Membrane-Based Oligonucleotide Array Developed from Multiple Markers for the Detection of Many *Phytophthora* Species

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ABSTRACT

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Most *Phytophthora* spp. are destructive plant pathogens; therefore, effective monitoring and accurate early detection are important means of preventing potential epidemics and outbreaks of diseases. In the current study, a membrane-based oligonucleotide array was developed that can detect *Phytophthora* spp. reliably using three DNA regions; namely, the internal transcribed spacer (ITS), the 5′ end of cytochrome *c* oxidase 1 gene (*cox*1), and the intergenic region between cytochrome *c* oxidase 2 gene (*cox*2) and *cox*1 (*cox*2-1 *spacer*). Each sequence data set contained ≈250 sequences representing 98 described and 15 undescribed species of *Phytophthora*. The array was validated with 143 pure cultures and 35 field samples. Together, nonrejected oligonucleotides from all three markers have the ability to reliably detect 82 described and 8 undescribed *Phytophthora* spp., including several quarantine or regulated pathogens such as *Phytophthora ramorum*. Our results showed that a DNA array containing signature oligonucleotides designed from multiple genomic regions provided robustness and redundancy for the detection and differentiation of closely related taxon groups. This array has the potential to be used as a routine diagnostic tool for *Phytophthora* spp. from complex environmental samples without the need for extensive growth of cultures.

Additional keywords: DNA hybridization.

Phytophthora is a genus in the order Peronosporales of the stramenopile lineage. It contains soil- or waterborne and airborne species, which use hyphae or a special structure called appressoria for the penetration of plant cell walls (37). *Phytophthora* infection at the roots or basal stem of a plant eventually blocks the transportation of nutrients and water within the host, resulting in severe impact to plant health and development, whereas some airborne species can cause leaf or stem blight (20,65). Thus far, 116 species have been listed in genus *Phytophthora* (24,39), most of which have been identified as phytopathogenic (10,18,21, 30,37). The most infamous diseases caused by *Phytophthora* include potato late blight caused by *Phytophthora infestans*, the disease responsible for the European potato famine in mid 19th century (55), and the recent sudden oak death epidemic caused by *P. ramorum* in California and Oregon in the United States (32, 47,57). *P. cinnamomi*, an aggressive saprophyte (21), has a worldwide distribution and is pathogenic to more than 2,000 host species (20,33,63).

The presence of *Phytophthora* spp. in an infested area is persistent over time, and accurate detection and identification of these plant destroyers is the key first step in disease control and is of vital importance for enforcing plant quarantines. Conventional detection and identification of *Phytophthora* spp. is a complex process that includes (i) examination of diseased plant material, (ii) plating of infected plant tissues on selective culture media, and (iii) detailed morphological examination of single isolates derived from environmental samples (12,26). Detection and identification from environmental samples such as soil or water is even more difficult. Such classic approaches are time consuming, labor intensive (especially when isolation is needed), and quite challenging for biologists who do not have a specialized background in taxonomy. Various molecular techniques, such as protein electrophoresis (25), including isoenzymes (49), as well as serological and biochemical assays (18,22,56), have been developed attempting to identify *Phytophthora* spp. that may not be easily distinguished by morphological characteristics. Assays based on polymerase chain reaction (PCR) and DNA sequencing expanded the capacity to detect species in this genus; however, applications were limited to detecting single or a few target pathogens simultaneously in a single reaction mixture (3–6,51,60). The next-generation sequencing technology is showing promise for multiplexed pathogen detection, yet it demands intensive bioinformatics assistance for data analysis and, as such, is considered expensive and impractical for routine diagnostics.

In the current study, we developed a DNA/oligonucleotide array based on a PCR and membrane-based DNA hybridization technique (42,43) to improve genus-wide diagnosis for the pathogen. This technique uses nylon membranes as a supporting platform to bind oligonucleotides that are taxon or group specific. When a labeled amplicon from the target genomic region hybridizes to the perfectly matched oligonucleotides on the DNA array, highly sensitive chemiluminescent signals are produced which indicate positive reactions. These signals can be captured by x-ray

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplemental figures and three supplemental tables. Figure 1 appears in color online.

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film or digital cameras (14,41). DNA array hybridization has proved to be a sensitive, reliable, fast, and high-throughput diagnostic molecular tool for the detection and identification of microbial organisms from environmental samples (41–43).

The objective of this study was to design a multiple-marker oligonucleotide array for the detection of many *Phytophthora* spp. simultaneously, based on nuclear ribosomal and mitochondrial genome regions, including the internal transcribed spacer (ITS) of the ribosomal RNA gene, the 5′ end of cytochrome *c* oxidase 1 gene (*cox*1), and the intergenic region between cytochrome *c* oxidase 2 gene (*cox*2) and *cox*1 (CS). This DNA array was validated using total DNA extracted from reference pure cultures as well as from complex environmental samples collected from North America and the United Kingdom, in which the presence of target species had been validated using alternative molecular or conventional approaches. We demonstrated the feasibility of designing a DNA array from multiple DNA regions and tested their discriminatory capacity for the detection of *Phytophthora* spp. The comparison of specificity, discrimination potential, and the application in testing field samples between each subarray is also discussed.

MATERIALS AND METHODS

Phytophthora and *Pythium* isolates used in this study are listed in Supplemental Table S1. All vouchers of these isolates are maintained at World *Phytophthora* Genetic Resource Collection. All oligonucleotides and primers used in this study are listed in Supplemental Table S2.

Amplification and sequencing of ITS, CS, and *cox*1 regions. Total DNA from each pure culture was extracted as described previously (8). Amplification of the ITS region was performed in 50 µl reaction mixtures containing 2 µl of total genomic DNA (≈20 ng), 3 µl of 25 mM $MgCl_2$, 5 µl of 10× Titanium *Taq* buffer (Clontech Laboratories Inc., Mountain View, CA), 2 µl of 2 mM dNTPs, 0.13 µl of each of the forward (TS5) (74) and reverse (ITS4) (74) primers (20 µM), 0.4 µl of Titanium *Taq*, and 36.88 µl of sterile high-performance liquid chromatography (HPLC) water. The amplification profile was conducted as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The amplification of the *cox*1 region was performed in 50 µl reaction mixtures containing 2 µl of total genomic DNA, 6 µl of 25 mM $MgCl_2$, 5 µl of 10× Titanium *Taq* buffer, 2.5 µl of 2 mM dNTPs, 1 µl of each of the forward (OomCox1Levup) (58) and reverse (FM85) (47) primers (20 µM), 0.4 µl of Titanium *Taq*, and 32.1 µl of sterile HPLC water. The amplification profile was conducted as follows: 94°C for 4 min; 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1min; and a final extension at 72°C for 5 min. The CS region was amplified using primer sets FMPh-8b and FMPh-10b as described by Martin et al. (47). Amplicons were then sequenced as described previously (46,59).

Sequences from each genomic region were aligned using a Linux version of MAFFT (36) with the L-INS-i algorithm for ITS and G-INS-I algorithm for *cox*1 and CS regions. Alignments were imported into Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (68) and a neighbor-joining (NJ) tree was reconstructed for each region, using maximum composite likelihood distance. The outgroup for the analyses contained seven *Pythium* isolates, including *Pythium sylvaticum* (P15580), *P. vexans* (P3980), and five undescribed isolates (P8204, P8201, P8204, P8209, and P8212). DNA sequence data of each region used in this study have been deposited in GenBank. A Statistic Analysis Software (SAS Institute Inc., Cary, NC) script was written to perform the distance matrix statistical analysis as described previously by Robideau et al. (59).

Design and *in silico* evaluation of oligonucleotides and the fabrication of the DNA array. Signature oligonucleotides with various levels of specificity were designed using two computer programs—SigOli (75) and Array Designer version 1.1 (Premier Biosoft International, Palo Alto, CA)—as described previously (14,62). The specificity of each oligonucleotide was verified by BLAST searches against both GenBank and our in-house database. Oligonucleotides preselected by Array Designer 1.1 were then subjected to *in silico* DNA hybridization experiments simulated by a commercial program: Visual OMP (DNA Software Inc., Ann Arbor, MI). Visual OMP helped identify oligonucleotides having the potential to hybridize with non-target amplicons by displaying the following four calculated parameters between the fixed probes and the target in the hybridization system: change in Gibbs Free Energy (dG), melting temperature (T_m), the concentration of the target bound to a specific probe at equilibrium (Concentration), and the percentage of the target bound a specific probe at equilibrium (PB). This program also provided information on folding and secondary structures of the amplicons (probes). Visual OMP calculated every possible interaction between any pair of oligonucleotide and target amplicon in a DNA hybridization experiment under preset conditions, such as hybridization temperature, Na^+ and $MgCl_2$ concentrations, and other conditions that could change in a reaction. By comparing the PB between an oligonucleotide with its target or non-target amplicons, the specificity of a fixed oligonucleotide was estimated.

The name of each oligonucleotide was designated with the species or clade and the genomic region it was designed for. For example, oligonucleotide "quercina_1_ITS" was designed for species or strains of *Phytophthora quercina* in the ITS region, whereas oligonucleotide "infestans_CLD4_ITS" was designed for a clade of five species with similar sequences, including *P. infestans* from the ITS region. The column named "location" in Supplemental Table S2 can be used to locate the spot of each oligonucleotide on the array. For example, oligonucleotide alni_1_ITS is spotted at B1R24C1 and B1R24C2, which indicates block 1, row 24, columns 1 and 2 in Supplemental Figure S1.

Oligonucleotides which passed *in silico* simulation tests were synthesized with 5′-end amine-modification and robotically spotted on to Immunodyne ABC membrane (PALL Europe Ltd., Portsmouth, England) at near microarray density, as described by Chen et al. (14), except for the following modifications, as indicated in the figure of the schematic arrangement of the array. The ITS subarray, blocks 1 and 4) was 2 by 6 cm in size, with duplicates of each oligonucleotide spotted horizontally side by side. On the CS and *cox*1 subarrays, a whole set of blanks, positive controls and all selected oligonucleotides were printed as a 16-by-24 array; then, a duplicate set were printed and arranged horizontally within a dimension of 3 by 5 cm. In total, 48 Immunodyne ABC membranes were printed for each subarray. Membranes were stored in 2× SSC (0.33 M sodium chloride, 0.1 M sodium citrate [pH 7.0], 0.5% [g/ml] skim milk powder, and 0.05% [wt/wt] Tween-20) at 4°C. The synthetic oligonucleotide ST1 (9) was an immobilized oligonucleotide spotted on each subarray, whose complimentary digoxygenin (DIG)-labeled strand ST3 was added to every DNA array hybridization reaction, acting as a positive control ensuring that proper hybridization to the array took place in each reaction (9,58).

Preparation of DIG-labeled PCR amplicons and hybridization with DNA from pure cultures. The three genomic regions of all DNA samples were amplified and labeled with DIG using the protocol described previously (14), with the following changes. To amplify ITS regions from DNA templates extracted from pure cultures, universal eukaryotic primer pairs UN_up18S42 (forward) and UN_lo28S22 (reverse) (66) were used with the following thermocycler profile: initial denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 1 min, 68°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 8 min. Primer pairs FM79 (forward) (46) and FM85mod (reverse) (59) were used to amplify the combined CS and *cox*1 regions. The amplifi-

cation profile was initiated with a hot start at 95°C for 2 min; followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. ST3 was 3′-end tailed with DIG-dUTP/dATP using terminal transferase following the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN). The concentration of PCR products was quantified using a Quant-iT dsDNA High-Sensitivity Assay Kit (Invitrogen Corporation, Carlsbad, CA). Hybridizations of DIG-labeled amplicons to the DNA array and the analysis of the results were done as described previously (14,28).

The sequence database for the species in this study contained more than one strain for most species, most of which shared exact or highly similar sequences at target regions. To save a significant amount of cost, laboratory labor, and time but still obtain the best coverage of species, amplicon mixtures of all three genomic regions from at least one strain of each species were hybridized to the array for validation, giving priority to species with some intraspecific variation for the testing of additional strains. In total, 143 pure cultures were amplified for the ITS and the combined CS and *cox*1 regions. The ITS and CS + *cox*1 amplicons were mixed in equal amounts (≈50 ng each) and then hybridized to the array.

Detection of *Phytophthora* spp. in field samples. Field samples for the testing of *P. ramorum* were collected on 2 June 2010 at Pfeiffer Big Sur State Park and Andrew Molera State Park, Monterey County, CA, and processed at the United States Department of Agriculture (USDA) Agricultural Research Service station in Salinas, CA (Table 1). Protocols for sample collection and handling were similar as the one used previously for DNA extraction and pathogen isolation from infected plant material (3,4). Leaf pieces with lesions and nonsymptomatic leaves were cut by a number 3 cork borer, with one-half of the leaf disk plated on pimaricin-ampicillin-rifampicin-pentachloronitrobenzene (PARP) agar V8 medium (34) for selective isolation of *Phytophthora* spp., while the other half was used for DNA extraction using the USDA Animal and Plant Health Inspection Service protocol with the Qiagen DNeasy Plant Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) (72). Cultures were checked after a few days for growth of any *Phytophthora* spp., especially *P. ramorum*. The 20 DNA extractions were diluted 1/10 in sterile water and tested with *Phytophthora* genus-specific and *P. ramorum*-specific plant *Taq*Man multiplex real-time PCR assay from a mitochondrial region (5). In total, 10 field samples were received by the Royal Horticultural Society (RHS) from across the United Kingdom as part of a survey of U.K. gardens (19). Traditional baiting of the soil and symptomatic plant material was undertaken using apple fruit (13) and hemp seed (52) followed by plating onto P_5ARP (26) plates. Mycelium from baited cultures, alongside symptomatic plant material, was flash frozen using liquid nitrogen and DNA was extracted using Qiagen DNeasy Plant Mini Kit following the manufacturer's instruction. An amplicon (≈900 bp) of the ITS region from both bait-derived cultures and directly extracted DNA samples was amplified following the semi-nested PCR protocol described by Cooke (17). The PCR products were run on a high-concentration Tris-borate-EDTA gel (1.7%). Each band was separately excised and cleaned using QIAquick Gel extraction kit (Qiagen Ltd.) before being sequenced using primers ITS4 and ITS6 through a commercial sequencing service (Genome Enterprise Limited–TGAC, Norwich, UK). Contigs were constructed using Lasergene software (DNASTAR, Inc., Madison, WI) and initially compared against the GenBank nucleotide collection database through BLAST, followed by an alignment with known *Phytophthora* spp. sequences for final designation, enabling species identification even where there was small sequence variation between species.

Total soil DNA collected from Canada by Agriculture and Agri-Food Canada (AAFC) was isolated using the PowerMax Soil DNA isolation kit (MoBIO Laboratories, Carlsbad, CA). For soybean roots and associated soil collected from the soybean nursery infested with *P. sojae*, total DNA was isolated from ≈10 mg of each sample.

Blind tests were done on all field samples using the DNA array (i.e., the technician who performed the DNA hybridization tests did not have the information of which field sample they processed). Total DNA extracted from all field samples were amplified and DIG-labeled for the ITS and combined CS and *cox*1 region as was done for pure cultures, except an oomycete-specific reverse primer, Oom-lo28S-345H (66), was used to replace the fungal universal primer UN_lo28S22 in the amplification of the ITS region. DNA hybridization on the array was carried out as described above. The DNA array results were compared with results obtained by other detection approaches mentioned above.

RESULTS

All supporting materials, including all supplemental files can be downloaded from the link for the online e-Xtra. To view the hybridization symbols in Supplemental Table S3 properly, the ZapfDingbats font is required.

Sequence and cluster analysis. The sequence data sets each included over 200 strains of *Phytophthora* representing 98 described species and 15 isolates that represent undescribed species based on mitochondrial multigene analysis (F. Martin, J. E. Blair, and M. D. Coffey, *unpublished data*) and a more detailed multigene analysis with nuclear and mitochondrial data (M. D. Coffey, M. Mansfield, F. Martin, and S. Kang, *unpublished data*). These multigene analyses also indicated that *P. cinnamomi* var. *robiniae* (P16351) and *P. cinnamomi* var. *parvispora* (P7154 and P8495) are distinct species and not a variety of *P. cinnamomi*; therefore, in this study, they were referred to as separate species.

The nucleotide composition of each region was significantly different, with the average percentage of (A+T) being 49% for ITS, 83% for CS, and 69% for *cox*1. The high AT content in the mitochondrial region forced the design of longer oligonucleotides in order to obtain suitable T_m for DNA hybridization. The mean intraspecific (within-species) variation for ITS, CS, and *cox*1 was 0.9, 1.0, and 0.9, respectively, whereas the mean interspecific (between-species) variation was 15.7, 10.0, and 6.6%, respectively. The NJ trees for each region are shown in Supplemental Figure S2 using seven *Pythium* strains as an outgroup. In general, most isolates were clustered into conspecific groups by all three regions due to high sequence similarity, and the three NJ trees agreed on the composition of major clades. Several notable exceptions, however, were observed. For example, ITS and CS sequences of *P. ramorum* strains were most similar to those of *P. hibernalis* (P3822) but not for the *cox*1 region. *P. sojae* isolates were clustered with *P. cinnamomi* var. *robiniae* (P16351) in the ITS tree but not in mitochondrial trees. All *P. frigida* strains clustered with *P. alticola* strains in the CS tree but, in ITS and *cox*1 trees, they did not. For the three *P. drechsleri* stains (P10331, P11638, and P1087), all were grouped together in ITS and *cox*1 NJ trees but the CS sequence of P1087 was more similar to that of *P. macrochlamydospora* strain P8017. In the latter example, it was only possible to design species-specific oligonucleotides from ITS and *cox*1 regions but not from the CS region, from which only strain-specific oligonucleotides could be designed for this species.

Design and validation of the DNA array. The SigOli program found 45 ITS, 40 CS, and 41 *cox*1 polymorphic sites that had the potential to discriminate *Phytophthora* spp. or complexes, from which Array Designer generated >1,000 oligonucleotides from each DNA region, with an ideal length (16 to 35 mer) for suitable T_m (54°C), and with the least opportunity to form hairpins and primer dimers. Only ≈350 oligonucleotides from each region, however, had passed the *in silico* specificity evaluation according to theoretical principles, and Visual OMP cross-hybridization

analysis as well as in-house and GenBank BLAST results with ITS and *cox*1 of all *Pythium* spp. (59) was done before oligos were synthesized and robotically spotted onto Immnodyne ABC membranes.

The array was validated with 143 pure cultures, representing 96 of 98 described and 9 of 15 undescribed *Phytophthora* spp., as well as four *Pythium* isolates. These oligonucleotides were grouped into four categories based on their performance in the DNA hybridization tests. (i) "Best" oligonucleotides: true positives (TPs) were easily detectable and stronger than false positives (FPs), with no FP stronger than faint (○) and ≤1 faint FP (e.g., pistaciae_1_ITS). (ii) "Good" oligonucleotides: TPs were detectable and stronger than FPs, with no FP stronger than weak (□) and ≤5 faint and weak FPs in total (e.g., ramorum_CLD1_ITS). (iii) "Acceptable" oligonucleotides: TPs were detectable and stronger or equal to FPs, with ≤10 faint and weak FPs in total and ≤1 FP stronger than weak (e.g., austrocedrae_CLD8_ITS). (iv) "Rejected" oligonucleotides: TPs were not detectable or weaker than FPs, and the oligonucleotide showed more or stronger FP than those in the other three categories (e.g., syringae_1_ITS).

Each subarray contained >200 oligonucleotides that are species or strain specific, among which 154 ITS, 149 CS, and 102 *cox*1 oligonucleotides were validated as well-performing ones ("Best," "Good," and "Acceptable") (Table 2). For example, oligonucleotides that can detect all or individual strains of *P. boehmeriae* were extracted from all three regions. Among the eight "Best" ITS oligonucleotides, two (boehmeriae_4_ITS and boehmeriae_6_ITS) are species specific (Table 2, listed as '4,6' in subcolumn 1 for ITS) and six are strain specific (Table 2, listed as 1*, 2*, 10*, 11*, 16*, and 18). In total, 62, 55, and 54 described *Phytophthora* spp. can be detected by ITS, CS, or *cox*1 subarray alone, respectively, among which 33 have at least one species-specific oligonucleotide from all three DNA regions. The combination of all three subarrays is able to reliably detect 82 described and 8 undescribed *Phytophthora* spp. in total. Three species (*P. alni*, *P. capsici*, and *P. macrochlamydospora*) only had reliable strain-specific oligonucleotides.

The combination of certain oligonucleotides helps to confirm the absence or presence of some species. For example, for *P. niederhauserii*, there was no species-specific ITS probes available but group oligonucleotide cinnamomi_CLD3_ITS showed positive reactions to both *P. cinnamomi* and *P. niederhauserii.* A negative reaction of species-specific probes for *P. cinnamomi* (cinnamomi_1_ITS, cinnamomi_CLD4_ITS, and cinnamomi_6_ITS) and a positive reaction of cinnamomi_CLD3_ITS would indicate the presence of *P. niederhauserii* or an unknown species from this clade. If all probes displayed positive reactions, the results would not be conclusive for the presence of *P. niederhauserii* by using the ITS subarray alone. In that case, both of the mitochondrial subarrays have "Best" oligonucleotides (niederhauserii_1_CS, niederhauserii_3_CS, niederhauserii_2_cox1, and niederhauserii_4_cox1) for the detection of *P. niederhauserii*.

The DNA array contains several well-performing *Phytophthora* genus-specific oligonucleotides, with two designed for the ITS region (ZCtrl_Phyto_gn-upITS-584_ITS and ZCtrl_Phyto3_ITS, designed previously by C. A. Lévesque, *unpublished data*) and three for the *cox*1 region (ZCtrl_PhyUni1_cox1, ZCtrl_PhyUni2_cox1, and ZCtrl_PhyUni3_cox1). DNA hybridization results showed that these five oligonucleotides have high specificity and broad coverage for *Phytophthora* spp., although BLAST results revealed that a perfect match of ZCtrl_Phyto3_ITS can also be found in some *Peronospora* spp. These oligonucleotides would allow the detection of a new species by the array if DNA from a culture or a field sample would only hybridize to the genus oligos and perhaps group oligos but not any individual species oligo. In cases like this, additional techniques could be used to generate sequence data that will be useful in characterizing the new species (47) but, ultimately, isolation in culture would still be required. The oligonucleotide ZCtrl_PhyUni4_cox1 hybridized to both *Phytophthora* and *Pythium* spp. in the tests, while the three CS genus oligonucleotides displayed low sensitivity and less species coverage for *Phytophthora* spp.

Approximately a quarter of the ITS and CS and half of the *cox*1 oligonucleotides were rejected after validation due to the lack of specificity or sensitivity. These "Rejected" oligonucleotides either did not generate positive signals with perfectly matched amplicons (e.g., hibernalis_3_cox1 and hibernalis_4_cox1) or had strong false positive signals (e.g., cuyabensis_1_cox1), or both scenarios had been observed for the same oligonucleotide.

Testing field samples with the DNA array. Oligonucleotides designed for the detection of *P. ramorum* and *P. sojae* proved to be sensitive and specific when tested with field samples. Well-performing oligonucleotides sojae_1_CS, sojae_1_cox1, and sojae_2_cox1 confirmed the existence of *P. sojae* in all AAFC samples collected from a *P. sojae*-infested nursery. Four oligonucleotides on the DNA array (ramorum_6_ITS, ramorum_7_ITS, ramorum_4_CS, and ramorum_3_cox1), together with *Taq*Man multiplex real-time PCR assay and culturing confirmed the existence of *P. ramorum* in six bay laurel leaf samples collected at two state parks in California (IDs: USDA_2, 3, 5, 6, 11, and 17) (Table 1), except culturing was not able to isolate this species from sample USDA_6. The other potential hosts (live oak and tan oak) were tested negative for this species using all three loci, and prior testing with the mitochondrial diagnostic markers (5) indicated that the pathogen was not present.

For field samples received by the RHS, the presence of *Phytophthora* and *Pythium* spp. detected by baiting methods and nested PCR was in agreement with results from the combination of three markers in most cases, although the array detected more species than baiting on apple fruit (Table 1). The results also showed increased detection redundancy by using an array developed from multiple loci. For example, in sample RHS_P15284_BW12_Root (Table 1), *P. megasperma* and *P. cinnamomi* were detected separately by baiting and nested PCR assays, respectively. In contrast, well-performing ITS oligonucleotides (Fig. 1A and B) detected *P. cinnamomi* (cinnamomi_1_ITS) (Fig. 1A, box1) and *P. megasperma* (megasperma_2_ITS) (Fig. 1A, box2) in one assay, plus *P. cinnamomi* var. *parvispora* (cinnamomiVparvispora_2_ITS) (Fig. 1A, box4) and the possible existence of any species that can be detected by group oligonucleotides (Fig. 1A and B, boxes 3, 5, 6, 7, and 8). The ITS and CS subarrays together confirmed the presence of *P. cinnamomi* (positive signals from cinnamomi_1_ITS, cinnamomi_2_CS, and cinnamomi_3_CS) (Table 3), while ITS and *cox*1 subarrays together confirmed the presence of *P. megasperma* (positive signals from megasperma_2_ITS and megasperma_4_cox1) (Table 3). Although cinnamomi_CLD3_cox1 was originally designed for two species (*P. cinnamomi* and *P. medicaginis*), the validation using pure cultures showed that *P. medicaginis cox*1 amplicons did not hybridize to this oligonucleotide; therefore, this oligonucleotide can be treated as *P. cinnamomi* specific as well.

All three subarrays contained a limited number of species-specific as well as group oligonucleotides for the detection of *Pythium* spp., such as "Good" oligonucleotide Py_sylv_29_CS that is *Pythium sylvaticum* specific and displayed positive signals when hybridized to a few RHS samples. However, because only four isolates from this genus were tested in the current study, how well these oligonucleotides will perform for the genus as a whole is inconclusive. Therefore, this array is not suitable for the detection of a specific *Pythium* sp. from a field sample.

DISCUSSION

Phylogenetic studies and the development of diagnostic assays for *Phytophthora* spp. have been based on either single genomic regions or multiple loci from both nuclear and mitochondrial

TABLE 1. *Phytophthora* and *Pythium* spp. identified and confirmed in field samples using different approaches

Isolate[a]	Plant host	Location[b]	Collection date	Sample	DNA array result[c]				
					Culture, baiting[d]	PCR, sequencing[e]	ITS	CS	COXI
USDA_1	Tan oak	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_2	Bay laurel	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*
USDA_3	Bay laurel	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*
USDA_4	Maple	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_5	Bay laurel	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*
USDA_6	Bay laurel	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*
USDA_7	Live oak	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_8	Live oak	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_9	Redwood	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_10	Redwood	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_11	Bay laurel	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*
USDA_12	Tan oak	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_13	Tan oak	Pfiffer SP, Big Sur	2010-Jun-02	Leaf	…	…	…	…	…
USDA_14	Maple	Andrew Molera SP	2010-Jun-02	Leaf	…	…	…	…	…
USDA_15	Bay laurel	Andrew Molera SP	2010-Jun-02	Leaf	…	…	…	…	…
USDA_16	Live oak	Andrew Molera SP	2010-Jun-02	Leaf	…	…	…	…	…
USDA_17	Bay laurel	Andrew Molera SP	2010-Jun-02	Leaf	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*	*P. ramorum*
USDA_18	Poplar	Andrew Molera SP	2010-Jun-02	Leaf	…	…	…	…	…
USDA_19	Live oak	Andrew Molera SP	2010-Jun-02	Leaf	…	…	…	…	…
USDA_20	Live oak	Andrew Molera SP	2010-Jun-02	Leaf	…	…	…	…	…
RHS_P116439_3R1_Root	Taxus	Newmarket, Suffolk, UK	2010-Mar-01	Root	*P. cryptogea*	*P. gonapodyides*	*P. gonapodyides*	*P. gonapodyides*	*P. gonapodyides*
					…	…	*P. cryptogea**	…	*P. cryptogea**
					…	…	*Pythium* spp.*	*Py. sylvaticum*	*Pythium* spp.*
RHS_P15284_BW12_Root	Hibiscus	Ashtead, Surrey, UK	2007-May-31	Root	*P. megasperma*	*P. cinnamomi*	*P. cinnamomi*	*P. cinnamomi*	*P. cinnamomi**
					…	…	*P. megasperma*	*P. megasperma**	*P. megasperma*
RHS_P28125_9W11_Root	Malus	London, UK	2007-Oct-09	Root	*P . plurivora*	*P. cactorum*	*P. cactorum*	…	…
					…	…	*Pythium* spp.*	…	…
RHS_P28131.7_9W13_Root	Skimmia	Bristol, Avon, UK	2007-Sep-10	Root	*P. cactorum*	*P. cryptogea*	*P. cryptogea**	*P. cryptogea*	*P. cryptogea*
					…	…	*Pythium* spp.*	…	…
RHS_P46329_5R5_Root	Hebe	Dorchester, Doset, UK	2008-Nov-04	Root	…	*P. megasperma*	*P. megasperma*	*P. megasperma**	…
					…	…	*P. citricola*	…	…
					Pythium spp.*	*Py. sylvaticum*	*Pythium* spp.*	…	…
RHS_P46329_5R6_Stem	Hebe	Dorchester, Doset, UK	2008-Nov-04	Stem	*P. multivora*	*P. multivora*	*P. multivora*	*P. multivora*	*P. multivora*
					…	…	*P. multivesiculata*	*P. multivesiculata*	…
RHS_P76047_1O3_Root	Taxus	Etchingham, East Sussex, UK	2009-Jul-01	Root	…	*P. cryptogea*	*P. cryptogea**	…	…
					Py. perplexum	*Py. perplexum*	*Pythium* spp.*	…	…
RHS_P80534.1_3cO4_Root	Rhododendron	Chester, Cheshire, UK	2009-Mar-19	Root	…	*P. PgChlamydo****	…	…	…
					Py. sylvaticum	*Py. intermedium*	*Pythium* spp.*	*Py. sylvaticum*	…
RHS_P93250.1_6Re13_Root	Helleborus	Yateley, Hampshire, UK	2009-Jun-26	Root	*P. cactorum*	*P. cactorum*	*P. cactorum*	*P .cactorum**	*P .cactorum*
							…	*P. salixsoil*	*P. salixsoil*
					Py. perplexum	*Py. sylvaticum*	*Pythium* spp.*	*Py. sylvaticum*	…
RHS_P93250.1_6Re14_Stem	Helleborus	Yateley, Hampshire, UK	2009-Jun-26	Stem	…	*P. cactorum*	*P. cactorum*	*P. cactorum*	…
AAFC_soil_2QCML6	Soybean	Quebec, Canada	2002	Soil	N/A	N/A	*P. sojae***	*P. sojae*	…
					…	…	*Pythium* spp.*	*Py. sylvaticum*	*Pythium* spp.*
AAFC_sr_2QCML6	Soybean	Quebec, Canada	2002	Root	N/A	N/A	*P. sojae***	*P. sojae*	…
					…	…	…	*Py. sylvaticum*	*Pythium* spp.*
AAFC_sr_2-ON-34-G	Soybean	Ontario, Canada	2002	Root	N/A	N/A	*P. sojae***	*P. sojae*	*P. sojae*
					…	…	*Pythium* spp.*	*Py. sylvaticum*	*Pythium* spp.*
AAFC_Range20_row3	Soybean	Ontario, Canada	2001	Root	N/A	N/A	*P. sojae***	*P. sojae*	*P. sojae*
AAFC_sr_infected_*P.sojae*	Soybean	Ontario, Canada	2010		N/A	N/A	*P. sojae***	*P. sojae*	*P. sojae*
					…	…	*Pythium* spp.*	*Py. sylvaticum*	*Pythium* spp.*

[a] USDA = United States Department of Agriculture; RHS = Royal Horticultural Society, UK; and AAFC = Agriculture and Agri-Food Canada, Canada.

[b] SP = state parks in California in the United States.

[c] PCR = polymerase chain reaction, ITS = internal transcribed spacer, and CS = intergenic region between cytochrome c oxidase 2 gene (*cox*2) and cytochrome c oxidase 2 gene (*cox*1). *Phytophthora* is abbreviated as *P.* and *Pythium* is abbreviated as *Py.* Asterisks: * = only group oligonucleotides showed positive signals; ** = species-specific ITS oligonucleotide for *P. sojae* (sojae_3_ITS) showed positive signal when testing the samples; however, this oligonucleotide was rejected due to some faint cross reactions with other *Phytophthora* spp.; and *** = *P. PgChlamydo* is a provisional species that has not been formally described (11). N/A = not applicable.

[d] For samples from the USDA, leave disks were plated on pimaricin-ampicillin-rifampicin-pentachloronitrobenzene agar; for samples from RHS, apple and hemp bait identification was applied.

[e] For samples from the USDA, *P. ramorum* was detected using a mitochondrial region; for samples from RHS, an amplicon (≈900 bp) of the ITS region was used for identification.

DNA (8,18,38,44,45,70). Regions (e.g., ITS and CS) as well as genes (e.g. β-tubulin and elicitin) have been used to develop PCR-based assays for the detection of *P. ramorum* (4,45), from which the authors stated that the use of multiple target regions can increase the reliability and confidence in the results. The current study also targeted multiple genomic loci (ITS, CS, and *cox*1) for the design of signature oligonucleotides in order to provide more accurate detection of *Phytophthora* spp. by DNA hybridization. Both the ITS and *cox*1 regions have been used for phylogenetic analyses of this genus solely or in conjunction with other genomic regions (1,17,29,40,46,73). A dual complementary DNA barcode system has been proposed for oomycetes (59) that includes the 5′ end of *cox*1, the default DNA barcode for eukaryotes sanctioned by the Consortium for the Barcode of Life and the National Center for Biotechnology Information, and the ITS, the *de facto* barcode in oomycetes. The hyper variable CS has been used to differentiate *Phytophthora ramorum*, *P. pseudosyringae*, and *P. nemorosa* through PCR-based assays (47,70) and, with the sequence polymorphisms observed when designing species-specific markers, it can also be used for species identification.

All three regions (ITS, CS, and *cox*1) are present in multiple copies per cell, producing higher sensitivity of the detection capacity of the array (4,45). These regions also have an appropriate level of DNA sequence variation between species which provides potential polymorphic sites for designing oligonucleotides. Both the *in silico* simulation and the laboratory experiments showed that the oligonucleotides from ITS and CS displayed less cross reactivity than those from *cox*1. Such observation once again stressed the importance of choosing the right genomic region or regions for the design of signature oligonucleotides. In general, a DNA region such as the *Phytophthora* ITS and CS region, with lower intraspecific variation and higher interspecific variation, is better for distinguish-

TABLE 2. *Phytophthora* spp. that can be detected using the oligonucleotide array designed in this study[a]

		Oligonucleotides from different regions with best three classes of hybridization results[c]								
		ITS			CS			*cox*1		
Phytophthora spp.[b]	Strain tested	Best	Good	Accep.	Best	Good	Accep.	Best	Good	Accep.
Phytophthora alni	P10564,P16202	…	…	10*	…	…	…	…	…	…
P. alticola	P16052	1	…	…	…	…	…	2	…	1
P. asparagi	P10690,P10707	2,3	…	…	1,3,4,5,6	…	…	1	2	…
P. austrocedrae	P15132,P16040	4	11*	10*	1,5	2,3,4	…	3,2*	5	…
P. bisheria	P10117	1	2	…	…	…	…	…	1	…
P. boehmeriae	P1257,P1378,P6950	4,6,1*,2*,10*, 11*,16*,17*	…	…	8*,10*,11*	…	…	2*	…	…
P. botryosa	P1044	2	…	…	…	…	…	…	…	…
P. brassicae	P10155		…	…	3	…	…	4,5	11,6	…
P. cactorum	P10365	1	…	…	…	…	…	…	…	…
P. cajani	P3105	…	…	…	…	…	…	1	…	…
P. cambivora	P0592	…	…	…	…	…	…	…	…	1
P. canalensis	P10456	2	1		3,7	1,4	…	…	5	…
P. capsici	P10386,P1319,P3375	…	1*	6*	…	…	…	…	…	…
P. captiosa	P10719,P10720	4*	1	…	…	6	5	3,7	…	…
P. cinnamomi	P2100,P2301	6*	1		2	…	3	…	…	1
P. cinnamomi var. *parvispora*	P7154	…	…	3,4	2	1	…	…	8,1	…
P. cinnamomi var. *robiniae*	P16351	…	1	…	1,3	2	…	…	2	…
P. citricola	P0716	…	…	…	10,4,9	…	6	…	…	…
P. clandestina	P3943	3	2	…	…	1	…	…	…	1
P. colocasiae	P6102	1		…	…	…	…	…	…	…
P. cuyabensis	P8213,P8218	3,4,7*	8*	…	2	…	…	2*	4	…
P. drechsleri	P1087	1,2	3	…	6,2	4	…	…	…	…
P. erythroseptica	P0340,P10382	…	…	…	…	…	…	…	…	1
P. europaea	P10324	…	2	…	…	…	…	1,2	…	…
P. fallax	P10722	1,2	…	…	…	2	…	2,4	1,3	…
P. foliorum	P10969,P10971	1,2	…	7*	1,4	…	…	…	…	…
P. fragariae	P11808,P3820	…	…	…	…	…	…	…	4	…
P. frigida	P16051,P16059	16,7,8	2	17*	…	…	…	…	1	…
P. glovera	P10618	2	…	…	…	…	…	…	…	…
P. gonapodyides	P7050	5	…	…	…	…	…	1	3	…
P. hedraiandra	P11678	1	…	…	…	…	…	…	…	…
P. heveae	P0578	2	…	…	…	…	…	…	…	2
P. hibernalis	P3822	1,2,5	…	…	3,4	…	2	2	…	…
P. humicola	P3826	…	…	…	…	2	…	…	2	…
P. hydropathica	P21281	…	…	3	…	1	…	…	…	…
P. idaei	P6767	…	1	2	…	…	…	…	…	…
P. ilicis	P6098	…	…	…	4,5	…	3	…	…	…
P. insolita	P6195,P6703	2	11*	12*	…	3,7	1,2,8,9	…	…	2
P. inundata	P8478	…	…	…	…	…	1	…	…	…
P. iranica	P3882	1	2	…	…	1,3	…	…	…	…
P. kernoviae	P10958	2,9	…	8	2,4,5	3	…	2	…	…
P. lacrimae	P15880	1	…	…	2	…	…	1,6	5	3

(*continued on next page*)

[a] Numbers in the table are extracted from the full oligonucleotide names in the manuscript, which are made of the species name, the numbers in the table, and the acronym for the region (e.g., asparagi_1_ITS, alticola_2_cox1). Oligonucleotides with good results for different region are organized in three categories: "Best" oligonucleotides, true positives (TPs) were detectable and stronger than false positives (FPs), with no FP stronger than faint and ≤1 faint FP; "Good" oligonucleotides, TPs were detectable and stronger than FPs, with no FP stronger than weak and ≤5 faint and weak FPs; and "Acceptable" (Accep.) oligonucleotides, TPs were detectable and stronger or equal to FPs, with ≤10 faint and weak FPs and ≤1 FP stronger than weak.

[b] Species can be reliably detected by selected oligonucleotides.

[c] ITS = internal transcribed spacer and CS = intergenic region between cytochrome *c* oxidase 2 gene (*cox*2) and cytochrome *c* oxidase 2 gene (*cox*1). Asterisks: * indicates strain-specific oligonucleotides and ** indicates oligonucleotides target at different strains, therefore the whole species can be reliably detected.

ing species, and the indels in its alignment provide optimum locations for the selection of oligonucleotides. The *cox*1 region, however, is extremely AT rich (≈70% average) with long homopolymers, which makes it difficult to hybridize effectively to oligonucleotides bound on a membrane (9). With the lowest interspecific variation, the highest intraspecific variation, and the lack of indels in alignment, oligonucleotides selected from *cox*1 can be long, with low signal intensity and low specificity, thereby making it more difficult to detect all strains of a species (9,14). Such a premise was confirmed by the observation that almost half of the *cox*1 oligonucleotides were rejected based on DNA hybridization results. These cross reactions may be eliminated by increasing the hybridization stringency (9,14); however, increased stringency of hybridization can also affect the intensity of true positive signals, resulting in increased occurrence of false negatives.

Due to the similarity in grouping of sequences at each of the three loci, it was possible to design oligonucleotides that were capable of detecting the same species or subclades using each of the markers, thereby providing redundancy and confidence in detection with a reduced number of false positives or negatives. In all, >80 *Phytophthora* spp., such as *P. clandestina*, *P. iranica*, and *P. tentaculata* (Table 2), had at least one well-performing oligonucleotide designed from one or two of the regions, making it possible to detect these species using at least one of the DNA markers. *P. asparagi*, *P. austrocedrae*, *P. multivora*, and another 30 *Phytophthora* spp. can be detected by all three markers. Group oligonucleotides were also able to be designed from all three loci for a clade of five taxa, including *P. infestans*, *P. andina* (some strains), *P. mirabilis*, *P. phaseoli*, and *P. ipomoeae*, and a clade of four, including *P. alni*, *P. fragariae*, *P. rubi*, and *P. cambivora*. Closely associated species in these two clades, however, shared the same or highly similar sequences at all three regions; as such, species- or strain-specific oligonucleotides for these two groups were only designed for *P. mirabilis* (mirabilis_2_CS), *P. phaseoli*

TABLE 2. (*continued from preceding page*)

		Oligonucleotides from different regions with best three classes of hybridization results[c]								
		ITS			CS			*cox*1		
Phytophthora spp.[b]	Strain tested	Best	Good	Accep.	Best	Good	Accep.	Best	Good	Accep.
P. lagoariana	P8220	2	1	…	…	…	…	…	…	…
P. macrochlamydospora	P10267,P8017	1*	…	9*	…	…	…	…	…	…
P. medicaginis	P10683,P7029	1	6*	3	1,2	…	…	…	…	…
P. megakarya	P1672	1,6	…	3	2,4,6	…	8	2	…	…
P. megasperma	P10340,P1679,P6957	…	…	2*	5*,8*	…	…	…	…	4,7*
P. melonis	P1748,P3609,P6870	2,3	…	…	2,4	3	…	4	…	…
P. mirabilis	P3005		…	…	2	…	…	…	…	…
P. multivesiculata	P10670	1,2	…	…	2,5	…	1,6	3	…	1
P. multivora	P7902	…	3	…	18	16	…	10,9	…	8
P. napoensis	P8221,P8222,P8225	7	4*	6	1,2	…	…	4	3	…
P. nemorosa	P16352	…	…	…	1,2		…	…	…	…
P. nicotianae	P6915	3	1,4,5	6	7,1,2	3,4	…	…	…	…
P. niederhauserii	P10279	…	…	…	1,3	…	…	…	4	2
P. ohioensis	P16050	…	…	2	2	1		2	…	…
P. palmivora	P0113,P10213,P6390	1,3*	4*	2	6	…	…	…	5	…
P. parsiana	P15164	…	…	…	…	…	…	…	1	…
P. personii	P11555	…	…	…	1,2,4	…	…	…		…
P. phaseoli	P10145,P10150,P6609	…	…	…		…	…	…	2	…
P. pinifolia	P16100	4	…	2	4,7	5,8	…	…	4	3
P. pistaciae	P6196	1	…	…	2,3,4	…	…	…	…	…
P. polonica	P15004,P15005	6,9	4*	12*,14*	1		3*	…	…	…
P. porri **	P6207,P7518	10*	5*,11*	…	2*,5*,6*	15*	13*,14*	5*,13*	3*,14*	4*,6*
P. primulae	P10220	4	2	…	…	…	…	…	5	4
P. pseudosyringae	P16355	…	…	…	1,2,3	…	…	3	1,2	…
P. pseudotsugae	P10218	4	3	…	1	…	…	…	…	…
P. psychrophila	P10434	…	1	…	…	4	…	3	…	…
P. quercetorum	P15555	1	…	…	…	…	4	…	…	…
P. quercina	P10441	…	1,2	3	1,2	…	…	…	…	…
P. quininea	P3247	…	2	1	…	…	…	…	…	…
P. ramorum	P10102	7	6	…	4	4	…	3	…	…
P. richardiae	P6875	…	…	…	…	…	…	3	…	…
P. rubi	P3289	…	…	…	…	…	…	…	6	…
P. salixsoil	P10337	2	…	…	8	10,5,9	…	13	11	…
P. sansomea	P3163	…	…	1,2,3	1	…	…	…	2	1
P. siskiyouensis	P15122	…	…	…	…	…	…	…	…	4
P. sojae	P0405,P6497	…	…	…	…	…	1	1	…	2
P. sulawesiensis	P6306	1	…	…	1,4	…	…	…	…	…
P. syringae	P10332	5,6	…	…	…	2,4	7	5,6	2,4	…
P. tentaculata	P8497	4,5	2	…	1,2	…	…	6	1	2
P. trifolii	P1462	1	…	3	1,2,3	…	…	3	…	1
P. tropicalis	P10329	8	…	7	…	…	…	…	…	…
P. uliginosa	P10328	1	…	…	…	4	7	…	…	1
P. vignae	P3019	1	…	…	2	…	…	…	…	…
Phytophthora sp. aff. *brassicae*2	P10728	1	2	…	…	8,9	…	18	…	…
Phytophthora sp. aff. *colocasiae*1	P10368	1,2,3	4	…	…	4	…	1	…	2
Phytophthora sp. aff. *heveae*	P1000	…	…	…	…	…	7	…	…	…
Phytophthora sp. aff. *katsurae*	P6921	…	4	…	4	3	…	…	2	…
Phytophthora sp. aff. *cryptogea*	P10705	…	…	1	…	1,2	…	2	…	…
Phytophthora sp. aff. *rosacearum*	P10678	…	…	…	…	…	14	…	…	…
Phytophthora sp. aff. *siskiyouensis*	P1212	6	5	…	…	…	…	…	…	…
Unknown *Phytophthora* sp.	P3600	3,5	…	…	…	…	…	1	…	…

(phaseoli_2_cox1), and *P. fragariae* (fragariae_4_cox1). It is noteworthy that the *Solanum* pathogen *P. andina* is heterozygous, with half of its alleles from *P. infestans* and the other half from an unknown *Phytophthora* sp. (7, 31). Goss (31) revealed two distinct haplotypes for *P. andina* isolates using cloned PCR products of four *Ras* genes, with one clade (including strain P13660, tested in this study) related to *P. infestans* and the other clade (no strains were available for this study) related but distinct from *P. mirabilis*. Therefore, we were not able to distinguish *P. andina* strain P13660 from several other species in this study. It is still possible, however, to design effective oligonucleotides for the detection of strains of *P. andina* in the other clade. For groups of taxa for which only group oligonucleotides could be designed, it may be necessary to select a fourth genomic region that has higher variation among the target species.

Several oligonucleotides designed in this study have high specificity for the detection of economically important *Phytophthora* spp. For example, all three regions contained robust species-specific oligonucleotides for the quarantine pathogens *P. kernoviae* and *P. pinifolia* and the causal agent of sudden oak death, *P. ramorum*. The capability of detecting multiple quarantine species with a single assay would be a significant asset in laboratories involved in regulatory testing. The positive reactions from the mitochondrial subarrays can also confirm the presence of *P. sojae*. Useful oligonucleotides were also available for the detection of *P. cinnamomi*, one of the most common *Phytophthora* spp. causing root rot in rhododendron, a host on which *P. cinnamomi* is often found along with *P. ramorum*.

For species such as *P. capsici* and *P. cryptogea* that are not monophyletic, it was only possible to design oligonucleotides for some isolates, so that it is possible that some strains in these species were present in an environmental sample but no oligonucleotides were available for their detection. As such, it is possible the array will not accurately detect all isolates of these species (but the genus-specific oligos will). It also should be noted that some of the phylogenetically distinct clades in these species complexes likely reflect putative new species, and the species concepts of the genus *Phytophthora* are continuously under revision and being refined through molecular systematic tools and databases (15). Some of the isolates used in this study are currently being subjected to taxonomic revisions. Supplemental Table S1 lists 15 putative new species based upon phylogenetic analysis of multiple loci, including *cox*2, subunit 9 of NADH dehydrogenase (*nad*9), ribosomal protein S10 gene (*rps*10), and phytoplasma translocation protein gene (*secY*) (F. Martin, *unpublished data*). For example, based on *cox*1 sequencing, P3103, P16165, and P10705 are the same as the isolates in the phylogenetic group II of *P. cryptogea* described by Mostowfizadeh-Ghalamfarsa et al. (50) and, therefore, were named *P. cryptogea*; however, according to the multigene analyses conducted recently, they now represent new species and were listed as *Phytophthora* sp. aff. *cryptogea*. However, even if the nomenclature of some of the isolates is likely to be changed, the oligonucleotides that detected these subclades with the proper resolution will remain valid under these new names, such as aff. cryptogea_P10705_1_ITS. As a result, the total number of *Phytophthora* spp. covered in this study and the number of species that can be detected by the array are inevitably subject to change as well, and some of these oligonucleotides will likely be useful for detection of additional species once the taxonomy of these complexes have been completed.

In a few cases, the oligonucleotides originally designed for a group of *Phytophthora* spp. displayed strong signals for only one species (e.g., alticola_CLD1_CS and alticola_CLD4_CS were designed for *P. alticola* and *P. frigida* but only displayed positive

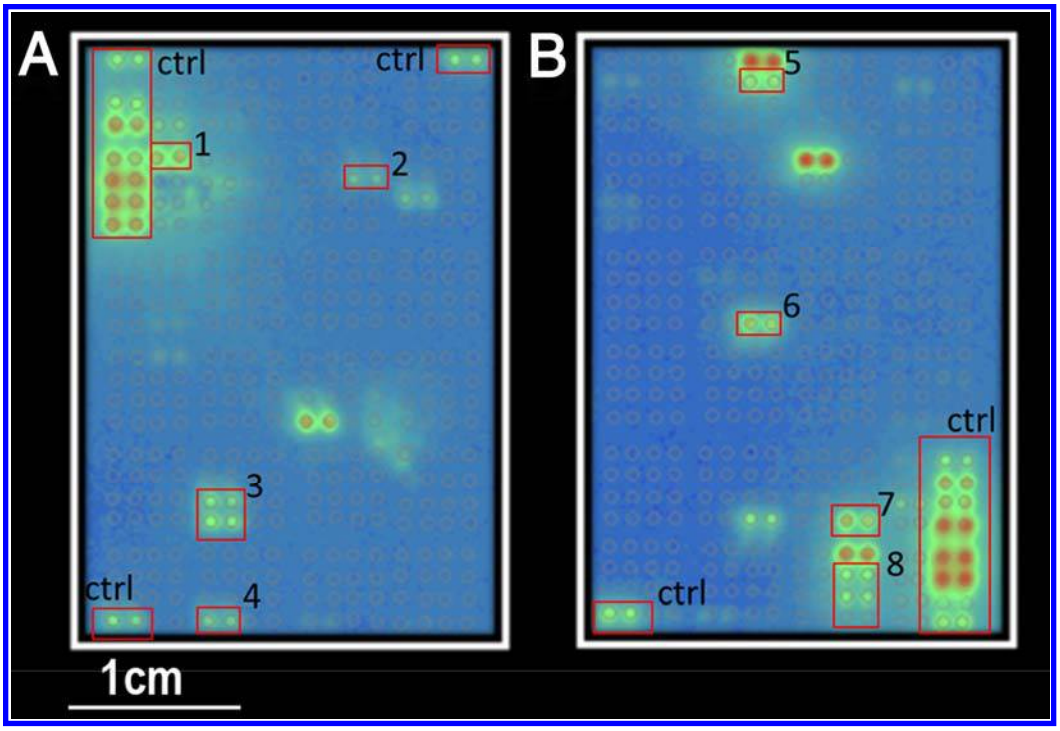
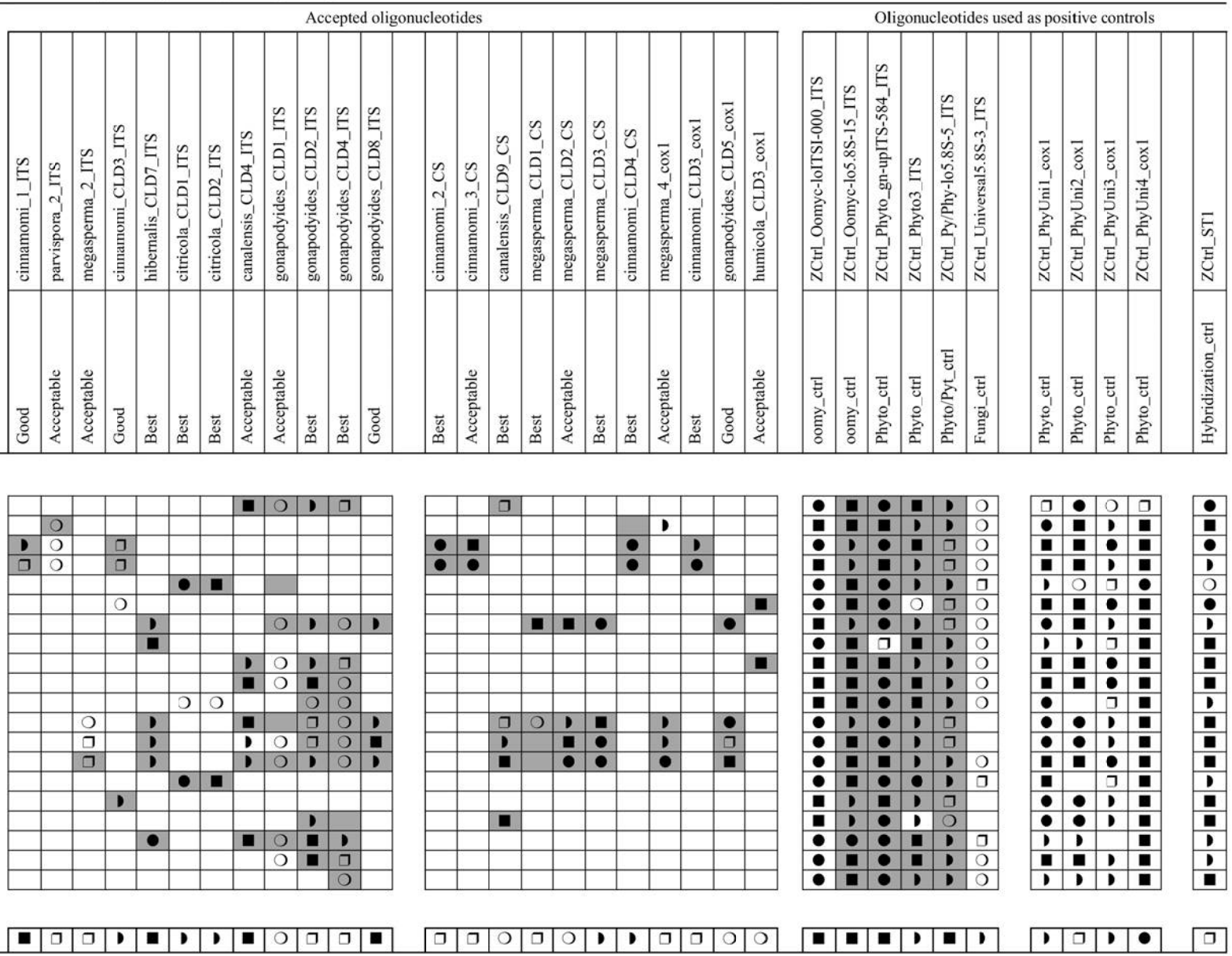


Fig. 1. DNA hybridization result that was processed by GenePix Pro software, representing the hybridization patterns of digoxigenin-labeled polymerase chain reaction amplicons from environmental sample RHS_P15284_BW12_Root to the internal transcribed spacer (ITS) subarray. The chemiluminescent image was captured by a 16-bit digital camera and was analyzed with GenePix Pro software. The exact location of each oligonucleotide on the membranes was overlaid with each feature in a grid created by the GenePix software. **A,** The GenePix Pro processed chemiluminescent image of the top of the ITS subarray hybridized to sample RHS_P15284_BW12_Root. Box 1, cinnamomi_1_ITS; box 2, megasperma_2_ITS; box 3, citricola_CLD1_ITS and citricola_CLD2_ITS; box 4, cinnamomiVparvispora_2_ITS; box ctrl, positive control oligonucleotides. **B,** The GenePix Pro processed chemiluminescent image of the bottom of the ITS subarray hybridized to sample RHS_P15284_BW12_Root. Box 5, cinnamomi_3_ITS; box 6, canalensis_CLD4_ITS; box 7, gonapodyides_CLD8_ITS; box 8, gonapodyides_CLD1_ITS, gonapodyides_CLD2_ITS, and gonapodyides_CLD4_ITS; box ctrl, positive control oligonucleotides. Oligonucleotides with name containing "CLD" are group oligonucleotides (e.g., canalensis_CLD4_ITS); oligonucleotides with name not containing "CLD" are species- or strain-specific oligonucleotides.

TABLE 3. Hybridization patterns of all three subarrays to sample RHS_P15284_BW12_Root[a]



[a] Cell with gray background indicates an *in silico* perfect match between the oligonucleotide and the corresponding DNA region of the *Phytophthora* spp./strain. The combination of the hybridization patterns of all three subarrays confirms the existence of *P. cinnamomi* and *P. megasperma* in this environmental sample. Intensities are indicated by the follow symbols: ■, very strong; ●, strong, ◗, moderate; ❒, weak; and ○, faint.

signals to *P. alticola*) Oligonucleotide medicaginis_CLD3_CS, designed for *P. trifolii* and *P. medicaginis*, did not hybridize to *P. trifolii*. Oligonucleotides drechsleri_3_CS, drechsleri_4_CS, drechsleri_5_CS, drechsleri_6_CS, and drechsleri_7_CS were originally designed to detect a group of four species—*P. drechsleri*, *P. macrochlamydospora*, *P. richardiae*, and *P. quinine*—but only strain P1087 of *P. drechsleri* reacted positively. Similarly, nicotianae_CLD1_cox1 and nicotianae_CLD2_cox1 reacted only with *P. nicotianae*, although they were designed for both *P. mengei* and *P. nicotianae*. The analyses of secondary structure using Visual OMP and sequence alignment did not find clear patterns to explain why PCR sequences did not hybridize to a perfect match oligonucleotide. Such observations suggested that the actual DNA-DNA hybridization results can be different than theoretical predictions (14,53,54).

Assays using conventional or real-time PCR have been considered to be the most sensitive means for the detection of pathogens thus far. The detection sensitivity for a number of forest *Phytophthora* spp. using multiplex real-time PCR ranges from 1 fg (gene with multiple copies) to 100 fg (single-copy genes) of target DNA (60,70). The membrane-based array, in comparison, was able to detect as few as 50 pg of PCR amplicon from pure cultures in our laboratory, yet the detection limit of a particular oligonucleotide varies due to the length, AT content, mismatch numbers, or even the actual arrangement of the sequence. To increase the testing sensitivity of an array for environmental samples, it is important to obtain high-quality DNA extracts and to minimize PCR inhibition for the target taxa groups (2,67). Serial dilutions of the total DNA from environmental samples can increase the amplification efficiency if PCR inhibitor factors (e.g., plant DNA or inhibitory chemicals) (51) are present but this also reduces the sensitivity of the assay. Using nested PCR has also been reported to increase the sensitivity of the classical PCR-based diagnostic methods (47,71). For the detection of *P. capsici* in pepper plant tissues, Silvar and coworkers (64) observed a 10,000 times increase of sensitivity of detection limit when using nested PCR (0.5 fg) compared with conventional PCR (5 pg). Nested PCR has been used for early diagnoses of quarantine *Phytophthora* spp. from environmental samples (infested media and infected hosts) (16,47,71) in which there is a low concentration of DNA from the targeted taxa groups relative to the total plant DNA present. For a broader coverage of *Phytophthora* spp. in either diversity assessment or pathogen diagnostics, genus-specific primers need to be designed and tested. Although the primers previously reported for amplification of just the CS region are *Phytophthora* specific and had been tested to not amplify DNA regions from *Pythium* spp. or plants (47), we used a combined CS and *cox*1 region as DNA hybridization probe, which was amplified using FM79 (forward primer) and FM85mod (reverse primer, a universal oomycetes primer for *cox*1 region); the sequencing and BLAST results confirmed that many *Pythium* spp. can be amplified by this primer pair. All ITS primers used in this study are universal fungal or oomycete primers. Amplifying the hybridization target regions with *Phytophthora*-specific primers (61) would reduce the potential effect caused by *Pythium* spp. in DNA hybridization when field samples are tested, so as to theoretically increase detection sensitivity and specificity. However, it is often important to know which *Pythium* and *Phytophthora* spp. are present when studying or diagnosing root rot. A *Phytophthora*-specific approach would not be conclusive when answering this question. Our next step would be merging this *Phytophthora* array with the *Pythium* array from Tambong et al. (66), which could be upgraded to include *Pythium* CS and *cox*1 markers as well.

Effectively and accurately detecting multiple *Phytophthora* spp. in one assay is especially important for disease management and the detection of quarantine species in local nurseries and forests, where they often co-exist in container mixes, soils on the same site, or different parts of the same plant (18,27). The potential of a DNA array to accommodate very large numbers of oligonucleotides with broad taxonomic coverage makes it a high-throughput and effective multiplex detection tool for monitoring plant pathogens from complex environmental samples (42). The oligonucleotides selected from different regions can be complementary with each other so as to increase detection redundancy and make it possible to significantly improve reliability and confidence, especially when environmental samples are tested. The disadvantage of the DNA array, as with any other DNA-based technologies, is the inability to distinguish living organisms from nonviable spores. This may be one of the reasons that more *Phytophthora* spp. are detected by array hybridization than by conventional methods such as baiting techniques. In addition, because *Phytophthora* spp. can be seasonally active and ephemeral and the population can vary from nondetectable to significant throughout a growing season, it is also possible that constitutive dormancy of resting spores can influence the ability to recover some species. Furthermore, differential colonization and competitive development on specific host tissue can reduce the possibility of species detection when using baiting technique.

The theoretical principle, simplicity, and effectiveness of the DNA array make this technique a good candidate for the development of a "lab-on-a-chip" (LOC) diagnostic device (23,48). The development of an LOC system for "bedside" detection of human pathogen DNA that was capable of automated sample processing and fast, sensitive, and accurate assays on miniature biosensors was an elusive goal for almost 20 years, mainly because of a wide range of engineering problems caused by the miniaturization of fluidic reactions (69). However, some of the technical difficulties have now been overcome and commercial miniaturized arrays are now available (48). A recent publication presented a prototype for an LOC device for the detection of some *Phytophthora* spp. (35). The oligonucleotides in this study were validated under somewhat similar hybridization conditions and should be easily integrated in such array-based devices. McGlennen (48) mentioned that probe densities on a DNA chip can be achieved as high as $10^6/cm^2$ indicating the possibility of enormous taxa coverage in one single assay. However, there can be trade-offs between expanded taxonomic coverage and the detection limit or sensitivity of a DNA array. To scan microbial biodiversity in an environmental sample will require the use of universal primers for amplification if multiplex PCR cannot be performed for targeted groups, which will, in turn, reduce the sensitivity for the detection of pathogens at low concentration levels due to competition for primers. Such trade-offs need to be balanced or shifted depending on the purpose of the assay.

In summary, *Phytophthora* spp. are among the most destructive pathogens to agricultural, forest, and natural ecosystems. The multiple-marker-based DNA array described in this study provides simple procedures to scan a range of suspected species in complex environmental samples without the requirement for isolation and culturing, adding to the toolbox to prevent and manage plant diseases caused by *Phytophthora* spp. Our results showed that combining three target regions into a single *Phytophthora* array can improve the capacity to rapidly and accurately detect or monitor multiple *Phytophthora* spp. in a wide range of environmental samples. With the discovery and description of more species and the addition of new DNA sequences to public databases, additional oligonucleotides could be designed and added to this array, so that the comprehensiveness of the current array would be maintained.

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