N. alba* (isolates MB-0202, MB-0203, and MB-0204); lanes 5–7: *N. perennans* (isolates MB-0274, MB-0278, and MB-0279); lanes 8–10: undescribed *Neofabraea* sp. (isolates MB-0222, MB-02109, and MB-02127); lanes 11–12: *N. malicorticis* (from cankers on Newtown apple trees, Hood River, OR); lane 13: negative control. A band at approximately 600 bp in lanes 2–12 corresponds to a fungal universal band used as control for positive DNA extraction.

Table 1. Molecular identification of pathogens isolated from bull's eye rot lesions on pear fruit from different sources in the western United States

Pathogen	Wenatchee, WA	Hood River, OR ^a	Medford, OR	California	Total
<i>Neofabraea alba</i>	0	42	236	4	282
<i>N. perennans</i>	18	28	49	0	95
<i>Neofabraea</i> undescribed species	0	0	18	0	18
<i>Cylindrocarpum magnusianum</i>	5	1	8	0	14
Not determined	0	0	20	0	20
Mixed reaction ^b	10	0	11	0	21
Total number of samples	33	71	342	4	450

^a Including the White Salmon area of Washington.

^b Isolates giving one band for *N. alba* and one band for *N. perennans*.

versal bands were obtained. Twenty-one isolates gave a mixed reaction where DNA bands specific for both *N. alba* and *N. perennans* were amplified. The primer sets failed to amplify DNA samples from 20 isolates, despite being screened twice. In addition, eight isolates from apple fruit of unknown cultivars were identified as *N. alba*, whereas one was identified as the undescribed *Neofabraea* sp. All of the apple fruit were grown in the orchard of SOREC in Medford.

Bull's eye rot lesions, caused by either *N. alba*, *N. perennans*, or the undescribed *Neofabraea* sp., were observed on nine different pear (*Pyrus communis*) cultivars (Bosc, Comice, d'Anjou, Packham's Triumph, Red Anjou, Red Comice, Rogue Red, Seckel, and Winter Nelis), quince (*Cydonia oblonga*), and Asian pear (*P. pyrifolia*, unknown cultivar) (data not shown).

Germinated macroconidia of *N. perennans* produced microconidia abundantly in culture media. In contrast, germinated

macroconidia of *N. alba* did not produce microconidia and their mycelia grew faster and branched more repeatedly than those of *N. perennans* (Fig. 2). These criteria were used in preliminary identification of 194 isolates as *N. perennans* or *N. alba*, when monocultures for molecular identification were prepared. An 85.1% match was obtained between identification based on morphological criteria and molecular identification as *N. perennans* or *N. alba*. Thirteen of the isolates morphologically identified as *N. perennans* were molecularly identified as the undescribed *Neofabraea* sp. Production of microconidia from germinating macroconidia of the undescribed *Neofabraea* sp. was confirmed (Fig. 2).

In 2001, 80.8% of the bull's eye rot samples collected were found to be infected by *N. alba* (Table 2). In contrast, *N. alba* was identified from only 8.3% of bull's eye rot lesions in the 2002 season, whereas most lesions yielded *N. perennans*. All orchards except one in 2002 had

a lower proportion of fruit infected by *N. alba*.

Sources of inoculum on pear trees. *N. alba* was positively identified in five samples of small cankers and four samples of pruning stubs (Fig. 3). None of the samples of dead bark or previous year's fruiting spurs gave a positive reaction. Neither *N. malicorticis*, *N. perennans*, nor the undescribed *Neofabraea* sp. were found in any woody sample from pear trees.

DISCUSSION

N. alba was the most common species isolated from bull's eye rot lesions on pear fruit. This species was listed previously by Shaw (23) by its anamorphic name *Phlyctesteria vagabunda* as found on *Malus sylvestris* Mill. in Washington State. In the present study, it was the prevalent species in 2001 in Oregon and the only one present in samples from California, although only a few samples were obtained from California. It was not found in isolates from Washington and, thus, may be more prevalent in the southern range of the area of study.

N. alba may have been present in the PNW for a long time. In 1925, Fischer (14) questioned the species composition of bull's eye rot fungi in apple fruit, because some isolates failed to produce spores in culture and could not be identified. Bompex (4) found that alternating light and temperature regimes were necessary to induce sporulation by *N. alba* in culture. It is likely that this species historically has been confused with *N. malicorticis*, because both produce curved macroconidia on fruit lesions. Most of the literature on bull's eye rot of apple and pear indicates *N. malicorticis* as the causal agent, an identification probably based on the shape of the macroconidia (22,25,28). *N. alba* was identified causing bull's eye rot of apple grown in Medford during the present study; therefore, this fungus also may be more important than previously considered as part of a pathogen complex that causes bull's eye rot of apple in the PNW. *N. perennans* was the second most prevalent species associated with bull's eye rot of pear, and its widespread distribution is consistent with what has been previously reported for apple (20).

The undescribed *Neofabraea* sp. was found in pear fruit from 10 different orchards. In all of these orchards sampled, at least one other *Neofabraea* sp. was present. In six of the orchards, all three species were found. In all cases, the undescribed *Neofabraea* sp. was the least frequent species, and was found only in southern Oregon. This species could be more restricted in geographic distribution, but a larger sampling of the areas where it was not found will be necessary in order to determine its presence or absence. Macroconidia of the undescribed *Neofabraea* sp. were similar in appearance to those of *N. perennans*. Microconidia were found

Fig. 2. Germinated macroconidia and production of microconidia by *Neofabraea* spp. causing bull's eye rot of apple and pear. **A**, *N. malicorticis*; **B**, *N. perennans*; **C**, *N. alba* (does not produce microconidia); **D**, undescribed *Neofabraea* sp. Ma = macroconidia and mi = microconidia. **A**, **B**, and **C**, $\times 400$; **D**, $\times 250$.

Table 2. Identity of *Neofabraea* isolates causing bull's eye rot of pear in 2001 and 2002^a

Orchard ^b	<i>N. alba</i>		<i>N. perennans</i>	
	2001	2002	2001	2002
Bybee (M)	20	2	0	36
Fairlane (M)	17	4	0	5
Hanley (M)	85	4	14	39
Klamath (M)	13	1	0	15
MCAREC (HR) ^c	42	6	28	93
Total	177	17	42	188

^a Identification of isolates from 2001 was based on DNA differences. Identification of isolates from 2002 was based on the morphology of macroconidia.

^b M = Medford, OR and HR = Hood River, OR.

^c Isolates from several Hood River orchards collected by the Mid-Columbia Agricultural Research and Extension Center (MCAREC) were screened in 2001 and from one orchard in 2002.

among macroconidia in acervuli and were observed being directly produced by germinating macroconidia. Superficial mycelia frequently grew over the bull's eye rot lesions caused by the undescribed *Neofabraea* sp. and *N. perennans*, but this was not observed for *N. alba*.

N. malicorticis was not found causing bull's eye rot of pear in this survey. This fungus was reported by de Jong et al. (8) on *Pyrus* spp. from British Columbia and was described by Kienholz (20) as restricted to areas of the PNW west of the Cascade Mountains. It is possible that this species is confined to higher rainfall areas of the PNW. A more extensive survey of fruit from the Hood River-White Salmon and Willamette Valley areas, where *N. malicorticis* has been reported, would be needed to verify the importance of *N. malicorticis* as a pathogen of pear.

N. perennans was not found in any sample of woody pear tissues, despite its association with cankers in apple (11), whereas *N. alba* was found in both small cankers and pruning stubs. Differential conditions for establishment and sporulation on plant surfaces and in infected tissue among growing seasons may determine the relative prevalence of *Neofabraea* spp. as agents of fruit rot. *N. perennans* was reported as less important than *N. alba* on apple in Great Britain in the 1930s, but then became the more prevalent cause of bull's eye rot by the 1950s. Wilkinson (27) speculated that this situation resulted from the progressive colonization of injured wood, especially pruning wounds; thus, *N. alba* subsequently became more prevalent as a fruit rot. The finding that *N. perennans* apparently does not induce cankers on pear trees under natural conditions could ex-

plain its relatively lower importance than *N. alba* in pear orchards. Climatic factors also may influence the prevalence of a given species of *Neofabraea*.

The production of microconidia by the germinating macroconidia of *N. perennans* but not by those of *N. alba* is consistent with the observations of Wilkinson (27). Combined with the morphological differences between the macroconidia of these species, this criterion should be adequate to differentiate these two species. However, germinating macroconidia of the undescribed *Neofabraea* sp. also produced microconidia, and their shape is similar to those of *N. perennans*; thus, differentiation of these two species appears to require application of molecular techniques. Germinating macroconidia of *N. malicorticis* produce microconidia as well, but it may be differentiated from *N. perennans* by the curvature of its macroconidia, and possibly by geographical distribution and host specificity.

Isolation of bull's eye rot pathogens from nine cultivars of European pear as well as Asian pear and quince suggests that bull's eye rot susceptibility may be widespread in *Pyrus* spp. and related genera. However, because the disease commonly appears only after four or more months in cold storage (25), practical importance of the disease excludes many pear cultivars that do not store for long periods of time, including Bartlett.

The identification of three species causing bull's eye rot of pear raises new questions regarding their comparative epidemiology and control. The different species coexist with each other and their relative importance from one season to another may be influenced by weather conditions or other factors. How cultural practices in the orchard affect fruit infections by each of the pathogens is important to know in order to design appropriate control measures.

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Fig. 3. Woody tissues from which *Neofabraea alba* was identified in a polymerase chain reaction with species-specific primers. **A**, Small superficial canker on a branch of a Bosc pear tree. **B**, Pruning stub showing a small necrotic zone on a branch of a Bosc pear tree.

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