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Dimeric oligonucleotide probes enhance diagnostic macroarray performance

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ABSTRACT

Disease management can be improved with rapid and accurate pathogen detection and identification techniques. Here we describe the development of a macroarray diagnostic technique with enhanced detection sensitivity and only small reduction in specificity. With probes designed based on the internal transcribed spacer sequences of the rRNA genes of fungal and oomycete strains, we produced a macroarray, which included five types of oligonucleotide probes: monomers (20–24 nt), dimers (40–48 nt), dimers with a poly-A spacer of 10 bases between the two repeats (50–58 nt), monomers with a poly-A tail of 10 (30–34 nt) and 20 (40–44 nt) bases. The use of repeat sequence probes (dimers) greatly improved the sensitivity of the macroarray. The dimeric probes could reliably detect 0.01 fg target genomic DNA, which is lower than the detection limits of most currently available molecular diagnostic methods, such as the conventional PCR and real-time PCR. Dimer probes also had lower signal variability, thereby increasing the macroarray signal uniformity. However, in a few cases, specificity was reduced in the dimer probes. Cross-hybridization occurred in highly similar sequences where the mismatched base was located near the end or in a chain of the same base, but this should be prevented in future array probe design.

1. Introduction

Detection and identification of microbial plant pathogens poses a challenge because different pathogens may infect the same host concurrently and may produce similar symptoms (Alvarez, 2004; Stowell and Gelernter, 2001; Uddin et al., 2003). In the absence of clear distinctive symptoms and signs, plant disease diagnosticians may use the host identity, time of the year and prevailing weather conditions to associate the pathogen with the disease. Accurate pathogen identification is the first step in disease management. Misidentification of a pathogen may lead to poor disease control, crop damage, and ultimately reduced yield. There is therefore a need for improved disease surveillance, rapid diagnoses, and accurate remedial measures in the shortest time possible. Traditionally, plant diagnosticians use direct observations and/or culturing of pathogens from diseased plant samples to make a diagnosis (van Doorn et al., 2009; Lievens and Thomma, 2005; López et al., 2003; Zhang et al., 2008). These methods are often time consuming and insufficient to identify pathogens to the species level. More recent advancements such as serology and PCR assays also have their drawbacks. For instance, immunoassay typing with antibodies has been found to be less specific than DNA-based methods (Ivnitski et al., 2003; Sergeev et al., 2004). Quantitative PCR (qPCR), a widely used technology in medical, agriculture, and the food industry, offers an alternative detection platform. However, it is limited in terms of throughput where only one or a few pathogens can be detected in a test reaction (van Doorn et al., 2009; Ivnitski et al., 2003; Uttamchandani et al., 2009; Lievens et al., 2005; Zhang et al., 2007). Considering the vast diversity of pathogens, an ideal pathogen detection tool would be characterized by its monitoring capacity for a wide range of pathogen groups as well as by its accuracy and sensitivity (Lee et al., 2010).

DNA diagnostic arrays are another molecular tool, which offers a fast, culture-independent alternative for the detection of microbes from field samples (van Doorn et al., 2009; Lee et al., 2010; Lievens et al., 2010; Lievens and Thomma, 2005; Zhang et al., 2008; Gilbert et al., 2008). The advantage of the array technique is its remarkably high throughput compared to other detection methods. Hundreds of different pathogens can be simultaneously detected with one array in one reaction in less than 12 h (Gilbert et al., 2008; Lievens and Thomma, 2005). Compared to the glass-based, high-density microarray, the membrane-based macroarray offers a cost-efficient and flexible platform and, therefore has been adopted by many disease diagnosis development projects (e.g., Gilbert et al., 2008; Lievens and Thomma, 2005; Maoka et al., 2010; Zhang et al., 2008). Moreover, macroarray results are visualized with an unaided eye, which offers simplicity compared to microarrays. Recently, the application of chromogenic technology in macroarrays has further increased their versatility for use in laboratories or diagnostic labs with limited resources (Abdullahi and Rott, 2009).

Like other DNA diagnostic array technologies, macroarrays combine the advantage of two molecular biological advancements. First, the growing amount of DNA sequences available in publically

Abbreviations: M, monomers; M-A10, monomers with a ten-adenine tail; M-A20, monomers with a 20 adenine tail; D, dimers; DA, dimers with a ten-adenine spacer.

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accessible databases (e.g., GenBank) allows for creation of signature probes specific to a species or infra-species target. Second, the high throughput capacity of the array technology permits hundreds of DNA oligomers to be queried simultaneously and produces signals indicative of matches between the oligomer and the query. The macroarray technique has been applied in a variety of areas. For example, in human biology it has been used for identification of different mRNA species present in human ejaculated spermatozoa (Dadoune et al., 2004) and in diagnosing ovarian cancer in epithelial cells (Chatterjee et al., 2006); while in veterinary science, macroarrays have been used for monitoring the Crimean–Congo Hemorrhagic fever virus, a tickborne zoonotic virus found across Africa, Eastern Europe and Asia (Wolfel et al., 2009). In Plant Pathology, membrane-based DNA arrays have been used for detection, identification, monitoring and quantification of phytopathogenic agents (Fessehaie et al., 2003; Lievens and Thomma, 2005; Zhang et al., 2008; Sholberg et al., 2005), such as phytopathogenic bacteria on potato (Fessehaie et al., 2003), pathogens of apples (Sholberg et al., 2005), and pathogenic viruses and fungi in different host plants (Lievens et al., 2005). At the infra-species level, DNA diagnostic arrays have been used for identification of races and biotypes of *Fusarium oxysporum* f. sp. *vasinfectum* on cotton (Gilbert et al., 2008) and the detection of members of the *F. solani* species complex in solanaceous crops (Zhang et al., 2007).

Probe design is the first step in the development of a diagnostic array. Parameters such as probe length and annealing temperatures (or GC content) play a role in array performance that have great impact on the fidelity of the assay, particularly with regard to the level of specificity and sensitivity attained (Barad et al., 2004; Goff et al., 2005; Uttamchandani et al., 2009; Loy and Bodrossy, 2006). If probes are not optimized for specificity, arrays can generate false positives due to cross-hybridization to similar sequences (Lievens and Thomma, 2005; López et al., 2003; Zhang et al., 2008; Selinger et al., 2000; Li and Stormo, 2001; Chou et al., 2004). On the other hand, high stringency often results in reduced signal intensity and may lead to false negatives. The occurrence of false positives and negatives is problematic because it is difficult to envisage whether a probe will attach efficiently to its target sequence and yield a good hybridization signal based on the sequence information alone (Li and Stormo, 2001; Chou et al., 2004). The design of effective probes is a challenge especially in related species where there is a high degree of sequence similarities (Schliep et al., 2003a, 2003b). Scientists have therefore resulted to engineering redundancy into the DNA array's systems to improve accuracy and analytical power simply by over-representation. That is, using multiple probes per target to achieve reliable and accurate detection (Ivnitski et al., 2003; Selinger et al., 2000; Chou et al., 2004).

A number of studies have been able to achieve high levels of specificity with DNA arrays, but sensitivity has remained elusive (Wong et al., 2007; Zhang et al., 2007). In clinical diagnostic studies, Wong et al. (2007) could achieve up to 100% specificity (94% accuracy score) and a sensitivity value of 76% using DNA arrays and a test subject of 36 patients. Similarly Zhang et al. (2007) could reduce the level of cross hybridization drastically at 55 °C and completely at 60 °C. However, the level of sensitivity was compromised, which made it difficult to detect species that were present at very low concentrations.

Increasing probe length will increase the array sensitivity, but the specificity is often sacrificed. A few studies on miRNA showed that tandem repeats of oligomer probes enhanced hybridization signals on the microarray platform (Barad et al., 2004; Goff et al., 2005). However, the impact of doubling (dimer) or tripling (trimer) a short probe sequence (~20 nt monomer) to maintain the array specificity has not been addressed. In this study, we designed and compared the performance of monomers, monomers with a ten-adenine tail (M-A10), monomers with 20 adenine tail (M-A20), dimers, and dimers with a ten-adenine spacer (DA), using a membrane-based macroarray platform (Fig. 1). We hypothesized that (1) Dimeric oligonucleotide probes are more sensitive than monomeric probes, (2) Poly-A spacer and tails would increase sensitivity of the probes, and that (3) Monomeric and dimeric probes would have equal specificity. The objectives of this study were to develop a novel technical approach that could increase the sensitivity of a macroarray to enhance its early pathogen detection power, and which could maintain the macroarray detection specificity to ensure accurate pathogen identification. Probes tested in this study were based on four important microbial pathogens of cereals, turfgrass and other plants-*Rhizoctonia solani* (basidiomycete), *Pythium aphanidermatum* (oomycete), *Fusarium solani* (ascomycete) and *F. oxysporum* (ascomycete) that cause brown patch, Pythium blight, root and vascular diseases, respectively.

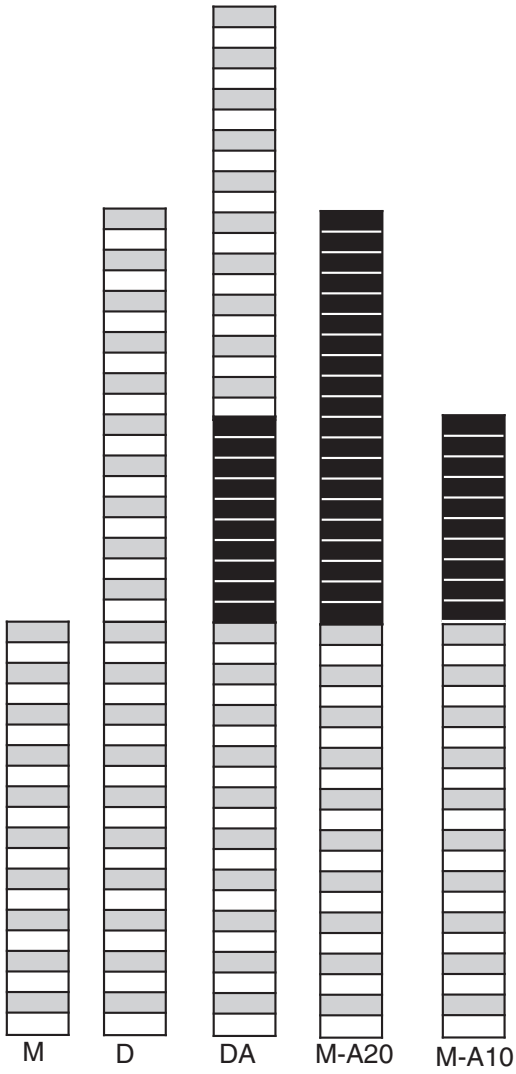

Fig. 1. Schematic representation of the five types of oligonucleotide probes used in the diagnostic macroarray. M = monomer (20–24 nt); D = dimer (40–48 nt); DA = dimer with poly-adenine spacer (black boxes) of 10 bases between the two repeats (50–58 nt); M-A20 = monomer with a poly-adenine tail (black boxes) of 20 bases (40–44 nt); and M-A10 = monomer with a poly-adenine tail (black boxes) of 10 bases (30–34 nt).

2. Materials and methods

2.1. Isolates

Test isolates used in this study are listed in Table 1. The identity of the target species, *P. aphanidermatum* and *R. solani* was confirmed by morphology and the internal transcribed spacer sequences of the rRNA

Table 1
Microbial, host species and substrate used for testing and validating the macroarray in this study.

Microbial/host species	Collection ID	Host/substrate	Origin	GenBank accession number
Fungi-Zygomycota				
Mortierella elongata	#141	*Poa annua*	Denville, NJ	–
Fungi-Ascomycota				
Curvularia trifolii	#185	*Agrostis* sp.	New Brunswick, NJ	–
Fusarium equisetti	#S2	Turfgrass soil	New Brunswick, NJ	–
F. oxysporum	#F2 (NRRL 54168)	*Lilium longiflorum* bulbs	New Brunswick, NJ	HQ379648
F. oxysporum	#S4	Turfgrass soil	New Brunswick, NJ	–
F. solani	#F3 (NRRL 54169)	*Lilium longiflorum* bulbs	New Brunswick, NJ	HQ379661
F. solani	#F19 (NRRL 54185)	*Lilium longiflorum* bulbs	New Brunswick, NJ	HQ379663
Trichoderma virens	#126-L	*Agrostis stolonifera*	New Brunswick, NJ	–
Fungi-Basidiomycota				
Rhizoctonia solani	#RH 20	Unknown	State College, PA	–
R. solani	#98	*Lolium multiflorum*	New Brunswick, NJ	–
Waitea circinata var. *circinata*	#10	*Agrostis* sp.	NJ	–
W. circinata var. *circinata*	#158	*Agrostis* sp./*Poa* sp.	Bedminster, NJ	HQ166071
W. circinata var. *zeae*	#1	*Poa annua*	New Brunswick, NJ	–
Stramenopiles-Oomycota				
Pythium aphanidermatum	#60	*Festuca arundinacea*	NJ	–
P. aphanidermatum	#99	*Lolium multiflorum*	New Brunswick, NJ	–
P. rostrum	#123p	*Agrostis* sp.	New Brunswick, NJ	–
P. torulosum	#122	*Poa annua*	New Brunswick, NJ	–
P. volutum	#124p-1	*poa annua*	New Brunswick, NJ	–
Host/substrate				
-[a]	#199	*Agrostis stolonifera*	New Brunswick, NJ	–
-[b]	NA	*Poa annua*	New Brunswick, NJ	–
-[c]	#176	*Poa annua*	New Brunswick, NJ	–
-[d]	NA	Soil	New Brunswick, NJ	–

NRRL = Agricultural Research Service Culture Collection (NCAUR, Peoria, IL).
NA = Not available.
[a] *Agrostis stolonifera* with symptoms of brown patch.
[b] Asymptomatic, disease free *Poa annua* from greenhouse.
[c] *Poa annua* with symptoms of Pythium blight.
[d] Soil substrates used were those associated with *P. aphanidermatum* and *R. solani.*

genes (ITS), while the *Fusarium* species were characterized in another study by partial sequences of translation elongation factor-1α (EF-1α), ITS, and β-tubulin (TUB) genes (Rajmohan et al., unpublished). In addition, non-target species of *Pythium, Rhizoctonia*, and *Fusarium* were used to test the array specificity. Three common co-inhabiting fungi in turfgrass soil, *Curvularia trifolii*, *Trichoderma virens*, and *Mortierella elongata* were also included in the study for validation and cross-reaction tests.

2.2. DNA extraction, amplification, purification and sequencing

Genomic DNA from all microbial isolates was extracted from 1- to 2-week-old cultures growing on PDA plates (Difco laboratories, Detroit, MI) using the UltraClean Soil DNA kit (MoBio Laboratories Inc., Solana Beach, CA, USA) or DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA, USA), following the manufacturer's protocol. The extracted genomic DNA was quantified using a NanoVue spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and diluted to 5 ng/μl before PCR. The ITS region was amplified with primers ITS1 and ITS4 (White et al., 1990). PCR was carried out in a 25 μl reaction volume containing 3 μl (15 ng) genomic DNA, 1x PCR buffer (Applied Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$, 200 μM each of the dNTPs, 0.5 μM of each forward and reverse primers, and 0.5 U *Taq* polymerase. The thermal cycling parameters were 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 56 °C for 1 min., and 72 °C for 1 min; followed by 10 min at 72 °C. PCR products were purified according to the manufacturer's protocol using the QIAquick PCR Purification Kit (Qiagen), quantified using NanoVue spectrophotometer and sequenced when identity of the isolates needed to be confirmed. Sequencing of the purified ITS PCR products was run on an Applied Biosystems 3730xl sequencer by GENEWIZ (GENEWIZ, Inc., South Plainfield, NJ, USA).

2.3. Oligonucleotide probe design and array development

Based on the 20–24 nt oligomer probes previously designed and validated by Lievens et al. (2003), Saiki et al. (1989), and Zhang et al. (2007, 2008), we designed two more sets of probes: dimers and dimers with a poly-A10 spacer of 10 bases for the four target species and the controls (Table 2). M-A10 and M-A20 were designed for *P. aphanidermatum* and *F. solani* only (Table 2), since we were more interested with the effect of duplexing but at the same time we wanted to disqualify sequence length as the only factor contributing to the enhanced sensitivity. The performance of the macroarray that contained the five types of probes was tested against the macroarray that contained only the monomeric probes.

The macroarray development followed the procedure described by Zhang et al. (2007). Briefly, 20 pmol of each detector oligonucleotide probe was spotted onto Hybond N+ nylon membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in quadruplicate using a 96-pin tool (V&P Scientific Inc., San Diego, CA, USA). Three types of controls were also spotted. First, the positive controls included the ITS4 primer, which is a universal primer for both fungi and oomycetes, and ITS2, which is a fungal universal primer (Table 2). Second, internal controls that differed from ITS2 at two bases were also spotted on the membrane. Negative controls were sterile water and the spotting buffer. The positive and internal controls also constituted dimers, DA, M-A10 and M-A20. The spotted membranes were air dried for 10 min and then fixed by UV exposure at 240 mJ/cm^2. After incubation in a 0.5%

Table 2
Probe name, type, sequence, length and targeted species used in this study.

Probe name	Probe type [a]	Probe sequence	Reference [b]	Probe length	Probe target
Specific target probe					
Pa1	M	GGAGAGAGATGGCAGAATGTGAG	Saiki et al. (1989)	23	*P. aphanidermatum*
Pa2	M	GGGAGAGAGATGGCAGAATGTGAG	Saiki et al. (1989)	24	*P. aphanidermatum*
Pa3	M	GAGGTGTACCTGAATTGTGTGAGG	Saiki et al. (1989)	24	*P. aphanidermatum*
Pa1R	D	GGAGAGAGATGGCAGAATGTGAGGGAGAGAGATGGCAGAATGTGAG		46	*P. aphanidermatum*
Pa2R	D	GGGAGAGAGATGGCAGAATGTGAGGGGAGAGAGATGGCAGAATGTGAG		48	*P. aphanidermatum*
Pa3R	D	GAGGTGTACCTGAATTGTGTGAGGGAGGTGTACCTGAATTGTGTGAGG		48	*P. aphanidermatum*
Pa1R-A	DA	GGAGAGAGATGGCAGAATGTGAGAAAAAAAAAAAGGAGAGAGATGGCAGAATGTGAG		56	*P. aphanidermatum*
Pa2R-A	DA	GGGAGAGAGATGGCAGAATGTGAGAAAAAAAAAAAGGGAGAGAGATGGCAGAATGTGAG		58	*P. aphanidermatum*
Pa3R-A	DA	GAGGTGTACCTGAATTGTGTGAGGAAAAAAAAAA GAGGTGTACCTGAATTGTGTGAGG		58	*P. aphanidermatum*
Pa1-A10	M-A10	GGAGAGAGATGGCAGAATGTGAGAAAAAAAAAA		33	*P. aphanidermatum*
Pa2-A10	M-A10	GGGAGAGAGATGGCAGAATGTGAGAAAAAAAAAA		34	*P. aphanidermatum*
Pa3-A10	M-A10	GAGGTGTACCTGAATTGTGTGAGGAAAAAAAAAA		34	*P. aphanidermatum*
Pa1-A20	M-A20	GGAGAGAGATGGCAGAATGTGAGAAAAAAAAAAAAAAAAAAAA		43	*P. aphanidermatum*
Pa2-A20	M-A20	GGGAGAGAGATGGCAGAATGTGAGAAAAAAAAAAAAAAAAAAAA		44	*P. aphanidermatum*
Pa3-A20	M-A20	GAGGTGTACCTGAATTGTGTGAGGAAAAAAAAAAAAAAAAAAAA		44	*P. aphanidermatum*
Rs2	M	CAGTGTTATGCTTGGTTCCACTC	Zhang et al. (2008)	23	*R. solani*
Rs3	M	TGTTGAAACTTAGTATTAGATGCGT	Zhang et al. (2008)	23	*R. solani*
Rs4	M	GAGTGGAACCAAGCATAACACTG	Zhang et al. (2008)	23	*R. solani*
Rs2R	D	CAGTGTTATGCTTGGTTCCACTCCAGTGTTATGCTTGGTTCCACTC		46	*R. solani*
Rs3R	D	TGTTGAAACTTAGTATTAGATGCGTTGTTGAAACTTAGTATTAGATGCGT		46	*R. solani*
Rs4R	D	GAGTGGAACCAAGCATAACACTGGAGTGGAACCAAGCATAACACTG		46	*R. solani*
Rs2R-A	DA	CAGTGTTATGCTTGGTTCCACTCAAAAAAAAAAACAGTGTTATGCTTGGTTCCACTC		56	*R. solani*
Rs3R-A	DA	TGTTGAAACTTAGTATTAGATGCGTAAAAAAAAAAATGTTGAAACTTAGTATTAGATGCGT		56	*R. solani*
Rs4R-A	DA	GAGTGGAACCAAGCATAACACTGAAAAAAAAAAGAGTGGAACCAAGCATAACACTG		56	*R. solani*
Fs4	M	TCGCGTAGTAGCTAACACCTCGC	Zhang et al. (2007)	23	*F. solani*
Fs6	M	CCTGTGAACATACCTAAACGTTG	Zhang et al. (2007)	23	*F. solani*
Fs13	M	TTATACAACTCATCAACCCTGTGA	Zhang et al. (2007)	24	*F. solani*
Fs4 R	D	TCGCGTAGTAGCTAACACCTCGCTCGCGTAGTAGCTAACACCTCGC		46	*F. solani*
Fs6R	D	CCTGTGAACATACCTAAACGTTGCCTGTGAACATACCTAAACGTTG		46	*F. solani*
Fs13R	D	TTATACAACTCATCAACCCTGTGATTATACAACTCATCAACCCTGTGA		48	*F. solani*
Fs4 R-A	DA	TCGCGTAGTAGCTAACACCTCGCAAAAAAAAAATCGCGTAGTAGCTAACACCTCGC		56	*F. solani*
Fs6R-A	DA	CCTGTGAACATACCTAAACGTTGAAAAAAAAAACCTGTGAACATACCTAAACGTTG		56	*F. solani*
Fs13R-A	DA	TTATACAACTCATCAACCCTGTGAAAAAAAAAAATTATACAACTCATCAACCCTGTGA		58	*F. solani*
Fs4-A10	M-A10	TCGCGTAGTAGCTAACACCTCGCAAAAAAAAAA		33	*F. solani*
Fs6-A10	M-A10	CCTGTGAACATACCTAAACGTTGAAAAAAAAAA		33	*F. solani*
Fs13-A10	M-A10	TTATACAACTCATCAACCCTGTGAAAAAAAAAA		34	*F. solani*
Fs4-A20	M-A20	TCGCGTAGTAGCTAACACCTCGCAAAAAAAAAAAAAAAAAAAA		43	*F. solani*
Fs6-A20	M-A20	CCTGTGAACATACCTAAACGTTGAAAAAAAAAAAAAAAAAAAA		43	*F. solani*
Fs13-A20	M-A20	TTATACAACTCATCAACCCTGTGAAAAAAAAAAAAAAAAAAAA		44	*F. solani*
Fo1	M	CGTTCCTCAAATTGATTGGCGGTC	Zhang et al. (2008)	24	*F. oxysporum*
Fox2	M	GTTGGGACTCGCGTTAATTCG	Lievens et al. (2003)	21	*F. oxysporum*
Fo1R	D	CGTTCCTCAAATTGATTGGCGGTCCGTTCCTCAAATTGATTGGCGGTC		48	*F. oxysporum*
Fox2R	D	GTTGGGACTCGCGTTAATTCGGTTGGGACTCGCGTTAATTCG		42	*F. oxysporum*
Fox2-A	DA	CGTTCCTCAAATTGATTGGCGGTCAAAAAAAAAACGTTCCTCAAATTGATTGGCGGTC		58	*F. oxysporum*
Fo1R-A	DA	GTTGGGACTCGCGTTAATTCGAAAAAAAAAAGTTGGGACTCGCGTTAATTCG		52	*F. oxysporum*
Positive control Probe					
ITS2	M	GCTGCGTTCTTCATCGATGC	White et al. (1990)	20	Fungi
ITS4	M	TCCTCCGCTTATTGATATGC	White et al. (1990)	20	Fungi & oomycete
ITS2R	D	GCTGCGTTCTTCATCGATGCGCTGCGTTCTTCATCGATGC		40	Fungi
ITS4R	D	TCCTCCGCTTATTGATATGCTCCTCCGCTTATTGATATGC		40	Fungi & oomycete
ITS2R-A	DA	GCTGCGTTCTTCATCGATGCAAAAAAAAAAGCTGCGTTCTTCATCGATGC		50	Fungi
ITS4R-A	DA	TCCTCCGCTTATTGATATGCAAAAAAAAAATCCTCCGCTTATTGATATGC		50	Fungi & oomycete
ITS2-A10	M-A10	GCTGCGTTCTTCATCGATGCAAAAAAAAAA		30	Fungi
ITS4-A10	M-A10	TCCTCCGCTTATTGATATGCAAAAAAAAAA		30	Fungi & oomycete
ITS2-A20	M-A20	GCTGCGTTCTTCATCGATGCAAAAAAAAAAAAAAAAAAAA		40	Fungi
ITS4-A20	M-A20	TCCTCCGCTTATTGATATGCAAAAAAAAAAAAAAAAAAAA		40	Fungi & oomycete
Internal control Probe					
ITS2-2-1	M	GCTGCGTTGATCATCGATGC	Zhang et al. (2008)	20	None
ITS2-2-1R	D	GCTGCGTTGATCATCGATGCGCTGCGTTGATCATCGATGC		40	None
ITS2-2-1R-A	DA	GCTGCGTTGATCATCGATGCAAAAAAAAAAGCTGCGTTGATCATCGATGC		50	None
ITS2-2-1-A10	M-A10	GCTGCGTTGATCATCGATGCAAAAAAAAAA		30	None
ITS2-2-1-A20	M-A20	GCTGCGTTGATCATCGATGCAAAAAAAAAAAAAAAAAAAA		40	None

[a] D = dimer, DA = dimer with 10 adenine nucleotides (poly-A) spacer, M = monomer, M-A10 = monomer with 10 adenine nucleotides (poly-A10) tail and M-A20 = monomer with 20 adenine nucleotides (poly-A20) tail.
[b] Reference for literature citations. Oligonucleotide probes that do not have a reference were modifications from referenced probes by the authors for this study.

sodium dodecyl sulfate (SDS) solution at 60 °C for an hour, membranes were rinsed in 100 mM Tris (pH 8.0) for 5 min, and kept moist at 4 °C until used. This last step was also used for stripping the array.

2.4. Hybridization

Hybridization was carried out as described previously (Zhang et al., 2007, 2008). The test ITS amplicons from either target or non-

target materials were labeled and hybridized using the Gene Images AlkPhos Direct Labeling (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and Detection System with CDP-Star (Topix Inc., Bedford, MA, USA). Before use, the arrays were pre-hybridized at 55 °C for 15 min and hybridized with 100 ng (10 μl of 10 μg/μl) of labeled ITS amplicon at 55 °C for 2 h. After two primary washes and two secondary washes, the detection reagent was added to the array to react for an hour, followed by 30 min of film exposure. Chemiluminescence was detected using Kodak Biomax Light film. Developed films were scanned by an Aficio MP C6000 Color Copier/Scanner (Ricoh Americas Corporation, West Caldwell, NJ, USA) and read with ImageJ 1.33u (National Institutes of Health, MD, USA).

2.5. Array sensitivity

The signal intensity for hybridization was measured as the average inverted gray value for the quadruplicate spots for each detector oligonucleotide on the array after the background gray values were subtracted. Since in 8-bit grayscale images, the darkest picture corresponds to the lowest value, we inverted each value by subtracting its reading from 255 (i.e. inverted gray value = 255-gray value readings). Each experiment was conducted at least twice. The signal intensity of the monomers was compared vis-à-vis those of the dimer, DA and where applicable, M-A10 and M-A20 for each isolate.

Two isolates, *R. solani* (#98) and *P. aphanidermatum* (#99) were used to determine the detection limit of the PCR-coupled macroarray method. The genomic DNA of #98 and #99 was 10× serially diluted from 1 ng/μl to 1×10^{-10} ng/μl prior to a standard ITS PCR. Following ITS amplification, hybridization was conducted as described above. 10 μl of purified ITS PCR product was used for hybridization and gel electrophoresis. The experiments were conducted twice. To determine whether DNA from the host and other co-inhabiting fungi would interfere with the target species detection, the target DNA was 10× serially diluted (from 1 ng/μl to 1×10^{-4} ng/μl) and mixed with 1 ng of plant DNA. 2 μl of the mixture were used for PCR amplification in a 25 μl reaction mix (Table 3). Plant DNA was derived from clean greenhouse grown *Poa annua* with no history of disease infection. Serially diluted genomic DNA of the target species (from 1 ng/μl to 1×10^{-2} ng/μl of #98 or #99) was mixed in same ratio with the serially diluted genomic DNA from three common turfgrass-associated fungi, *C. trifolii, T. virens,* and *M. elongata* (Table 3). PCR amplification and hybridization were done as explained above. Control experiments were done to test if the observed results were due to skewed amplification by PCR, by mixing 2.5 ng of purified PCR products of each of the test sample (*P. annua, C. trifolii, T. virens*, *M. elongata*, and the target species) to final volume of 10 μl before macroarray hybridization.

Table 3
Amount of target species DNA, common co-inhabiting non-target fungal species DNA, and host DNA used in 25 μl-PCR reactions for assessment of hybridization interference.

Reaction number	Target DNA amount (ng)[a]	*C. trifolii* DNA amount (ng)	*M. elongata* DNA amount (ng)	*T. virens* DNA amount (ng)	Host DNA amount (ng)	Hybridization signal observed
1	10^{-4}	0	0	0	1	yes
2	10^{-3}	0	0	0	1	yes
3	10^{-2}	0	0	0	1	yes
4	10^{-1}	0	0	0	1	yes
5	10^{-2}	10^{-2}	10^{-2}	10^{-2}	1	yes
6	10^{-1}	10^{-1}	10^{-1}	10^{-1}	1	yes
7	1	1	1	1	1	yes

Yes implies that a hybridization signal was observed.
[a] Target species used were *Rhizoctonia solani* isolate #98 or *Pythium aphanidermatum* isolate #99. The non-target fungal species used were *Curvularia trifolii, Trichoderma virens*, and *Mortierella elongata* while the host species was *Poa annua*.

2.6. Array validation

The macroarray was validated with DNA extracted from plant tissues or soils infested by the target species. DNA was extracted using UltraClean Soil DNA kit (MoBio Laboratories, Inc., Solana Beach, CA, USA) or DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA, USA) depending on the material. Microscopic observation was also performed to check for presence of fungal or oomycete structures in the substrate.

2.7. Array specificity

To assess the specificity of detection by the array, fungal and oomycete strains that differ from the target species by 1 to 3 bases of the probe sequence were tested for cross-reaction against the array (Table 6). DNA extraction, purification and hybridization were done as described above. Other fungal and oomycete species often found associated with turfgrasses were also included in the experiments to test the array's ability to discriminate non-target species.

2.8. Statistical analysis

Every probe was spotted four times on the macroarray and measured individually and the hybridization experiment was repeated at least once. Quantitative data were analyzed using a two-way ANOVA (isolate and probe type) with SAS (version 9.2) statistical software. A P value<0.05 was considered significant. Specifically, signal intensities of hybridization of monomers printed on a membrane with and without other probe types were analyzed using a 2×4 factorial design.

3. Results

3.1. Probe sensitivity

Dimers and DA had a significantly higher sensitivity than those of the M, M-A10 and M-A20 (Fig. 3). To test whether the monomers were being outcompeted in the race for hybridization targets, we printed monomers on a separate membrane. Results showed that there was no significant difference in signal intensities between monomers printed on a separate array and monomers printed in same array with dimers (Table 5).

3.2. Signal uniformity

Signal intensities of monomeric probes were most variable compared to the other types of probes (Figs. 2 and 4). The signal intensities for monomers were so diverse that *F. oxysporum* had one probe with an inverted gray value of 89 while the other was showing only 4. On the same array, dimers and dimers with poly-A spacer derived from those monomers had lesser inverted gray value disparity (117–108 and 117–114, respectively). The coefficient of variation (CV), defined here as the standard deviation divided by the means of the hybridization signals was used as a measure of the variation of signal intensity (Chou et al., 2004; Fig. 4). Overall, the dimers displayed the lowest variability, followed by the dimers with poly-A and then the monomers M-A20 and M-A10. Monomers alone had the greatest variability.

3.3. Limit of detection and validation

The dimer probes could reliably detect up to 0.01 fg genomic DNA, which is a thousand times lower than using PCR product visualization with gel electrophoresis (Fig. 5). A simulation based on a condition where the pathogen DNA was serially diluted while holding the host grass DNA at 1 ng showed that dimeric probes could detect target DNA at all levels tested (Table 3), including 0.1 pg at a ratio of $1{:}10^4$ pathogen to host DNA for *R. solani* and *P. aphanidermatum*. Mixing the DNA of the target species, commonly found co-inhabiting fungal species and the

Table 4
DNA sequence mismatches between target and closely related non-target species at the ITS region where the probe was designed.

Target			Non-target					
Species	Probe	Probe sequence	Species with 1 nt mismatch	Mismatch position	Species with 2 nt mismatch	Mismatch position	Species with 3 nt mismatch	Mismatch positions
Pa	Pa1	GGAGAGAGATGGCAGAATGTGAG	*P. torulosum* (#122)	GGAGAGAAATGGCAGAATGTGAG	*P. volutum* (#124p-1)	GGAGAGAAATGGCAGA-TGTGAG	–	NA
	Pa2	GGGAGAGAGATGGCAGAATGTGAG	–	NA	*P. torulosum* (#122)	AGGAGAGAAATGGCAGAATGTGAG	*P. volutum* (#124p-1)	AGGAGAGAAATGGCAGA-TGTGAG
	Pa3	GAGGTGTACCTGAATTGTGTGAGG	–	NA	*P. volutum* (#124p-1), *P. torulosum* (#122)	GAGGTGTACCTGTCTTGTGTGAGG	–	NA
Rs	Rs2	CAGTGTTATGCTTGGTTCCACTC	–	NA	-	NA	–	NA
	Rs3	TGTTGAAACTTAGTATTAGATCGCGT	–	NA	–	NA	–	NA
	Rs4	GAGTGGAACCAAGCATAACACTG	–	NA	–	NA	–	NA
Fs	Fs4	TCGCGTAGTAGCTAACACCTCGC	–	NA	*F. equiseti* (#S2)	TAGCGTAGTAGCTAACACCTCGT	–	NA
	Fs6	CCTGTGAACATACCTAAACGTTG	*F. solani* (#F19)	**CCTGTGAACATACCTAAAACGTTG**	*F. equiseti* (#S2)	CCTGTGAACATACCTA-CGTTG	–	NA
	Fs13	TTATACAACTCATCAACCCTGTGA	*F. solani* (#F3)	**TTATTCAACTCATCAACCCTGTGA**	*F. oxysporum* (#F2, #S4), *F. equiseti* (#S2)	TTATACAACTCATCAAACCCCTGTGA	–	NA
Fo	Fo1	CGTTCCTCAAATTGATTGGCGGTC	–	NA	*F. equiseti* (#S2)	**CGTCCCTCAAATCGATTGGCGGTC**	–	NA
	Fox2	GTTGGGACTCGCGTTAATTCG	–	NA	–	NA	*F. equiseti* (#S2)	GTTGGGACTCGCGGTAACCCG

– indicates no candidate species was available with the specified mismatch, NA = not applicable, sequences that caused cross-hybridization are in boldface.
Pa = *Pythium aphanidermatum*, Rs = *Rhizoctonia solani*, Fs = *Fusarium solani*, and Fo = *Fusarium oxysporum*.

host plant did not interfere with the hybridization reactions for all reactions tested (Table 3). The macroarray was also successfully validated with target species infected plant or soil materials. The array detection and identification results matched with the identification based on traditional microscopic observation, culture isolation and ITS DNA sequence (Table 1).

3.4. Array specificity

There was no cross hybridization to *P. aphanidermatum* and *R. solani* probes from any of the non-target isolates. Cross-hybridization was observed in three sets of dimers and DA probes (Table 6) when reacting with non-target species that had one or two nucleotide sequence mismatches (Table 6). The mismatches between the cross-hybridized probes and the corresponding non-target sequences were located either in chain of A or G (*F. solani* #F19/probe Fs6 and *F. equiseti* #S2/probe Fo1, Tables 6) or near the end of the probe sequence (*F. solani* #F3/probe Fs13 Table 4). The cross-hybridization signal intensity values were 44% or lower compared to the perfect-match signals. False negatives were only observed in a monomer probe for *P. aphanidermatum* (Table 6).

4. Discussion

Rapid and early diagnosis of microbe-causing diseases requires a technique capable of detecting low quantity of causal agents from the natural host environment. This study demonstrates an improved macroarray detection technique that provides enhanced and consistent signals of detection with small reductions in specificity. The tandem-repeat dimeric probes (40–48 nt) had significantly higher sensitivity and lower signal variability compared to the monomers (20–24 nt).

The diagnostic array technique for microbe detection requires both high sensitivity and specificity. While developing macroarrays for the detection of solanaceous plant pathogens, Zhang et al. (2007) optimized the hybridization temperature to reduce cross-hybridization. However, this improvement in specificity came with a considerable sacrifice in signal intensity, which is also determined by the probe length. Long-sequence probes are known to decrease the array specificity; therefore we tested the hypothesis that dimeric probes containing two short identical sequences would enhance the DNA array sensitivity without sacrificing the specificity. At the same time we considered that two monomers in tandem might interfere with hybridization of targets to the two matching sequences. We thus, tested the possibility that addition of a spacer in between may facilitate the binding of long target DNA fragments to the probes without unnecessary tangling. However, our results showed that overall, dimers with poly-A spacer did not produce stronger signals than the dimers. This suggests that the proximity of two monomers to each other doesn't have a significant effect on hybridization. Previous work has shown that an addition of spacers can have a large effect on hybridization signals for 15–30 mer oligonucleotide probes, but indicated that the signal decreases with spacer length after an optimal length is reached (Guo et al., 1997; Shchepinov et al., 1997;

Table 5
Two-way ANOVA for hybridization results of monomers printed on a membrane with and without other probe types, tested against four isolates (N = 24).

ANOVA					
Source of variation	*SS*	*df*	*MS*	*F*	*P-value*
Monomer type	192.13	1	192.13	0.21	0.65
Isolate	5154.76	3	1718.25	1.92	0.17
Interaction (monomer type × isolate)	257.45	3	85.82	0.09	0.96
Error	14349.09	16	896.82		
Total	19953.44	23			

Table 6
Comparison of hybridization results between target and non-target species based on monomer, dimer, and dimer with poly-A spacer probes.

Test species[b]	ID #[c]	Probe target species/type/name[a]																															
		Pythium aphanidermatum									*Rhizoctonia solani*									*Fusarium solani*									*Fusarium oxysporum*					
		M	M	M	D	D	D	DA	DA	DA	M	M	M	D	D	D	DA	DA	DA	M	M	M	D	D	D	DA	DA	DA	M	M	D	D	DA	DA
		Pa1	Pa2	Pa3	Pa1R	Pa2R	Pa3R	Pa1R-A	Pa2R-A	Pa3R-A	Rs2	Rs3	Rs4	Rs2R	Rs3R	Rs4R	Rs2R-A	Rs3R-A	Rs4R-A	Fs4	Fs6	Fs13	Fs4R	Fs6R	Fs13R	Fs4R-A	Fs6R-A	Fs13R-A	Fo1	Fox2	Fo1R	Fox2R	Fo1R-A	Fox2R-A
Ct	#185	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fe	#S2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	⊕	−	⊕	−
Fo	#F2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Fo	#S4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Fs	#F3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	⊕	+	+	⊕	−	−	−	−	−	−
Fs	#F19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	⊕	+	+	⊕	+	−	−	−	−	−	−
Me	#141	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Poa	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pa	#60	⊗	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pa	#99	⊗	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pr	#123p	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pt	#122	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pv	#124p-1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Wc	#10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Wc	#158	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rs	#RH 20	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rs	#98	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Tv	#126-L	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

− indicates no hybridization signal was observed, + indicates a hybridization signal was observed. All isolates were tested on three probes for each target in quadruplicate. False negatives (⊗) were observed in M probe Pa1 for *P. aphanidermatum* test isolates (#60 and #99). False positives (⊕) between *F. oxysporum* probes (D and DA) and *F. equiseti*, and between *F. solani* probes (D and DA) and non-target *F. solani* strains.

[a] A list of 33 probe names derived from 4 target species and 3 probe types, where M = monomer, D = dimer, and DA = dimer with 10 adenine (poly-A) spacer.

[b] Ct = *Curvularia trifolii,* Fe = *Fusarium equiseti*, Fo = *Fusarium oxysporum*, Fs = *Fusarium solani,* Me = *Mortierella elongata,* Poa = *Poa annua,* Pa = *Pythium aphanidermatum*, Pr = *Pythium rostrum*, Pt = *Pythium torulosum*, Pa = *Pythium volutum*, Wc = *Waitea circinata* var. *circinata*, Rs = *Rhizoctonia solani, and* Tv = *Trichoderma virens.*

[c] Collection ID/accession number.

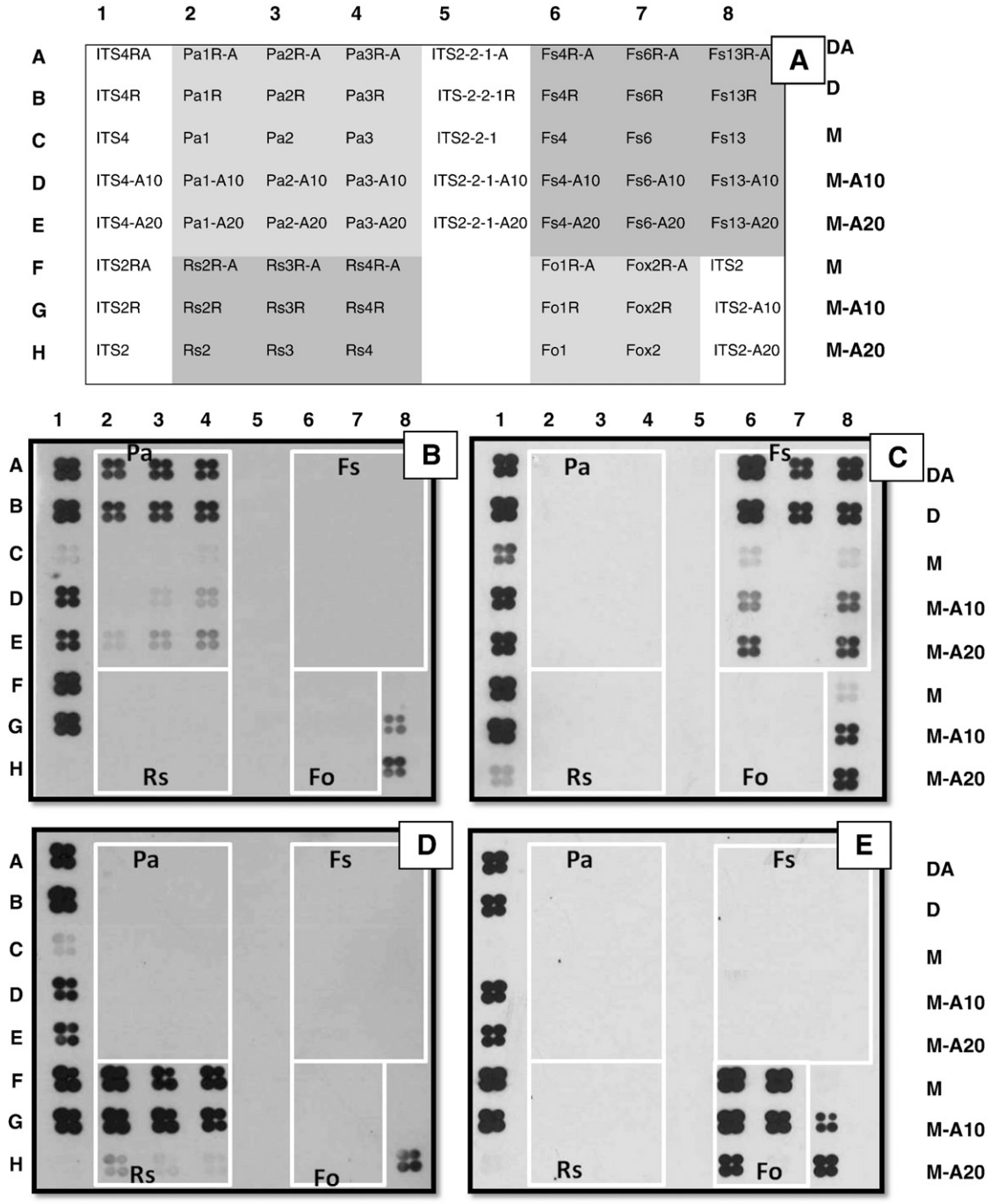

Fig. 2. Macroarray design and hybridization results. A, Macroarray design. Specific probes for *Pythium aphanidermatum* (Pa), *Rhizoctonia solani* (Rs), *Fusarium solani* (Fs), and *F. oxysporum* (Fo, Fox) were spotted in the four shaded regions. Each of the five types of oligonucleotide probes was spotted in a row as follows: row A, DA; B, dimer; C, monomer; D, M-A10; E, M-A20; F (except F8 spotted with monomer), DA; G (except G8 spotted with M-A10), dimer; H (except H8 spotted with M-A20), monomer. Positive controls were spotted in A1, B1, C1, D1, E1, G1, H1, F8, G8, and H8. Internal controls were spotted in A5, B5, C5, D5 and E5. B, C, D and E show macroarray hybridization results with *P. aphanidermatum, F. solani, R. solani*, and *F. oxysporum*, respectively.

Southern, et al., 1999). Shchepinov et al. (1997) observed that longer spacers tend to reduce the effective concentration of the probe by creating sufficient space to mitigate sterical and electrostatic effects on hybridization.

Previous studies (Chou et al., 2004; Shchepinov et al., 1997) demonstrated that longer probes usually had higher signal intensities than shorter probes. This was exemplified by our treatment where monomers with poly-A20 tail gave stronger signal than those with poly-A10 tail, whereas pure monomer had the weakest signal intensity. The high signal intensity produced by dimer than monomer oligos confirms with earlier work by Goff et al. (2005) who observed enhanced hybridization signals for dimers compared to monomers while developing multimeric miRNA microarray from 18 to 24 mer oligos. They also observed that signal intensities of trimers were comparable to dimers. This was in contrast to the work by Barad et al. (2004) who while comparing 60-mer probes containing a single miRNA sequence (about 22 nt) to the duplex and triplex miRNA probes, observed that there was negligible difference in signal intensity between the three.

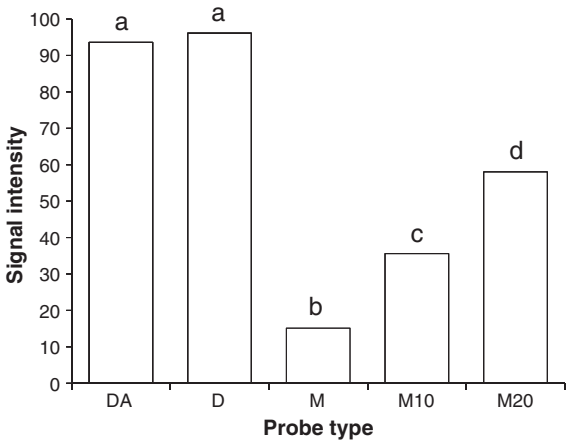

Fig. 3. Signal intensity comparison of different oligonucleotide probe types. Y-axis represents the mean signal intensity levels as measured with inverted grayscale values. For each probe type (X-axis), there are three independent probes targeting each species (four species tested) except for *F. oxysporum* which had two probes per probe type, making a total of 11 unique probes for M, D and DA oligonucleotide probe types. Only two species (6 unique probes) were tested for M-A10 and M-A20 probe types. Two isolates were tested for each species except for *F. oxysporum*, which had only one isolate available. The experiment was conducted twice. Means with the same letter do not differ significantly (Tukey test, $P<0.05$, $n=11$ for M, D, and DA whereas $n=6$ for M-A10 and M-A20).

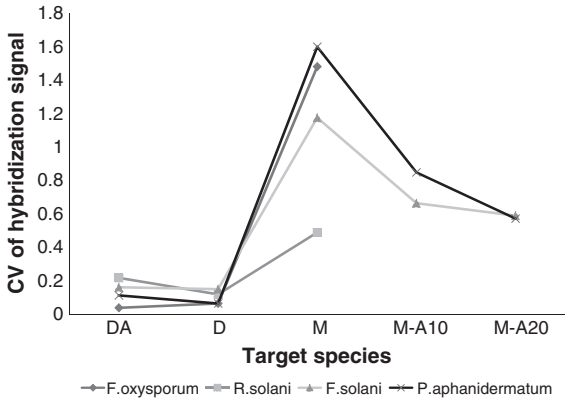

Fig. 4. Coefficient of variation of hybridization signal intensity among different probe types for the four target species. The coefficient of variation (CV), is defined as the standard deviation divided by the means of the hybridization signals (Chou et al., 2004) and is used here as a measure of the variation of signal intensity.

Their explanation was that 60-mers are in excess over the matching miRNA sequence.

To assess the reproducibility of our results, eleven sets of probes for DA, D, and M types (two to three independent sets for each of the four species) in addition to the controls were examined in this study. For the diagnostic arrays, multiple probes for each target species also ensure positive detection of genetically diverse target species. Random signal variation (noise) and the systematic deviation of the measurement from the true signal due to probe-specific or other confounding technical effects can interfere with the results (Ivnitski et al., 2003; Selinger et al., 2000; Chou et al., 2004). Signal variation, expressed as a coefficient of variation (CV), showed that dimer probes had lowest variation while the monomers showed wide variability. Based on the statistical principles of sampling, the smaller the CV for the hybridization signals, the more reliable or reproducible the results are (Chou et al., 2004). This inherently implies that fewer probes are needed when using dimer probes.

Although dimeric probes provided a low measurement variation and superior signal intensity, some dimeric probes tested here were relatively poor in discriminating sequences with high level of similarity (especially 1 nt differences). Cross-homology is a predicator of cross-hybridization (Chou et al., 2004; Evertsz et al., 2001; Kothapalli, et al., 2002). The concept of using dimers as probes does not change the level of similarity compared to the monomers. Therefore, the cross-hybridization observed maybe a consequence of other factors such as the binding energy accruing from longer probes. Longer probes typically have higher binding energy than shorter probes (Kreil et al., 2006). Cross-hybridization observed here was only limited to members of same genus and occurred in highly similar sequences where the mismatch base was located near the end or in a chain of same base. This should be prevented in future array probe design by avoiding such regions of a gene. Our results showed that in all cases, the dimeric probes were able to distinguish strains that differed from target by 3 or more nucleotides. Full discrimination also achieved for most cases of 2-nucleotide mismatches and one case of single nucleotide match. Therefore, with improved probe design strategy, the dimer array system is expected to distinguish between closely related pathogens, at species and infra species levels, such as race and subspecies.

Even though this macroarray system displayed some false-positives, it was remarkable in detecting low quantities of pathogen DNA. The dimer probes were able to detect as low as 0.1 pg target DNA in raw or mixed biological samples with plant extract despite the possibility of PCR bias in amplification. The dimers also reliably detected 0.01 fg target DNA from pure cultures on the array, while gel electrophoresis required a thousand fold more DNA for a positive detection. Assessment of low concentrations of target DNA that is only present in few plant cells can be very elusive (Lievens et al., 2003). Understanding pathogen biology is of paramount importance in disease diagnosis, since some pathogens are localized in certain parts of the plant tissue, while others are systemic in nature or cause symptoms in advance of tissue pathogen ingress. Visualization of pathogen structures using dissecting microscope to locate tissue with signs of the disease followed by targeted isolation of DNA from these tissues may improve the power of molecular detection.

In summary, we report here that dimeric probes (40–48 nt) enhanced macroarray performance. The optimized dimer macroarray system demonstrated significantly higher sensitivity and consistency than the conventional monomer oligonucleotide arrays. Its detection sensitivity is also higher than many other currently available molecular diagnostic methods, such as PCR and real-time PCR. Moreover, this method is faster (<12 h) than the traditional culture-based diagnostic method, which often takes days or even weeks. Therefore, this

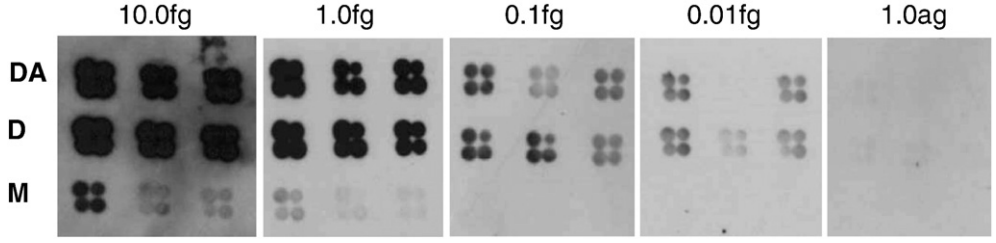

Fig. 5. Macroarray results when probes were hybridized with amplified *Rhizoctonia solani* from 10× serial diluted genomic DNA (10 fg to 1 ag).

technique should be useful for early disease diagnosis when only trace amounts of target microbes are present in a sample. Our finding should aid in the development of a multiplex diagnostic macroarray system to facilitate early disease diagnosis and management. The technique also can be adapted and applied to microbial ecological studies and other research areas.

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References

Abdullahi, I., Rott, M., 2009. Microarray immunoassay for the detection of grapevine and tree fruit. J. Virol. Methods 160, 90–100.
Alvarez, A.M., 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. Annu. Rev. Phytopathol. 42, 339–366.
Barad, O., Meiri, E., Avniel, A., Aharonov, R., Barzilai, A., Bentwich, I., Einav, U., Gilad, S., Hurban, P., Karov, Y., Lobenhofer, E.K., Sharon, E., Shiboleth, Y.M., Shtutman, M., Bentwich, Z., Einat, P., 2004. MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. Genome Res. 14, 2486–2494.
Chatterjee, M., Mohapatra, S., Ionan, A., Bawa, G., Ali-Fehmi, R., Wang, X., Nowak, J., Ye, B., Nahhas, F.A., Lu, K., Witkin, S.S., Fishman, D., Munkarah, A., Morris, R., Levin, N.K., Shirley, N.N., Tromp, G., Abrams, J., Draghici, S., Tainsky, M.A., 2006. Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. Cancer Res. 66, 1181–1190.
Chou, C.C., Chen, C.H., Lee, T.T., Peck, K., 2004. Optimization of probe length and the number of probes per gene for optimal microarray analysis of gene expression. Nucleic Acids Res. 32, e99.
Dadoune, J.P., Pawlak, A., Alfonsi, M.F., Siffroi, J.P., 2004. Identification of transcripts by macroarrays, RT-PCR and in situ hybridization in human ejaculate spermatozoa. Mol. Hum. Reprod. 11, 133–140.
Evertsz, E.M., Au-Young, J., Ruvolo, M.V., Lim, A.C., Reynolds, M.A., 2001. Hybridization cross-reactivity within homologous gene families on glass cDNA microarrays. Biotechniques, 31, 1182, 1184, 1186 passim.
Fessehaie, A., De Boer, S.H., Lévesque, C.A., 2003. An oligonucleotide array for the identification and differentiation of bacteria pathogenic on potato. Phytopathology 93, 262–269.
Gilbert, C.A., Zhang, N., Hutmacher, R.B., Davis, R.M., Smart, C.D., 2008. Development of a DNA-based macroarray for the detection and identification of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue. J. Cotton Sci. 12, 165–170.
Goff, L.A., Yang, M., Bowers, J., Getts, R.C., Padgett, R.W., Hart, R.P., 2005. Rational probe optimization and enhanced detection strategy for microRNAs using microarrays. RNA Biol. 2, E9–E16.
Guo, Z., Liu, Q., Smith, L.M., 1997. Enhanced discrimination of single nucleotide polymorphisms by artificial mismatch hybridization. Nat. Biotechnol. 15, 331–335.
Ivnitski, D., O'Neil, D.J., Gattuso, A., Schlicht, R., Calidonna, M., Fisher, R., 2003. Nucleic acid approaches for detection and identification of biological warfare and infectious disease agents. Biotechniques 35, 862–869.
Kothapalli, R., Yoder, S.J., Mane, S., Loughran Jr., T.P., 2002. Microarray results: how accurate are they? BMC Bioinformatics 3, 22.
Kreil, D.P., Russell, R.R., Russell, S., 2006. Microarray oligonucleotide probes. Methods Enzymol. 410, 73–98.
Lee, D.-Y., Seto, P., Korczak, R., 2010. DNA microarray-based detection and identification of waterborne protozoan pathogens. J. Microbiol. Methods 65, 453–467.
Li, F., Stormo, G.D., 2001. Selection of optimal DNA oligos for gene expression arrays. Bioinformatics 17, 1067–1076.
Lievens, B., Thomma, B.P.H.J., 2005. Recent developments in pathogen detection arrays: implications for fungal plant pathogens and use in practice. Phytopathology 95, 1374–1380.
Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Lévesque, C.A., Cammue, B.P.A., Thomma, B.P.H.J., 2003. Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. FEMS Microbiol. Lett. 223, 113–122.
Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Lévesque, C.A., Cammue, B.P.A., Thomma, B.P.H.J., 2005. Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. Environ. Microbiol. 7, 1698–1710.
Lievens, B., van Kerckhove, S., Justé, A., Cammue, B.P.A., Honnay, O., Jacquemyn, H., 2010. From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi. J. Microbiol. Methods 80, 76–85.
López, M.M., Bertolini, E., Olmos, A., Caruso, P., Gorris, M.T., Llop, P., Penyalver, R., Cambra, M., 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. Int. Microbiol. 6, 233–243.
Loy, A., Bodrossy, L., 2006. Highly parallel microbial diagnostics using oligonucleotide microarrays. Clin. Chim. Acta 363, 106–119.
Maoka, T., Sugiyama, S., Maruta, Y., Hataya, T., 2010. Application of cDNA macroarray for simultaneous detection of 12 potato viruses. Plant Dis. 94, 1248–1254.
Saiki, R.K., Walsh, P.S., Levenson, C.H., Erlich, H.A., 1989. Genetic-analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc. Natl. Acad. Sci. U. S. A. 86, 6230–6234.
Schliep, A., Schönhuth, A., Steinhoff, C., 2003a. Using hidden Markov models to analyze gene expression time course data. Bioinformatics 19, I264–I272.
Schliep, A., Torney, D.C., Rahmann, S., 2003b. Group testing with DNA chips: generating designs and decoding experiments. Proceedings of the Computational Systems Bioinformatics, August 11–14, Stanford, CA, pp. 84–91.
Selinger, D.W., Cheung, K.J., Mei, R., Johansson, E.M., Richmond, C.S., Blattner, F.R., Lockhart, D.J., Church, G.M., 2000. RNA expression analysis using a 30 base pair resolution *Escherichia coli* genome array. Nat. Biotechnol. 18, 1262–1268.
Sergeev, N., Volokhov, D., Chizhikov, V., Rasooly, A., 2004. Simultaneous analysis of multiple staphylococcal enterotoxin genes by an oligonucleotide microarray assay. J. Clin. Microbiol. 42, 2134–2143.
Shchepinov, M.S., Case-Green, S.C., Southern, E.M., 1997. Steric factors influencing hybridisation of nucleic acids to oligonucleotide arrays. Nucleic Acids Res. 25, 1155–1161.
Sholberg, P., O'Gorman, D., Bedford, K., Lévesque, C.A., 2005. Development of a DNA macroarray for detection and monitoring of economically important apple diseases. Plant Dis. 89, 1143–1150.
Southern, E., Mir, K., Shchepinov, M., 1999. Molecular interactions on microarrays. Nat. Genet. 21, 5–9.
Stowell, L.J., Gelernter, W.D., 2001. Diagnosis of turfgrass diseases. Annu. Rev. Phytopathol. 39, 135–155.
Uddin, W., Viji, G., Schumann, G.L., Boyd, S.H., 2003. Detection of *Pyricularia grisea* causing gray leaf spot of perennial ryegrass turf by a rapid immuno-recognition assay. Plant Dis. 87, 772–778.
Uttamchandani, M., Neo, J.L., Ong, B.N.Z., Moochhala, S., 2009. Applications of microarrays in pathogen detection and biodefence. Trends Biotechnol. 27, 53–61.
van Doorn, R., Slawiak, M., Szemes, M., Dullemans, A.M., Bonants, P., Kowalchuk, G.A., Schoen, C.D., 2009. Robust detection and identification of multiple oomycetes and fungi in environmental samples by using a novel cleavable padlock probe. Appl. Environ. Microbiol. 75, 4185–4193.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.
Wolfel, R., Paweska, J.T., Petersen, N., Grobbelaar, A.A., Leman, P.A., Hewson, R., Georges-Courbot, M.-C., Papa, A., Heiser, V., Panning, M., Gunther, S., Drosten, C., 2009. Low-density macroarray for rapid detection and identification of Crimean-Congo hemorrhagic fever virus. J. Clin. Microbiol. 47, 1025–1030.
Wong, C.W., Heng, C.L.W., Yee, L.W., Soh, S.W.L., Kartasasmita, C.B., Simoes, E.A.F., Hibberd, M.L., Sung, W.K., Miller, L.D., 2007. Optimization and clinical validation of a pathogen detection microarray. Genome Biol. 8, R93.
Zhang, N., Geiser, D.M., Smart, C.D., 2007. Macroarray detection of solanaceous plant pathogens in the *Fusarium solani* species complex. Plant Dis. 91, 1612–1620.
Zhang, N., McCarthy, M.L., Smart, C.D., 2008. A macroarray system for the detection of fungal and oomycete pathogens of solanaceous crops. Plant Dis. 92, 953–960.