A Universal Microarray Detection Method for Identification of Multiple *Phytophthora* spp. Using Padlock Probes

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ABSTRACT

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The genus *Phytophthora* consists of many species that cause important diseases in ornamental, agronomic, and forest ecosystems worldwide. Molecular methods have been developed for detection and identification of one or several species of *Phytophthora* in single or multiplex reactions. In this article, we describe a padlock probe (PLP)-based multiplex method of detection and identification for many *Phytophthora* spp. simultaneously. A generic TaqMan polymerase chain reaction assay, which detects all known *Phytophthora* spp., is conducted first, followed by a species-specific PLP ligation. A 96-well-based microarray platform with colorimetric readout is used to detect and identify the different *Phytophthora* spp. PLPs are long oligonucleotides containing target complementary sequence regions at both their 5′ and 3′ ends which can be ligated on the target into a circular molecule. The ligation is point mutation specific; therefore, closely related sequences can be differentiated. This circular molecule can then be detected on a microarray. We developed 23 PLPs to economically important *Phytophthora* spp. based upon internal transcribed spacer-1 sequence differences between individual *Phytophthora* spp. Tests on genomic DNA of many *Phytophthora* isolates and DNA from environmental samples showed the specificity and utility of PLPs for *Phytophthora* diagnostics.

Phytophthora spp. are responsible for serious disease worldwide and can occur on a wide range of different crops (12). The genus *Phytophthora* currently encompasses more than 103 species (22) that are classified within the diploid, algae-like Oomycetes in the Stramenopile clade of the Chromista (14). The number of species is still increasing due to intensive monitoring in nurseries but also in forests and public greens, mainly due to the appearance of *Phytophthora ramorum* (sudden oak death). The most well-known species in the genus include *P. infestans*, *P. cinnamomi*, *P. sojae*, *P. capsici*, and *P. ramorum* and cause major losses on potato, soybean, pepper, and ornamental and forest trees. *P. infestans* was the first species in the genus described (10), caused tremendous devastation on potato in many parts of the world during the potato famine, and is a current threat to food security worldwide.

Some *Phytophthora* spp. have narrow host ranges, such as *P. fragariae* on strawberry or *P. sojae* on *Glycine max*, but others have wide host ranges, such as the root pathogen *P. cinnamomi* that can infect more than 3,000 host species. Early detection, accurate identification, traceability, and elimination are important aspects in the control of *Phytophthora* spp.

Traditionally, baiting has been used to detect *Phytophthora* spp. in soil, water, and plant material (11). Different materials can be used as baits, including rhododendron leaves or punches of leaves, pine needles, lupine seed, or whole plants (e.g., strawberry). Isolates are recovered from symptomatic areas of the baits onto (semi)selective media and, once obtained as a pure culture classical morphology using discrete structures and growth characteristics on specific media, are used as the main methods for identification of the *Phytophthora* spp. isolated (32,40,41). Identification based upon morphological characteristics is important but may take a considerable amount of time, and specific taxonomical expertise is required to correctly identify the isolate to species level.

Molecular techniques using DNA sequence analysis of different parts of the genome have been performed for identification and detection of *Phytophthora* spp. and many other plant pathogens (42). The most widely studied sequences are the introns of the ribosomal genes (internal transcribed spacer [ITS] regions) (42). Based upon these sequences, Cooke et al. (8) described 10 clades in the genus *Phytophthora*. Other genes were studied by Kroon et al. (23), Martin et al. (26), Blair et al. (4), and Schena et al. (30), and sequence analysis of several mitochondrial and nuclear loci have shown a similar relatedness between the different *Phytophthora* spp.

Based upon ITS and other gene sequences, many specific detection methods have been developed for *Phytophthora* spp. using the polymerase chain reaction (PCR) (1,5,18,28,37) or quantitative PCR (TaqMan) (3,15–17,20,21,31,36). Martin et al. (27) evaluated many of the conventional and real-time PCR based assays developed for *P. ramorum* and concluded that all the tests accurately identified the pathogen in field samples. However, all described methods detect only one single species, and unknown *Phytophthora* spp. are not detected with specific primers.

A universal method for detection of all *Phytophthora* spp. would be very useful for research and regulatory laboratories and could be tailored for downstream use in the field (31). Different fungal pathogens have been detected using PCR amplification with universal ribosomal primers coupled to hybridization on a microarray, on which species-specific probes are spotted in a multiplex detection assay (24,44). Limitations in these systems include cross-hybridization.

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To improve the currently available protocols for multiplex detection of various *Phytophthora* spp. in different environmental substrates, including soil, roots, water, and plant tissues (5,6,9, 24,25,35,43,44), we combined a generic TaqMan PCR, developed to detect all currently known *Phytophthora* spp. (21), with individual species-specific padlock probe (PLP) detection on a dedicated universal microarray.

PLPs are circularizing probes (Fig. 1) which combine specific molecular recognition and universal amplification, thereby increasing sensitivity and multiplexing capabilities without limiting the detection range of potential target organisms. PLPs are long oligonucleotides of approximately 100 bases, containing target complementary sequence regions (T1 and T2) at both their 5′ and 3′ ends (Fig. 1), which recognize adjacent sequences on the target DNA. Between the target complementary sequence regions, universal primer sites (P1 and P2) and a unique sequence identifier, the so-called ZipCode, are situated (Fig. 1). Upon hybridization, the 5′and 3′ end of the probes get into adjacent positions and can be joined by enzymatic ligation converting the probe into a circular molecule. This ligation and the formation of a circular molecule can only take place when both end segments recognize their target sequences correctly (point mutation specificity). Noncircularized probes are removed by exonuclease treatment whereas the circularized PLPs are amplified by PCR with the universal primers P1 and P2. This mechanism ensures reaction specificity even in complex DNA extracts with a large number of PLPs and targets. Subsequently, the target-specific products are detected by a universal cZipCode spotted on a microarray which identifies the detected *Phytophthora* spp.

Some PLPs for *Phytophthora* spp. have already been developed in our laboratory (e.g., *P. infestans, P. nicotianae*, *P. cactorum*, and *P. fragariae*) (34,38,39). A general "All *Phytophthora"* PLP, which detects and identifies many *Phytophthora* spp., was designed based upon ITS-1 sequence homology between all currently reported *Phytophthora* spp. (22). Sequence differences within the ITS-1 region were then used to develop additional species-specific PLPs for a range of *Phytophthora* spp. For this design, we used ITS-1 sequences for all known *Phytophthora* spp. currently available in different online databases. Identification is based upon ligation of a mixture of PLPs to generic amplified DNA isolated from pure cultures or a range of different substrates infected with one or more *Phytophthora* spp.

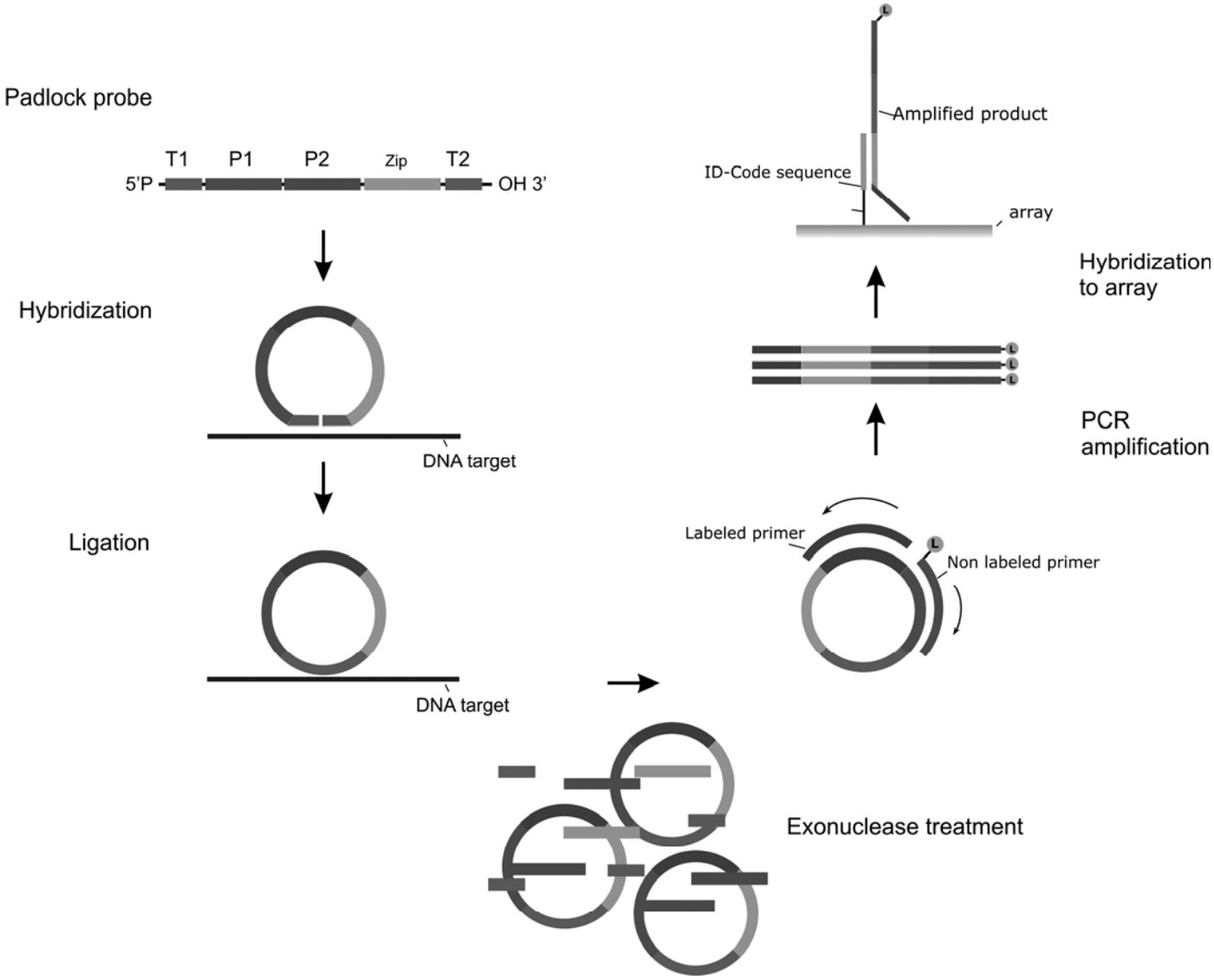

Fig. 1. Padlock probe (PLP) design and detection principle. The PLP is a long oligonucleotide of approximately 100 bases, containing five regions: target complementary sequence regions (T1 and T2) at both their 5′ and 3′ ends, universal primer sites (P1 and P2), and a unique sequence identifier, the so-called ZipCode. Upon hybridization, the 5′and 3′ end of the probes are ligated, converting the probe into a circular molecule. Noncircularized probes are removed by exonuclease treatment, while the circularized PLPs are amplified by polymerase chain reaction with the universal primers P1 and P2. Subsequently, the target-specific products are detected by a universal cZipCode microarray which identifies the detected *Phytophthora* spp.

The objectives of this research were to test the specificity and sensitivity of a PLP-based diagnostic method combined with a colorimetric microtiter plate array detection device using reference *Phytophthora* cultures as well as mixed infected material collected from field surveys, including airborne inoculum, roots, water, and plant tissue samples.

MATERIALS AND METHODS

Nucleic acids. *Phytophthora* and *Pythium* isolates were derived from the culture collection of the Central Bureau of Schimmelcultures (CBS), Plant Research International BV (PRI), or the Dutch Plant Protection Service (PPS) (Table 1). Isolates were

TABLE 1. Species of *Phytophthora* or *Pythium*, isolate number, padlock probe (PLP) name, and internal transcribed spacer (ITS)-1 sequences used to design the PLPs[a]

Species	Isolate[b]	PLP name[c]	Complete sequence (5′–3′)
Phytophthora alni	CBS 117376	PLP_1	AGGGCTACTGGCTCAGTTCCCCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATC GTAAGAGCATCGACGCGGATCATATTCAAGAATGGGTTTAAAAGATA
P. andina	EC 3425	PLP_2	AAAAAAGCTACTAGCTCAGACCGAAGTCCTCGACCGTTAGCAGCATGACCGAGATGTAC CGCTATCGTGACGCTCGTTAGTCGGCTATTCAGTATTAAGTAAAGGGTTTAAAAT
P. bisheria	PD 98/8/9022	PLP_3	AGCCGCACACCAAGCAAATCCCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATC GTGCGATGTCTCTACGTGTCTCGCCAATAAAGGCCAGCT
P. palmivora	CBS 533.92	PLP_4	CCCAACAGCAGCCGCCAGCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATCGTC ATCTAGCTCGTCGGTCAGATCGCCATGATAGAGCTCT
P. brassicae	CBS 112277	PLP_5	AAGGGTTGATACGGTTCACGTGGACTCGACCGTTAGCAGCATGACCGAGATGTACCGCT ATCGTGTGCCCAGTGGTTCGCATAACAGCAGCCCCCAATAATC
P. cactorum	CBS 294.29	PLP_6	GACTTTCGTCCCCACAGTATAATCAGTATTAAGGAATCTCGACCGTTAGCAGCATGACC GAGATGTACCGCTATCGTGTGATGCGTTCTGATACATGTATCTAGTTAAAAGCAAG
P. cambivora	CBS 114085	PLP_7	GCCCCCAACTAAGTGGGTTGATACCTCGACCGTTAGCAGCATGACCGAGATGTACCGCT ATCGTTAGGACTTGCGTCTCTGTGCAGCCGCCGGGACTA
P. capsici	CBS 111333	PLP_8	ACCCTATCATGGCGAATGTTTGGACTTCCTCGACCGTTAGCAGCATGACCGAGATGTAC CGCTATCGTCCTAGTGTGACGGCCTGATAAGTTGGGGGTCTTGT
P. cinnamomi	CBS 402.48	PLP_9	CAGAATGGGTTTAAAAGAGAGGCTACTAGCTCGACCGTTAGCAGCATGACCGAGATGT ACCGCTATCGTAGCGTGACTCGACATTAGCTCCCCACAGTATGTTCAGTATTA
P. citricola	CBS 11337	PLP_10	AGAATGGGTTTAAAACAAAAAGCTACTAGCTCGACCGTTAGCAGCATGACCGAGATGT ACCGCTATCGTATGCCGTAGCTGCGATAACGCCCACAGTATAATCAGTATTGT
P. citrophthora	CBS 111338	PLP_11	TAAATGGGTTTAAAACAAAAAGCTACTCGCTCGACCGTTAGCAGCATGACCGAGATGTA CCGCTATCGTGTCCCACATGGTGCTTCGTACACAGTATAATCAGTATTGT
P. cryptogea	CBS 307.62	PLP_12	TACGCTACTAGCCCAGGCCGACTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATC GTAGATTACGGACTGTGCTTTGAGTAATTAGGAATGGGTTTAAAAAA
P. fragariae	CBS 309.62	PLP_13	TGGGTTGATACGGTTCACGTGGACTCGACCGTTAGCAGCATGACCGAGATGTACCGCTA TCGTACATCCTGGACACGAGTGACACAGGCCCCCAACTAAG
P. hibernalis	CBS 119904	PLP_14	CTACTAGCTCAGACCCGAAGGCCCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTA TCGTAGCCGCAATGCACGACTGATTATTTAAGAAAGGGTTTAAAAGAAGA
P. humicola	CBS 114082	PLP_15	TCAGTAATTAAAGAAGGGGTTTAAAAGAACTCGACCGTTAGCAGCATGACCGAGATGT ACCGCTATCGTCTGTCCGATGCTCTGATCTACTTTCGTCCCCACAGTATT
P. infestans	PIC 99189	PLP_16	AGCCGCCAGCAATAAAGCCAGCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATC GTGCTCGGAATTACTGCGTGTTTCGTCGCCCAGCAGT
P. lateralis	CBS 102608	PLP_17	GCGCTCGCCATGATAGAGCCCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATCG TATGATCCATGAGCGCCGTGAAGACCCGAAGGCCCAT
P. megasperma	CBS 118733	PLP_18	GCCGACCCATTACAGGCCAGCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATCG TGACAGCGATGTGAGCACTCTGCCCAGCAGCAGCAC
P. multivesiculata	CBS 101593	PLP_19	CCAATAAAGGCCAGCAGCCGCCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATC GTCTGGTCAATGCTCGTCGCTTCACGCAGCCAGCCAA
P. nicotianae	CBS 304.29	PLP_20	AAGACCCCCAACTATTGGGTTGAAACCTCGACCGTTAGCAGCATGACCGAGATGTACCG CTATCGTGTGATTAGTTCGCTGTCCGCAGCCGCCGCCAAAT
P. ramorum	CBS 101553	PLP_21	CAAGCGCTCGCCATGATAGAGCCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTAT CGTATGACGCTGCATCGCTCAGTCTCAGACCCGAAGGCT
P. tentaculata	CBS 412.96	PLP_22	CACCGCCAAACAAGACCCCCCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATCG TGTGAGGTCTTCGTGCTATCCAGCAATAAAGCCAGCAGT
Phytophthora sp.		PLP_All_ Phytophthora	TATCTAGTTAAAAGCAGAGACTTTCGTCCTCGACCGTTAGCAGCATGACCGAGATGTAC CGCTATCGTGTCACGTATGGTTCGCTGCTCTGCTGAAAGTTGC
Ligation control		PLP_LC	AGCGCATAGACCACGTATCGACTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATC GTGTCTCGTCTCGTCTTCGTGAGTGCATCTCCAACTACGTCT
Ligation template	LT		TCCTCCGCTTATTGATATGCACCTAGCTTCGATACGTGGTCTATGCGCTAGACGTAGTTG GAGACCCTTGTAACGATCCGCCGCAGGTTCACCTACGGA
Pythium aphanidermatum	CBS 118.80		
P. intermedium	CBS 266.38		
P. myriotylum	CBS 114.77		
P. oedochilum	PD 96/9770		
P. paroecandrum	CBS 157.64		
P. sylvaticum	CBS 453.67		
P. ultimum	P9 99		
P. undulatum	CBS 157.69		
P. violae	CBS 178.86		

[a] The ITS-1 region was used to design the species-specific PLPs.

[b] Isolates were deposited into either the Central Bureau of Schimmelcultures (CBS), Plant Research International BV (PRI), or the Dutch Plant Protection Service (PPS) culture collections.

[c] PLP name refers to the number assigned to the PLP. PLPs are long oligonucleotides of approximately 100 bases, containing target complementary sequence regions (T1 and T2) at both their 5′ and 3′ ends which recognize adjacent sequences on the target DNA.

grown for 7 days in pea broth medium at 20°C before harvesting the mycelium. Prior to the DNA extraction, mycelium was freeze-dried and disrupted in a bead beating mill. Genomic DNA extractions were performed with the DNeasy 96 Plant Kit (Qiagen, Valencia, CA) and the Wizard Magnetic DNA Purification System for Food (Promega Corporation, Madison, WI) according to the manufacturers' instructions. DNA concentration was determined using gel electrophoresis and GelRed staining. Lamba DNA was used to calibrate DNA concentration.

Design of PLP. The ITS-1 sequences of multiple isolates of different *Phytophthora* spp. (Table 1) were aligned with MegAlign software (DNASTAR, Madison, WI) in order to identify polymorphic sites in the ITS-1 region of rDNA that could serve as a potential ligation sites for the PLPs. The ITS-1 sequences were obtained from the National Center for Biotechnology Information (NCBI) database as well as from the PRI database. Based on the alignment of at least 10 sequences per species, the consensus ITS-1 sequences of 88 *Phytophthora* spp. were aligned. Then, unique sites within the ITS-1 region were ascertained for each of the selected species of *Phytophthora*. Based upon these polymorphic sites, target complementary ligation arms T1 and T2 (Fig. 1) were designed according to previously described design criteria (34,38) using the software package ProbeMaker (33). Sequences of ligation arms were adjusted to obtain equal T_m values, checked for intraspecies variation, and blasted against the NCBI database for specificity in order to eliminate unspecific target recognition. Afterward, the sequences were connected with generic complementary primers sites P1 and P2 and a PLP-specific sequence (ZipCode) for array hybridization (Fig. 1). The length of the designed PLPs varied slightly because the target sequences had to meet the required T_m. Probes were tested with Visual OMP 7.5.1.0 (DNA Software Inc., Ann Arbor, MI) to exclude the formation of secondary structures and homo- and hetero-dimerization. A set of PLPs was selected for detection and identification of 22 *Phytophthora* spp. important in agriculture (Table 1) after in silico analysis and were synthesized by Integrated DNA Technologies (Coralville).

TaqMan PCR. TaqMan PCR amplification of the ITS-1 regions of selected Oomycetes isolates was performed with primer pairs FITS_15Ph and RITS_279Ph and an All *Phytophthora* probe (Table 2) as described by Kox et al. (21). Amplification of gDNAs was followed in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the TaKaRa kit Premix Ex Taq (Perfect Real Time; Takara Shuzo Co. Ltd., Kyoto, Japan). The 30-µl reaction mixture consisted of 15 µl of 2× Premix Ex Taq (Perfect Real Time), 0.6 µl of 50× ROX Reference Dye II, 83 nM TaqMan probe, 250 nM each primer, and 2 µl of gDNA (in a range between 100 ng/µl and 1 pg/µl).

Ligation and exonuclease treatment. TaqMan PCR-amplified products were used as templates for ligation. The ligation was performed in buffer as described by van Doorn et al. (38) with 2 U of *Pfu* ligase (Stratagene, La Jolla, CA), 10 pM PLP mix, 1 ng of Ligation Control phosphorylated PCR product, and 1 µl of the 10× diluted TaqMan PCR preamplified DNA (in a range of 4 to 10 ng). A mixture of 10 pM each PLP was prepared for multiplex detection. Reaction mixtures were prepared on ice and transferred rapidly into a thermal cycler set with the following parameters: 5 min at 95°C, 10 ligation cycles of 30 s at 95°C and 5 min at 65°C, followed by 15 min of inactivation at 95°C. Exonuclease mixture (10 µl of 67 mM glycine-KOH [pH 9.4], 2.5 mM $MgCl_2$, bovine serum albumin at 50 µg/ml, and 1 U of λ exonuclease) (New England BioLabs, Ipswich, MA) was added to each reaction after ligation to degrade unreacted PLPs and DNA, and the samples were incubated at 37°C for 10 min, followed by inactivation of the enzyme at 75°C for 10 min.

To monitor the ligation efficiency, a ligation control consisting of a phosphorylated PCR product and the corresponding PLP was added to the reaction. The PCR product was amplified from a 99-bp artificial ligation template LT (Table 1) using the LC-forw and LC-rev primer pair (Table 2). The corresponding PLP, PLP_LC (Table 1), was added to the 10 pM PLP mix at the same concentration.

SYBR Green PCR. Amplification of ligated PLPs was performed in the ABI Prism 7500 Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). A reaction of 25 µl contained 12.5 µl of optimized real-time buffer containing SYBR Green 1 Dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP and Passive Reference 1 ROX Dye, 300 nM each primers (P1-f19 5′-biotinylated and P2-r20) (Table 2), and 3 µl of exonuclease treated ligation mix. The reaction mixture was initially incubated at 50°C for 2 min, followed by 10 min of denaturation at 95°C and 20 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence was recorded in the extension phase of each cycle.

Microarray preparation. Epoxy-coated microarray plates containing 4-by-24 subarrays with epoxy surface modification (Greiner Bio-One GmbH, Frickenhausen, Germany) were spotted by PolyAn GmbH (Berlin) with 5′-amino-C12-linker DNA cZipCode oligonucleotides (IDT) complementary to a set of unique sequences included in each PLP (Fig. 2). Oligonucleotides, diluted to 10 µM in 150 mM phosphate-buffered saline (PBS) spotting buffer, pH 8.1, were spotted in a specific pattern (Fig. 2) using a QArray 2 microarrayer (Genetix GmbH, München-Dornach, Germany) with a split pin needle. Every subarray was spotted with 11 CH_2 immobilization controls, four control oligonucleotide cZipCodes (C), and PLP-complementary cZipCodes spotted seven times. Microarrays were spotted at 65% humidity, and then the cZipCodes were left on the surface to immobilize for 3 days. Nonreacted epoxy groups were blocked with 25 mM ethanolamine in 100 mM sodium borate buffer (pH 8.5) for 2 h and finally incubated in 100 mM NaOH for 10 min.

Microarray hybridization. The arrays were washed with water and incubated for 2 min at 50°C with 1× TMAC buffer (4.5 M tetramethylammonium chloride, 0.15% N-lauroylsarcosine, 75 mM Tris-HCl [pH 8.0], and 6 mM EDTA) in a humidity chamber prior to hybridization. The hybridization mixtures contained a 5× dilution of the PCR-amplified PLPs in 1× TMAC buffer and a biotinylated 10 µM hybridization control oligo-

TABLE 2. Primer or probe name, sequence, and modifications to the primer or probes used in the design of the padlock probes (PLPs) used in this study

Primer or Probe Name	Sequence (5′–3′)	Modifications
FITS_15Ph[a]	TGCGGAAGGATCATTACCACACC	5′-Phosphorylated
RITS_279Ph[a]	GCGAGCCTAGACATCCACTG	5′-Phosphorylated
All_Phytophthora TaqMan probe	TTGCTATCTAGTTAAAAGCA	5′-FAM/NFQ-MGB
LC-forw[b]	TCCGTAGGTGAACCTGCGG	3′-Phosphorylated
LC-rev[b]	TCCTCCGCTTATTGATATGC	None
P1-f19[c]	CGAGATGTACCGCTATCGT	5′-Biotinylated
P2-r20[c]	TCATGCTGCTAACGGTCGAG	5′-Phosphorylated

[a] Primers used in the forward and reverse direction with the All TaqMan probe to amplify the internal transcribed spacer (ITS)-1 region from *Phytophthora* using real time polymerase chain reaction (PCR).
[b] Primers used in the forward and reverse direction to amplify a ligation control consisting of a phosphorylated PCR product and the corresponding PLP.
[c] Primers used for amplification of the ligated PLPs.

nucleotide (C) as corner spot. The mixtures were heated for 5 min at 95°C and rapidly cooled on ice. After adding 50 µl of the hybridization mixture to each subarray, the plate was sealed and incubated at 50°C for 2 h in a humidity chamber on a shaker. The microarray was subsequently washed three times, once with prewarmed 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) per 0.1% sodium dodecyl sulfate for 2 min at 50°C, followed by 2× SSC for 2 min and, finally, with 0.2× SSC for 2 min; the latter two solutions were at room temperature (RT). Blocking of unspecific binding sites was done by a 15-min incubation in blocking buffer (6× saline-sodium phosphate-EDTA buffer [SSPE], 2% powdered milk, and 0.05% Triton X-100) at RT. In the following step, a 1:5000 dilution (6× SSPE and 0.01% Triton X-100) of Pierce High Sensitivity Streptavidine-conjugated Horseradish Peroxidase at 1 mg/ml (Thermo Scientific, Waltham, MA) was added to each subarray and incubated at RT for 1 h. After the incubation, the microarray was washed twice with 1× PBS buffer at RT for 1 min and once with 22 mM citric buffer (pH 4.3) at RT for 30 s. Staining of the microarray was done with SeramunGrün Spot o-dianisidine substrate solution ready to use (Seramun Diagnostica GmbH, HeideSee-Wolzig, Germany) for at least 1 min.

Analysis of microarray data. Microarrays were analyzed using a colorimetric scanner TLR500 (MicroDiscovery, Berlin) with StrixAluco image acquisition and analysis software (version 2.3; Strix Diagnostics, Berlin). The detection was carried out using a highly sensitive interline CCD-based transmission detector with a resolution of 10 µm/pixel and dynamic range of 12+ bit. A halogen light source with a continuous spectrum was used in the device. The measurements were obtained at a wavelength of 635 nm. The x-y axis system was used with a resolution of 25 µm/step and flexible detection area definition. The median value of the pixels in the spots area was measured for each of the samples. The background signal was determined from the corners outside the spots while the signal from the edge of the spots was ignored in the evaluation. After the measurement signals were averaged for each target, they were presented in a graph.

Specificity. To determine the specificity of the developed method, the 23 PLPs were first ligated in simplex with target and closest non-target *Phytophthora* spp. Then, individual *Phytophthora* spp. were tested with a mixture of all PLPs. After TaqMan PCR, PLP ligation, exonuclease treatment, SYBR Green PCR, and microarray hybridization, each individual *Phytophthora* sp. gave a "target signature" on the microarray based on the ITS-1 sequence of the species involved.

Sensitivity and dynamic range. In order to evaluate the assay sensitivity, 10-fold dilution series of genomic DNA of eight different *Phytophthora* spp. (*P. cactorum, P. capsici, P. cinnamomi, P. citricola, P. infestans, P. megasperma, P. ramorum*, and *P. tentaculata*) were preamplified in the TaqMan PCR and subsequently ligated with the 23 developed PLPs. After exonuclease treatment and SYBR Green PCR, samples were analyzed on the microarray and the signatures of the samples were determined. To study the dynamic range of the assay, 4×10^6 copies of *P. cinnamomi* amplicon and 4×10^{10} copies of *P. andina* amplicon were mixed and tested with the mixture of PLPs. After ligation, exonuclease treatment, and SYBR Green PCR, samples were analyzed on the microarray and the signatures of the samples were determined.

Assay validation. The developed method was evaluated with DNA samples from different environmental studies collected during previous *Phytophthora* spp.-monitoring programs (e.g., for *P. ramorum* monitoring) supplied by the Dutch PPS and by PRI. Environmental samples consisted of symptomatic leaves, leaves from bait tests, water samples, and air samples on cellotape. DNA

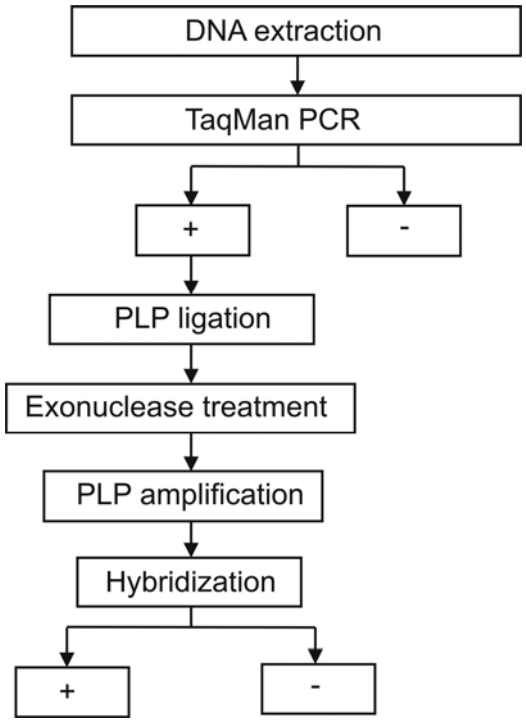

Fig. 3. Flow chart of the assay. All samples are first run through a real-time TaqMan polymerase chain reaction (PCR) for *Phytophthora* spp. Then, positive samples were ligated with a mixture of padlock probes (PLPs) followed by treatment with exonuclease to degrade unreacted PLPs. After SYBR Green PCR amplification of ligated PLPs, amplicons were hybridized on the microarray and visualized.

C	IC	11	11	11	11	11		4	4	4	4	4	IC	C
IC	22	22	22	22	22	11		4	2	2	2	2	2	IC
16	16	16	16	16	22	11		4	2	10	10	10	10	10
19	19	19	19	16	16	22		2	10	10	3	3	3	3
15	15	15	15	19	19	19		3	3	3	18	18	18	18
14	14	14	14	15	15	15		18	18	18	17	17	17	17
21	21	21	21	14	14	14		17	17	17				
IC	5	5	5	21	21	21	IC							IC
5	5	5	5	9	9	9	8							
9	9	9	9				8							
				1	1	1	8	7	7	7				
1	1	1	1	13	13	20	8		12	12	7	7	7	7
13	13	13	13	13	20	23	8	6		12	12	12	12	12
IC	20	20	20	20	20	23	8	6						IC
C	IC	23	23	23	23	23	8	6	6	6	6	6	IC	C

C	Corner Spot
1	PLP_1
2	PLP_2
3	PLP_3
4	PLP_4
5	PLP_5
6	PLP_6
7	PLP_7
8	PLP_8
9	PLP_9
10	PLP_10
11	PLP_11
12	PLP_12

13	PLP_13
14	PLP_14
15	PLP_15
16	PLP_16
17	PLP_17
18	PLP_18
19	PLP_19
20	PLP_20
21	PLP_21
22	PLP_22
23	PLP_All_Phyt_2004
IC	CH2 (Immob control)

Fig. 2. Configuration of the microarray. cZipCode sequences were spotted seven times on the array. Corner spots (C) and immobilization control spots (IC) were spotted for correct orientation and as controls.

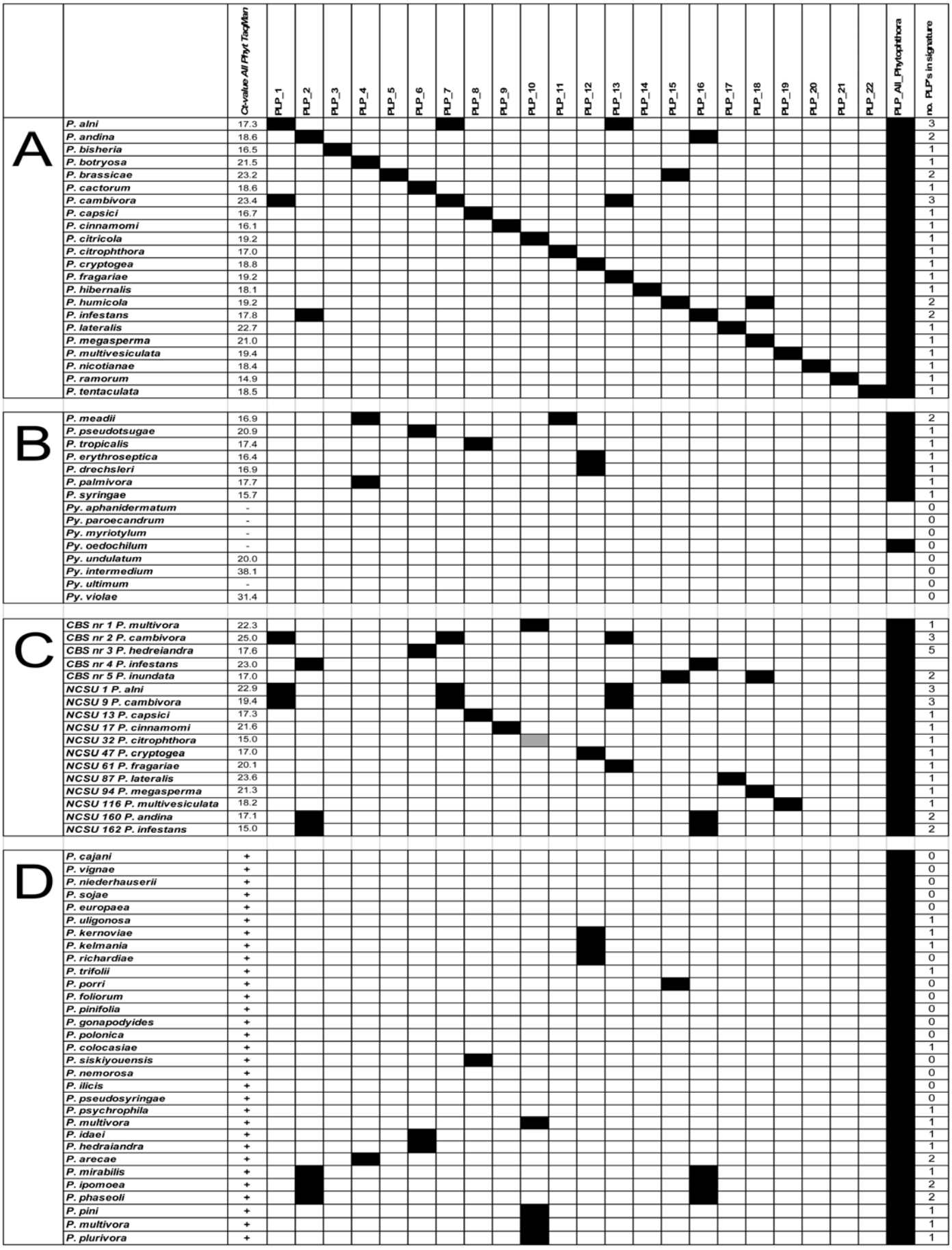

Fig. 4. Specificity of 23 *Phytophthora* padlock probes (PLPs) for different *Phytophthora* spp. Black = correct signature and gray = incorrect signature. **A,** *Phytophthora* spp. for which PLPs were developed; **B,** other *Phytophthora* spp. for which no PLPs were developed and nine *Pythium* spp.; **C,** samples of pure cultures from the Central Bureau of Schimmelcultures or North Carolina State University which were assayed blindly; **D,** predicted signatures for other *Phytophthora* spp. that were not extensively validated in this work.

was isolated as previously described (21). Samples were assayed by isolation on selective PARP culture media (12), use of a *P. ramorum* specific real-time PCR (15,16), or via the All Phytophthora TaqMan PCR (21) and PLPs (this study).

RESULTS

Specificity. The detection system (Fig. 3) was experimentally tested for specificity using the PLPs designed to detect several *Phytophthora* spp. (Table 1). Genomic DNA samples of *Phytophthora* and *Pythium* spp. were first preamplified in TaqMan PCR with general *Phytophthora* spp. primers (Table 2); then, positive DNA samples were subsequently ligated with the developed mixture of PLPs. Circularized PLPs, specifically ligated to the TaqMan PCR amplicons, were amplified in SYBR Green PCR (Table 2) and subsequently hybridized to the microarray.

Based on the ITS-1 sequence alignment, individual *Phytophthora* spp. tested with the mixture of the developed PLPs gave a "target signature" for each *Phytophthora* sp. analyzed on the microarray (Fig. 4). *P. ramorum* could easily be distinguished from the closest non-target *Phytophthora* (*P. lateralis*) despite the ITS-1 sequence similarities between the two species (*P. ramorum*: GCTCTATCATGGCGAGCGCTTGAGCCTTCGGGTCTGAG and *P. lateralis*: GCTCTATCATGGCGAGCGCATGGGCCTTC GGGTCT). Differences in the ligation sites (shown in dark gray) allowed specific PLP ligation of both *P. ramorum* and *P. lateralis*, respectively (Fig. 5). The PLP_All_Phytophthora reacted with DNA from both species.

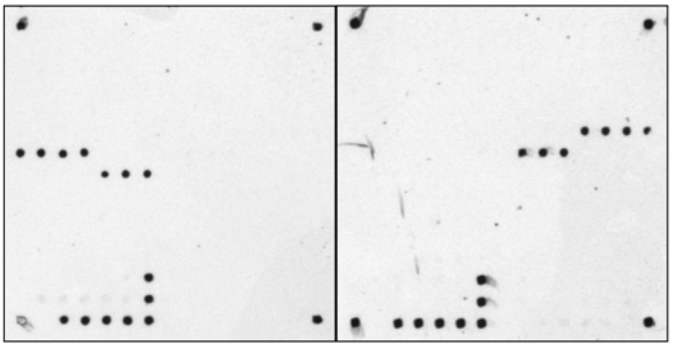

Fig. 5. Hybridized microarray displaying the target signatures for *Phytophthora ramorum* (left) and *P. lateralis* (right).

Most of the *Phytophthora* spp. displayed a unique target signature, indicating the specificity of the method (Fig. 4A). It was not possible to obtain a simple target signature for several *Phytophthora* spp., because some of these species share the same ITS-1 sequence that is recognized by the designed PLP. The signatures for *P. andina* and *P. infestans* (PLP_2 and PLP_16) were the same because both species have identical ITS-1 sequences.

A more complex signature was obtained for the hybrid species *P. alni*. This species is a putative hybrid of *P. cambivora* and a *P. fragariae*-like *Phytophthora* sp. *P. alni* gave a signal derived from PLP_1 (*P. alni*) together with positive signals for both PLP_7 (*P. cambivora*) and PLP_13 (*P. fragariae*). Further analysis of more *P. alni* isolates revealed that two isolates (CBS 117377 and CBS 117376) reacted with the same three PLPs (PLP_1, PLP_7, and PLP_13) and one *P. alni* isolate (PD 92/1471) reacted with two of the three probes (PLP_1 and PLP_13). Sequence and trace-file analysis of the ITS-1 sequence confirmed the obtained difference in signatures.

Other *Phytophthora* spp., for which no PLPs were designed, were tested and showed signatures according their ITS-1 sequence (Fig. 4B). Because of the high ITS-1 sequence similarity within the Oomycetes, several *Pythium* spp. were also amplified in the All Phytophthora TaqMan PCR. Hence, a set of *Pythium* spp. (Table 1) was tested in the assay as well (Fig. 4B). A clear, positive TaqMan PCR signal was observed only for *Pythium undulatum*, while no PLP signal was detected for this species. *P. oedichulum* reacted only with the PLP_All_Phytophthora, although the TaqMan PCR was not positive (Fig. 4B).

DNA samples from pure cultures were tested anonymously from CBS and North Carolina State University (NCSU) (Fig. 4C).

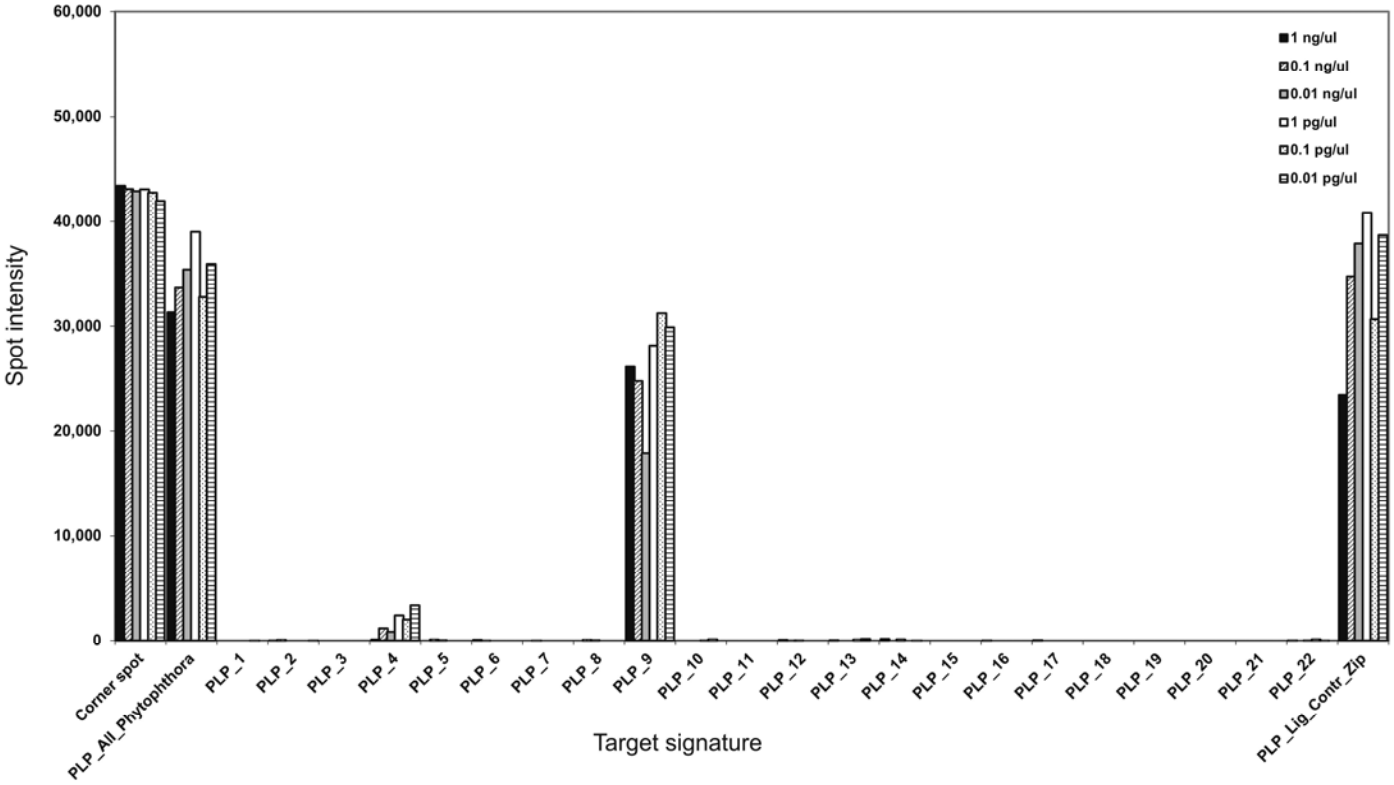

Fig. 6. Threshold of sensitivity of *Phytophthora cinnamomi* (PLP_9) gDNA. Tenfold serial dilutions (1 ng/µl to 0.01 pg/µl) of gDNA were detected.

Sample 1 from CBS was *Phytophthora multivora* and reacted with PLP_10 (*P. citricola*), because there was no sequence difference between those two species at the ligation site for PLP_10. CBS sample 2 was correctly identified as *P. cambivora*. CBS sample 3 (*P. hedreiandra*) reacted with the same PLP as *P. cactorum* and has an identical sequence for the ligation site of PLP_6. CBS sample 4 (*P. infestans*) reacted as previously with PLP_2 (*P. andina*) and PLP_16 (*P. infestans*). CBS sample 5 (*P. inundata*) showed the same signature as for *P. humicola*, which could be explained by the sequence similarity on both PLPs target sites (PLP_15 and PLP_18).

Results from the anonymous DNA extracts obtained from NCSU are also shown in Figure 4C. All the DNA samples from NCSU (NCSU 1, 9, 13, 17, 32, 47, 61, 87, 94, 116, 160, and 162) were correctly identified and contained one *Phytophthora* sp. (single signature or the double signature for *P. infestans* and *P. andina* or triple signature for *P. alni* and *P. cambivora*), as previously described. Sample NCSU 32 reacted with PLP_10 (for *P. citricola*) and was misidentified as *P. citrophthora*. Sequence analysis of the ITS region confirmed that this isolate was, in fact, *P. citricola,* and was mislabeled.

The predicted signatures of DNA from other *Phytophthora* spp. are shown in Figure 4D. ITS-1 sequences of these species were retrieved from databases and some were identical with sequences of species already analyzed, resulting in the same signature on the microarray. Other species only reacted with the PLP_All_Phytophthora.

Sensitivity and dynamic range. A dilution series (1 ng/µl to 0.01 pg/µl) of DNA from eight *Phytophthora* spp. was tested in order to determine the sensitivity of the assay. All species showed the correct "signature" and the limit of detection for the different *Phytophthora* spp. was 0.01 pg of gDNA (Fig. 6). In some diagnostic applications, more than one individual *Phytophthora* sp. can be expected in different concentrations of the individual species. Therefore, mixtures of DNA from several *Phytophthora* spp. were prepared in order to resemble potential environmental samples. As an example, 4×10^6 copies of *P. cinnamomi* amplicon and 4×10^{10} copies of *P. andina* amplicon were mixed. Signatures of both targets could clearly be detected (data not shown). The maximum dynamic range of the assay was four log scales (4×10^6 versus 4×10^{10} copies).

Assay validation. Environmental samples obtained by the PPS (Wageningen, The Netherlands) in their survey for *P. ramorum* (Table 3) were used to test the PLP assay selectivity (Tables 3 and 4). Results obtained from pure reference cultures and the standard *P. ramorum* TaqMan PCR assay were compared with the PLP ligation assay. In most cases, the presence of *P. ramorum* in the sample was confirmed by both TaqMan PCR and PLPs. More than one *Phytophthora* sp. (*P. ramorum* and *P. citricola*) was detected and identified in three samples (PPS 9, 11, and 14) (Table 3). Sequence analysis of the TaqMan PCR amplicons confirmed a mixed sequence of both *Phytophthora* spp. The PPS conducts analysis of quarantine species and, after isolation in culture, a *P. ramorum*-specific TaqMan PCR is run; therefore, only *P. ramorum* was detected from these samples (Table 3). Both *P. citricola* and *P. megasperma* were detected in sample PPS 16 using the PLP assay. The presence of *P. cinnamomi* was detected in sample PPS 18 with the PLP assay where, previously, no *Phytophthora* sp.

TABLE 3. Results obtained with environmental samples from the Dutch Plant Protection Service (PPS)[a]

				Results PPS			Results PRI				
Sample	Number	Host	Origin	Culture[b]	Ct All[c]	Ct *Pr*[d]	Ct All[c]	Detected *Phytophthora* spp. (PLP name)[e]			
PPS 1	3950618	Rhododendron	Netherlands	*Phytophthora ramorum*	NT	19.8	19.9	All Phyt *P. ramorum* (-21)			
PPS 2	4452397	Fagus	Netherlands	*P. ramorum*	28.7	28.2	29.0	All Phyt *P. ramorum* (-21)			
PPS 3	4170357-1	Rhododendron	Netherlands	*P. ramorum*	NT	25.7	26.7	All Phyt *P. ramorum* (-21)			
PPS 4	4170357-2	Rhododendron	Netherlands	*P. ramorum*	NT	19.9	20.9	All Phyt *P. ramorum* (-21)			
PPS 5	4373808	Fagus	Netherlands	*P. ramorum*	31.9	31.3	31.3	All Phyt *P. ramorum* (-21)			
PPS 6	4373795	Fagus	Netherlands	*P. ramorum*	27.3	26.0	27.7	All Phyt *P. ramorum* (-21)			
PPS 7	4373787	Fagus	Netherlands	*P. ramorum*	23.2	24.1	23.3	All Phyt *P. ramorum* (-21)			
PPS 8	4612012	*Quercus rubra*	Netherlands	*P. ramorum*	34.5	35.1	35.2	All Phyt *P. ramorum* (-21)			
PPS 9[f]	4757174	Rhododendron	Netherlands	*P. ramorum*	NT	27.4	21.9	All Phyt *P. ramorum* (-21)	*P. citricola* (-10)		
PPS 10	4442471-B	Rhododendron	Netherlands	*P. ramorum*	23.4	22.6	24.1	All Phyt *P. ramorum* (-21)			
PPS 11[f]	4167203	Rhododendron	Germany	*P. ramorum*+ *Phytophthora* sp.	NT	21.0	21.8	All Phyt *P. ramorum* (-21)	*P. citricola* (-10)		
PPS 12	4578715	Rhododendron	Netherlands	*P. ramorum*	25.4	21.8	25.0	All Phyt *P. ramorum* (-21)			
PPS 13	4178690	Rhododendron	Belgium	*P. ramorum*	29.8	25.8	29.2	All Phyt *P. ramorum* (-21)			
PPS 14[f]	4917932	Unknown	Netherlands	No *Phytophthora*/ *P. ramorum**	NT	28.8	26.7	All Phyt *P. ramorum* (-21)	*P. citricola* (-10)		
PPS 15	4917967	Unknown	Netherlands	*P. ramorum*	NT	22.1	25.9	All Phyt *P. ramorum* (-21)			
PPS 16[f]	3598474	Rhododendron	Bulgaria	*P. citricola*	24.7		26.0	All Phyt *P. citricola* (-10)	*P. megasperma* (-18)		
PPS 17	4707371	Pachysandra	Netherlands	*P. citrophthora*	24.3	NT	25.1	All Phyt *P. citrophthora* (-11)			
PPS 18	4708663	*Vaccinium vits-ideae* L.	Netherlands	No *Phytophthora*	31.8		32.0	All Phyt *P. cinnamomi* (-9)			
PPS 19[f]	4482166	Rhododendron	Netherlands	No *Phytophthora*	33.6		31.5	All Phyt *P. cinnamomi* (-9)	*P. fragariae* (-13)	*P. cambivora* (-7)	*P. palmivora* (-4)
PPS 20	4707881	*Chamaecyparis lawsoniana*	Netherlands	*P. gonapodyides*	27.7		26.5	All Phyt *P. cryptogea* (-12)			

[a] Samples were obtained from different host plants and identified by (PPS) and the Plant Research International (PRI). Cultures were isolated using baiting on environmental samples and identified by morphological characters. The All *Phytophthora* spp. TaqMan PCR (21), the *P. ramorum* TaqMan PCR (15,16), padlock probes (PLPs), or sequence analysis of obtained amplicons were performed on DNA extracts from the environmental samples. NT = not tested; * indicates that, based on molecular results, the sample was cultured a second time.

[b] Culture or morphology.

[c] Cycle threshold (Ct) value, All *Phytophthora* TaqMan.

[d] Ct value, *P. ramorum* TaqMan.

[e] PLP names are indicated in Table 1.

[f] Multiple species were detected by PLPs in some samples (PPS 9,11,14,16, and 19) where either no *Phytophthora* spp. were previously detected or only a single species (*P. ramorum*) was identified.

could be isolated. Sample PPS 19 gave a complex PLP signature, indicating several *Phytophthora* spp., while both PPS and PRI obtained a high cycle threshold value using TaqMan PCR. Sample PPS 20 showed a clear signal for PLP_12, suggesting the presence of *P. cryptogea*, whereas this sample was previously identified as *P. gonapodyides* by PPS after baiting and culturing. Sequence analysis of the TaqMan PCR amplicon confirmed the presence of *P. cryptogea* in this sample.

DNA extracts obtained from samples collected during PRI experiments on *P. infestans, P. cactorum, P. cryptogea*, and *P. ramorum* and previously identified with species-specific TaqMan probes or ITS-1 sequence analysis of the PCR amplicon were tested in the PLP detection system (Table 4). The microarray results confirmed the previous TaqMan PCR and sequencing identification. Additionally, in samples PRI 2, 23, and 24, *Phytophthora* isolates were identified to species level (*P. cryptogea, P. infestans*, and *P. cactorum*) where, previously, the species were unknown. Samples PRI 5 and 7 showed no or low signals in TaqMan PCR but gave a signal with the PLP_All_Phytophthora, indicating the presence of a *Phytophthora* sp., even though no other PLP reacted. In samples PRI 1 and 4, *Phytophthora* spp. could not be detected although, in a previous analysis, *P. cambivora* was observed. Other samples (PRI 9, 10, 12, 13, and 19) from the *P. ramorum* survey confirmed the presence of *P. ramorum*.

DISCUSSION

In this article, we present a rapid colorimetric multiplex detection and identification method for *Phytophthora* spp. The method utilizes the advantages of a generic *Phytophthora* TaqMan PCR followed by the high specificity of PLPs in a multiplex detection on a microarray. A broad range of different *Phytophthora* spp. may be found in natural ecosystems, nurseries, and greenhouses, causing damage to many plant species. In the past, many different TaqMan PCR protocols have been developed that detect only a single *Phytophthora* sp. Multiplex TaqMan PCR, in which multiple *Phytophthora* spp. can be detected, has severe limitations due to the limited amount of fluorophores which can be combined. In monitoring field samples, one can expect different *Phytophthora* spp. in the same sample, and the ability to detect multiple *Phytophthora* spp. in one single test would be welcomed by diagnosticians, regulatory agencies, and inspection services.

We developed a PLP-based identification test upon ITS-1 sequences of 88 *Phytophthora* spp. Care was taken to examine intraspecies variation of the ligation sites and, therefore, ITS-1 sequences of at least 10 isolates of a given species were used. The consensus sequence of those species was used in designing the PLPs. For 22 *Phytophthora* spp., different PLPs and one generic *Phytophthora* PLP were designed and tested. In a previous study, we observed disadvantages of PLP ligation directly to genomic DNA extracts, which can lead to nonspecific background signals (34). Therefore, a generic All *Phytophthora* TaqMan PCR assay was used to preamplify *Phytophthora* sp. DNA in extracts prior to the PLP ligation assay.

The specificity of the assay is introduced by a ligation reaction of the PLPs. In silico tests as well as ligation reactions with selected target and non-target DNA samples, derived from *Phytophthora* cultures (Table 1), gave correct target signatures. Furthermore, ligation reactions performed in the presence of nontarget organisms with very similar ligation target regions did not result in a detectable signal, demonstrating the specificity of the designed PLP probes. Because the test used point-mutation-specific ligation of PLPs, specific detection of very closely related species such as *P. ramorum* and *P. lateralis* is possible. Other multiplex detection systems, using microarray hybridization, do not use point mutation specificity to separate species (24,44).

Nine PLPs were species specific for *Phytophthora* spp., including *P. bisheria* (PLP_3), *P. brassicae* (PLP_5), *P. cinnamomi* (PLP_9), *P. hibernalis* (PLP_14), *P. lateralis* (PLP_17), *P. multivesiculata* (PLP_19), *P. nicotianae* (PLP_20), *P. ramorum* (PLP_21), and *P. tentaculata* (PLP_22). Several *Phytophthora* spp. share identical ITS-1 sequences (e.g., *P. infestans*, *P. mirabilis*, *P. phaseoli*, *P. ipomoeae*, and *P. andina*). The PLP for *P.*

TABLE 4. Results obtained with environmental samples from Plant Research International (PRI)[a]

Name	Substrate[b]	Previously tested with[c]	Previous identification	Ct PCR[d]	Detected *Phytophthora* spp. (PLP name)[e]			
PRI 1	Leaf bait	All Phyt	*Phytophthora cambivora*	ND	No	…	…	…
PRI 2	Recirculation	All Phyt	*Phytophthora* sp.	29.5	All Phyt	*P. cryptogea* (-12)	…	…
PRI 3	Cellotape	*P. infestans*	*P. infestans*	31.2	All Phyt	*P. infestans* (-2,-16)	*P. andina* (-2,-16)	…
PRI 4	Leaf bait	All Phyt	*P. cambivora*	ND	No	…	…	…
PRI 5	Leaf bait	All Phyt	*P. citrophthora*	ND	All Phyt	…	…	…
PRI 6	Cellotape	*P. infestans*	*P. infestans*	30.4	All Phyt	*P. infestans* (-2,-16)	*P. andina* (-2,-16)	…
PRI 7	Leaf bait	All Phyt	*P. citrophthora*	34.7	All Phyt	…	…	…
PRI 9	Water sample from contaminated nursery	*P. ramorum*	*P. ramorum*	29.2	All Phyt	*P. ramorum* (-21)	…	…
PRI 10	Water sample from contaminated nursery	*P. ramorum*	*P. ramorum*	26.1	All Phyt	*P. infestans* (-2,-16)	*P. andina* (-2,-16)	*P. ramorum* (-21)
PRI 12	Lesion Rhododendron leaf greenhouse exp	*P. ramorum*	*P. ramorum*	31.0	All Phyt	*P. ramorum* (-21)	…	…
PRI 13	Water sample from contaminated nursery	*P. ramorum*	*P. ramorum*	29.4	All Phyt	*P. ramorum* (-21)	…	…
PRI 17	Leaf bait	All Phyt	*P. cambivora*	20.0	All Phyt	*P. cambivora* (-7)	*P. fragariae* (-13)	*P. alni* (-1)
PRI 19	Water sample from contaminated nursery	*P. ramorum*	*P. ramorum*	34.5	All Phyt	*P. ramorum* (-21)	…	…
PRI 23	Leaf bait	All Phyt	*Phytophthora* sp.	30.2	All Phyt	*P. infestans* (-2,-16)	*P. andina* (-2,-16)	…
PRI 24	Water from bait	All Phyt	*Phytophthora* sp.	26.7	All Phyt	*P. cactorum* (-6)	…	…

[a] Previous identification was performed by PRI using *Phytophthora* spp. TaqMan polymerase chain reaction (PCR) (21) on DNA extracts from the environmental samples, and sequence analysis of the obtained amplicon.

[b] Environmental samples were collected from floating Rhododendron leaf baits (Leaf bait), recirculation water from a (non-)contaminated nursery (Recirculation), water sample from a *P. cactorum* bait test (Water from bait), and sporangia collected in Burkardt spore traps on cellotape (Cellotape).

[c] All Phyt = All *Phytophthora* TaqMan, *P. infestans* = *P. infestans* PCR, and *P. ramorum* = *P. ramorum* TaqMan PCR done following methods previously reported (15,16).

[d] Cycle threshold (Ct) value, All *Phytophthora* TaqMan PCR; ND = not done.

[e] PLP names are indicated in Table 1.

infestans (PLP_16) and the PLP for *P. andina* (PLP_2) did not discriminate between these species. PLP_12 reacted with *P. cryptogea* but also with *P. erythroseptica* and *P. drechsleri*, and this PLP is predicted to react also with *P. kernoviae*, *P. kelmania*, and *P. richardiae*. Therefore, the use of this probe is not specific and care should be taken to use it in nurseries and for quarantine purposes. Perhaps combining PLPs using both ITS and *Cox-1* genes would allow further discrimination of these species but further work is needed to confirm this.

Our results indicate that some species complexes of *Phytophthora* should be analyzed in more detail (e.g., *P. megasperma, P. cryptogea*, and *P. citricola*). The *P. citricola* complex was recently split into several new species, including *P. pini, P. multivora*, and *P. plurivora* (13,19). The designed PLP probe for *P. citricola* (PLP_10) will also react with all these new species. Some *Phytophthora* spp. are recognized by the same PLPs and gave an identical signature; therefore, more genes need to be used for species specificity. Recently, *Cox-1* has proven to be useful for *Phytophthora* diagnostics and can be used to discriminate between closely related *Phytophthora* spp. (29).

High similarities in the ITS-1 sequence within the Oomycetes may also create problems in specific detection of *Phytophthora* spp. at the first step of the protocol. As shown in our results, some *Pythium* spp. were detected in the TaqMan PCR, due to the similarity of the sequence recognized by the TaqMan probe or primers. In a recent report, the new genus *Phytopythium* has been described (2). Organisms belonging to this particular genus originate from the clade K of *Pythium* genus, and are morphologically and phylogenetically situated between *Pythium* and *Phytophthora*. Only *Pythium oedochilum* was tested from this new genus and scored negative in the TaqMan PCR but gave a signal with the PLP_All_Phytophthora. Of the *Pythium* spp. tested, only *P. undulatum* showed a clear, positive TaqMan PCR result but this species didn't react with any of the PLPs developed.

In environmental samples, target organisms may appear in a wide concentration range, often making detection of less abundant organisms problematic. We demonstrated that the PLP system developed could detect and identify as little as 0.01 pg of target gDNA, which is less than the genomic DNA content of a single zoospore. Moreover, detection and identification of several organisms appearing in diverse ratios (0.01% relative abundance) was possible, making the system useful for pathogen screening. The applicability of the system was demonstrated by analyzing samples acquired from environmental studies by PPS and PRI. These samples had previously been scored positive for *Phytophthora* spp. TaqMan PCR and microarray data confirmed the earlier identification of the *Phytophthora* spp. involved. Results with the PLPs were obtained to identify *Phytophthora* to the species level, making the identification more reliable than previously used methods. In several PPS samples, more than one *Phytophthora* spp. was detected and identified based on the unique target signature obtained. The *Phytophthora* sp. identity was confirmed by sequencing the TaqMan PCR amplicons. Experiments with *Phytophthora alni* isolates also showed the usefulness of the system. *P. alni* isolates, which are reported to be *Phytophthora* hybrids (7), reacted with multiple PLPs depending on the sequence present in the ITS-1 region.

The system developed uses detection of the PLPs on low-density, epoxy-coated plastic microarray plates spotted with universal ZipCode oligonucleotides. Because the oligonucleotide detector probes are not target specific, it makes the system flexible and open for new tasks. Using additional PLPs with other ZipCodes, we can easily expand the system for more *Phytophthora* spp. or for additional genes that may be useful for identification. We can also develop this system for other (groups) of pathogens using the universal ZipCode microarray. A generic fungal, bacterial, or insect amplification system based upon ITS, 16S, or Cox-1 primers is already available and can be used with the same microarray system. Therefore, the same ZipCodes can be incorporated into fungal, bacterial, or insect species-specific PLPs. Application of the colorimetric detection makes the method cheap and simple, because the detection can be done with a low-density CCD detector or even with a binocular microscope or digital camera for prescreening.

We have shown that the PLP method developed can be used in *Phytophthora* diagnostics. Nine *Phytophthora* spp. could be discriminated to the species-specific level, while other species were detected in groups. With the ZipCode-based PLP detection procedure, the discrimination between the different *Phytophthora* spp. can be easily expanded using additional more informative genetic markers, such as the *Cox-1* gene. The system developed will be a useful tool for plant diagnosticians and researchers interested in the specific identification of *Phytophthora* spp.

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