

Species specific identification of the *Neofabraea* pathogen complex associated with pome fruits using PCR and multiplex DNA amplification

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Received 6 December 2002; accepted 30 March 2003.

Five species of pathogenic fungi belong to *Neofabraea*. One of these, *N. krawtzevii* (syn. *N. populi*), is responsible for bark lesions on poplar (*Populus*) trees. The other four species cause post-harvest bull's eye rot of pome fruits, and at least two of these also cause bark cankers on pome fruit trees. Morphological variation among these species is slight, and overlap in geographic range sometimes occurs. As a consequence, identification based on conventional criteria can be tenuous. PCR primers with putative species specificity were developed following genetic analysis of the β -tubulin gene for isolates of each of the five species of *Neofabraea*. PCR conditions required to achieve specificity of the primer sets were determined, and a multiplex PCR protocol was developed to optimize their diagnostic utility on apple fruits. A protocol with higher annealing temperatures in the initial PCR cycles followed by lower temperatures in later cycles gave complete species-specificity when the primer sets were used individually and in multiplex, resulting in successful detection of the pathogens from axenic culture and infected apple fruits.

INTRODUCTION

Several members of the genus *Neofabraea* are causal agents of tree cankers and post-harvest rots of apples and pears. In recent decades, these species were considered members of the genus *Pezizula*, but recent data and revisions have shown that they belong to *Neofabraea* (Verkley 1999, Abeln, de Pagter & Verkley 2000). Many of these species have complex nomenclatural histories, and the various synonyms of these species are outlined in Verkley (1999). Phylogenetic relationships among the fungi belonging to *Neofabraea* have been established, and genetic analysis of ITS nuclear rDNA, mitochondrial rDNA, and the β -tubulin gene allowed the delineation of five separate species within this genus (de Jong *et al.* 2001). Historically, *N. malicorticis* and *N. perennans* are responsible for anthracnose canker and perennial canker, respectively, in the Pacific north-west (Kienholz 1939). These fungi are also two of the causal agents of post-harvest bull's eye rot of pome fruits. *Neofabraea*-induced tree cankers and associated fruit rots in western North America have been mainly attributed to *N. malicorticis* and *N. perennans* (Farr

et al. 1989, Jones & Aldwinckle 1990), whereas *N. alba* is most commonly found on apple in eastern North America (Lockhart & Ross 1961, de Jong *et al.* 2001). However, *Phlyctema vagabunda*, the anamorph of *N. alba*, was reported once in Washington state (Shaw 1973), and more recently was identified as the causal agent of coin canker of ash trees in Michigan (Rossman *et al.* 2002). A fourth *Neofabraea* apple pathogen, an as yet formally undescribed species, has been found in Portugal and Nova Scotia (de Jong *et al.* 2001). The fifth *Neofabraea* species, *N. populi*, does not produce disease symptoms on apple (*Malus*) or pear (*Pyrus*), but causes bark cankers on *Populus*. Morphological and physiological characteristics of these species are similar (Dugan 1992, Dugan, Grove & Rogers 1993, de Jong *et al.* 2001) making identifications based on conventional criteria tenuous. In addition to the limited morphological variation among these fungi, there is also an overlap in geographic range, such that two or more of these pathogens may occur in the same geographic region. This makes unambiguous identification of these pathogens difficult.

In recent years, there has been increased use of techniques employing species-specific DNA primers in the detection of plant pathogens. These have been reviewed by Henson & French (1993), Ward (1994), and Martin, James & Lévesque (2000). The development

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of species-specific molecular diagnostic tools for *Neofabraea* species would allow rapid and accurate identification of the causal agents of *Neofabraea*-associated tree cankers and fruit rots, even in areas of geographic overlap.

Sequence data generated in a recent phylogenetic analysis of the genus *Neofabraea* (de Jong *et al.* 2001) were used to design PCR primers with putative specificity for each of the five *Neofabraea* species. This study was initiated to determine the amplification conditions required for specificity of these primer sets, and to optimize their diagnostic utility by combining primers for all five *Neofabraea* species in a multiplex DNA assay to simultaneously detect distinct *Neofabraea* species from axenic culture and infected apple fruits.

MATERIALS AND METHODS

Acquisition of material

Putative species-specific primers for the five members of the genus *Neofabraea* were designed based on sequences of the β -tubulin gene. These primer sets use the same forward primer in combination with species-specific reverse primers (Tables 1–2). Isolates of each of the five species belonging to *Neofabraea* were used to demonstrate operational specificity of the primer sets. Details pertaining to the isolates obtained are presented in Table 3. Total DNA from seven isolates of *N. alba*, six isolates of *N. malicorticis*, four isolates of *N. perennans*, two isolates of *N. populi*, two isolates of *Neofabraea* sp. nov., one of *Pezizula corticola*, and one of *Neonectria galligena* (syn. *Nectria galligena*) were obtained. *Neonectria galligena* causes European canker of apple trees and was chosen based on its prevalence in the Pacific north-west. *Pezizula corticola* has also been identified in areas where *Neofabraea* occurs and is genetically closely related to the genus *Neofabraea* (Verkley 1999, de Jong *et al.* 2001). As such, it was included to challenge the specificity of the five *Neofabraea* PCR primer sets.

DNA extraction

All fungal isolates were grown in 20% V8 broth (Lévesque *et al.* 1993). Total genomic DNA from mycelia was extracted using the FastDNA kit (Q-Biogene, Carlsbad, CA) as per the manufacturer's instructions for DNA extraction from fungi. Note that extractions from apple rots (described later) were performed using the instructions for DNA extraction from plant tissue.

Amplification of total DNA using species-specific primers

PCR reactions were performed in a PE GeneAmp 2400 PCR machine (Perkin Elmer, Foster City, CA). The 25 μ l reaction mixtures contained 0.2 mM dNTPs, 0.1 μ M of the universal fungal primers UN-UP18S-42

Table 1. Nucleotide sequences for *Neofabraea* primers.

Primer name	Primer sequence (5'-3')
Neofab-upTub-100	TGA TGA GAC CTT CTG TAT GC
Neo_alba-loTub-439	AGC AGA GCA GGT CAA GTA A
Neo_mal-loTub-262	GAC AGC CAA CTT GCG G
Neo_per-loTub-382	GGG TCG AAC ATC TGT TGT
Neo_pop-loTub-475	AAG AGT AGC ACC ATA AGA AAG T
Neo_spnov-loTub-319	TGG TGA GAG GAG CGA AC

and UN-LO28S-22 (Bakkeren, Kronstad & Lévesque 2000), 0.4 μ M of Neofab-upTub-100, 0.4 μ M of the appropriate species-specific reverse primer, and 0.5 μ l of 50 \times Titanium Taq DNA polymerase (CLONTECH, Palo Alto, CA) in a 10 \times Titanium Taq PCR buffer (CLONTECH, CA). Approximately 1–10 ng of total DNA template was used per reaction. The universal fungal primers served as internal positive controls to demonstrate that fungal DNA capable of amplification was present in each reaction.

The PCR conditions used for the amplification of *Neofabraea* DNA were as follows: an initial denaturation step of 3 min at 95 °C, followed by 40 cycles of 45 s at 95 °, 45 s at a given annealing temperature (specified below), and 2 min at 72 °. A final extension period of 8 min at 72 ° followed the completion of the 40 cycles, and products were then rapidly cooled to 4 °. Annealing temperatures began at 72 ° for the initial five cycles and were subsequently decreased by 2 ° every five cycles from 72 ° to 68 °. This was followed by an additional 25 cycles at an annealing temperature of 66 °, for a total of 40 cycles.

The specificity of each primer pair was evaluated by amplifying each isolate of a given species of *Neofabraea* using the appropriate primer set in addition to one isolate of each of the other four *Neofabraea* species, one isolate of *Neonectria galligena* and one isolate of *Pezizula corticola*.

Multiplex PCR amplification

In addition to the amplification of species-specific fragments of *Neofabraea* DNA using the primers designed for each of the five species, amplification was also attempted using a combination of all five primer sets in a single PCR reaction mixture (multiplex PCR). Multiplex reaction mixtures contained the same reagents listed above, except that each reaction mixture contained 0.1 μ M of each of the species-specific lower primers in addition to 0.4 μ M of the upper primer, Neofab-upTub-100. DNA from one isolate of each of the five species of *Neofabraea* was used to test the applicability of this approach, and PCR conditions were as described above.

PCR detection from artificially infected apple fruits

Apple fruits were wound-inoculated with mycelium from actively growing cultures of one isolate of each of

Table 2. *Neofabraea* primer sets and their respective species-specific fragment sizes.

<i>Neofabraea</i> species	Upper primer	Lower primer	Fragment size (bp)
<i>N. alba</i>	Neofab-UpTub-100	Neo_alba-loTub-439	358
<i>N. malicorticis</i>	Neofab-UpTub-100	Neo_mal-loTub-262	178
<i>N. perennans</i>	Neofab-UpTub-100	Neo_per-loTub-382	300
<i>N. populi</i>	Neofab-UpTub-100	Neo_pop-loTub-475	397
<i>N. sp. nov.</i>	Neofab-UpTub-100	Neo_spnov-loTub-319	236

Table 3. Culture and total DNA acquisitions of fungi used in this research, including species, collection reference number, type of acquisition and location (adapted from de Jong *et al.* 2001).

Species	Reference number ^a	Acquisition	Location
<i>Neofabraea alba</i>	135 (CAL 135)	DNA	Canada (NS)
<i>N. alba</i>	142 (CAL 142)	DNA	Canada (NS)
<i>N. alba</i>	167 (DAOM 227091)	Culture and DNA	Canada (NS)
<i>N. alba</i>	189 (ATCC 16504)	DNA	USA (PA)
<i>N. alba</i>	109 (ATCC 38338)	DNA	Poland
<i>N. alba</i>	194 (CBS 452.64)	DNA	UK
<i>N. alba</i>	195 (CBS 628.72)	DNA	Yugoslavia
<i>N. malicorticis</i>	102 (CAL 102)	DNA	Canada (BC)
<i>N. malicorticis</i>	103 (DAOM 227083)	DNA	Canada (BC)
<i>N. malicorticis</i>	105 (DAOM 227085)	Culture and DNA	Canada (BC)
<i>N. malicorticis</i>	158 (DAOM 227090)	DNA	Canada (BC)
<i>N. malicorticis</i>	198 (CAL 198)	DNA	Canada (BC)
<i>N. malicorticis</i>	234 (RGR 90.0081)	DNA	USA (WA)
<i>N. perennans</i>	152 (DAOM 227089)	Culture and DNA	Canada (BC)
<i>N. perennans</i>	106 (ATCC 26206)	DNA	Germany
<i>N. perennans</i>	236 (DAOM 227093)	DNA	USA (WA)
<i>N. perennans</i>	177 (CBS 453.64)	DNA	UK
<i>N. populi</i>	149 (DAOM 227087)	Culture and DNA	Norway
<i>N. populi</i>	150 (DAOM 227088)	DNA	Norway
<i>N. sp. nov.</i>	107 (DAOM 227086)	Culture and DNA	Canada (NS)
<i>N. sp. nov.</i>	180 (CBS 355.72)	DNA	Portugal
<i>Neonectria galligena</i>	129 (CAL 129)	DNA	N/A
<i>Pezizula corticola</i>	179 (CBS 259.31)	DNA	Denmark

^a ATCC, American Type Culture Collection; CAL, Collection of C. André Lévesque, Agriculture and Agri-Food Canada, Ottawa; CBS, Centraalbureau voor Schimmelcultures; DAOM, Canadian National Collection of Fungal Cultures, Ottawa.

the four *Neofabraea* species that are able to cause post-harvest bull's-eye rot. Apple fruits of the variety Jonagold were surface sterilized by soaking the fruits in 70% ethanol for 3 min. Following sterilization, two equally spaced v-shaped notches approximately 1 cm in length were excised on the apple surface midway between the calyx and stem using a sterilized scalpel (Guthrie 1959). Pieces of V8 agar containing actively growing hyphae from the edge of fungal colonies were aseptically placed under the flaps of the v-shaped notches. Each apple was inoculated in this manner with one isolate of *N. alba*, *N. malicorticis*, *N. perennans*, and *Neofabraea* sp. nov., respectively. Apples treated in the same manner but inoculated with sterile agar plugs served as controls. The four *Neofabraea* isolates and the sterile agar controls were each inoculated into three apple fruits. All fruits were placed individually in Ziploc Sandwich bags, incubated at 15 ° for 4–6 wk, and monitored for the development of associated rots.

Following incubation, tissue samples of approximately 200 mg were removed from the edge of the bull's eye rot tissue of the apples and placed in 2 ml screw-cap tubes. Total DNA was extracted using the FastDNA

Kit (Q-Biogene, CA), following the manufacturers instructions for DNA extraction from plant tissue. Extracted DNA from each isolate was amplified using the protocols for amplification of *Neofabraea* DNA using species-specific primers and multiplex PCR.

Agarose gel electrophoresis and visualization of PCR products

All PCR products were visualized on 1.5% agarose gels (Gibco BRL, Carlsbad, CA) stained with 0.5 µg ml⁻¹ ethidium bromide. DNA fragments resulting from PCR amplification were visualized by UV transillumination. Band sizes yielded from the PCR reaction were estimated based on Gibco BRL's Low Mass DNA ladder.

RESULTS

Amplification of total DNA using species-specific primers

Under the 'touch-down' PCR conditions imposed by ramping the annealing temperature from 72 ° to 66 °,

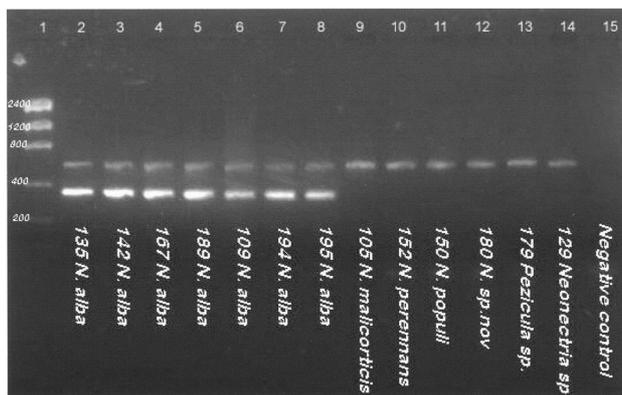


Fig. 1. PCR amplification of 358 bp *Neofabraea alba*-specific fragments from total DNA using Neofab-upTub-100 and Neo_alba-loTub-439 and an approximately 600 bp fragment amplified using the universal fungal primers UN-UP18S-42 and UN-LO28S-22.

the individual primer sets yielded the expected species-specific fragment sizes consistent with those indicated in Table 2.

Neofabraea alba

As shown in Fig. 1, the primer combination designed for *Neofabraea alba* yielded 358 bp fragments for all seven isolates analyzed. In addition, this primer set failed to amplify DNA from representative isolates of each of the other four *Neofabraea* species and DNA from the non-*Neofabraea* species included in this study (*Neonectria galligena* and *Pezicula corticola*). Universal fungal primers serving as internal positive controls yielded the expected 600 bp fragment they were designed to amplify for all of the fungal isolates analyzed.

Neofabraea malicorticis

Using the combination of universal fungal primers and species-specific primers generated for *Neofabraea malicorticis*, the expected 600 and 178 bp fragments, respectively, were observed (Fig. 2). All six isolates of *N. malicorticis* were successfully amplified, and none of the other *Neofabraea* species nor *N. galligena* or *P. corticola* were amplified by the *N. malicorticis* primers. The 600 bp fragment generated by the universal fungal primers was observed for all isolates analyzed in this reaction.

Neofabraea perennans

The predicted 300 bp fragment was successfully amplified from DNA of all four isolates of *Neofabraea perennans* using the *N. perennans* specific primer set (Fig. 3). This fragment was not amplified from DNA of the other four species of *Neofabraea*, nor from *Neonectria galligena* or *Pezicula corticola*. The 600 bp fragments generated using universal fungal primers as an internal positive control were amplified for all fungal DNA analyzed in these reactions.

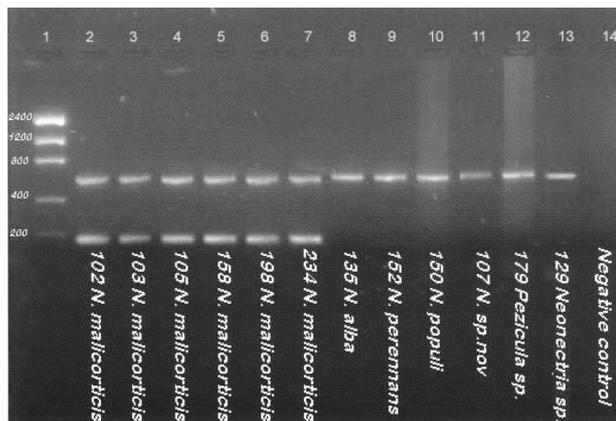


Fig. 2. PCR amplification of a 178 bp *Neofabraea malicorticis*-specific fragments from total DNA using Neofab-upTub-100 and Neo_mal-loTub-262 and an approximately 600 bp fragment amplified using the universal fungal primers UN-UP18S-42 and UN-LO28S-22.

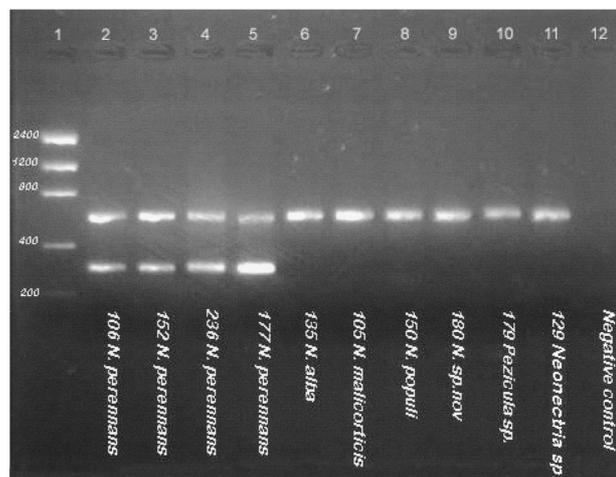


Fig. 3. PCR amplification of 300 bp *Neofabraea perennans*-specific fragments from total DNA using Neofab-upTub-100 and Neo_per-loTub-382, and an approximately 600 bp fragment amplified using the universal fungal primers UN-UP18S-42 and UN-LO28S-22.

Neofabraea populi

Amplification of total DNA from both *Neofabraea populi* isolates with Neofab-upTub-100 and Neo_pop-loTub-475 produced a 397 bp fragment specific to *N. populi* (Fig. 4). These primers did not produce amplification products from DNA of *N. alba*, *N. malicorticis*, *N. perennans*, *Neofabraea* sp. nov., *Pezicula corticola*, or *Neonectria galligena* under the PCR conditions applied. The 600 bp fragment generated using the universal fungal primers was present for all species analyzed in these reactions.

Neofabraea sp. nov.

Exclusive amplification of the two isolates of *Neofabraea* sp. nov. was achieved using Neofab-upTub-100 in combination with Neo_spnov-loTub-319, yielding

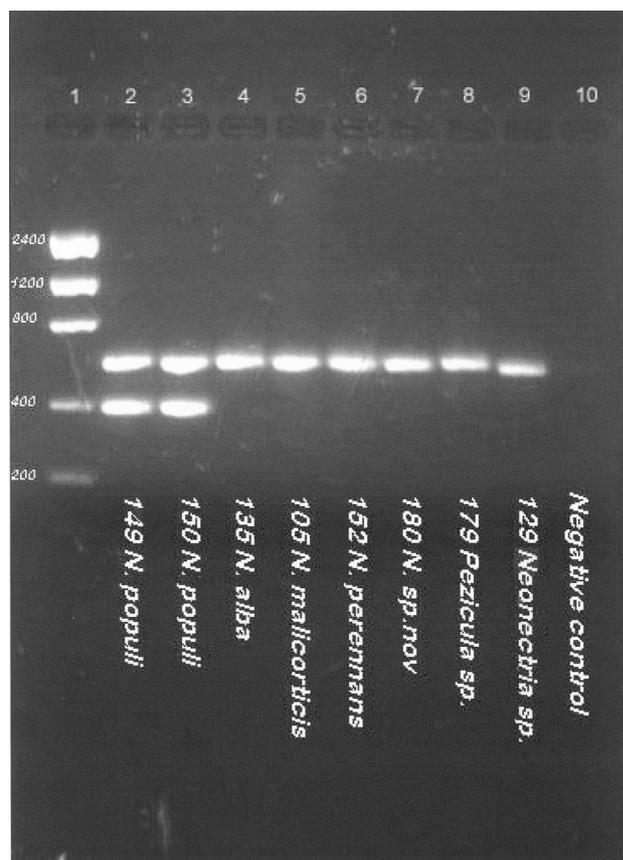


Fig. 4. PCR amplification of 397 bp *Neofabraea populi*-specific fragments from total DNA using Neofab-upTub-100 and Neo_pop-loTub-475 and an approximately 600 bp fragment amplified using the universal fungal primers UN-UP18S-42 and UN-LO28S-22.

a 236 bp fragment (Fig. 5). All fungal DNA analyzed in this experiment amplified with the universal fungal primer combination, giving the expected 600 bp fragments.

Amplification using multiplex PCR

The combination of all five primer sets in the multiplex reaction mixture yielded one clear species-specific band of the expected size for DNA of each of the five *Neofabraea* species used in this experiment (Fig. 6). Fragment sizes of 358 bp for *N. alba*, 178 bp for *N. malicorticis*, 300 bp for *N. perennans*, 397 bp for *N. populi*, and 236 bp for *Neofabraea* sp. nov. were produced in these reactions and were consistent with the results obtained using the respective species-specific primer sets individually.

PCR detection from artificially infected apple fruits

All apple fruits inoculated with the four *Neofabraea* species associated with bull's eye rot developed characteristic symptoms. None of the apples that served as uninoculated controls developed bull's eye rot symptoms. Amplification of DNA extracted from the

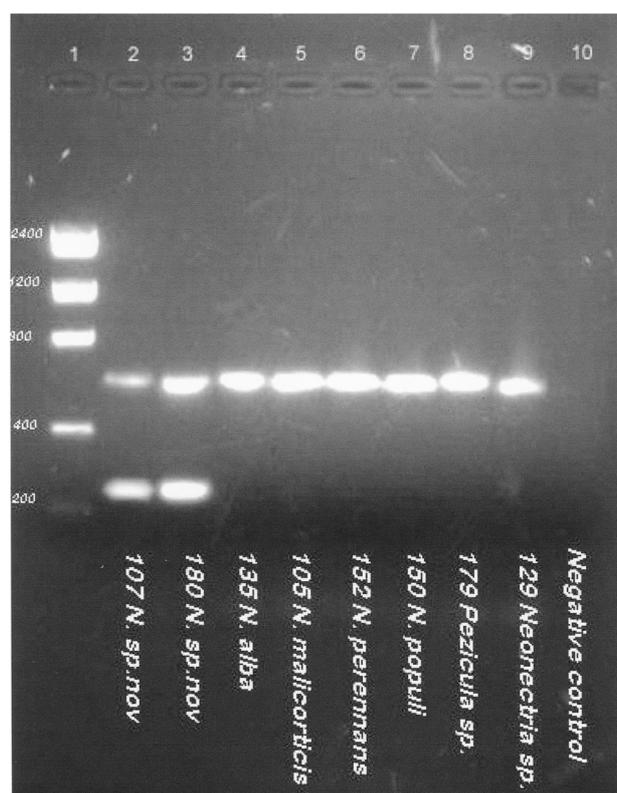


Fig. 5. PCR amplification of 236 bp *Neofabraea* sp. nov.-specific fragments from total DNA using Neofab-upTub-100 and Neo_spnov-loTub-319, and an approximately 600 bp fragment amplified using the universal fungal primers UN-UP18S-42 and UN-LO28S-22.

symptomatic tissue using species-specific primer sets individually (Fig. 7), and in multiplex (Fig. 8), yielded fragment sizes that corresponded to those expected for the *Neofabraea* species inoculated into the apple fruits. No fragments were observed when the extraction and amplification procedures were applied to DNA extracted from the uninoculated apples serving as controls.

DISCUSSION

The apparent overlap in geographic occurrence of *Neofabraea* species, coupled with their lack of morphological variation underlies the need for a molecular diagnostic tool for the identification of the five species in the genus *Neofabraea*. Sequencing of the β -tubulin gene of representatives of all five species of *Neofabraea* by de Jong *et al.* (2001) permitted the design of putative species-specific primers for *N. alba*, *N. malicorticis*, *N. perennans*, *N. populi*, and *Neofabraea* sp. nov. Under the stringent PCR parameters used in this research, the species-specific primers designed for the five species of the genus *Neofabraea* proved specific for the fungi for which they were designed. While only two isolates are known to exist for each of *N. populi* and *Neofabraea* sp. nov., the primers successfully detected both isolates of each species exclusively. It may be prudent to try to

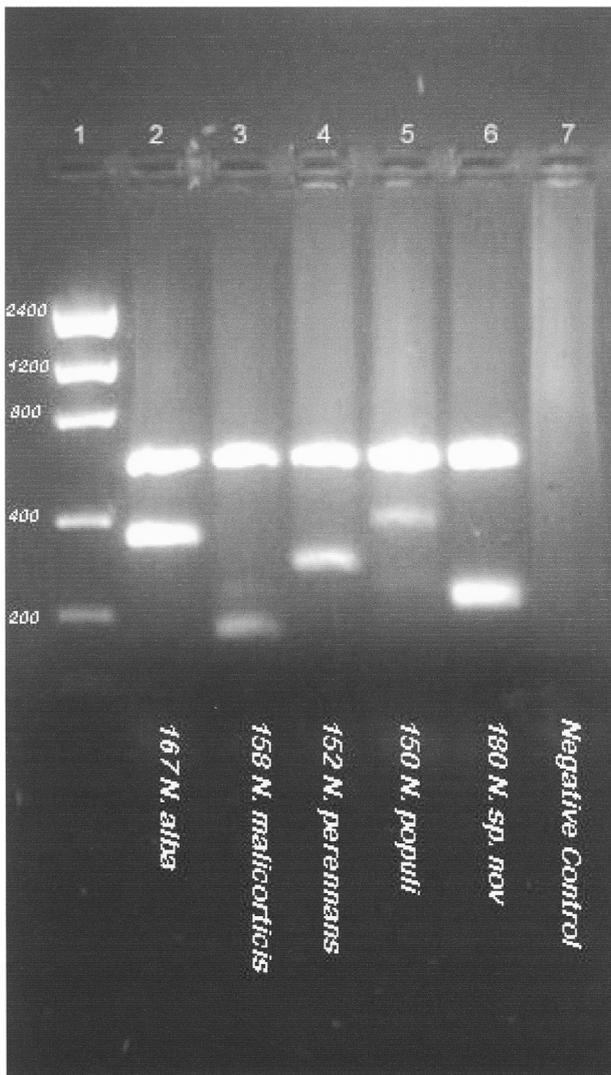


Fig. 6. Amplification of each of the five species of *Neofabraea* using a combination of the primers Neofab-upTub-100 with Neo_alba-loTub-439, Neo_mal-loTub-262, Neo_per-loTub-382, Neo_pop-loTub-475 and Neo_spnov-loTub-319 in a single reaction mixture. The universal fungal primers UN-UP18S-42 and UN-LO28S-22 used as internal positive controls amplified an approximately 600 bp fragment.

find more isolates of these two species to ensure that specificity is consistent over a broad range of isolates obtained from various locations.

When used in multiplex, these primer sets retained their specificity, yielding fragment sizes consistent with those obtained using the species-specific primer sets individually. Diagnostically, the application of this technology is quite valuable, providing a rapid, accurate, and specific technique for the identification of these pathogens that transcends the difficulties commonly encountered using conventional techniques. When dealing with a large number of samples that must be assayed for a variety of different pathogens, using one set of species-specific primers per reaction can be impractical. Multiplexing allows the screening of a given sample for several organisms simultaneously

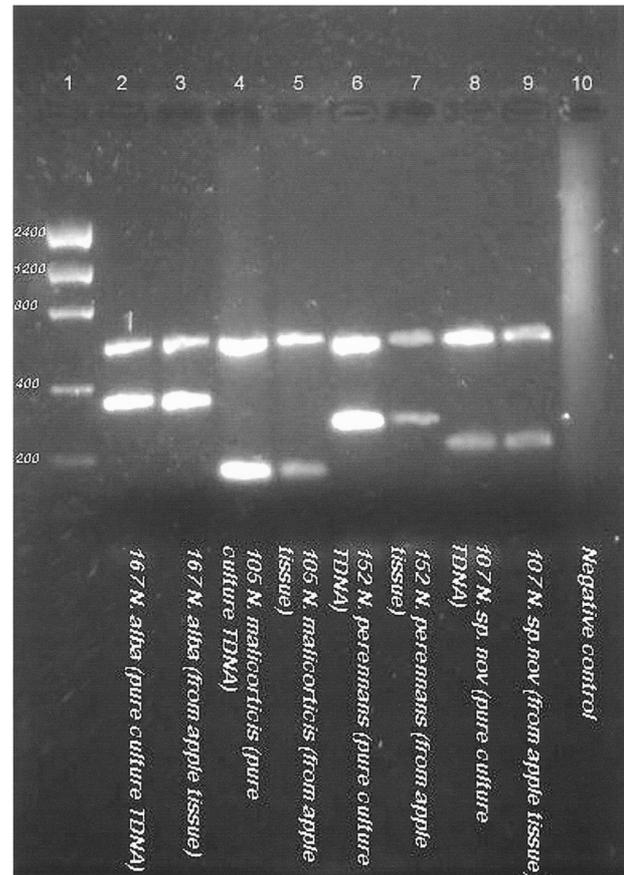


Fig. 7. PCR of *Neofabraea* DNA isolates 167 (*N. alba*), 105 (*N. malicorticis*), 152 (*N. perennans*), and 107 (*Neofabraea* sp. nov.) extracted from infected apple fruits (Lanes 3, 5, 7 and 9, respectively). Each isolate was amplified using the primer combination designed to be specific for that species. Lanes 2, 4, 6 and 8 represent pure culture total DNA (TDNA) of *N. alba*, *N. malicorticis*, *N. perennans*, and *Neofabraea* sp. nov., respectively, amplified with the appropriate species-specific primer set. Universal fungal primers UN-UP18S-42 and UN-LO28S-22 were used in each reaction as internal positive controls, generating an approximately 600 bp band for all isolates.

(Bej, McCarty & Atlas 1991, Henson & French 1993), allowing for concurrent identifications of the organisms in question. While this technique was developed to aid diagnosis of Duchenne muscular dystrophy (Chamberlain *et al.* 1988), it has since been successfully applied in various fields, and has earned applications in the identification of various pathogens. Examples of the application of this technique in microbiology include the detection of bacterial pathogens from environmental samples (Bej *et al.* 1990, 1991), and of multiple waterborne *Giardia* species (Mahbubani *et al.* 1992), as well as the simultaneous detection of various *Listeria* species from food samples (Border *et al.* 1990).

Multiplex PCR approaches have also found a niche in phytopathology. Multiplex PCR has been applied to the identification of mating types of *Septoria passerinii*, the causal agent of barley speckled leaf blotch (Goodwin *et al.* 2002) and *Magnaporthe grisea*, a pathogen of

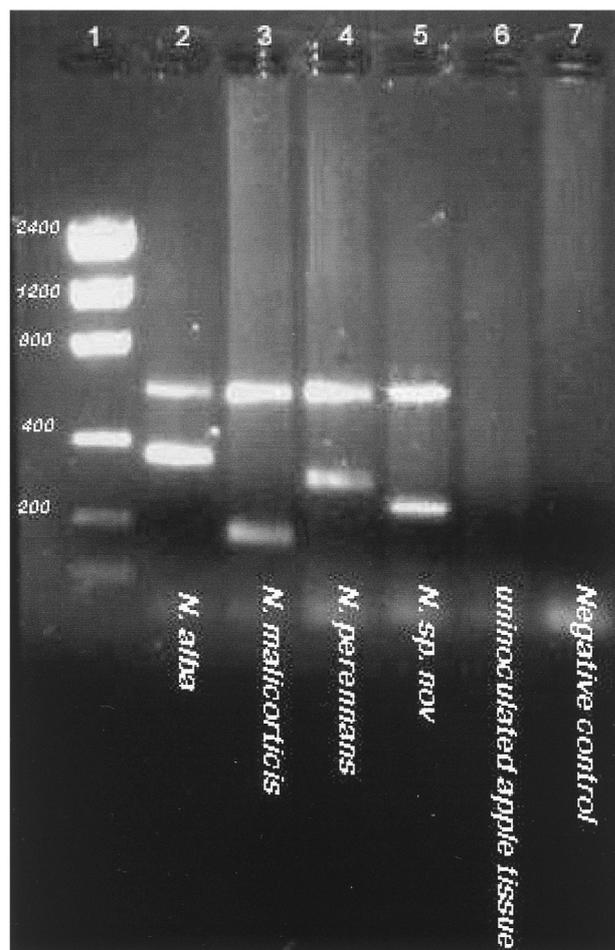


Fig. 8. PCR of *Neofabraea alba* (167), *N. malicorticis* (105), *N. peremans* (152), and *Neofabraea* sp. nov. (107) extracted from infected apple tissues using a multiplex protocol combining the *Neofabraea* primers Neofab-upTub-100 with Neo_alba-loTub-439, Neo_mal-loTub-262, Neo_per-loTub-382, Neo_pop-loTub-475, and Neo_spnov-loTub-319 in a single reaction mixture. The universal fungal primers UN-UP18S-42 and UN-LO28S-22 used as internal positive controls amplified an approximately 600 bp fragment.

grasses (Tredway *et al.* 2001). In addition, the technique has facilitated the accurate race determination of *Fusarium oxysporum* f. sp. *gladioli* (de Haan *et al.* 2000) and f. sp. *dianthi* (Chiocchetti *et al.* 1999). The detection and quantification of the foliar wheat pathogens *Septoria tritici*, *Stagonospora nodorum*, *Puccinia striiformis*, and *P. recondita* using multiplex PCR has resulted in the development of a method to assess varietal resistance of wheat to these pathogens (Fraaije *et al.* 2001). And finally, multiplex PCR has also been used to simultaneously identify two closely related root rot pathogens, *Cylindrocarpon floridanum* and *C. destructans* (Hamelin *et al.* 1996), and three potato pathogens, *Helminthosporium solani*, *Colleotrichum coccodes*, and *Streptomyces scabies* (Cullen *et al.* 2000). Other putative uses of this technique include the standardized screening of seeds for seed-borne pathogens, as discussed by Taylor *et al.* (2001).

While the use of these methods in plant pathogen identification have been successful, to date they have rarely been used for the detection of more than three plant pathogens in a single reaction (Cullen *et al.* 2000, Fraaije *et al.* 2001), and have never been used to test for more than two plant pathogens of the same genus simultaneously (Audy *et al.* 1996, Hamelin *et al.* 1996). The number of pathogens that can be detected using multiplex PCR is often limited by the number of primer pairs that can be incorporated into a single reaction, as well as the number of species for which bands can be clearly distinguished in a single reaction using standard agarose gel visualization techniques.

In the case of a species complex such as bull's eye rot, rather than running a series of five separate reactions to determine the *Neofabraea* species in question, a single reaction containing all five primer sets allows the accurate identification of the pathogen in question based on the fragment size generated. The diagnostic technique described here successfully incorporates five sets of species-specific primers into a single PCR reaction, and produces distinct bands for each pathogen, facilitating accurate identification of all five members of the genus *Neofabraea* to species. The potential for this technique to successfully identify multiple species in a single assay is immense. An indication of this potential is provided by Wang *et al.* (1998), who performed multiplex PCR assays for single nucleotide polymorphisms in the human genome analyses with one hundred primer pairs per reaction.

The diagnostic potential of the multiplex PCR technique is further enhanced when applied to DNA extracted directly from infected plant tissues, as was accomplished in this study. The ability to detect pathogens *in planta* from infected hosts could facilitate the identification process considerably, eliminating the need to isolate the pathogen in pure culture prior to extraction, and the potential for error inherent in this step. The diagnostic assays described here for the identification and detection of *Neofabraea* species provide a tool that enables discrimination amongst members of the genus *Neofabraea* and boasts several potential applications. As suggested with regards to brown rot of stone fruits, species-specific primers could be used to predict and monitor the incidence of specific pathogens in packing houses prior to the onset of rot symptoms (Boehm, Ma & Michailides 2001). This level of clarity would facilitate the development of species-specific control measures, and allow for the early removal of infected material from packing houses, minimizing associated losses. Furthermore, in cases where it is unclear as to which species is the cause of the bull's eye rot symptoms, species-specific primers could be used to discriminate between post harvest rot caused by *N. malicorticis*, *N. perennans*, *N. alba*, and *Neofabraea* sp. nov. Early and specific identification of *Neofabraea* species causing post-harvest bull's eye rot of pome fruits could allow for responsible, integrated pest management control measures to be applied. The same

would be true for the identification of fungal agents causing cankers on limbs and branches of pome fruit trees. Early identification and removal of infected tissues would limit associated tissue damage, provide the necessary information for varietal selection, and the development of effective control measures. It has historically been suggested that control measures for *N. malicorticis* are different than for *N. perennans*; the former relying on applications of Bordeaux mixture and the latter relying on the control of woolly apple aphid using insecticides (Kienholz 1939, 1951). While more recent research has shown that fungicides are not effective in anthracnose canker control (Rahe 1997), it does suggest that differences in the life cycle of these two fungi, and presumably other members of the genus *Neofabraea*, result in potential differences in their control. The primary control method currently suggested for both anthracnose canker and perennial canker involves removal of cankered bark in order to eliminate the source of inoculum for bull's eye rot. However, according to Kienholz (1951), some pear growing regions in Oregon and Washington have historically had post harvest bull's eye rot problems with fruit infections occurring on varieties that show no evidence of the tree cankers normally associated with *N. malicorticis* and *N. perennans*, the two pathogens claimed to be responsible for these diseases in the Pacific Northwest. Confirmation of the pathogen or pathogens in question in these areas may provide insight into alternative options for control of bull's eye rot in regions where cankers are not evident. Finally, species-specific primers could be used to clarify and delineate the geographic boundaries of each of these pathogens, and to elucidate geographic regions where these species overlap.

Accurate identification and diligent monitoring are the cornerstones of pest management. The lack of fast, reliable methods to detect and identify disease organisms is an important limitation in integrated disease management programs implementing low-impact cultural and chemical alternatives and breeding for resistance. The management of diseases caused by *Neofabraea* pathogens has been plagued by a lack of information pertaining to their epidemiology, which is in part due to a lack of morphological distinctiveness that would allow accurate identification to species. Use of the molecular diagnostic tools described herein would serve to abate diagnostic confusion and provide a path forward for the development of effective pest management strategies for members of the genus *Neofabraea*.

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