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Coupling Spore Traps and Quantitative PCR Assays for Detection of the Downy Mildew Pathogens of Spinach (*Peronospora effusa*) and Beet (*P. schachtii*)

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Abstract

Downy mildew of spinach (*Spinacia oleracea*), caused by *Peronospora effusa,* is a production constraint on production worldwide, including in California, where the majority of U.S. spinach is

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grown. The aim of this study was to develop a real-time quantitative polymerase chain reaction (qPCR) assay for detection of airborne inoculum of *P. effusa* in California. Among oomycete ribosomal DNA (rDNA) sequences examined for assay development, the highest nucleotide sequence identity was observed between rDNA sequences of *P. effusa* and *P. schachtii,* the cause of downy mildew on sugar beet and Swiss chard in the leaf beet group (*Beta vulgaris* subsp. *vulgaris*). Single-nucleotide polymorphisms were detected between *P. effusa* and *P. schachtii* in the 18S rDNA regions for design of *P. effusa*- and *P. schachtii*-specific TaqMan probes and reverse primers. An allele-specific probe and primer amplification method was applied to determine the frequency of both *P. effusa* and *P. schachtii* rDNA target sequences in pooled DNA samples, enabling quantification of rDNA of *P. effusa* from impaction spore trap samples collected from spinach production fields. The rDNA copy numbers of *P. effusa* were, on average, ≈3,300-fold higher from trap samples collected near an infected field compared with those levels recorded at a site without a nearby spinach field. In combination with disease-conducive weather forecasting, application of the assays may be helpful to time fungicide applications for disease management.

Downy mildews are widespread and destructive plant diseases caused by obligate oomycete pathogens. These pathogens produce large quantities of short-lived, asexual spores on host plant leaves which become airborne to cause secondary plant infections. Downy mildew on spinach (*Spinacia oleracea*), caused by *Peronospora effusa* (13), is a major disease constraint on production worldwide (16) and, in particular, in California and Arizona, where ≈85% of U.S. fresh-market spinach is produced (2). In the cool coastal Salinas Valley, CA, a region which produces roughly half of the fresh-market spinach grown in the state, downy mildew outbreaks are common. Although spinach downy mildew can usually be controlled with fungicide applications in conventional production, the disease can be devastating for organic spinach production, in which fungicide use is excluded. This, in turn, can accentuate inoculum availability for downy mildew epidemics in conventional production fields. The appearance of new races of *P. effusa*, which has occurred more frequently in recent years (16,18), has complicated efforts aimed at breeding host resistance to downy mildew. The rapid appearance of these new races also emphasizes a need for alternative disease management practices.

Early and accurate warnings of the increases in airborne inoculum of plant pathogens can help manage disease outbreaks (35) and is recognized as a major grower priority (21). The ability to detect and quantify airborne spores may reduce costs associated with disease control in conventional production (number and frequency of fungicide applications) and contribute to reduced or delayed fungicide resistance development. Spore trapping systems such as Burkard traps, tapes, and impaction samplers, in combination with DNA amplification using conventional or real-time quantitative polymerase chain reaction (qPCR)-based methods, have been deployed successfully for the detection of plant pathogens in air samples (9–11,17,19,29–31). The airborne powdery mildew pathogen, *Erysiphe necator*, was detected in vineyards using an impaction sampler and qPCR methodology (17). Similarly, detection and quantification were achieved for the airborne pine pitch canker pathogen, *Fusarium circinatum* (30), and Botryosphaeriaceae spp. that cause grapevine canker (31). PCR-based detection of *Pseudoperonospora humuli* in air samples from commercial hop yards has been useful in the timing of fungicide applications

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(22). These studies have highlighted the potential utility of spore trap and DNA-based assays in the analyses of airborne inoculum dispersal for economically important plant pathogens.

For the development of an assay for airborne downy mildew inoculum, a key requirement is species-specific detection. Although ≈50 species have been reported to cause downy mildew on members of the Chenopodiaceae family (15), these have been classified as a single species, *Peronospora farinosa* (36). Observations of phylogenetic and morphological differences within *P. farinosa* (12,13,33) as well as differences in host specificity (8) indicate that three formae speciales of *P. farinosa* may be more appropriately elevated into individual species, because they are not monophyletic. For example, phylogenetic and morphological analyses indicate that *P. effusa* is a distinct *Peronospora* sp. (13), although the designation as *P. farinosa* f. sp. *spinaciae* remains in common usage to refer to the causal agent of downy mildew on spinach (16,18).

Closely related downy mildew pathogens that do not cause disease on spinach could potentially interfere with an assay designed for specific detection of *P. effusa*. For example, *Beta vulgaris* subsp. *vulgaris*, which includes cultivated leaf beet (Swiss chard) and sugar beet groups (26), is infected by the downy mildew pathogen *P. schachtii* (20,27), also known as *P. farinosa* f. sp. *betae* (8). Sequence analyses indicate that *P. schachtii* is a distinct species (Y.-J. Choi, S. J. Klosterman, and M. Thines, *unpublished data*). *P. schachtii* does not cause disease on spinach, nor does *P. effusa* cause disease on *B. vulgaris* subsp. *vulgaris* (8,27). However, *P. schachtii* is closely related to *P. effusa*, as determined by both phylogenetic analyses of sequences of the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA (13,24) and morphological studies (13). *P. effusa* and *P. schachtii* have overlapping geographic ranges, as in the Salinas Valley, CA, where spinach and Swiss chard are grown in proximity and harvested for their edible leaves. Because PCR methods rely on DNA-based detection, these techniques can increase the specificity of detection, potentially enabling differentiation between *P. effusa* and closely related species, such as *P. effusa* and *P. schachtii*. *Peronospora* sp.-specific PCR primers have been developed for *P. aborescens* infecting poppy (25), *P. tabacina* infecting tobacco (6), and *P. belbarhrii* infecting sweet basil (3). In each of these examples, the primers for development of these assays were designed from sequences of the ITS region in the rDNA.

The primary objectives of this study were to (i) develop a real-time qPCR assay for detection of *P. effusa* and (ii) apply the qPCR assay for the detection of *P. effusa* on impaction spore trap samples collected from fields. A single-nucleotide polymorphism (SNP) was the focal point for the development of *P. effusa*- and *P. schachtii*-specific TaqMan probes and primer sets for SNP allele-frequency determination, as previously applied in other systems (4,23). In theory, nonspecific amplification of the nontarget SNP sequence is not expected when using the SNP-specific primers. In practice, however, there is typically nonspecific amplification (23). Therefore, SNP allele frequencies can be used to determine the difference in quantification cycle (Cq) values between two amplified SNPs in two independent reactions and calculate the frequency of each SNP in a pooled DNA sample. The method was applied to determine the frequency of *P. effusa* and *P. schachtii* rDNA amplification from impaction spore trap samples collected from the field.

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MATERIALS AND METHODS

Sequence analyses for assay development

The ribosomal RNA gene and ITS DNA sequences from *P. effusa*, *P. schachtii*, and those of other oomycetes were aligned and examined for differences and similarities using DNasis software (version 2.09; MiraiBio, Inc., Alameda, CA). These alignments included sequences available at the National Center for Biotechnology Information (NCBI). For additional sequence comparisons, DNA from downy-mildew-infected leaves was amplified using primers UF1 (5′-TGAATGCGCATCGTGC-3′) and UR2 (5′-AGATGCCA CACAACCGAAG-3′), designed to amplify the rDNA sequence spanning a portion of the 18S RNA gene, ITS1, 5.8S RNA gene, and a portion of ITS2 from *Peronospora* spp. DNA was extracted from downy-mildew-infected leaf tissue using a NucleoSpin Plant II kit (Machery-Nagel, Bethlehem, PA). The rDNA sequence was amplified using an MJ Research PTC200 thermal cycler (Bio-Rad, Hercules, CA) using 200 nM each of primers UF1 and UR2 and 1× GoTaq Mix (Promega Corp., Madison, WI). The reaction conditions consisted of an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A final extension at 72°C was for 5 min. The PCR products were cloned into pCR4.0-TOPO (Life Technologies, Carlsbad, CA) and sequenced (Eton Biosciences, San Diego, CA). All of those listed in Supplemental Table 1 were sequenced and submitted to GenBank.

Primer and probe design for TaqMan assays

TaqMan probe-based assays were designed for SNP allele-frequency determination (23) using the allele-specific probe and primer amplification assay (ASPAA) method (4). For the *P. effusa*-specific assay, forward primer AS1 (5′-GCACCTACCGATTGAATGAC-3′) was combined with the FAM dye-labeled TaqMan-minor-groove-binding (MGB) probe with a nonfluorescent quencher (NFQ) Pef1 (5′FAM-ACTGTGACCCCG**C**T-MGB-NFQ-3′) and a *P. effusa*-specific reverse primer PeR1 with the SNP (bold) and a mismatch (italics and underlined) incorporated at the 3′ end (5′-CGCTCGCAATTACTTC*C*A**G**-3′). The *P. schachtii*-specific assay consisted of AS1 and the VIC dye-labeled TaqMan-minor-groove-binding (MGB) probe Pbe1 (5′VIC-ACTGTGACCCCG**T**T-MGB-NFQ-3′) combined with reverse primer PbR1 containing the *P. schachtii*-specific SNP (bold) and a mismatch (italics and underlined) incorporated at the 3′ end (5′-TCCGCTCGCAATTACT*C*CAA**A**-3′). Probes were labeled differently with FAM or VIC reporter dyes for use in multiplexing but the multiplexed reaction was deemed unusable due to the nonspecific amplification between the two sets of probes at higher Cq values (data not shown). A single base pair was added to the 3′ end of the original primers to generate P1EF (5′-GCACCTACCGATTGAATGACT-3′) and P1BF (5′-GCACCTACCGATTGAATGACA-3′) in replacement of forward primer AS1.

Assay efficiencies and detection limits

All real-time qPCR experiments in this study were performed using a LightCycler 480 II (Roche Diagnostics, Basel, Switzerland). TaqMan assay efficiencies were determined using a 10-fold dilution standard curve from 600,000 copies to 600 copies using *Pst*I-linearized pCR4.0-TOPO (Life Technologies) containing the PCR products from *P. effusa* or *P. schachtii* that had been generated with primer pair AS1 and PeR1 or with pair AS1 and

PbR1, respectively. Limits of detection were determined by performing serial 10-fold dilutions of genomic DNA from 1 ng to 1 fg and a standard curve of 600,000 to 6 copies of the linearized plasmid containing the respective rDNA fragment.

Tests of TaqMan assay specificity

Specificity of the TaqMan assays was examined by testing both the *P. effusa*- and *P. schachtii*-specific assays against DNA samples of downy-mildew-infected plant leaves obtained from multiple sources (Table 1). Controls included leaf DNA samples from noninfected beet or spinach. DNA was extracted for this purpose using either the NucleoSpin Plant II kit (Machery-Nagel) or a previously described method (28). All DNA extracted for this purpose was quantified with a Qubit fluorometer (Life Technologies) and the Qubit dsDNA BR Assay Kit, using 2 μl of extraction per reaction. The integrity of *Peronospora* spp. DNA from the infected leaf samples tested was confirmed by qPCR using the rDNA primer pair UF1 and UR1 in a reaction containing 200 nM each primer, 1× IQ SYBR Green (Bio-Rad) and 2 ng DNA with a reaction profile of 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s using the LightCycler 480 II (Roche Diagnostics). Melt peaks were examined to confirm amplification of the correct product.

Downy mildew pathogenicity tests

To test whether other local downy mildews from non-spinach Chenopodiaceae plants could infect spinach, a series of inoculation experiments were conducted. Symptomatic plants exhibiting abaxial sporulation were collected near spinach-growing areas in Monterey County (lambsquarters weed, *Chenopodium album*; and nettleleaf goose-foot weed, *C. murale*) and neighboring Santa Cruz County (commercial Swiss chard, *B. vulgaris* subsp. *vulgaris*; and commercial epazote, *Dysphania ambrosioides*). A set of 10 differential spinach cultivars used for identifying races of spinach downy mildew, including the universally susceptible 'Viroflay' (18), were used in the experiments. Fungicide-free seed of the 10 cultivars was planted into two 25-by-50-cm^2 plastic trays containing Sunshine mix number 4 (Sun Gro Horticulture, Agawam, MA). Seed were arranged in five rows per tray and 25 seed per row. Plants were inoculated when 21 days old. Sporangial inoculum was prepared by agitating collected, diseased leaves in cold (4°C) water, filtering the mixture through a layer of cheesecloth, and adjusting inoculum concentration to $\approx 1 \times 10^4$ sporangia/ml. Plants were inoculated with a hand-held mister until runoff and incubated in a dew chamber (100% relative humidity, 18°C) for 24 h (18). Plants were then moved to a humidity chamber that was periodically misted (3-min duration every 12 min) and examined daily for chlorotic symptoms and signs of the downy mildew. Inoculations were completed two times, with the exception of the nettleleaf goosefoot downy mildew sample, which did not provide enough inoculum for a second inoculation.

Spore trap sample collection

Custom solar- or battery-powered impaction spore traps, similar to Rotorods (Sampling Technologies Inc., Minnetonka, MN), for detection of windborne inoculum were obtained from Dr. Walt Mahaffee (United States Department of Agriculture–Agricultural Research Service [USDA-ARS], Corvallis, OR). The application of similar traps in the field for

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detection of *Erysiphe necator* was described previously (17). Pairs of 1.2-by-40-mm stainless-steel rods were cut from welding wire (316LSi stainless steel; Harris Products Group, Mason, OH), and coated with a layer of high-vacuum grease (Dow Corning, Midland, MI) using gloved hands. The rods were positioned on the rotating arm ≈53 cm above the soil surface at each sampling site. To ensure that the rods from the impaction spore traps were not contaminated by spores trapped in residual grease and debris, the rotating arm of the spore trap that holds the impaction rods was washed with isopropanol and wiped with a clean paper towel after each sampling, before replacement with new clean rods. The rods were collected at ≈48- or 72-h intervals and placed into 1.75-ml screw-cap centrifuge tubes and stored at 4°C until DNA extraction. Rods were not reused.

Spore traps were placed at three separate sites for this study. First, spore traps were placed near a spinach field during 1 through 8 September 2012, by Watsonville, in Santa Cruz County, CA. The other two sites in Monterey County, designated as Salinas and Soledad, were monitored during 13 March to 3 April 2013. At the Salinas site, located ≈35 km from the Soledad site, two spore traps were placed side by side (≈0.5 m apart) in a USDA-ARS equipment yard where there were no nearby spinach fields during the period of study. At the Soledad site, one spore trap was placed at the north end and one ≈750 m apart at the south end of a downy-mildew-infected spinach field.

DNA extraction from spore trap impaction rods

The NucleoSpin Plant II kit (Machery-Nagel) was used to extract DNA from each 40-mm stainless-steel spore trap rod individually. A single 1.5-ml microcentrifuge tube cap full (≈300 mg) of acid-washed 425- to 600-μm glass beads (Sigma Aldrich, St. Louis) and 300 μl of buffer PL1 were added to each tube and vortexed horizontally for 5 min, incubated at 65°C for 10 min, and vortexed once again horizontally for 5 min. After a brief centrifugation, a 100-μl aliquot of chloroform was added, mixed by vortexing, and centrifuged for 15 min. The supernatant was applied to a spin column and further processed following the manufacturer's protocol, with the exception that DNA elution was performed with one 65-μl aliquot of buffer PE incubated at 65°C for 5 min. The elution (5 μl) was quantified with the Qubit fluorometer using the Qubit dsDNA HS Assay Kit (Life Technologies), which has a detection range of 10 pg to 100 ng/μl.

Cloning and sequencing of PCR products obtained from spore traps

The *Peronospora*-conserved primers UF1 and UR1 were used to amplify sequences from selected spore trap samples. The PCR product was sequenced directly but some selected qPCR products were purified and cloned into pCR4.0-TOPO (as described above) and sequenced in both directions with T7 and T3 universal primers (Eton BioSciences).

qPCR with spore trap samples

The qPCRs were run in triplicate using one DNA extraction per spore trap. Reaction volumes of 20 μl contained 200 nM probe, 900 nM each primer, 1× TaqMan Environmental Master Mix 2.0 (Life Technologies), and 2 ng of DNA or 6 μl of eluate when the DNA concentration was <0.33 ng/μl. The LightCycler 480 II (Roche Diagnostics) with LightCycler 480 software (release 1.5.0) and the Fit Points Absolute Quantification Analysis

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was used to conduct the assays with a reaction profile of 10 min at 95°C initial denaturation followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Both assays were run on each sample and the relative frequency of the *P. effusa* SNP (allele 1) was calculated based on the following equation, as described (23): frequency of $allele_1 = 1/(2^{Cq} + 1)$ where Cq = (Cq of $allele_1$ – specific PCR) – (Cq of $allele_2$ – specific PCR). The use of absolute quantification for calculating the relative frequency of the amounts of *P. effusa* and *P. schachtii* is possible because the TaqMan assays used for each have nearly identical efficiencies, at 99 and 102%, respectively.

Standard curves were used to determine copy number in spore trap extractions. The standard curves were used to establish thresholds of 0.8 for the *P. effusa* assay and 2.0 for the *P. schachtii* assay, which gave the slightly higher efficiency. After initial testing, a 600,000 rDNA copy standard was run on each plate using these standard thresholds, and the same slope and *y*-intercept were used for the linear regressions for the copy number calculations (3.378 slope, 43.77 *y*-intercept for the *P. effusa* assay and 3.226, 41.92 for the *P. schachtii* assay).

Analyses of qPCR inhibition

The effect of spore trap grease on PCR inhibition was analyzed by comparing the amplification of rDNA in the presence or absence of spore trap extract using *P. effusa* genomic DNA as template. The DNA extractions were performed as described for the DNA extraction on impaction spore trap rods coated in grease. Buffer elution was performed with a single 65-μl buffer aliquot on either a single column or as elutions on two columns representing two separate rod extractions with the same 65-μl buffer volume. *P. effusa* genomic DNA was diluted 10-fold, 100 pg to 100 fg. qPCR was performed on the genomic DNA or genomic DNA combined with 6 μl of eluate from either single-rod extractions or extractions from two rods. Differences in Cq values between the single-rod extraction or two-rod extraction were compared.

To calculate the amount of PCR inhibition on the impaction spore trap samples, the copy number standard curve generated initially was modified by adjusting the threshold so that the efficiency used in the standard curve for the calculation of the DNA copy number amplification matched the efficiency of the genomic DNA standard curve plus inhibitors (91.6%). Then, the slope and *y*-intercept from the changed copy number curve was applied to all of the spore trap samples.

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Spore counts under microscopy

Fresh spores of *P. effusa* were obtained for counting under light microscopy 1 or 2 days after collection from greenhouse-grown infected spinach leaf samples (grown and inoculated as described above). A single human hair, ≈2 cm in length, was brushed across the gray spore masses typical of *P. effusa* on the underside of the infected leaf to collect spores. Decreased numbers of spores could be obtained by rubbing only the tip of the hair or increased numbers by rubbing the entire length of the hair in the spore masses. Spores were counted along the length of the hair and immediately placed in microfuge tubes containing a steel rod coated with grease. Sporangiophores were rarely encountered on the hairs in this process

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and, if present, the sample was discarded. This was repeated 20 times to obtain 20 samples differing in spore number. These 20 samples were used to correlate *P. effusa* spore counts by microscopy and DNA copy number determined by qPCR. Negative control samples were prepared by DNA extraction from a grease-coated rod in the presence of a single hair, also of ≈2 cm in length.

Data analysis

Linear regression analysis was used to evaluate the relationships between the DNA concentration and Cq values and between the numbers of *P. effusa* spores counted by microscopy and DNA copy number.

RESULTS

Sequence analyses for TaqMan assay probe and primer development

Initial analyses were aimed at developing a *P. effusa*- specific probe and primer set by focusing on the ITS 1 and 2 regions of the rDNA. ITS sequence alignments did not reveal potential primer sites that could uniformly distinguish between *P. effusa* from all related oomycetes surveyed (data not shown). However, alignments of the upstream (5′) 18S ribosomal RNA gene (18S rDNA) revealed potential target sequences that could be used in probe and primer development to differentiate *P. effusa* from related oomycetes. The alignments of a 67-bp region of the *P. effusa* 18S rDNA from representative oomycetes revealed sequence variation between *P. effusa* and other *Peronospora* spp. and oomycetes (Fig. 1). BLASTn analysis (1) of the NCBI non-redundant database with the 67-bp 18S rDNA sequence of *P. effusa* yielded only two sequences with 100% identity (accessions AF528560.1 and AF528559.1), both of which were derived from *P. effusa* (13).

The highest interspecies sequence homology observed between the partial 18S rDNA sequences in this analysis (Fig. 1) was recorded between *P. effusa* and *P. schachtii* (97%), although two SNPs were identified in the 18S rDNA sequence to differentiate *P. effusa* from *P. schachtii* (Fig. 1). The rDNA sequences of *P. effusa* deposited in the GenBank database are often partial, with only the two *P. effusa* accessions mentioned above with the up-stream 18S rDNA area containing the two SNPs for further evaluation. Therefore, 10 additional rDNA sequences from *P. effusa* and 10 additional sequences from *P. schachtii* were obtained, revealing the presence of the same two SNPs in all of the sequences. The single SNP difference depicted in Figure 1 with an asterisk was incorporated into the sequence of the reverse primer (PeR1) and probe (Pef1) for the purpose of generating a *P. effusa*-specific TaqMan assay. *P. schachtii*-specific reverse primer (PbR1) and the probe (Pbe1) were prepared similarly. The two assays were used in two separate reactions for SNP allele-frequency determination (23). The reverse primers and probes for both assays were designed to intentionally overlap (Fig. 1) and a mismatch was introduced following the ASPAA method described by Billard et al. (4) to enhance species-specific detection.

Assay efficiencies, limits of detection, and specificity

Standard curves for each of the TaqMan assays designed for *P. effusa* or *P. schachtii* detection are shown in Figure 2 For the *P. effusa* TaqMan assay, the limit of detection was 10

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fg of genomic DNA or 60 copies on the standard curve (Fig. 2A). The TaqMan assays used for each had nearly identical efficiencies, at 99 and 102%, respectively. When determining efficiency level of the VIC dye-linked *P. schachtii* assay, there was increased background interference at the highest Cq values and, therefore, the 60 copy number was not included in this curve (Fig. 2B). However, the limit of detection of the *P. schachtii* TaqMan assay was also determined at 10 fg using a *P. schachtii* genomic DNA standard.

Although the assays were designed for species specificity, nonspecific amplification of *P. schachtii* DNA was evident (Fig. 3) using the *P. effusa*-specific assay on a range of plasmid concentrations containing the rDNA fragment. Additional forward primers P1EF and P1BF, incorporating the second SNP difference (Fig. 1, not labeled with an asterisk) between *P. effusa* and *P. schachtii* were tested in an effort to further increase specificity of amplification. Tests of these primers also resulted in nonspecific amplification of the 18S rDNA sequence (data not shown).

Specificity testing of the TaqMan assays

Further specificity tests of the TaqMan assays using both the *P. effusa*-specific and *P. schachtii*-specific probe and primer sets were conducted on a range of downy-mildew-infected leaf samples from different geographic locations, including those outside of California (Table 1). *P. schachtii*-nonspecific amplification was observed when using the *P. schachtii*-infected leaf tissue DNA extract as template (Table 1). Aside from that, the *P. effusa*-specific assay was specific when tested against a range of other downy mildew DNA samples extracted from various infected hosts, including *C. album* (lambsquarters), a common weed in and around spinach fields in the Salinas Valley. Application of the TaqMan assays with DNA template from *C. album* infected with *P. variabilis* (12) did not yield a detectable DNA amplification signal using either the *P. effusa*- or *P. schachtii*-specific DNA probes and primer pairs (Table 1). Among the infected leaf samples tested, all of those from *Rumex acetosa* (garden sorrel) infected with *P. rumicis* were positive when tested with the *P. schachtii*-specific TaqMan assay, with a Cq range of 21.1 to 25.12 (Table 1). DNA samples derived from noninfected spinach and beet were negative for detection using either the *P. effusa*- or *P. schachtii*-specific TaqMan assay (data not shown)

Pathogenicity tests of downy mildew isolates on spinach

Because the TaqMan assays for *P. effusa* or *P. schachtii* were designed for species-specific DNA amplification, the assays would exclude pathogens from other hosts which could potentially contribute to disease on spinach. All of the differential cultivars tested, including the universal susceptible spinach Viroflay, exhibited no downy mildew symptoms and no sporulation when inoculated with *Peronospora* spp. isolates collected from the weeds (lambsquarters and nettleleaf goose-foot) or from the cultivated plants (Swiss chard and epazote) (Supplemental Table 2). All inoculations resulted in the same lack of disease.

Detection of downy mildew inoculum from spore traps

The configurations of primers and probes for specific detection of either *P. effusa* or *P. schachtii* (Fig. 1) were used for the experiments to test application of the TaqMan assays with spore trap samples obtained from the field. Initial tests of the impaction spore trap

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samplers for the detection of *P. effusa* and *P. schachtii* were conducted at a location near a commercial spinach field by Watsonville, CA. No downy mildew infection of the spinach field was observed during this period but Cq values of 25.55 ± 0.12 standard deviation (SD) and 31.38 ± 0.19 SD, such as those from trap samples collected on 8 September 2012, suggested that the traps were positive. To assess whether *P. effusa* or *P. schachtii* was present, DNA was extracted from the rods from these two trap samples and sequenced, revealing the 67- or 69-bp 18S rDNA sequences identical to those of *P. effusa* and *P. schachtii* (Fig. 1), respectively. All of the no-DNA template control samples prepared with grease-coated rods and extracted for DNA were negative for amplification (data not shown).

Because the primer–probe combination for *P. effusa* yielded a detection signal for *P. schachtii* from qPCRs using the rDNA fragment from *P. schachtii*- (Fig. 3) or *P. schachtii*-infected leaf tissue DNA Table 1), dual qPCR analyses were subsequently performed on each sample. The differences in Cq values were determined for the two independent PCRs and the frequency of nonspecific amplification was calculated as described in Materials and Methods, following the method of Germer et al. (23) shown in Figure 4. Multiplication of frequency by the total *P. effusa* 18s rDNA copy number detected for each trap sample enabled calculation of the 18S rDNA copy number of *P. effusa* for each sample (Table 2). The average 18S rDNA copy number for *P. effusa* detected at the Soledad site on both traps across all sampling dates was 785,723. In contrast, the average rDNA copy number for *P. effusa* was 236, detected at the Salinas site, where the traps were not placed near a downy-mildew-infected spinach field. Based upon these average values, the amount of *P. effusa* DNA detected was ≈3,300-fold higher at the Soledad site during the entire period of investigation (Table 2).

Though spore traps were not placed near downy-mildew-infected fields that contained cultivated beet groups, the average frequency of *P. schachtii* 18S rDNA amplification from the Salinas site spore trap samples was 0.51 and 0.30 for *P. effusa* (including detection dates with values of 0 in the average calculation) during the sampling period (Table 2). There were no nearby spinach fields at the Salinas site during the sampling period. However, the Salinas spore trap site was located ≈1.5 km north of a site with known downy mildew infections of Swiss chard during the sampling period.

Correlation between DNA copy number of *P. effusa* and spore counts

The correlation between DNA copy number of *P. effusa* detected and actual spore counts of *P. effusa* from microscopy analyses (Fig. 5) provided an estimate of the number of spores detected on the steel impaction rods obtained from the field. Correlation was observed (R^2 = 0.7603) between DNA copy number of *P. effusa* derived from a standard curve of the amplification of rDNA and counts of spores obtained from light microscopy (Fig. 5). Removal of one outlier (Fig. 5, marked with the arrow) resulted in a coefficient of determination (R^2) = 0.8644. Using either standard curve estimate, application of the qPCR assay on spore trap samples yielded detection values corresponding to >200 spores of *P. effusa* on half of the dates tested (5 of 10), when the traps were placed adjacent to the infected spinach field (Table 2, copy number values >575,000). Within the same period, spore traps set at the separate Salinas location, without a nearby spinach field, yielded <20

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spores per sampling rod (Table 2, copy number values <20,000). In contrast to the high copy numbers recorded for the Soledad trap site, the maximum copy number recorded for the Salinas site was well below 20,000 copy number (<2,339) on 20 March 2013 (Table 2).

Analyses of qPCR inhibition

Inhibition of the TaqMan assay with the *P. effusa*-specific probe and *P. effusa* genomic DNA as template was detected in the presence of grease-coated spore trap rods. This inhibition was detected whether or not DNA was extracted from a single spore trap rod with a single elution of buffer volume, or from two spore trap rods in which the DNA extract was eluted through the column twice with the same extraction buffer volume (Table 3). However, the level of qPCR inhibition was reduced by ≈1 Cq value using a single rod with a single elution of the silica membrane spin column with buffer, rather than elution of two membranes with the same buffer volume (Table 3). Because of evidence of qPCR inhibition by spore trap grease, all the copy number values were adjusted by changing the threshold to the efficiency of the *P. effusa* genomic DNA standard curve plus inhibitors (91.6% data not shown). All of the copy numbers shown in Table 2 represent these adjusted values.

DISCUSSION

The primary objectives of this study were to develop a real-time qPCR assay for *P. effusa* and to apply the assay for detection of *P. effusa* on impaction spore trap samples. In the process, the rDNA gene and ITS sequences were compared for the development of TaqMan probes to differentiate *P. effusa* from related oomycetes. Because of the high sequence identity between rDNA sequences of *P. effusa* and *P. schachtii*, employment of SNP allele-frequency determination by qPCR (4,23) allowed calculations of the frequency of *P. effusa* and *P. schachtii* on impaction spore trap samples collected from the field.

Individual probes and reverse primers were designed for detection of each pathogen, *P. schachtii* and *P. effusa*, taking advantage of a SNP identified in the 18S rDNA target sequence. Even with the SNP differences incorporated into the probe and reverse primer, nonspecific amplification of the target rDNA was detected from *P. effusa*- or *P. schachtii*-infected leaf samples or purified DNA. This necessitated subtraction of nonspecific amplification of one assay from the other TaqMan assay. This need arose especially in view of the findings that *P. schachtii* does not infect spinach (8,27, and results from this study) and that *B. vulgaris* subsp. *vulgaris* is grown in a range that closely overlaps that of spinach production in California. The presence of *P. schachtii* could potentially interfere with an assay designed for specific detection of *P. effusa*, which only causes downy mildew on spinach (8).

Nonspecific amplification may be negligible from impaction spore trap samples, such as at the Soledad trap site, where high copy numbers of *P. effusa* rDNA were recorded. In these instances, the amount of nonspecific *P. effusa* or *P. schachtii* DNA present per reaction would be reduced substantially. Nevertheless, rather than using a Cq cut-off limit for each primer–probe set, individual PCRs from the pooled DNA sample (i.e., spore traps) would be advisable for testing the amount of rDNA amplification from either pathogen. For example, Cq values of 31.48 ± 0.33 SD and 30.49 ± 0.75 SD were recorded for *P. effusa* and *P.*

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schachtii, respectively, on the same date (20 March 2103) at the same sampling site (Salinas). These Cq values reflect similar quantities of each of the pathogens on that date; therefore, nonspecific amplification must be taken into account.

The TaqMan assays were designed for specific amplification of the rDNA of *P. effusa* or *P. schachtii*. However, all leaf samples infected with *P. rumicis* tested positive using the *P. schachtii*-specific assay, and one of three samples infected with *P. boni-henrici* was positive when tested using the *P. schachtii*-specific assay. Although the *P. schachtii*-specific TaqMan assay showed nonspecificity in the amplification of DNA of two other *Peronospora* spp., this may not affect the assay specificity in the geographic regions of interest for the following reason. The hosts for these two pathogens, *R. acetosa* (garden sorrel, in the family Polygonaceae) and *C. bonus-henricus* (good King Henry, in the family Chenopodiaceae), respectively, are the only two host plants tested in this study that have not been reported in the states of California or Arizona in the western United States (USDA Natural Resources Conservation Service, National Plant Data; http://plants.usda.gov). Therefore, these particular pathogens should not interfere with specificity of detection of *P. schachtii* in these states. *P. schachtii* is phylogenetically close to *P. rumicis* (13) and likely explains the cross-reactivity using the *P. schachtii*-specific probe and primer set. In contrast, the *P. effusa*-specific TaqMan assay allowed for the differentiation of *P. effusa* from other downy mildew pathogens infecting various other plant hosts. The testing of additional primer sets and sequences to delineate intra- or interspecific variation within and between *P. effusa* and *P. schachtii* based upon geographic origin, or even races of the pathogen, may be highly informative. Analyses of mitochondrial sequences have revealed intraspecific differentiation of *P. effusa* (14), which could probably also help to further delineate the closely related species of Chenopodiaceae, including *P. effusa*, *P. schachtii*, and *P. rumicis*.

The pathogenicity tests on the differential spinach cultivars using downy-mildew-infected samples obtained from other plants in the family Chenopodiaceae indicate that downy mildew on those hosts does not contribute to downy mildew observed on spinach. These samples included those from locally collected downy-mildew-infected weed (*C. album*, lambsquarters) and crop (*D. ambrosioides*, epazote) plants in Monterey County or neighboring Santa Cruz County. Previous studies (8,27) and related studies reviewed therein also indicate that both *P. effusa* and *P. schachtii* are host specific. Taken together, these results are highly informative for development of assays designed to detect and quantify the amounts of airborne inoculum from these pathogens that contribute to disease outbreaks only on spinach or beet. Also, in agreement with previous results (8,27), downy mildew from cultivated beet did not cause disease on spinach in this study.

Among the potential drawbacks of qPCR strategies for disease forecasting, qPCR detects DNA from both live and dead spores. However, given the adaptations of windborne pathogens for the dissemination of short-lived spores, the most important increases of airborne inoculum during a growing season are likely derived almost entirely from live spores (35). The use of rDNA sequences for the purpose of quantifying the amount of airborne inoculum is beneficial because the detection target is multicopy and, thus, increases sensitivity. However, the rDNA copy number varies approximately two- to threefold between isolates of fungi, such as in *Verticillium dahliae* (5) and *Leptosphaeria maculans* (32), and

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probably in the oomycetes as well. Nevertheless, in the case of *L. maculans*, the amount of ITS DNA amplified was proportional to the numbers of ascospores (32). The results of this current study also indicate that the amount of *P. effusa* rDNA amplified is proportional to the amount of *P. effusa* spores counted by microscopy. Furthermore, for airborne inoculum of *Peronospora* spp., the average rDNA copy number (between isolates within a population) is likely the number of most relevance for predictive purposes.

Inhibition of PCR in DNA extracts may increase false-negative readings and could detrimentally affect a qPCR results by reducing amplification efficiency. DNA purification with commercial kits and dilution of the DNA extract can minimize the effect of inhibitors on PCRs (7). The findings herein of spore trap grease-mediated qPCR inhibition indicate the importance of factoring in the amount of qPCR inhibition to accurately assess target DNA copy number in environmental sampling. Importantly, qPCR inhibition was decreased by reducing the number of buffer passes through the silica membrane column used with the Nucleospin DNA extraction kit (Machery-Nagel).

The TaqMan assays developed herein are sensitive detection tools for the downy mildew pathogens of spinach and *B. vulgaris* subsp. *vulgaris*. The total DNA content per nucleus for *Peronospora* spp. was previously estimated at 47 to 138 fg (34). The detection sensitivity limit of 10 fg using the TaqMan assays for *P. effusa* and *P. schachtii* suggested that the amounts of genomic DNA corresponding to a single spore were detectable. Although the focus of this research was on the development and deployment of a detection assay for *P. effusa*, known numbers of spores of both species could also be calculated from spore trap samples. These sensitive tools are projected to provide data on patterns of windborne inoculum levels in the Salinas Valley and to yield insights on the environmental conditions that favor outbreaks of downy mildew.

Predictions based on the optimal conditions favoring disease have been used for the timing of fungicide applications for the cucumber downy mildew pathogen *Pseudoperonospora cubensis* (22). Similarly, knowledge acquired for forecasting grape powdery mildew caused by *E. necator* may be useful in reducing the number of fungicide applications per growing season (17). As noted for other spore-trapping systems deployed for the purpose of disease forecasting (10), a future challenge will be in determining relationships between inoculum amount and disease development. To assess these relationships, spore trap data collection and analyses from multiple locations within the Salinas Valley, CA are currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Design of TaqMan assay primers and probes for detection of the spinach downy mildew pathogen, *Peronospora effusa*, and the downy mildew pathogen of *Beta vulgaris* subsp. *vulgaris*, *P. schachtii*. Sequences shown in the alignment are DNA sequence fragments of the 18S ribosomal RNA (rRNA) gene of each of the pathogens. Positions of the forward and reverse primers and probe sites are shown above the pathogen DNA sequence of *P. effusa*. Representative sequences are from *Peronospora* and *Phytophthora* (*Ph*) species and a single sequence of each *Pseudoperonospora cubensis* (*Ps*) and *Hyaloperonospora* sp. (*Hy*). GenBank accessions are shown following each of the species names. The lone asterisk marks the site of the single-nucleotide polymorphism (SNP) used to generate the *P. effusa* (C) and *P. schachtii* (T)-specific probes. The respective SNP (C or T) was incorporated into both the reverse primer and the probe to increase probe or primer binding specificity. Shaded nucleotides depict those that are different from those in the *P. effusa* 18S rDNA sequence alignments. AS1 = nonspecific forward primer, Pef1 = *P. effusa*-specific TaqMan probe, Pbe1 = *P. schachtii*-specific TaqMan probe, PeR1 = *P. effusa*-specific reverse primer, PbR1 = *P. schachtii*-specific reverse primer. The shaded circle and diamond on the TaqMan probe indicate the positions of the fluorescent dye and nonfluorescent quencher, respectively.

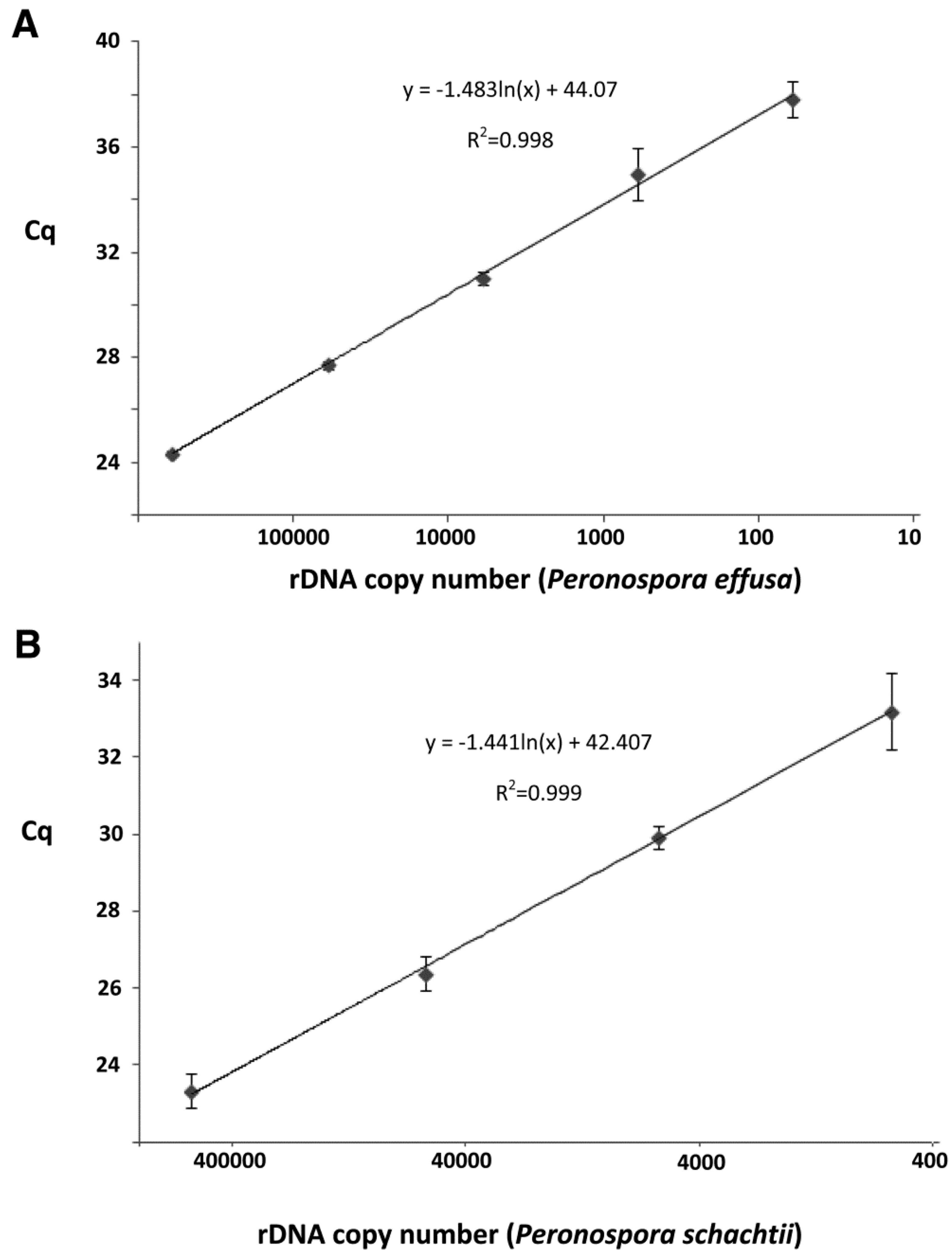

Fig. 2.

Standard curves for the quantification of the 18S ribosomal DNA fragment (rDNA copy number) from the downy mildew pathogens *Peronospora effusa* and *P. schachtii* of spinach and *Beta vulgaris*, respectively. **A,** Five-point standard curve based on TaqMan assay amplification of plasmid DNA containing a single copy of the 18S ribosomal RNA (rRNA) gene fragment from *P. effusa*. Copy dilution was from 600,000 to 60 copies. **B,** Four-point standard curve based on TaqMan assay amplification of plasmid DNA containing a single copy of the 18S rRNA gene fragment from *P. schachtii*. In both A and B, the log rDNA copy

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number is plotted against the quantification cycle (Cq) values. All data points are from an average of three technical replicates.

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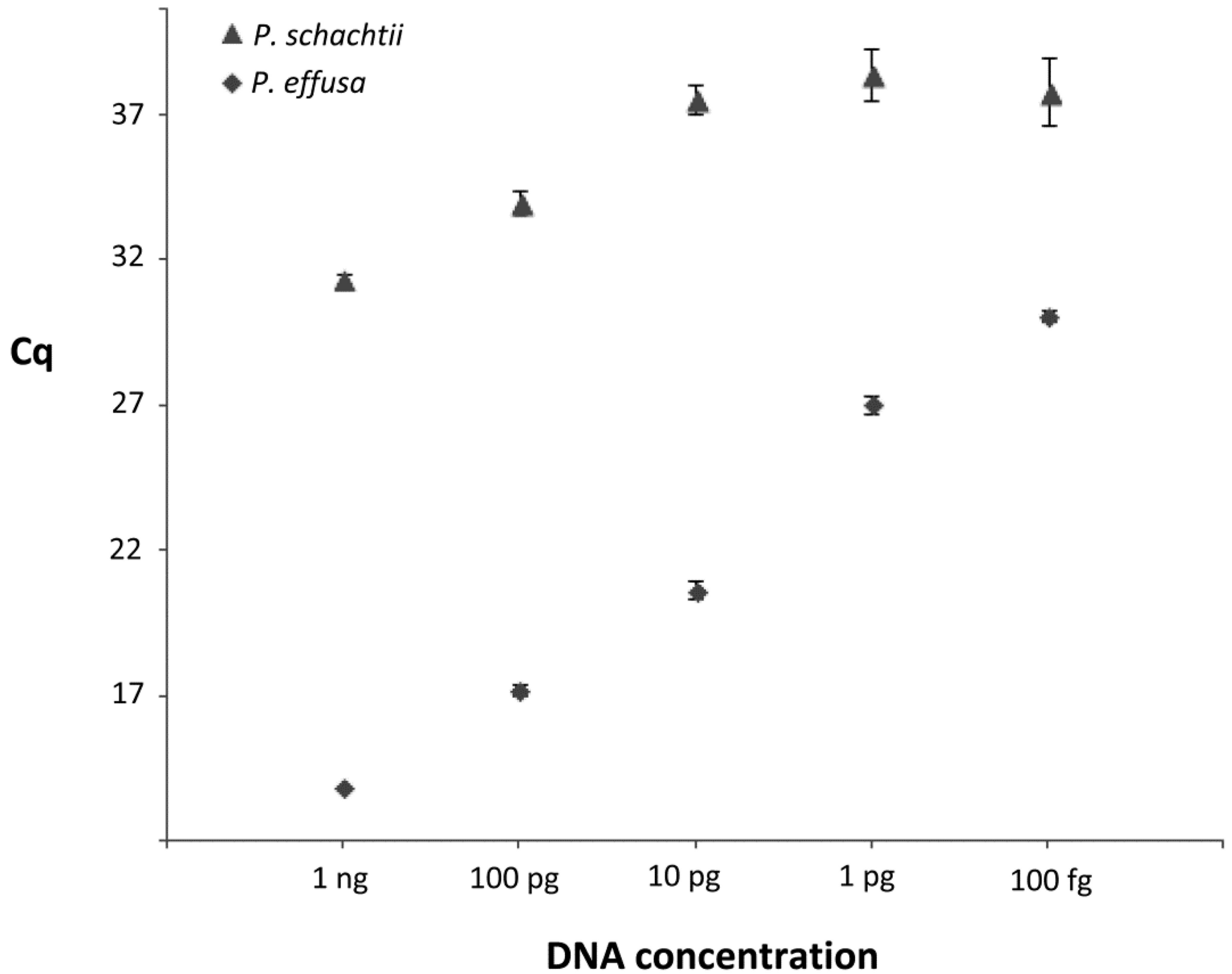

Fig. 3.

Nonspecific amplification of the rDNA 18S ribosomal RNA gene fragment of *Peronospora schachtii* when using the TaqMan assay designed for specific detection of *Peronospora effusa.* Quantification cycle (Cq) values were obtained from the amplification of purified plasmid DNA at the concentrations indicated from *P. effusa* and *P. schachtii.* The *P. effusa*-specific probe Pbe1 and primer PeR1 and nonspecific primer AS1 were used for all reactions. Error bars are ± standard deviation from three technical replicates.

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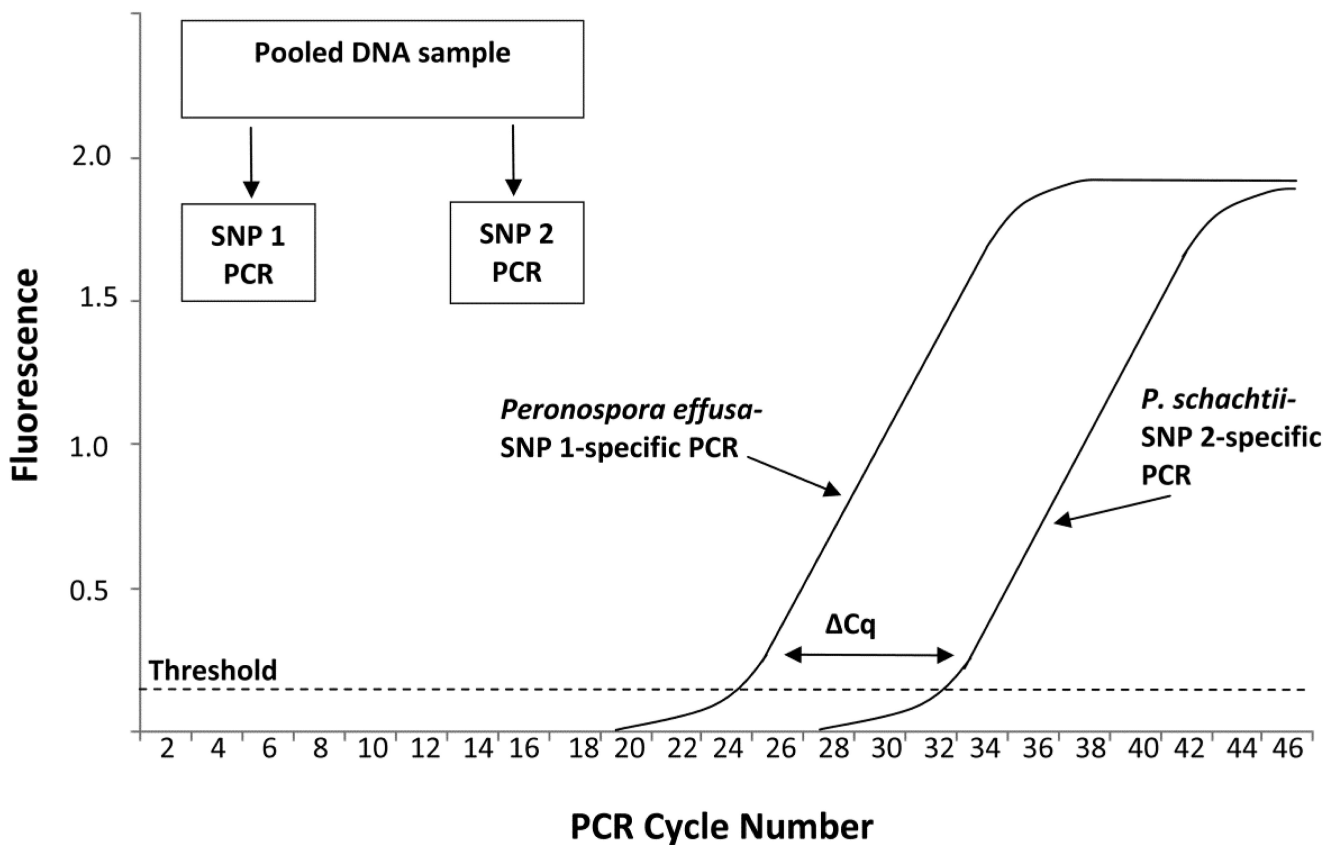

Fig. 4.

Illustration of *Peronospora*-single-nucleotide polymorphism (SNP)-specific polymerase chain reaction to determine the frequency of amplification of the 18S ribosomal DNA sequence of *Peronospora effusa* or *P. schachtii.* The illustration is adapted from Germer et al. (23) and reproduced with permission from Cold Spring Harbor Laboratory Press.

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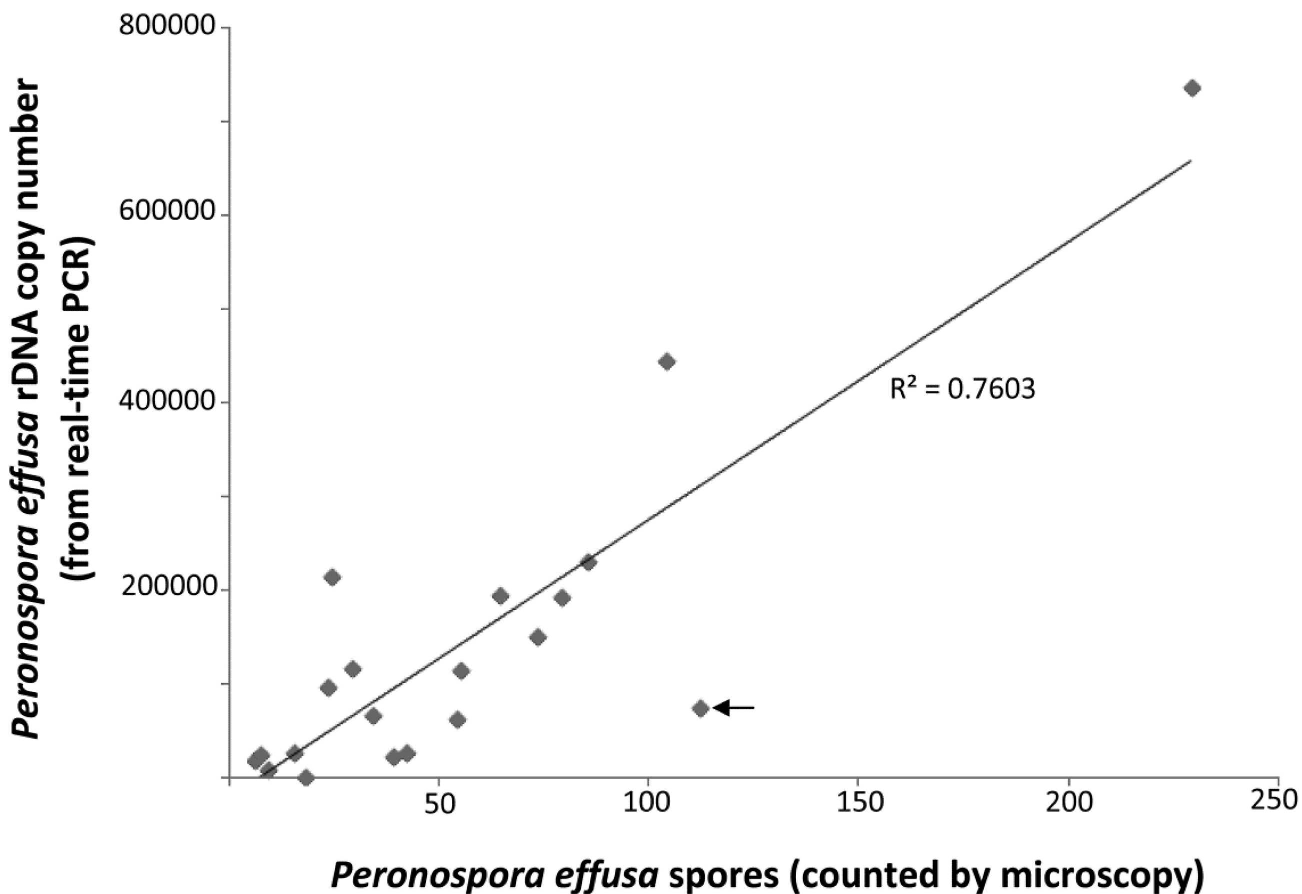

Fig. 5.

Correlation between DNA copy number of *Peronospora effusa* derived from a standard curve of the amplification of ribosomal DNA (rDNA) with counts of spores obtained from light microscopy. The equation for the line with $R^2 = 0.7603$ is $y = 2960.4x - 20261$. Removal of a single outlier (112 spores; marked by the arrow) resulted in $R^2 = 0.864$.

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TABLE 1.

TaqMan assay specificity tests for detection of *Peronospora effusa* and *P. schachtii* using DNA from downy-mildew-infected leaves of multiple plant hosts

Host plant sample (species or common names)	Pathogen	Sample location[a]	Year	*P. effusa* assay (Cq ± SD)[b]	*P. schachtii* assay (Cq ± SD)
Spinacia oleracea (spinach)[c]	*Peronospora effusa*	Imperial County	2012	19.95 ± 0.07	30.10 ± 0.09
S. oleracea	*P. effusa*	San Benito County	2012	19.60 ± 0.19	30.61 ± 0.16
S. oleracea	*P. effusa*	San Benito County	2012	19.28 ± 0.17	30.38 ± 0.15
S. oleracea	*P. effusa*	Monterey County	2012	22.25 ± 0.16	33.54 ± 0.50
Beta vulgaris subsp. *vulgaris* (beet)[d]	*P. schachtii*	Monterey County	2012	34.66 ± 0.23	19.92 ± 0.13
B. vulgaris (beet)	*P. schachtii*	Santa Maria County	2012	37.09 ± 0.42	21.03 ± 0.15
B. vulgaris (Swiss chard)	*P. schachtii*	Santa Cruz County	2012	36.87 ± 0.93	19.89 ± 0.08
B. vulgaris (Swiss chard)	*P. schachtii*	Monterey County	2012	36.60 ± 0.45	20.11 ± 0.13
Atriplex oblongifolia (oblong orache)	*Peronospora* sp.[e]	Germany	2005	–	NT
A. oblongifolia	*Peronospora* sp.	Germany	2004	–	NT
A. patula (spear saltbush)	*P. minor*	Austria	2011	–	–
A. patula	*P. minor*	Austria	2005	–	–
A. prostrata (triangle orache)	*P. atriplicis-hastatae*	Germany	2003	–	–
A. prostrata	*P. atriplicis-hastatae*	Germany	2003	–	–
A. sagittata (hoary orache)	*Peronospora* sp.[e]	Germany	2002	–	–
Bassia scoparia (burning bush)	*P. kochiae-scopariae*	Germany	1998	–	–
B. scoparia	*P. kochiae-scopariae*	Germany	1996	–	–
B. scoparia	*P. kochiae-scopariae*	Austria	2011	–	–
Chenopodium album (lamb's quarters)	*P. variabilis*	Germany	2008	–	–
C. album	*P. variabilis*	Germany	2007	–	–
C. album	*P. variabilis*	Austria	2011	–	–
C. album	*P. variabilis*	Monterey County	2013	–	–
C. bonus-henricus (good King Henry)	*P. boni-henrici*	Austria	1991	–	–
C. bonus-henricus	*P. boni-henrici*	Italy	2012	–	34.08 ± 0.50
C. bonus-henricus	*P. boni-henrici*	Austria	2000	–	–
C. polyspermum (manyseed goosefoot)	*P. chenopodii-polyspermi*	Germany	1998	–	–
C. polyspermum	*P. chenopodii-polyspermi*	Germany	2004	–	NT
C. polyspermum	*P. chenopodii-polyspermi*	Austria	2011	–	–
Dysphania ambrosioides (epazote)	*Peronospora* sp.[f]	Santa Cruz County	2013	–	–
Rumex acetosa (garden sorrel)	*P. rumicis*	Germany	2008	–	22.80 ± 0.00
R. acetosa	*P. rumicis*	Austria	2011	–	25.12 ± 0.19
R. acetosa	*P. rumicis*	United Kingdom	2011	–	21.10 ± 0.21
Spergula arvensis (corn spurry)	*P. obovata*	Germany	2003	–	–
S. arvensis	*P. obovata*	Germany	2005	–	–
S. arvensis	*P. obovata*	Austria	2008	–	–
S. arvensis	*P. obovata*	Austria	2011	–	–

[a]Counties located in California in the United States.

[b]SD = standard deviation from three replicates; – denotes no detection by quantitative polymerase chain reaction using the *P. effusa*- or *P. schachtii*-specific TaqMan assays with DNA from infected leaf tissue (quantification cycle [Cq] values > 38 were considered negative for these specificity tests); and NT = not tested.

[c]DNA was extracted from *Peronospora* spp.-infected leaves and 2 ng was used as template for all reactions. DNA integrity was confirmed with a SYBR green assay (see Materials and Methods), and the *Peronospora* spp. ribosomal DNA of the samples from *Spinacia oleracea* and *B. vulgaris* subsp. *vulgaris* and the single one from *D. ambrosioides* were cloned and sequenced.

[d]*B. vulgaris* subsp. *vulgaris* includes the cultivated beet groups of table beet and Swiss chard.

[e]Phylogenetically different from *Peronospora* species known previously on *Atriplex* spp. (12).

[f]The *Peronospora* sp. on *D. ambrosioides* is phylogenetically different from other *Peronospora* spp. (12).

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TABLE 2.

Relative frequency of ribosomal DNA (rDNA) amplification from *Peronospora effusa* and *P. schachtii* from impaction spore traps at two locations in the Salinas Valley, CA

Sample location[a]	Date of sample collection	*P. effusa* Cq ± SD[b]	*P. schachtii* Cq ± SD	Frequency *P. effusa*	Frequency *P. schachtii*	DNA copy number (*P. effusa* 18S rDNA)[c]
Salinas	March 13	37.33 ± 1.52	34.24 ± 0.15	0.01	0.99	2
Salinas	–	35.34 ± 0.41	29.67 ± 0.36	0.00	1.00	0
Soledad[1]	–	30.04 ± 0.15	NA	1.00	0.00	18,129
Soledad[2]	–	29.15 ± 0.26	34.40 ± 4.48	0.98	0.02	31,683
Salinas	March 15	36.42 ± 1.17	38.60 ± 0.00	0.99	0.01	282
Salinas	–	37.90 ± 1.05	NA	1.00	0.00	109
Soledad[1]	–	24.91 ± 0.14	36.85 ± 0.44	1.00	0.00	508,689
Soledad[2]	–	23.39 ± 0.43	35.44 ± 0.41	1.00	0.00	1,368,163
Salinas	March 18	NA	36.06 ± 0.32	0.00	1.00	0
Salinas	–	NA	NA	0.00	0.00	0
Soledad[1]	–	28.45 ± 0.28	35.69 ± 0.08	0.99	0.01	50,359
Soledad[2]	–	23.01 ± 0.14	28.57 ± 1.15	0.98	0.02	1,711,320
Salinas	March 20	31.48 ± 0.33	30.49 ± 0.75	0.33	0.67	2,339
Salinas	–	35.37 ± 0.91	31.72 ± 0.92	0.07	0.93	39
Soledad[1]	–	21.36 ± 0.49	30.97 ± 0.53	1.00	0.00	5,131,043
Soledad[2]	–	21.71 ± 0.61	30.53 ± 0.00	1.00	0.00	4,073,110
Salinas	March 22	37.31 ± 0.00	NA	1.00	0.00	159
Salinas	–	36.06 ± 0.65	36.60 ± 1.51	0.59	0.41	212
Soledad[1]	–[d]	31.69 ± 1.31	32.15 ± 0.00	0.58	0.42	3,592
Salinas	March 25	34.51 ± 0.00	34.51 ± 0.00	0.50	0.50	494
Salinas	–	34.61 ± 1.96	30.56 ± 0.37	0.05	0.95	46
Soledad[1]	–	26.94 ± 0.40	35.01 ± 0.00	1.00	0.00	135,353
Soledad[2]	–	27.05 ± 0.47	36.43 ± 0.64	1.00	0.00	126,327
Salinas	March 27	36.51 ± 0.00	NA	1.00	0.00	269
Salinas	–	36.91 ± 0.00	36.91 ± 0.00	0.05	0.95	207
Soledad[1]	–	32.66 ± 0.72	39.52 ± 0.00	0.98	0.02	95,593
Soledad[2]	–	36.02 ± 1.43	36.88 ± 0.32	0.65	0.35	11,492
Salinas	March 29	32.92 ± 0.25	29.27 ± 0.63	0.07	0.93	194
Salinas	–	35.58 ± 1.40	30.51 ± 0.04	0.03	0.07	15
Soledad[1]	–	28.04 ± 0.29	37.72 ± 0.21	1.00	0.00	66,285
Soledad[2]	–	27.08 ± 0.28	37.11 ± 0.41	1.00	0.00	123,627
Salinas	April 1	34.43 ± 0.47	29.38 ± 0.41	0.03	0.97	31
Salinas	–	32.82 ± 0.38	29.65 ± 0.30	0.10	0.90	297
Soledad[1]	–	23.68 ± 0.41	32.48 ± 0.42	1.00	0.00	1,131,390
Soledad[2]	–	26.02 ± 0.62	36.32 ± 0.69	1.00	0.00	246,283
Salinas	April 3	36.37 ± 0.00	32.48 ± 0.72	0.06	0.94	18

Sample location[a]	Date of sample collection	*P. effusa* Cq ± SD[b]	*P. schachtii* Cq ± SD	Frequency *P. effusa*	Frequency *P. schachtii*	DNA copy number (*P. effusa* 18S rDNA)[c]
Salinas	–	NA	NA	0.00	0.00	0
Soledad[1]	–	27.81 ± 0.48	31.33 ± 1.61	0.92	0.08	71,063
Soledad[2]	–	29.50 ± 0.22	34.97 ± 0.00	0.98	0.02	25,239

[a]Cq = quantification cycle, SD = standard deviation calculated with three technical replicates, and NA = no amplification recorded.

[b]Trap 1 from the Soledad location was positioned at the north end of a downy mildew-infected spinach field, while trap 2 was positioned at the south end of the same infected field. The two traps at the Salinas location were placed side-by-side in an equipment yard, where there was not a nearby spinach field.

[c]Derived from frequency *P. effusa* × total 18S DNA copy number for each reaction.

[d]here was a single sample collected from the Soledad location on March 22 because impaction spore trap rods were missing from trap 2.

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TABLE 3.

Analyses of quantitative polymerase chain reaction inhibition using DNA of *Peronospora effusa* and the *P. effusa*-specific TaqMan assay in the absence (No extract) or presence (2× or 1× elution) of grease-coated spore trap rods

DNA concentration	Cq ± SD (no extract)[a]	Cq (2× elution, 2 rods)	Cq (1× elution, 1 rod)	Cq difference[b]
100 pg[c]	24.80 ± 0.70	27.04 ± 0.48	…	2.24
10 pg	28.36 ± 0.22	30.14 ± 0.16	…	1.78
1 pg	31.39 ± 0.08	33.53 ± 0.07	…	2.14
100 fg	34.43 ± 0.40	36.54 ± 0.23	…	2.11
100 pg	24.63 ± 0.32	…	25.56 ± 0.28	0.93
10 pg	27.79 ± 0.32	…	28.76 ± 0.36	0.97
1 pg	30.53 ± 0.60	…	32.27 ± 0.44	1.74
100 fg	33.76 ± 0.49	…	35.01 ± 0.49	1.25

[a]Cq = quantification cycle and SD = standard deviation.

[b]Difference between the average Cq values of three technical replicates.

[c]Separate standard curves were prepared at the indicated concentrations of genomic DNA.