A Comparison of Standard and High-Fidelity PCR: Evaluating Quantification and Detection of Pathogen DNA in the Presence of Orchid Host Tissue

Robert A. Cating, Twyford International – Biotechnology, Apopka, FL 32703; **Marjorie Ann Hoy**, University of Florida – IFAS – Entomology & Nematology, Gainesville; and **Aaron J. Palmateer**, University of Florida – Tropical Research & Education Center, Homestead

Abstract

Cating, R. A., Hoy, M. A., and Palmateer, A. J. 2012. A comparison of standard and high-fidelity PCR: Evaluating quantification and detection of pathogen DNA in the presence of orchid host tissue. *Plant Dis.* 96:480-485.

The polymerase chain reaction (PCR) has been used with increasing frequency for detecting and identifying plant pathogens. Although PCR is sensitive, research has shown that amplification of target microbial DNA from within another organism, such as an arthropod or plant, can be inhibited by the presence of host DNA. In this study, the sensitivity of standard and high-fidelity PCR, which incorporates a second DNA polymerase with proofreading ability, to detect and amplify DNA from the fungal pathogen *Pseudocercospora odontoglossi* while in the presence of *Cattleya* orchid DNA, was compared. Different dilutions of

plasmids containing internal transcribed spacer (ITS)1, 5.8S, and ITS2 rDNA from *P. odontoglossi* were spiked with *Cattleya* orchid plant DNA. The high-fidelity PCR could detect and amplify as few as 207 plasmids containing the fungal DNA, whereas the standard PCR required over 200 million copies. The high-fidelity PCR was more efficient than conventional PCR in detecting *Sclerotium rolfsii* and a *Dickeya* sp. from freshly inoculated orchid plants, demonstrating its increased sensitivity in early detection of fungal and bacterial pathogens that are difficult to discriminate early in disease development.

The polymerase chain reaction (PCR) has become widely used in phylogenetic studies (8,9,13,31,34), genomic analyses (22,27), and plant-disease diagnosis (1,23,25,38,44), as well as to examine genetic diversity in plant pathogens (40,47,48). However, standard PCR usually does not amplify sequences of more than 5 kb, and those between 2 and 5 kb are amplified inefficiently (2). Unlike standard PCR, high-fidelity PCR (=long PCR) can amplify longer sequences, making it possible to amplify whole viruses and plasmids up to 35 kb (2).

High-fidelity PCR differs from standard PCR by the addition of a small amount of a second, proofreading DNA polymerase. Barnes (2) demonstrated that the addition of the proofreading enzyme removed mismatched base pairs that can stop the extension of the DNA strand, allowing strand synthesis to proceed (2). In addition to producing longer sequences than standard PCR, high-fidelity PCR efficiently amplifies target DNA in the presence of large amounts of non-target DNA, which can potentially inhibit detection of target DNA. Vickers and Graham (42) used high-fidelity PCR to consistently amplify a single-copy gene (*Bar*), a marker for the selection of transgenic plants, in the presence of barley genomic DNA, whereas standard PCR only occasionally amplified the target gene. High-fidelity PCR can detect bacterial infections and microbial associations in arthropods; Jeyaprakash and Hoy (20) demonstrated that high-fidelity PCR could detect as little as 1 fg of plasmid DNA containing the *wsp* gene from *Wolbachia* spp. versus 1 ng of plasmid with standard PCR. High-fidelity PCR also was more sensitive than standard PCR in detecting the citrus greening pathogen, '*Candidatus Liberobacter asiaticum*', in the presence of genomic DNA from the Asian citrus psyllid, *Dia-*

phorina citri, or parasitoids of the citrus psyllid (19). It has been used to detect and characterize a new microsporidium species from the predatory mite *Metaseiulus occidentalis* (3); identify and distinguish two parasitoids of the brown citrus aphid (32); examine the microbial diversity of *M. occidentalis* and its spider mite prey, *Tetranychus urticae* (18); and amplify 16S ribosomal sequences of endotoxin-producing bacteria in varying amounts of dust mite DNA (41). Because high-fidelity PCR is more sensitive than standard PCR, it could prove to be an important tool for plant-disease diagnosis, especially when the target DNA is mixed with plant genomic DNA.

When compared with most horticultural crops, very little is known about orchid diseases. The scientific literature contains only limited information concerning pathogenicity tests, new diseases, and host ranges of orchid pathogens. Based on the economic importance of orchid plants and their increasing popularity, resulting in large-scale commercial production, more efficient methods of diagnosing orchid diseases are warranted. In order to implement management strategies for orchid diseases and reduce plant losses, it is necessary to detect the pathogen and diagnose the problem accurately and as early as possible (1,11,16,24).

Diagnoses in the clinic or field can be complicated by the fact that some bacterial diseases can be confused with fungal diseases. For example, bacterial soft rots caused by *Pectobacterium carotovorum* or *Dickeya* sp. (*Erwinia chrysanthemi*) can be confused with early symptoms caused by the fungus *Sclerotium rolfsii*; all of these pathogens can cause substantial losses in orchid production facilities (Fig. 1) (4–7). The rapid identification of disease-causing organisms that produce similar symptoms or those that cannot be cultured could potentially be improved by the use of a high-fidelity PCR protocol.

The objectives of this study were to (i) determine whether high-fidelity PCR is more sensitive than standard PCR in detecting fungal DNA in the presence of plant genomic DNA by using serially diluted plasmids containing a portion of the internal transcribed spacer (ITS) region of *Pseudocercospora odontoglossi*, a common leaf spot pathogen of orchid (9), as a model system; and (ii) deter-

Corresponding author: A. Palmateer, E-mail: ajp@ufl.edu

Accepted for publication 25 October 2011.

<http://dx.doi.org/10.1094/PDIS-08-11-0705>
© 2012 The American Phytopathological Society

mine whether high-fidelity PCR is more sensitive than standard PCR in detecting *S. rolfii* and *Dickeya* sp. directly from *Phalaenopsis* orchid tissues early in the infection process.

Materials and Methods

Evaluation of high-fidelity PCR by use of plasmids. DNA extraction. *Cattleya* orchid leaf tissue (3.866 g) was frozen in liquid nitrogen and ground in 8 ml of cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris [pH 7.5], 20 mM EDTA, 1% polyvinyl pyrrolidone, and 1.4 M NaCl) for 10 min and aliquoted into eight tubes. The samples were then incubated at 60°C for 16 h. After two chloroform extractions, the eight DNA aliquots were combined into four, precipitated in 2-propanol, and resuspended in 100 µl of sterile water. All samples were pooled to make a 400-µl sample of genomic orchid DNA. Plant DNA quantity and quality was estimated using an Eppendorf BioPhotometer G131 V1.35 (Eppendorf, Hamburg, Germany).

The leaf spot fungus *P. odontoglossi* was isolated from a *Cattleya* hybrid and identified based on morphological characters, sequence analysis, and host (9,10,15,34). Single spores cultures were grown on V8 juice agar for 2 weeks at 25°C under artificial light under a photoperiod of 12 h of light and 12 h of darkness. A section of mycelium (approximately 2 by 2 cm) was scraped from the surface of the plate with a sterile wooden applicator stick and placed in a 0.5-ml tube. Extraction solution (100 µl; Extract-n-Amp; Sigma-Aldrich, St. Louis) was added and the sample was ground for 5 min with a sterile plastic pestle and heated to 95°C for 10 min. Dilution solution (100 µl; Extract-n-Amp; Sigma-Aldrich) was added to the sample, after which the sample was briefly vortexed. In all, 30 µl of the extracted DNA was added to 270 µl of sterile water (17).

High-fidelity PCR protocol. To compare high-fidelity and standard PCR, plasmids that contained ITS1, 5.8S, and ITS2 rDNA sequences were generated with DNA extracted from *P. odontoglossi* as described above with high-fidelity PCR. The 50-µl reaction volume contained 50 mM Tris, pH 9.2; 16 mM ammonium sulfate; 1.75 mM MgCl₂; 350 µM each dATP, dGTP, dCTP, and dTTP; 800 pmol of primers (ITS4, 5'-TCCTCCGCTTATTGATATGC-3' and ITS5, 5'-GGAAGTAAAAGTCGTAACAAGG-3') (45); 1 unit of Accuzyme (Bioline, Taunton, MA); and 5 units of *Taq* DNA polymerase (Bioline) (2). Samples were covered with 100 µl of sterile mineral oil and amplified using three linked temperature profiles: (i) 94°C for 2 min; (ii) 10 cycles consisting of denaturing at 94°C for 10 s, annealing at 53°C for 30 s, and extension at 68°C for 1 min; and (iii) 25 cycles consisting of 94°C for 10 s, annealing at 53°C for 30 s, and extension at 68°C for 1 min plus an additional 20 s during each consecutive cycle (20).

Molecular cloning. The ITS1, 5.8S, and ITS2 rDNA sequences amplified by high-fidelity PCR from *P. odontoglossi* were cloned using the TOPO T/A cloning kit (Invitrogen Corp., Carlsbad, CA). Before ligation into the cloning vector, PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's recommendations and eluted in 50 µl of sterile glass-distilled, glass-collected water. A 3' A-overhang was added to the PCR product after purification to facilitate ligation into the cloning vector by mixing the 50-µl DNA sample with 5.75 µl of 10× high-fidelity buffer (50 mM Tris [pH 9.2], 16 mM ammonium sulfate, and 1.75 mM MgCl₂), 100 mM dATP, and 1 unit of *Taq* polymerase (Bioline). The reaction was placed in a thermal cycler (Perkin Elmer DNA Thermal Cycler 480) at 72°C for 45 min. The product was immediately cloned into the TOPO T/A cloning vector following the manufacturer's recommendations (Invitrogen Corp.). Plasmids were extracted using the Qiagen Plasmid Mini Prep Kit (Qiagen) and digested with *Eco*RI followed by gel electrophoresis on a 2% agarose Tris-acetate-EDTA gel stained with ethidium bromide to confirm the correct size of the insert. Purified plasmids (pRC17) were sent to the Interdisciplinary Center for Biotechnology Research Core Facility at the University of Florida, Gainesville for DNA sequencing.

Comparison of standard and high-fidelity PCR. *Escherichia coli* cells containing plasmid pRC17 constructed above were grown in Luria-Bertani broth overnight at 37°C, then extracted and purified using the Qiafilter Plasmid Midi Prep Kit (Qiagen) and quantified using a BioPhotometer G131 V1.35 (Eppendorf). To quantify the differences between standard and high-fidelity PCR, serially diluted (1,000 ng to 1 fg) plasmid pRC17 DNA was spiked with 10 ng of *Cattleya* genomic DNA (Fig. 2). The high-fidelity PCR was the same as described above, and the standard PCR was performed in a 25-µl reaction volume and contained 2.5 µl of 10× PCR buffer + Mg (Boehringer, Mannheim, Germany); 200 µM dATP, dGTP, dCTP, and dTTP; 400 pM primers ITS4 and ITS5 (45); and 0.2 units of *Taq* DNA polymerase (Bioline). Samples were covered with 50 µl of sterile mineral oil and amplified using the following temperature profile: (i) 94°C for 5 min and (ii) 35 cycles consisting of denaturing at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min. Two negative controls were used in each set of high-fidelity and standard PCR reactions, one containing plant genomic DNA alone, the other without DNA.

As a comparison, a set of standard PCR reactions was performed using the same serially diluted plasmid pRC17 DNA but without the addition of 10 ng of plant DNA. All reactions were replicated twice on different days.

Comparison of high-fidelity and standard PCR in detecting *S. rolfii* and *Dickeya* sp. (*Erwinia chrysanthemi*) from *Phalaenopsis* orchid. At three times on different days, *Phalaenopsis* orchid plants were artificially inoculated with two economically important orchid pathogens: *S. rolfii* and a *Dickeya* sp. The *S. rolfii* isolate was recovered from an *Ascocenda* orchid, identified based on morphological characteristics and ITS1, 5.8S, and ITS2 rDNA sequences (6), grown for 7 days on V8 medium, and used to inoculate five *Phalaenopsis* plants, each with a 6-mm plug that was placed mycelium side down on the youngest leaf. Plants were placed in a greenhouse under a photoperiod of 16 h of light and 8 h of darkness at 25 to 30°C and 56 to 88% relative humidity (RH). After 2 days, inoculated leaves were removed and washed in a 10% household bleach solution for 30 s followed by two 1-min rinses with sterile water. Leaf tissue was patted dry with sterile paper towels and a 5-by-5-mm section was removed from the margin of the lesion from each of the inoculated plants. With primer pair ITS4/ITS5 (45), high-fidelity and standard PCR were performed with DNA that was extracted from each of the five samples using the Extract-n-Amp (Sigma-Aldrich) protocol described above. Identification of the pathogen was confirmed by cloning and sequencing the PCR product using the procedures described above and comparing the sequence with that deposited previously in GenBank (GQ358518) (6).

The *Dickeya* sp. was isolated from a *Phalaenopsis* orchid and identified based on characteristics described by Samson et al. (33), including 16S rDNA sequence. The isolate was grown on nutrient agar for 24 h at 27°C, suspended in sterile tap water, and adjusted to a concentration of 1×10^8 with a BioPhotometer G131 V1.35 (Eppendorf); 100 µl of the suspension was injected into the youngest leaf of five *Phalaenopsis* plants. Plants were incubated in a greenhouse under a photoperiod of 16 h of light and 8 h of darkness at 26 to 30°C and 56 to 90% RH. After 24 h, soft-rot symptoms developed and DNA was extracted using the procedure described above for *S. rolfii*. High-fidelity and standard PCR were performed with primer pair ADE1 (5'-GATCAGAAAGCCCGCAGCCAGAT-3') and ADE2 (5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3') (28). With the exception of a 63°C annealing temperature for the *Dickeya* sp. samples, the parameters were the same as those used for the *S. rolfii* samples. The identity of the pathogen was confirmed by cloning and sequencing the PCR product following the above procedures. To confirm that reactions worked properly, two positive controls were used in each set of reactions that consisted of genomic DNA from the *S. rolfii* or the *Dickeya* sp. isolates extracted using the PUREGENE DNA extraction protocol (Qiagen) according to the manufacturer's recommendations. All reactions were replicated twice on different days.

Results

Evaluation of high-fidelity PCR by use of plasmids. High-fidelity PCR was six orders of magnitude more sensitive than standard PCR when detecting *P. odontoglossi* DNA in the presence of orchid DNA. Bright bands of the correct size were generated with high-fidelity PCR using 1 fg of target sequence (Fig. 2A), whereas standard PCR detected only 1 ng of plasmid DNA (Fig. 2B). In the absence of host DNA, standard PCR detected target sequences in the plasmid DNA at concentrations as low as 10 fg (Fig. 2C). No amplification was observed in either the no-DNA or plant-alone reactions and the same results were obtained both times the experiment was conducted.

Comparison of high-fidelity and standard PCR in detecting *S. rolfii* and *Dickeya* sp. (*Erwinia chrysanthemi*) from *Phalaenopsis* orchid. High-fidelity PCR detected *S. rolfii* DNA sequences in all five plants that had been inoculated 2 days previously in each of the three replicates and both positive controls (Fig. 3A). When the same samples were amplified using the standard PCR protocol, only the positive controls produced a band of the expected size (Fig. 3A). Similarly, high-fidelity PCR detected *Dickeya* sp. DNA from each of five inoculated plants in each of the three replicates but standard PCR only amplified the positive control (Fig. 3B). To confirm the identity of the pathogens, amplicons from the positive reactions, including the controls, were cloned and sequenced according to the above method and determined to be from the correct pathogen (*data not shown*).

Discussion

In our plasmid dilution series study, the high-fidelity PCR consistently detected as little as 1 fg of target DNA in the presence of host DNA, which is equivalent to 207 copies of the ITS1, 5.8S, and ITS2 rDNA. High-fidelity PCR was shown to be

more sensitive than the standard PCR in detecting both a fungal and a bacterial pathogen of *Phalaenopsis* orchid within 2 days of inoculation.

These results are similar to those obtained by Jeyaprakash and Hoy (20), who detected as little as 1 fg (= 100 copies) of the *Wolbachia* *wsp* sequence in the presence of genomic DNA of the parasitoid wasp *Tamarixia radiata* with high-fidelity PCR. Similarly, Hoy et al. (19) demonstrated that the high-fidelity PCR could detect as little as 1 fg of citrus greening pathogen DNA while in the presence of citrus DNA or citrus psyllid (*D. citri*) DNA.

Although standard PCR frequently is used to detect plant pathogens, there are clearly limits to its sensitivity. For example, Nielsen et al. (29) could only detect between 1 and 10 pg of pure fungal DNA of *Botrytis* spp. that cause neck rot of onion, and Moricca et al. (26) could only detect between 1 and 50 ng of *Fusarium oxysporum* f. sp. *vasinfectum* DNA from infected cotton. Tsai et al. (39) could not amplify less than 1 pg of *Phytophthora palmivora* DNA but amplified as little as 10 fg when a nested PCR protocol was used. Diallo et al. (14) found that DNA of *Pectobacterium carotovorum* subsp. *carotovorum* or *P. atrosepticum* reduced detection limits of *Dickeya* sp. from 0.1 to 1.0 ng when added to PCR reactions. In the present study, the high-fidelity PCR amplified as little as 1 fg of target DNA while in the presence of host genomic DNA, an increase in sensitivity of sixfold when compared with standard PCR. Because high-fidelity PCR can detect pathogen DNA while in the presence of host DNA more efficiently than standard PCR, it should be useful for early pathogen detection when symptoms are not characteristic for the disease.

Latent infections can considerably reduce a plant inspector's or diagnostician's ability to detect a given pathogen. Although data are not shown in the present study, we have used high-fidelity PCR for detecting latent infection by a *Dickeya* sp. from asymptomatic *Vanda* orchid imported from Thailand (A. Palmateer, unpublished).

Fig. 1. Difficulties in orchid disease diagnosis. **A**, *Ascocenda* orchid and **B**, *Phalaenopsis* orchid with southern blight caused by *Sclerotium rolfii*; **C**, *Oncidium* orchid with a soft-rot caused by a *Dickeya* sp. (*Erwinia chrysanthemi*); **D**, *Phalaenopsis* orchid with soft-rot caused by *Pectobacterium carotovorum*. As is evident in these photos, early symptoms can appear similar and can be difficult to diagnose without the aid of molecular tools.

and this allowed for the importer to reject the shipment, potentially avoiding a major disease outbreak in the nursery. Environmental factors such as temperature and moisture are critical in disease development, and pathogens can remain latent in the host after infection if disease-conducive conditions are not met (21,30, 35,37). Because latent pathogens can be difficult to detect on artificial media or with standard PCR protocols, false negatives or escapes from detection are not uncommon.

The present results indicate that the high-fidelity PCR protocol could be an important tool for diagnosticians and regulatory agencies, especially when pathogens are of quarantine significance, occur at low titers, are latent, or cannot be cultured. The rapidity and sensitivity of this method, its utility with impure or mixed DNA samples, and the ability to amplify target DNA directly from plant tissue are valuable attributes that allow its use in a wide range of conditions.

According to Vincelli and Tisserat (43), nucleic acid-based technology should be used more frequently for detecting plant pathogens in order to provide data to support a diagnosis, particularly for nonculturable organisms, for pathogen species determination, and to contribute sequence data to GenBank. However, when using PCR in a diagnostic setting, particularly when detecting plant pathogens directly from plant tissue, inhibited DNA amplification can be a major problem. Plant cellular contents (organic and inorganic compounds) interfere with the efficiency of standard PCR (42,43,46), making the diagnosis of plant pathogens directly from plant tissue difficult and one of the most common factors that limit obtaining accurate results.

To increase the applicability and reliability of the PCR in microbe detection, Wilson (46) indicated that methods to remove PCR inhibitors were needed. For example, large amounts of genomic DNA, whether from the host or target organism, can inhibit

Fig. 2. Comparison of high-fidelity and standard polymerase chain reaction (PCR) using plasmid pRC17 containing the 571-bp internal transcribed spacer (ITS)1, 5.8S, and ITS2 rDNA of *Pseudocercospora odontoglossi* as a template and primers ITS4/ITS5. **A**, High-fidelity PCR amplified as few as 207 copies (1 fg) of template in the presence of 10 ng of *Cattleya* DNA, whereas **B**, the standard PCR required at least 200 million copies (1 ng) of template for amplification. **C**, In the absence of host genomic DNA, the standard PCR protocol amplified as little as 10 fg of template. M = molecular marker IV (Roche).

Fig. 3. Comparison of high-fidelity and standard polymerase chain reaction (PCR) in the detection of two important orchid pathogens from five inoculated *Phalaenopsis* plants. **A**, *Sclerotium rolfssii* could be detected in all five plants using fungal internal transcribed spacer (ITS)4/ITS5 and the high-fidelity PCR protocol (lanes 5–9), whereas it could be detected in no plants with standard PCR (lanes 14–18). **B**, Similarly, the high-fidelity PCR could detect *Dickeya* sp. in inoculated plants using primers ADE1/ADE2 (lanes 5–9) whereas it could be detected in no plants with standard PCR (lanes 14–18). M = molecular marker XIV (Roche).

PCR reactions (19,36,46). Using the high-fidelity PCR protocol in this study, which incorporates a second DNA polymerase with proof-reading ability, reduces false negatives when detecting fungal and bacterial plant pathogens in the presence of PCR amplification inhibitors.

Many techniques exist to increase the sensitivity of standard PCR. These include nested PCR (44), immunocapture (24,44), multiple displacement amplification (12), and real-time PCR, as well as others (43). Although real-time PCR is more sensitive than standard PCR, it requires the purchase of expensive equipment and necessitates additional training. High-fidelity PCR is relatively simple and requires only the addition of small amounts of a proof-reading enzyme to the *Taq* DNA polymerase (2), a slight increase in primer and dNTP concentrations, and modifications to the heating and cooling protocol to increase sensitivity. High-fidelity PCR kits and DNA polymerase blends are readily available from companies such as Clontech, Roche, and Invitrogen. Plant disease clinics

and diagnosticians that already own thermal cyclers can adopt high-fidelity PCR without the purchase of new equipment or additional training.

Acknowledgments

We thank all of the people who provided orchid samples: K. Herndon (Kerry's Nursery, Homestead, FL), Goodwin Orchids (Leesburg, FL), B. Peters (Whimsy Orchids, Homestead, FL), M. Motes (Motes Orchids, Homestead, FL), and R. Fuchs (R.F. Orchids, Homestead, FL), and Dr. Ayyamperumal Jeyaprakash for his assistance and advice on molecular techniques. Part of this study was supported by the Redland Orchid Festivals Inc., the USDA/TSTAR (USDA 2009-34135-20109), and the Davies, Fischer and Eckes Endowment in Biological Control to M. A. Hoy.

Literature Cited

1. Alvarez, A. M. 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. *Annu. Rev. Phytopathol.* 42:339-366.
2. Barnes, W. 1994. PCR amplification of up to 35-kb DNA with high fidelity

- and high yield from λ bacteriophage templates. PNAS 91:2216-2220.
3. Becnel, J. J., Jeyaprakash, A., Hoy, M. A., and Shapiro, A. 2002. Morphological and molecular characterization of a new microsporidium species from the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Acari, Phytoseiidae). J. Invert. Pathol. 79:163-172.
4. Cating, R. A., Hong, J. C., Palmateer, A. J., Stiles, C. M., and Dickstein, E. R. 2008. First report of *Dickeya chrysanthemi* (*Erwinia chrysanthemi*) on *Vanda* orchids in Florida. Plant Dis. 92:977.
5. Cating, R. A., and Palmateer, A. J. 2011. Bacterial soft rot of *Oncidium* orchids caused by a *Dickeya* sp. (*Pectobacterium chrysanthemi*) in Florida. Plant Dis. 95:74.
6. Cating, R. A., Palmateer, A. J., and McMillan, R. T., Jr. 2009. First report of southern blight caused by *Sclerotium rolfsii* in *Ascoenda* and *Ascozentrum* orchids in Florida. Plant Dis. 93:963.
7. Cating, R. A., Palmateer, A. J., McMillan Jr., R. T., and Dickstein, E. R. 2009. First report of a soft rot in *Tolumnia* orchids caused by a *Dickeya* sp. Plant Dis. 93:1354.
8. Chaverri, P., Samuels, G. J., and Hodge, K. T. 2005. The genus *Podocrella* and its nematode-killing anamorph *Harposporium*. Mycologia 97:433-443.
9. Crous, P. W., and Braun, U. 2003. *Mycosphaerella* and Its Anamorphs: 1. Names Published in *Cercospora* and *Passalora*. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
10. Crous, P. W., Kang, J. C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequence and morphology. Mycologia 93:1081-1101.
11. Daughtrey, M. A., and Benson, D. M. 2005. Principles of plant health management for ornamental plants. Annu. Rev. Phytopathol. 43:141-169.
12. Dean, F. B., Hosono, S., Fang, L., Wu, X., Faruqi, A., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S. F., Egholm, M., and Lasken, R. S. 2002. Comprehensive human genome amplification using multiple displacement amplification. PNAS 99:5261-5266.
13. Dettman, J. R., Jacobson, D. J., and Taylor, J. W. 2006. Multilocus sequence data reveal extensive phylogenetic species diversity within the *Neurospora discreta* complex. Mycologia 98:436-446.
14. Diallo, S., Latour, X., Groboillot, A., Smadja, B., Copin, P., Orange, N., Feuilloley, M. G. J., and Chavalier, S. 2009. Simultaneous and selective detection of two major soft rot pathogens in potato: *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atrosepticum*) and *Dickeya* spp. (*Erwinia chrysanthemi*). Eur. J. Plant Pathol. 125:349-354.
15. Ellis, M. B. 1976. More Dematiaceae Hyphomycetes. CAB International, New York.
16. Finetti Sialer, M. M., and Rosso, L. 2007. Molecular detection in integrated pest and disease management. Pages 305-328 in: General Concepts in Integrated Pest and Disease Management. A. Ciancio and K. G. Mukerji, eds. Springer, Dordrecht, The Netherlands.
17. Harmon, P. F., Dunkle, L. D., and Latin, R. 2003. A rapid PCR-based method for the detection of *Magnaporthe oryzae* from infected perennial ryegrass. Plant Dis. 87:1072-1076.
18. Hoy, M. A., and Jeyaprakash, A. 2005. Microbial diversity in the predatory mite *Metaseiulus occidentalis* (Acari: Phytoseiidae) and its prey, *Tetranychus urticae* (Acari: Tetranychidae). Biol. Control 32:427-441.
19. Hoy, M. A., Jeyaprakash, A., and Nguyen, R. 2001. Long PCR is a sensitive method for detecting *Liberobacter asiaticum* in parasitoids undergoing risk assessment in quarantine. Biol. Control 22:278-287.
20. Jeyaprakash, A., and Hoy, M. A. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9:393-405.
21. Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot erwinias. Annu. Rev. Phytopathol. 25:405-430.
22. Lashkari, D. A., McCusker, J. H., and Davis, R. W. 1997. Whole genome analysis: experimental access to all genome sequenced segments through larger-scale efficient oligonucleotide synthesis and PCR. PNAS 94:8945-8947.
23. Lévesque, C. A. 2001. Molecular methods for detection of plant pathogens—what is the future? Can. J. Plant Pathol. 24:333-336.
24. Louws, F. J., Rademaker, J. L. W., and De Bruijn, F. J. 1999. The three Ds of PCR-based genomic analysis of phyto-bacteria: diversity, detection, and disease diagnosis. Annu. Rev. Phytopathol. 37:81-125.
25. McCartney, H. A., Foster, S. J., Fraaije, B. A., and Ward, E. 2003. Molecular diagnostics for fungal plant pathogens. Pest Manage. Sci. 59:129-142.
26. Moricca, S., Ragazzi, A., Kasuga, T., and Mitchelson, K. R. 1998. Detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue by polymerase chain reaction. Plant Pathol. 47:486-494.
27. Nadeau, J., Bedigian, H. G., Bouchard, G., Denial, T., Kosowsky, M., Norberg, R., Pugh, S., Sargeant, E., Turner, R., and Paigen, B. 1992. Multilocus markers for mouse genome analysis: PCR amplification based on single primers of arbitrary nucleotide sequence. Mamm. Genome 3:55-64.
28. Nassar, A., Parrasse, A., Lemattre, M., Kotoujansky, A., Dervin, C., Vedel, R., and Bertheau, Y. 1996. Characterization of *Erwinia chrysanthemi* by pectinolytic isozyme polymorphism and restriction fragment length polymorphism analysis of PCR-amplified fragments of *pel* genes. Appl. Environ. Microbiol. 62:2228-2235.
29. Nielsen, K., Yohalem, D. S., and Jensen, D. F. 2002. PCR detection and RFLP differentiation of *Botrytis* species associated with neck rot of onion. Plant Dis. 86:682-686.
30. Palacio-Bielsa, A., Cambra, M. A., and López M. M. 2006. Characterisation of potato isolates of *Dickeya chrysanthemi* in Spain by a microtitre system for biovar determination. Ann. Appl. Biol. 148:157-164.
31. Palmateer, A. J., McLean, K. S., and Morgan-Jones, G. 2003. Concerning *Phomopsis gossypii*, the causal organism of boll rot of cotton. Mycotaxon 87:157-172.
32. Persad, A. B., Jeyaprakash, A., and Hoy, M. A. 2004. High-fidelity PCR assay discriminates between immature *Lipolexis oregmae* and *Lysiphlebus testaceipes* (Hymenoptera: Aphidiidae) within their aphid hosts. Fla. Entomol. 87:18-24.
33. Samson, R., Legendre, J. B., Christen, R., Fischer-Le Saux, M., Achouax, W., and Gardan, L. 2005. Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. Int. J. Syst. Evol. Microbiol. 55:1415-1427.
34. Stewart, E. L., Liu, Z., Crous, P. W., and Szabo, L. J. 1999. Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. Mycol. Res. 103:1491-1499.
35. Swanson, J. K., Montes, L., Mejia, L., and Allen, C. 2007. Detection of latent infections of *Ralstonia solanacearum* race 3 biovar 2 in geranium. Plant Dis. 91:828-834.
36. Tebbe, C. C., and Vahjen, W. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl. Environ. Microbiol. 59:2657-2665.
37. Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. J. 2003. Soft-rot erwiniae: from genes to genomes. Mol. Plant Pathol. 4:17-30.
38. Trout, C. L., Ristaino, J. B., Madritch, M., and Wangsomboondee, T. 1997. Rapid detection of *Phytophthora infestans* in late-blight infected potato and tomato using PCR. Plant Dis. 81:1042-1048.
39. Tsai, H. L., Huang, L. C., Ann, P. J., and Liou, R. F. 2006. Detection of orchid *Phytophthora* disease by nested PCR. Bot. Stud. 47:379-387.
40. Urena-Padilla, A. R., MacKenzie, S. J., Bowen, B. W., and Legard, D. E. 2002. Etiology and population genetics of *Colletotrichum* spp. causing crown and fruit rot of strawberry. Phytopathology 92:1245-1252.
41. Valerio, C. R., Murray, P., Arlian, L. G., and Slater, J. E. 2005. Bacterial 16S ribosomal DNA in house dust mite cultures. J. Allergy Clin. Immun. 118:1296-1300.
42. Vickers, J. E., and Graham, G. C. 1996. A protocol for the efficient screening of transformed plants for *bar*, the selectable marker gene, using the polymerase chain reaction. Plant Mol. Biol. Rep. 14:363-368.
43. Vincelli, P., and Tisserat, N. 2008. Nucleic acid-based pathogen detection in applied plant pathology. Plant Dis. 92:660-669.
44. Ward, E., Foster, S. J., Fraaije, B. A., and McCartney, H. A. 2004. Plant pathogen diagnostics: immunological and nucleic acid-based approaches. Ann. Appl. Biol. 145:1-16.
45. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
46. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741-3751.
47. Winton, L. M., Hansen, E. M., and Stone, J. K. 2006. Population structure suggests reproductively isolated lineages of *Phaeocryptopus gaeumannii*. Mycologia 98:781-791.
48. Zhang, J. X., Fernando, W. G. D., and Remphrey, W. R. 2005. Genetic diversity and structure of the *Apiosporina morbosus* populations on *Prunus* spp. Phytopathology 95:859-866.