Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis

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Abstract

The lack of a rapid and reliable means for routine pathogen identification has been one of the main limitations in plant disease management, and has pushed the development of culture-independent, molecular approaches. Currently, DNA array technology is the most suitable technique for high-throughput detection and identification, as well as quantification, of multiple pathogens in a single assay. Closely related pathogens that may have completely different host ranges or pathogenicity often differ in only a single to a few base pairs in genes that may be targeted for identification. Therefore, the ability to discriminate single nucleotide polymorphisms (SNPs) should be pursued in any diagnostic assay. In this paper, we demonstrate the utility of DNA array technology to detect SNPs while accounting for specific criteria such as the position of the mismatch, the sequence of the oligonucleotide, and the length and amount of labeled amplicons that are hybridized. When disregarding mismatches at the extreme ends of the oligonucleotides, cross hybridization to single mismatch oligonucleotides is rare when processing environmental samples that contain genetic material from unknown sources. In addition to plant pathology, this study is relevant for any field of research where DNA arrays are used to detect mutations or polymorphisms, ranging from human diagnostics to environmental microbiology and microbial ecology.

Introduction

Accurate detection, identification, and quantification of plant pathogens are fundamental to plant disease management. The failure of traditional methods to identify plant pathogenic microorganisms quickly and adequately on a routine basis has led to the development of culture-independent, highly specific molecular detection and identification techniques (McCartney *et al*., 2003; Lievens *et al*., 2005b). DNA array technology is currently the only technology that enables detection and identification of many pathogens in a single assay (Martin *et al*., 2000; Lévesque, 2001; Lievens *et al*., 2003; Lievens & Thomma, 2005). With this technology, detector oligonucleotides are immobilized on a solid support and used for target microorganism detection (Martin *et al*., 2000; Lévesque, 2001; Lievens *et al*., 2003; Lievens & Thomma, 2005). Generally, target DNA is PCR amplified and labeled using consensus primers spanning a genomic region harboring microorganism-specific sequences. Subsequently, labeled amplicons are hybridized to the array. In theory, an unlimited amount of organisms can be identified using a single PCR, provided that sufficient polymorphism(s) exist within the genomic target region. DNA array technology was originally developed to screen for human genetic disorders (Saiki *et al*., 1989; Kawasaki & Chehab, 1994), and has since then also been applied for plant pathogen diagnosis (Lévesque *et al*., 1998; Uehara *et al*., 1999; Boonham *et al*., 2003; Fessehaie *et al*., 2003; Lievens *et al*., 2003, 2004; Nicolaisen *et al*., 2005). Recently, target quantification has been established using DNA arrays (Lievens *et al*., 2005a), even enhancing their appeal for plant disease management.

Generally, ubiquitously conserved genes are targeted for molecular diagnostics (McCartney *et al*., 2003; Lievens *et al*., 2005b; Lievens & Thomma, 2005). Currently, the ribosomal RNA (rRNA) gene and internal transcribed spacer (ITS) regions are most commonly targeted for several reasons. First, the rRNA gene occurs in high copy numbers, allowing sensitive detection. Second, rRNA gene sequences have been extensively used in phylogenetic studies, and large numbers of ribosomal sequences are publicly available, facilitating the validation of the reliability of the assay. Third, variable ITS

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regions allowing discrimination over a wide range of taxonomic levels are flanked by highly conserved sequences, allowing the design of consensus primers targeting many (unrelated) species (White *et al*., 1990).

The high discriminatory potential of immobilized detector oligonucleotides is crucial for diagnostic applications, as closely related microbial species may differ in only a single base pair [single nucleotide polymorphism (SNP)] for a target gene (Nazar *et al*., 1991; Cooke *et al*., 2000). As a consequence, discrimination of SNPs should be pursued for diagnostic assays. Factors such as the number and type of the mismatch, as well as its position, are believed to play an important role in hybridization kinetics, and it is generally accepted that center mismatches are the most destabilizing (Kawasaki & Chehab, 1994; Bodrossy *et al*., 2003). However, it has never been explored as to the number or position at which mismatches significantly influence the diagnostic assay. In general, relatively high amplicon concentrations are used to generate strong, unambiguous hybridization signals (Lévesque *et al*., 1998; Uehara *et al*., 1999; Fessehaie *et al*., 2003; Lievens *et al*., 2003). However, potentially, the lack of oligonucleotide specificity combined with the hybridization of an excess of amplicons increases the risk of 'false positives'.

In this paper, the discriminating power of immobilized oligonucleotides is assessed, aiming at the discrimination of SNPs. Multiple oligonucleotides were mutated at one or more positions and used for hybridization using different concentrations of labeled PCR products. In addition, hybridization assays were carried out using DNA dilutions representing those from environmental samples, and with DNA extracts from naturally infested plant, soil, and water samples.

Materials and methods

Fungal isolates and cultivation

The fungal isolates *Fusarium oxysporum* f. sp. *lycopersici* CBS 645.78, *Verticillium dahliae* CBS 381.66 (CBS, Utrecht, the Netherlands), *Phytophthora nicotianae* MUCL 28775, and *Pythium ultimum* MUCL 16164 (MUCL, Louvain-la-Neuve, Belgium) were cultured on potato dextrose agar in the dark at 24 °C.

DNA extraction

Genomic DNA from pure cultures was extracted as described previously (Lievens *et al*., 2003). For DNA isolation from soil and plant samples, genomic DNA was extracted from 0.75 g sample material using the UltraClean Soil DNA Isolation Kit and the UltraClean Plant DNA Isolation Kit, respectively, according to the manufacturer's protocol (Mo Bio Laboratories, Solana Beach, CA), and subsequently diluted 10-fold. For water samples, DNA was isolated using the UltraClean Water DNA Isolation Kit, as described by the manufacturer (Mo Bio Laboratories). DNA yield was determined spectrophotometrically.

Selection of oligonucleotides

In order to test the discriminatory potential of arrayed detector oligonucleotides, oligonucleotides were selected from ITS sequences and altered at various positions (Table 1). To generalize the results obtained, perfect match oligonucleotides were selected from either ITS I or ITS II sequences, and originated from four unrelated species: *F. oxysporum*, *Phytophthora nicotianae*, *Pythium ultimum*, and *V. dahliae* (Fig. 1). The length of these oligonucleotides was adjusted to obtain detector sequences with a melting temperature of 55 ± 5 °C, calculated using the nearest-neighbor method. The ability to form dimers and hairpin structures was verified using Vector NTI software, and oligonucleotides with the lowest tendency to form such structures were chosen. For each species, initially several perfect match detector oligonucleotides were designed, of which oligonucleotides that provided the most consistent hybridization signals were selected for this study. These encompassed Fox1 and Fox2, Pni1 and Pni2, Pul1 and Pul2, and Vda1, to detect *F. oxysporum*, *Phytophthora nicotianae*, *Pythium ultimum*, and *V. dahliae*, respectively. Although these oligonucleotides differ in length, GC content, and origin, these oligonucleotides all provide uniform and strong hybridization signals upon hybridization with 10 ng labeled target amplicons per milliliter of hybridization buffer (data not shown), and were therefore selected. To explore the discriminatory power of immobilized oligonucleotides, nucleotides were substituted (Table 1). In addition to these oligonucleotides, a digoxigenin-labeled control oligonucleotide Dig1 (Lievens *et al*., 2003, 2005a) was used as a reference for detection and calibration.

DNA array production

Oligonucleotides were synthesized with a 5′ NH_2 group followed by a C6 linker, and DNA arrays were produced as previously described (Lievens *et al*., 2003, 2005a). Briefly, 8.0 fmol of the oligonucleotides was spotted in duplicate on Immunodyne ABC membrane strips (PALL Europe Limited, Portsmouth, UK) using a 384-pin replicator (V & P Scientific, San Diego, CA) to create different DNA arrays (Table 1). For the reference oligonucleotide Dig1, 2.0 fmol was printed. Membranes were air-dried, blocked for 30 min at room temperature, again air-dried, and stored at room temperature until use.

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Table 1. Sequences of match[*] and mismatch[†] detector oligonucleotides used in this study[‡]

Array[‡]	Code[*]	Sequence[†] (5′–3′)	Substitution[§]	Length	T_m[¶] (°C)	GC (%)	Target[‖]
1	Fox1[*,**]	TTGGGACTCGCGTTAATTCG		20	55.4	50.0	ITS II
	Fox1-1	**A**TGGGACTCGCGTTAATTCG	T1A	20	55.2	50.0	
	Fox1-2	**C**TGGGACTCGCGTTAATTCG	T1C	20	55.9	55.0	
	Fox1-3	**G**TGGGACTCGCGTTAATTCG	T1G	20	56.2	55.0	
	Fox1-4	TTGG**C**ACTCGCGTTAATTCG	G5C	20	56.1	50.0	
	Fox1-5	TTGG**A**ACTCGCGTTAATTCG	G5A	20	53.3	45.0	
	Fox1-6	TTGG**T**ACTCGCGTTAATTCG	G5T	20	52.8	45.0	
	Fox1-7	TTGGGACTC**C**CGTTAATTCG	G10C	20	54.7	50.0	
	Fox1-8	TTGGGACTC**A**CGTTAATTCG	G10A	20	52.6	45.0	
	Fox1-9	TTGGGACTC**T**CGTTAATTCG	G10T	20	52.1	45.0	
	Fox1-10	TTGGGACTCGCGTT**T**ATTCG	A15T	20	55.4	50.0	
	Fox1-11	TTGGGACTCGCGTT**C**ATTCG	A15C	20	57.9	55.0	
	Fox1-12	TTGGGACTCGCGTT**G**ATTCG	A15G	20	57.9	55.0	
	Fox1-13	TTGGGACTCGCGTTAATTC**C**	G20C	20	55.2	50.0	
	Fox1-14	TTGGGACTCGCGTTAATTC**A**	G20A	20	54.4	45.0	
	Fox1-15	TTGGGACTCGCGTTAATTC**T**	G20T	20	54.1	45.0	
	Fox1-16	**AA**GGGACTCGCGTTAATTCG	T1A; T2A	20	55.2	50.0	
	Fox1-17	TTGG**CT**CTCGCGTTAATTCG	G5C; A6T	20	55.6	50.0	
	Fox1-18	TTGGGACTC**CG**GTTAATTCG	G10C; C11G	20	54.7	50.0	
	Fox1-19	TTGGGACTCGCGTT**TT**TTCG	A15T; A16T	20	56.3	50.0	
	Fox1-20	TTGGGACTCGCGTTAATT**GC**	C19G; G20C	20	56.0	50.0	
	Fox1-21	**A**TGG**C**ACTCGCGTTAATTCG	T1A; G5C	20	56.0	50.0	
	Fox1-22	**A**TGGGACTC**C**CGTTAATTCG	T1A. G10C	20	54.6	50.0	
	Fox1-23	**A**TGGGACTCGCGTT**T**ATTCG	T1A; A15T	20	55.2	50.0	
	Fox1-24	**A**TGGGACTCGCGTTAATTC**C**	T1A; G20C	20	55.1	50.0	
	Fox1-25	TTGG**C**ACTC**C**CGTTAATTCG	G5C; G10C	20	55.5	50.0	
	Fox1-26	TTGG**C**ACTCGCGTTA**T**TTCG	G5C; A15T	20	56.1	50.0	
	Fox1-27	TTGG**C**ACTCGCGTTAATTC**C**	G5C; G20C	20	56.0	50.0	
	Fox1-28	TTGGGACTC**C**CGTTA**T**TTCG	G10C; G20C	20	54.7	50.0	
	Fox1-29	TTGGGACTC**C**CGTTAATTC**C**	G10C; G20C	20	54.5	50.0	
	Fox1-30	TTGGGACTCGCGTT**T**ATTC**C**	A15T;G20C	20	55.2	50.0	
	Fox1-31	**AA**GGGACTCGCGTTAATTC**C**	T1A; T2A; G20C	20	55.0	50.0	
	Fox1-32	**A**TGGGACTCGCGTTAATT**GC**	T1A; C19G; G20C	20	55.8	50.0	
	Fox1-33	**AA**GGGACTCGCGTTAATT**GC**	T1A; T2A; C19G; G20C	20	55.7	50.0	
2	Fox2[*,**]	GTTGGGACTCGCGTTAATTCG		21	56.4	52.4	ITS II
	Fox2-1	GTTG**C**GACTCGCGTTAATTCG	G5C	21	56.9	52.4	
	Fox2-2	GTTGGGACT**G**GCGTTAATTCG	C10G	21	56.5	52.4	
	Fox2-3	GTTGGGACTCGCGT**A**AATTCG	T15A	21	56.4	52.4	
3	Pni1[*]	AAAAAAGACTACTAAATCAGGCC		23	51.0	34.8	ITS I
	Pni1-1	AAAA**T**AGACTACTAAATCAGGCC	A5T	23	50.2	34.8	
	Pni1-2	AAAAAAGAC**A**ACTAAATCAGGCC	T10A	23	51.9	34.8	
	Pni1-3	AAAAAAGACTACTA**T**ATCAGGCC	A15T	23	50.2	34.8	
	Pni1-4	AAAAAAGACTACTAAATCA**C**GCC	G20C	23	51.4	34.8	
4	Pni2[*]	TTTGGGAACTTAATGTGTACTTC		23	51.0	34.8	ITS II
	Pni2-1	TTTG**C**GAACTTAATGTGTACTTC	G5C	23	51.6	34.8	
	Pni2-2	TTTGGGAAC**A**TAATGTGTACTTC	T10A	23	51.0	34.8	
	Pni2-3	TTTGGGAACTTAAT**C**TGTACTTC	G15C	23	50.5	34.8	
	Pni2-4	TTTGGGAACTTAATGTGTA**G**TTC	C20G	23	51.0	34.8	
5	Pul1[††,‡‡]	TGCTGACTCCCGTTCCAGTG		20	59.6	60.0	ITS I
	Pul1-1	**A**GCTGACTCCCGTTCCAGTG	T1A	20	59.3	60.0	
	Pul1-2	**C**GCTGACTCCCGTTCCAGTG	T1C	20	60.5	65.0	
	Pul1-3	**G**GCTGACTCCCGTTCCAGTG	T1G	20	60.4	65.0	
	Pul1–4	TGCT**C**ACTCCCGTTCCAGTG	G5C	20	59.6	60.0	
	Pul1-5	TGCT**A**ACTCCCGTTCCAGTG	G5A	20	57.0	55.0	
	Pul1-6	TGCT**T**ACTCCCGTTCCAGTG	G5T	20	57.0	55.0	
	Pul1-7	TGCTGACTC**G**CGTTCCAGTG	C10G	20	60.1	60.0	
	Pul1-8	TGCTGACTC**A**CGTTCCAGTG	C10A	20	57.3	55.0	

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Table 1. Continued.

Array[‡]	Code[*]	Sequence[†] (5′–3′)	Substitution[§]	Length	T_m[¶] (°C)	GC (%)	Target[‖]
	Pul1-9	TGCTGACTC**T**CGTTCCAGTG	C10T	20	56.9	55.0	
	Pul1-10	TGCTGACTCCCGTT**G**CAGTG	C15G	20	60.3	60.0	
	Pul1-11	TGCTGACTCCCGTT**A**CAGTG	C15A	20	56.9	55.0	
	Pul1-12	TGCTGACTCCCGTT**T**CAGTG	C15T	20	57.4	55.0	
	Pul1–13	TGCTGACTCCCGTTCCAGT**C**	G20C	20	59.3	60.0	
	Pul1-14	TGCTGACTCCCGTTCCAGT**A**	G20A	20	58.0	55.0	
	Pul1-15	TGCTGACTCCCGTTCCAGT**T**	G20T	20	58.8	55.0	
	Pul1-16	**AC**CTGACTCCCGTTCCAGTG	T1A; G2C	20	59.1	60.0	
	Pul1-17	TGCT**CT**CTCCCGTTCCAGTG	G5C; A6T	20	59.1	60.0	
	Pul1-18	TGCTGACTC**GG**GTTCCAGTG	G10C; C11G	20	59.6	60.0	
	Pul1-19	TGCTGACTCCCGTT**GG**AGTG	C15G; C16G	20	59.6	60.0	
	Pul1-20	TGCTGACTCCCGTTCCAG**AC**	T19A; G20C	20	59.3	60.0	
	Pul1-21	**A**GCT**C**ACTCCCGTTCCAGTG	T1A; G5C	20	59.3	60.0	
	Pul1-22	**A**GCTGACTC**G**CGTTCCAGTG	T1A; C10G	20	59.9	60.0	
	Pul1-23	**A**GCTGACTCCCGTT**G**CAGTG	T1A; C15G	20	60.1	60.0	
	Pul1-24	**A**GCTGACTCCCGTTCCAGT**C**	T1A; G20C	20	59.1	60.0	
	Pul1-25	TGCT**C**ACTC**G**CGTTCCAGTG	G5C; C10G	20	60.1	60.0	
	Pul1-26	TGCT**C**ACTCCCGTT**G**CAGTG	G5C; C15G	20	60.3	60.0	
	Pul1-27	TGCT**C**ACTCCCGTTCCAGT**C**	G5C; G20C	20	59.3	60.0	
	Pul1-28	TGCTGACTC**G**CGTT**G**CAGTG	C10G; C15G	20	60.8	60.0	
	Pul1-29	TGCTGACTC**G**CGTTCCAGT**C**	C10G; G20C	20	59.9	60.0	
	Pul1-30	TGCTGACTCCCGTT**G**CAGT**C**	C15G;G20C	20	60.0	60.0	
	Pul1-31	**ACG**TGACTCCCGTTCCAGTG	T1A; G2C; C3G	20	59.4	60.0	
	Pul1-32	TGCT**CTG**TCCCGTTCCAGTG	G5C; A6T; C7G	20	59.6	60.0	
	Pul1-33	TGCTGACTC**GGC**TTCCAGTG	C10G; C11G, G12C	20	59.8	60.0	
	Pul1-34	TGCTGACTCCCGTT**GGT**GTG	C15G; C16G; A17T	20	60.0	60.0	
	Pul1-35	TGCTGACTCCCGTTCCA**CAC**	G18C; T19A; G20C	20	59.8	60.0	
	Pul1-36	**A**GCT**C**ACTC**G**CGTTCCAGTG	T1A; G5C; C10G	20	59.9	60.0	
	Pul1-37	TGCT**C**ACTC**G**CGTT**G**CAGTG	G5C; C10G; C15G	20	60.8	60.0	
	Pul1-38	TGCTGACTC**G**CGTT**G**CAGT**C**	C10G; C15G; G20C	20	60.6	60.0	
	Pul1-39	**AC**CTGACTCCCGTTCCAGA**C**	T1A; G2C; G20C	20	58.8	60.0	
	Pul1-40	**A**GCTGACTCCCGTTCCAG**AC**	T1A; T19A; G20C	20	59.1	60.0	
	Pul1-41	**AC**CTGACTCCCGTTCCAG**TC**	T1A; G2C; A19T; G20C	20	58.8	60.0	
	Pul1-42	**ACG**TGACTCCCGTTCCA**CAC**	T1A; G2C; C3G; G18C; T19A; G20C	20	59.7	60.0	
	Pul1-43	**AGC**T**C**ACTC**G**CGTTC**G**AGT**C**	T1A; C2G; G3C; G5C; C10G; C15G; G20C	20	59.5	60.0	
6	Pul2[*]	TGTATGGAGACGCTGCATTT		20	54.5	45.0	ITS II
	Pul2-1	TGTA**A**GGAGACGCTGCATTT	T5A	20	54.4	45.0	
	Pul2-2	TGTATGGAG**T**CGCTGCATTT	A10T	20	54.5	45.0	
	Pul2-3	TGTATGGAGACGCT**C**CATTT	G15C	20	53.7	45.0	
7	Vda1[*,**]	AACAGAGAGACTGATGGACCG		21	56.2	52.4	ITS I
	Vda1-1	AACA**C**AGAGACTGATGGACCG	G5C	21	56.7	52.4	
	Vda1-2	AACAGAGAG**T**CTGATGGACCG	A10T	21	56.2	52.4	
	Vda1-3	AACAGAGAGACTGA**A**GGACCG	T15A	21	56.1	52.4	

[*]100% match oligonucleotides.

[†]Nucleotide substitutions are in bold and underlined.

[‡]In total seven arrays were designed. Specificity of the oligonucleotides was tested with labeled amplicons from *Fusarium oxysporum* (arrays 1 and 2), *Phytophthora nicotianae* (arrays 3 and 4), *Pythium ultimum* (arrays 5 and 6), and *Verticillium dahliae* (array 7).

[§]Notation is as follows: the first character indicates the original and substituted nucleotide at the position indicated by the second character. The third character indicates the nucleotide by which the original one is substituted.

[¶]Melting temperature calculated using the nearest-neighbor method.

[‖] Target of the 100% match oligonucleotide.

[**]Lievens *et al*. (2003).

[††]Lévesque *et al*. (1998).

[‡‡]Lievens *et al*. (2005a).

ITS, internal transcribed spacer.

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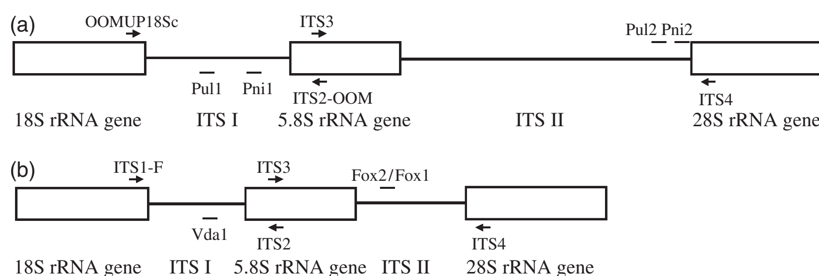

Fig. 1. Schematic representation of an oomycete (a) and fungal (b) ribosomal cistron showing the location of the PCR primers (→) and the detector oligonucleotides (—) used in this study. Sense sequences are indicated at the top and antisense sequences at the bottom of the cistron.

PCR amplification and labeling

Target ITS regions were amplified and simultaneously labeled with alkaline-labile digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany). The region between the small and the large subunit of the rRNA gene was amplified using ITS1-F and ITS4 (Gardes & Bruns, 1993) or OOMUP18Sc and ITS4 (Lievens *et al.*, 2004), for fungi or oomycetes, respectively. Depending on the target's origin, ITS I sequences were amplified using the fungal primer set ITS1-F and ITS2 (White *et al.*, 1990) or the oomycete primer set OOMUP18Sc and ITS2-OOM (5′-GCAGCGTTCTTCATCGATGT-3′). In order to amplify the ITS II region, ITS3 was combined with ITS4 (White *et al.*, 1990). Samples were amplified in 20 μL, containing 5 ng genomic DNA from a pure fungal culture or 1 μL DNA from an environmental sample. Amplification was performed using 1 U Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA) in the presence of 7.5 μM digoxigenin-11-d-UTP (Dig-dUTP; Roche Diagnostics GmbH). Before amplification, DNA samples were denatured at 94 °C for 2 min. Next, 35 cycles were run consisting of 45 s at 94 °C, 45 s at 59 °C, and 45 s at 72 °C, with a final extension at 72 °C for 10 min. After gel electrophoresis, the resulting Dig-dUTP-labeled amplicons were quantified by comparison with a DNA ladder (Smartladder SF, Eurogentec, Seraing, Belgium) using Labworks 4.0 Image Acquisition and Analysis Software (UVP, Upland, CA).

DNA array hybridization

Hybridization was carried out as previously described (Lievens *et al.*, 2003, 2005a). Prior to hybridization, membranes were prehybridized for 1.5 h at 54 °C in hybridization buffer [6 × SSC, 0.1% sarcosine, and 0.02% sodium dodecyl sulfate (SDS)] amended with 1% casein. Labeled amplicons were denatured by boiling for 10 min and subsequently hybridized overnight to the array in 6 mL of hybridization buffer at 54 °C. Hybridization was followed by two washes in stringency buffer (6 × SSC and 0.1% SDS) at hybridization temperature and three final washes in washing solution (0.1 M maleic acid and 0.15 M sodium chloride; pH 7.5) at room temperature. Detection of digoxigenin was performed using antidigoxigenin alkaline phosphatase conjugate and CDP-Star substrate (both from Roche Diagnostics GmbH). Chemiluminescence was detected cumulatively within 45 min at intervals of 30 s using a highly sensitive digital CCD camera (BioChemi System; UVP). Hybridization signals were quantified and analyzed using Labworks 4.0 Image Acquisition and Analysis Software. Hybridization strength was reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). All assays were conducted at least twice.

Results and discussion

Hybridization of amplicons from pure cultures

DNA array technology is a powerful tool for rapid identification of multiple microbial species in a single sample (Lévesque *et al.*, 1998; Uehara *et al.*, 1999; Fessehaie *et al.*, 2003; Lievens *et al.*, 2003). However, not much is known about the discriminatory potential of the detector oligonucleotides, especially under the high amplicon concentrations used to ensure sensitivity. In this study, ITS I- or ITS II-specific oligonucleotides were mutated (mismatch oligonucleotides; Table 1) and tested for hybridization. Initially, one or more nucleotides were substituted in the *Pythium ultimum* ITS I oligonucleotide Pul1 (Lévesque *et al.*, 1998; Lievens *et al.*, 2005a) (Table 1). In addition to Pul1, 43 mismatch oligonucleotides were arrayed. For hybridization, *Pythium ultimum* ITS I amplicons from different PCR reactions were pooled and used at 1, 10, 100, or 200 ng mL^{-1} of hybridization buffer. With increasing amounts of amplicon, hybridization signals increased along with cross hybridization for the mismatch oligonucleotides (Fig. 2). When only a single nucleotide was substituted, mismatches at the fifth nucleotide were the most selective (Pul1-4, Pul1-5, and Pul1-6; Fig. 2), allowing SNP discrimination irrespective of amplicon amount or the nucleotide used in the substitution. In contrast with mismatches at the extreme 5′ or 3′ end (Pul1-1, Pul1-2, Pul1-3 and Pul1-13, Pul1-14, Pul1-15, respectively), oligonucleotides were the least discriminatory. With two adjacent substitutions at any location, amplicons did not cross hybridize to the mismatch oligonucleotides,

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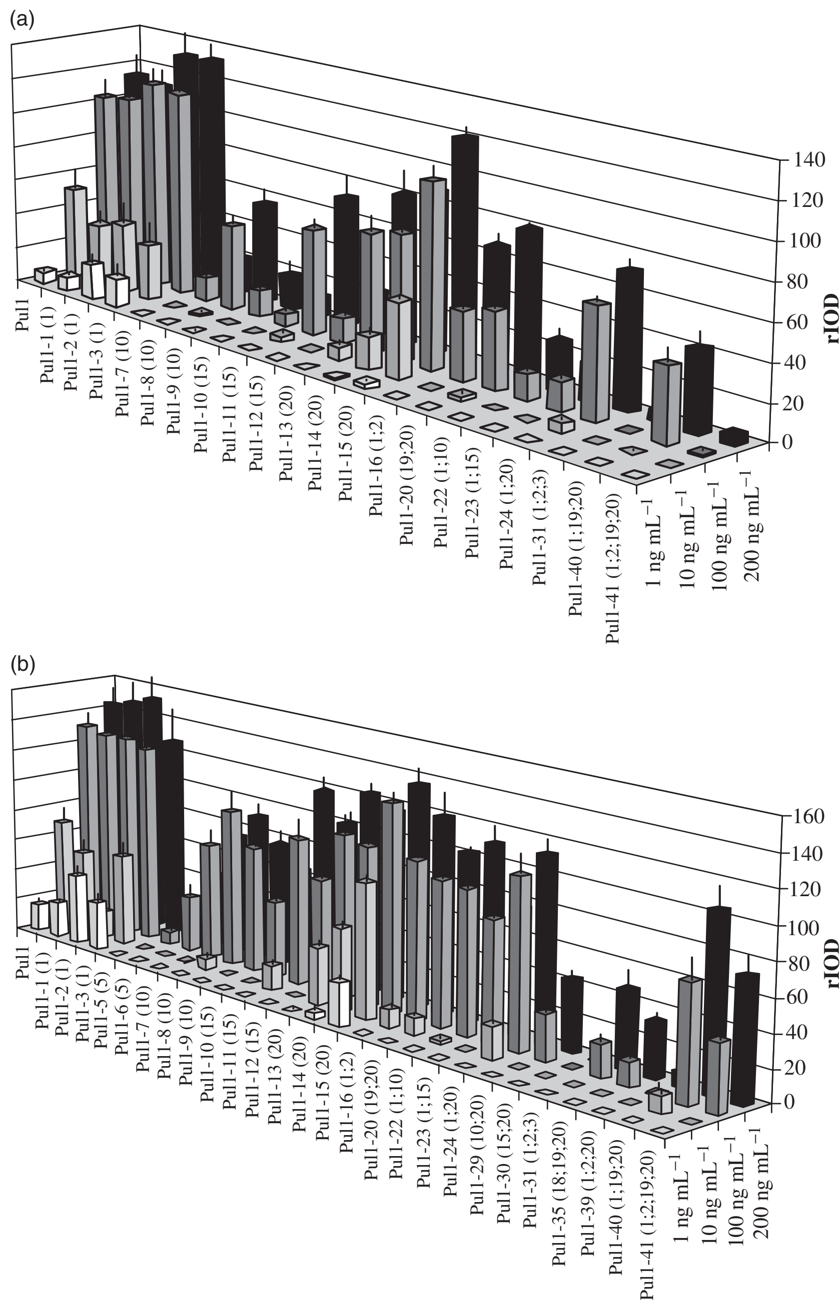

Fig. 2. Discriminatory potential of Pul1-derived mismatch detector oligonucleotides upon hybridization with different amounts of internal transcribed spacer (ITS) I (a) and ITS I-5.8S rRNA gene-ITS II (b) amplicons from *Pythium ultimum*. Results are only shown for those oligonucleotides that resulted in detectable hybridization signals. Mismatch positions are indicated following the code of the oligonucleotide. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means from three hybridization runs ($n = 6$). Error bars indicate standard errors. Before hybridization, amplicons from different PCR reactions were pooled to minimize variability because of differences in DNA amplification.

except when they were positioned at the extreme 5′ end (Pul1-16) or 3′ end (Pul1-20). Similar observations were made with multiple mismatches that were spread throughout the oligonucleotide (Pul1-22, Pul1-23, Pul1-24, Pul1-31, Pul1-40, Pul1-41; Fig. 2). However, no cross hybridization occurred in combination with a mismatch at the fifth nucleotide (Pul1-21), again demonstrating the high selectivity of this nucleotide (Fig. 2).

To test whether amplicon length affects specificity, amplicons were generated from the ITS I-5.8S rRNA gene-ITS II region (*c.* 900 bp, compared with 300 bp for the ITS I amplicons). In all cases, when longer amplicons were hybridized, signal intensities increased slightly because of the larger number of labeled nucleotides incorporated per amplicon, but oligonucleotides also provided lower specificity (Fig. 2). Specificity is enhanced by hybridizing less amplicon. However, although highly specific at an amplicon concentration of 1 ng mL^{-1}, hybridization signals produced by olignucleotide Pul1 were rather weak (Fig. 2).

A similar experiment was conducted for a set of oligonucleotides derived from an *F. oxysporum* ITS II detector sequence, Fox1 (Lievens *et al.*, 2003). As shown in Fig. 3,

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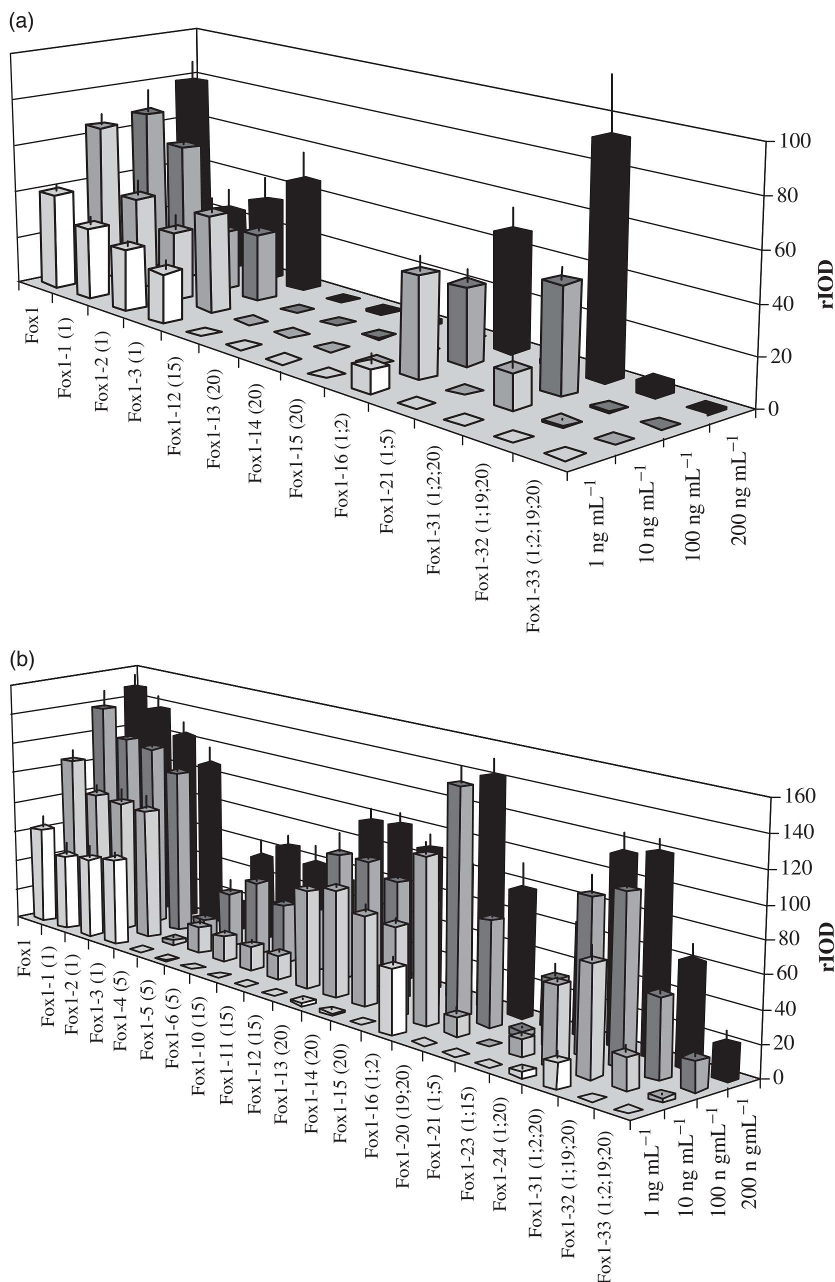

Fig. 3. Discriminatory potential of Fox1-derived mismatch detector oligonucleotides upon hybridization with different amounts of internal transcribed spacer (ITS) II (a) and ITS I-5.8S rRNA gene-ITS II (b) amplicons from *Fusarium oxysporum*. Results are only shown for those oligonucleotides that resulted in detectable hybridization signals. Mismatch positions are indicated following the code of the oligonucleotide. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means from three hybridization runs ($n = 6$). Error bars indicate standard errors. Before hybridization, amplicons from different PCR reactions were pooled to minimize variability because of differences in DNA amplification.

similar results as for Pul1 substitutions were obtained for mutations of Fox1. However, whereas the fifth position was the most selective for the Pul1-derived oligonucleotide, for Fox1 the highest specificity was obtained with a center mismatch oligonucleotide (Fox1-7, Fox1-8, and Fox1-9; Fig. 3). This illustrates that the selectivity of a specific SNP oligonucleotide also depends on its sequence. These observations were confirmed by experiments with other SNP oligonucleotides designed on ITS sequences from *Phytophthora nicotianae* (Pni1 and Pni2) and *V. dahliae* (Vda1) and additional oligonucleotides for *F. oxysporum* (Fox2) and *Pythium ultimum* (Pul2) (Table 2). For Pni2 and Pul2, the 15th nucleotide was found to be the most selective (Pni2-3 and Pul2-3) and for Pni1 it was the 20th nucleotide (Pni1-4). For Fox2 and Vda1 mutations, specificity could not be obtained when hybridizing complete ITS I-5.8S rRNA gene-ITS II amplicons. However, when ITS I amplicons were hybridized, specificity was obtained for all mismatch oligonucleotides under all conditions (Table 2).

Hybridization of amplicons from naturally infested samples

In addition to pathogen identification from pure cultures, DNA array technology is currently the only technology

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Table 2. Discriminatory potential of different single nucleotide mismatch detector oligonucleotides upon hybridization with different amounts of amplicons generated from several fungal isolates

		Hybridization signal (ng mL^{-1})[†]							
		ITS I or ITS II amplicon[‡,§]				ITS I–5.8S rRNA gene–ITS II amplicon[‡]			
Oligonucleotide*	Target organism	200	100	10	1	200	100	10	1
Fox1	*Fusarium oxysporum*	79.1 ± 8.3	68.7 ± 9.2	66.0 ± 4.3	40.4 ± 2.6	148.8 ± 7.2	138.2 ± 9.7	105.7 ± 6.4	62.5 ± 6.2
Fox1-4 (5)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18.3 ± 5.6	11.7 ± 2.3	2.8 ± 0.8	0.0 ± 0.0
Fox1-7 (10)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Fox1-10 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	58.6 ± 10.0	37.2 ± 8.5	15.0 ± 2.2	0.0 ± 0.0
Fox2		67.9 ± 2.3	75.5 ± 5.0	78.5 ± 4.2	57.2 ± 2.8	115.9 ± 1.7	106.1 ± 4.0	81.3 ± 10.8	85.6 ± 5.6
Fox2-1 (5)		0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	84.8 ± 4.2	78.3 ± 3.7	36.4 ± 11.1	2.8 ± 0.9
Fox2-2 (10)		0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	68.8 ± 6.3	60.7 ± 8.6	30.1 ± 9.2	1.1 ± 0.5
Fox2-3 (15)		4.0 ± 0.9	0.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	58.6 ± 15.6	66.2 ± 11.8	61.5 ± 5.3	7.5 ± 2.8
Pni	*Phytophthora nicotianae*	102.2 ± 2.3	99.6 ± 6.7	89.4 ± 7.3	81.9 ± 8.9	135.4 ± 2.0	119.7 ± 1.1	63.6 ± 8.3	68.4 ± 2.7
Pni1-1 (5)		30.5 ± 6.0	33.6 ± 5.8	15.1 ± 2.2	1.6 ± 0.4	98.3 ± 4.5	90.6 ± 6.6	9.6 ± 3.3	0.4 ± 0.1
Pni1-2 (10)		10.8 ± 3.8	14.0 ± 2.2	3.8 ± 0.7	0.4 ± 0.3	79.6 ± 8.8	76.8 ± 5.1	7.6 ± 1.5	0.0 ± 0.0
Pni1-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	38.9 ± 9.5	24.8 ± 5.4	0.0 ± 0.0	0.0 ± 0.0
Pni1-4 (20)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.6
Pni2		88.9 ± 5.1	94.5 ± 2.7	76.9 ± 6.0	40.3 ± 7.7	99.3 ± 7.7	101.6 ± 6.6	93.8 ± 4.1	32.1 ± 5.7
Pni2-1 (5)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	29.0 ± 6.4	13.0 ± 5.5	0.8 ± 0.4	0.0 ± 0.0
Pni2-2 (10)		1.1 ± 0.2	1.2 ± 0.3	0.0 ± 0.0	0.1 ± 0.1	43.0 ± 3.4	14.5 ± 4.1	2.1 ± 0.6	0.0 ± 0.0
Pni2-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pni2-4 (20)		1.4 ± 0.5	1.8 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	55.7 ± 5.6	29.8 ± 4.5	6.9 ± 2.1	0.0 ± 0.0
Pul1	*Pythium ultimum*	114.3 ± 10.2	104.3 ± 9.0	52.1 ± 10.2	6.2 ± 1.1	143.9 ± 6.2	131.8 ± 6.2	69.3 ± 8.0	16.7 ± 2.0
Pul1-4 (5)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pul1-7 (10)		20.4 ± 2.7	13.2 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	75.2 ± 7.5	72.6 ± 7.5	0.4 ± 0.2	0.0 ± 0.0
Pul1-10 (15)		11.3 ± 4.0	6.3 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	64.9 ± 8.3	48.0 ± 8.3	0.0 ± 0.0	0.0 ± 0.0
Pul2		87.3 ± 5.1	108.1 ± 2.8	94.8 ± 0.9	59.8 ± 5.9	119.3 ± 4.2	104.1 ± 4.8	99.8 ± 5.8	75.7 ± 0.9
Pul2-1 (5)		2.0 ± 0.7	9.7 ± 1.1	7.9 ± 2.5	0.2 ± 0.1	73.6 ± 10.3	51.7 ± 5.1	35.2 ± 6.1	0.6 ± 0.2
Pul2-2 (10)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	35.9 ± 8.4	20.5 ± 5.3	10.5 ± 3.0	0.0 ± 0.0
Pul2-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Vda1	*Verticillium dahliae*	85.4 ± 2.7	73.7 ± 4.7	79.8 ± 3.9	63.5 ± 4.3	134.6 ± 5.5	134.1 ± 8.1	88.6 ± 12.2	72.1 ± 13.7
Vda1-1 (5)		0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	105.6 ± 6.0	106.4 ± 4.4	41.8 ± 15.2	2.8 ± 0.9
Vda1-2 (10)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	85.5 ± 5.2	92.2 ± 4.8	29.7 ± 14.1	0.0 ± 0.0
Vda1-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	84.7 ± 5.1	89.9 ± 5.2	45.5 ± 12.4	0.1 ± 0.1

*Mismatch positions are indicated between brackets.

†Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). Values are means ± standard errors ($n = 6$ from three hybridization runs).

‡Before hybridization, amplicons from different PCR reactions were pooled to minimize variability owing to differences in DNA amplification.

§Pul1-, Pni1-, and Vda1-derived detector oligonucleotides were hybridized with ITS I amplicons from the respective fungi whereas Fox1-, Fox2-, Pul2-, and Pni2-derived detector oligonucleotides were hybridized with ITS II amplicons.

ITS, internal transcribed spacer.

allowing identification of many pathogens in environmental samples (Lievens *et al*., 2003, 2004, 2005a; Nicolaisen *et al*., 2005). To investigate whether cross hybridizations to mismatch oligonucleotides are relevant when analyzing environmental samples, practical conditions were mimicked by DNA dilutions reflecting those typically encountered in horticultural practice (Lievens *et al*., 2005a). A 10-fold dilution series of genomic DNA from *F. oxysporum* f. sp. *lycopersici* and *Pythium ultimum*, ranging from 1 ng to 0.1 pg, was amplified and 10 μL of labeled amplicons were hybridized. When disregarding mismatches at the extreme ends, cross hybridization to mismatch oligonucleotides was rare, especially with template amounts equal to or less than 100 pg (Fig. 4). When *Pythium ultimum* amplicons were hybridized, cross hybridization to single mismatch oligonucleotides was only observed for Pul1-8 and Pul1-11, both carrying a C to A mutation, at the tenth and fifteenth positions, respectively (Fig. 4). When *F. oxysporum* amplicons were hybridized, again no cross hybridization was observed for the center mismatch oligonucleotides Fox1-7, Fox1-8, and Fox1-9. Whereas weak signals were obtained for PCR amplification of 1 ng or 100 pg DNA with the oligonucleotides mutated at position 5 or 15, no cross hybridization was observed when amplifying 10 pg DNA or less. Moreover,

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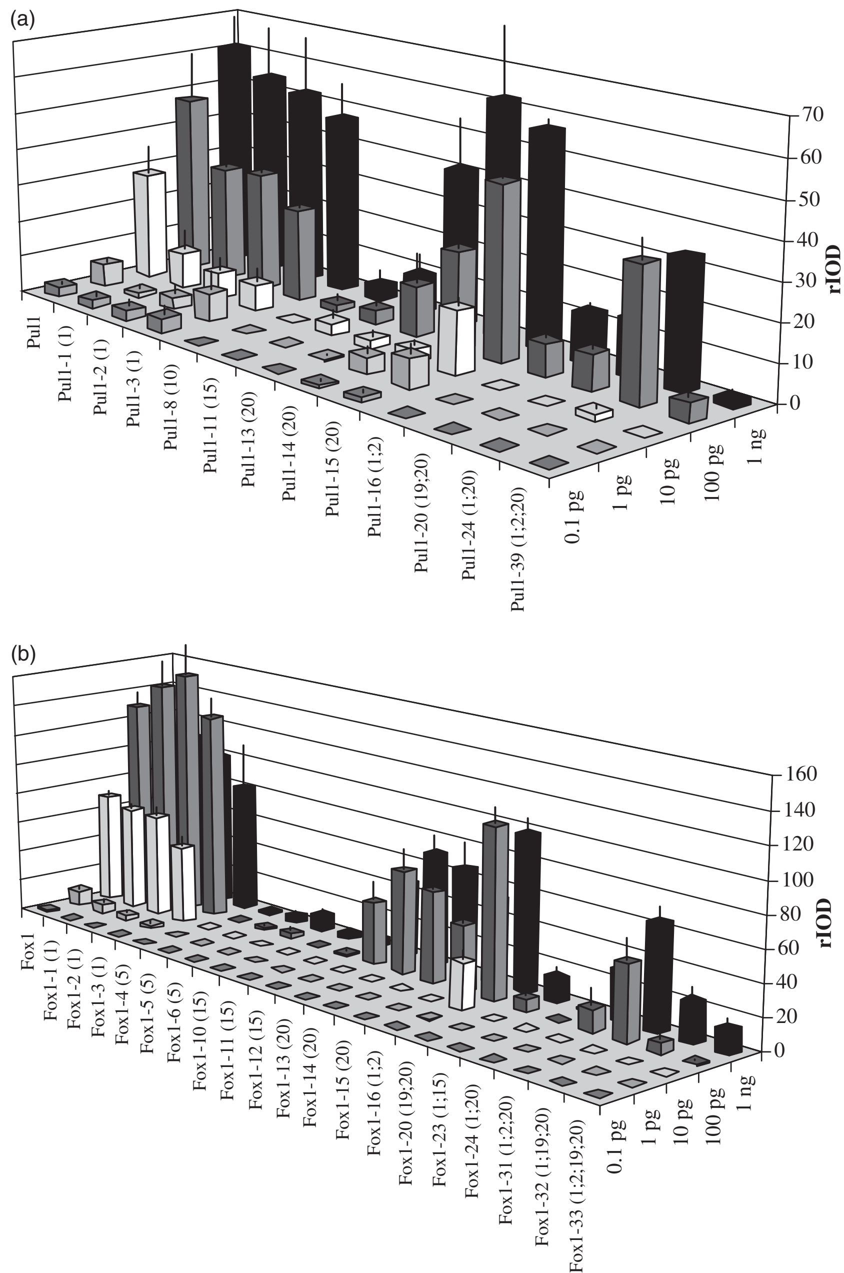

Fig. 4. Discriminatory potential of Pul1- (a) and Fox1- (b) derived detector oligonucleotides after PCR amplification of DNA dilutions. Results are only shown for those oligonucleotides that resulted in detectable hybridization signals. Mismatch positions are indicated following the code of the oligonucleotide. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means from two hybridization runs ($n = 4$). Error bars indicate standard errors.

in these cases, cross hybridization was limited to the oligonucleotides with a 5′ end mismatch (Fig. 4).

Finally, the mismatch *F. oxysporum* and *Pythium ultimum* oligonucleotides were tested for hybridization with amplicons from naturally infested plant, soil, and water samples (Table 3). Ten microliters of labeled amplicons were hybridized in 6 mL of hybridization buffer. As shown in Table 3, cross hybridization was strictly limited to the oligonucleotides with mismatches at one or both extreme ends, except for the analysis of plant sample 04-376B, which was heavily infected with *Pythium ultimum*. For this sample, amplicons additionally hybridized at low levels to Pul1-8 and Pul1-11. Nevertheless, the overall results of these experiments suggest that cross hybridization to oligonucleotides with a mismatch that is not located at one or both extreme ends should not be a major concern when analyzing environmental samples. In addition, real-time PCR quantification of the amount of DNA from the two species tested previously revealed that the hybridization signals for the perfect match oligonucleotides strongly correlated ($R^2 > 0.9$) to the actual amount of DNA (Lievens *et al.*, 2005a), illustrating its power for reliable target quantification. When amplicons corresponding to one of the two ITS regions were hybridized, signals were very weak, even for the signals produced

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Table 3. Discriminatory potential of Pul1- or Fox1-derived detector oligonucleotides* upon hybridization of ITS I–5.8S rRNA gene-ITS II amplicons generated from environmental samples naturally infested with *Pythium ultimum* or *Fusarium oxysporum*, respectively

	Sample ID								
	Soil			Plant				Water	
Oligonucleotide[†]	04–285	04–336C	04–495B	04–349	04–376B	04–398	04–446	04–495E	
Pul1	31.9 ± 6.3[‡]	27.3 ± 5.3	16.1 ± 3.3	21.6 ± 5.8	58.0 ± 10.2	7.3 ± 3.8	57.0 ± 5.4	8.4 ± 1.5	
Pul1-1 (1)	40.5 ± 7.2	26.5 ± 5.1	18.3 ± 5.0	15.0 ± 3.3	72.0 ± 13.6	7.6 ± 4.1	66.7 ± 4.2	10.6 ± 5.2	
Pul1-2 (1)	32.1 ± 5.2	37.2 ± 10.1	17.3 ± 2.0	25.0 ± 8.0	75.0 ± 16.1	7.0 ± 4.2	61.4 ± 8.8	12.6 ± 7.5	
Pul1-3 (1)	23.1 ± 6.2	30.9 ± 10.1	14.8 ± 3.3	27.0 ± 6.6	63.7 ± 17.0	8.8 ± 4.6	41.4 ± 10.9	9.6 ± 5.9	
Pul1-8 (10)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	4.9 ± 2.9	0.2 ± 0.2	0.8 ± 0.7	0.0 ± 0.0	
Pul1-11 (15)	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 2.9	0.0 ± 0.0	0.8 ± 0.7	0.0 ± 0.0	
Pul1-13 (20)	2.6 ± 0.9	0.4 ± 0.4	0.3 ± 0.3	0.1 ± 0.1	28.5 ± 7.1	0.1 ± 0.1	6.2 ± 2.4	0.0 ± 0.0	
Pul1-14 (20)	5.9 ± 1.5	4.5 ± 1.8	1.2 ± 1.1	0.9 ± 0.6	34.4 ± 6.9	0.4 ± 0.4	11.3 ± 4.0	1.4 ± 1.3	
Pul1-15 (20)	20.3 ± 4.4	11.9 ± 3.1	3.4 ± 1.7	6.9 ± 1.6	55.6 ± 7.9	0.5 ± 0.3	51.5 ± 3.6	4.1 ± 0.8	
Pul1-16 (1;2)	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	11.1 ± 2.5	0.0 ± 0.0	10.8 ± 0.7	0.0 ± 0.0	
Pul1-20 (19;20)	0.2 ± 0.1	0.0 ± 0.0	1.1 ± 1.1	0.0 ± 0.0	6.9 ± 3.7	0.0 ± 0.0	5.2 ± 3.0	0.0 ± 0.0	
Pul1-24 (1;20)	0.8 ± 0.3	0.9 ± 0.6	0.0 ± 0.0	0.2 ± 0.1	30.7 ± 5.2	0.0 ± 0.0	16.1 ± 6.0	0.5 ± 0.5	
Pul1-39 (1;2;20)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 2.2	0.0 ± 0.0	5.8 ± 3.0	0.0 ± 0.0	
	04–285			04–348	04–411	04–507		04–494	04–569C
Fox1	11.5 ± 2.2[‡]			33.4 ± 8.1	74.1 ± 12.9	3.3 ± 1.3		36.0 ± 5.2	17.9 ± 8.0
Fox1-1 (1)	8.1 ± 0.7			12.6 ± 2.8	58.9 ± 14.6	1.2 ± 0.7		19.6 ± 3.7	11.0 ± 6.4
Fox1-2 (1)	3.4 ± 0.7			33.8 ± 7.8	79.6 ± 9.7	1.0 ± 0.8		11.6 ± 5.0	3.9 ± 2.3
Fox1-3 (1)	2.3 ± 0.9			49.9 ± 11.2	87.3 ± 8.7	0.7 ± 0.6		2.2 ± 1.3	2.5 ± 0.8
Fox1-13 (20)	0.0 ± 0.0			5.7 ± 3.1	0.1 ± 0.0	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0
Fox1-14 (20)	0.0 ± 0.0			5.3 ± 2.4	0.1 ± 0.1	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0
Fox1-15 (20)	0.0 ± 0.0			7.5 ± 2.7	0.3 ± 0.2	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0
Fox1-16 (1;2)	0.0 ± 0.0			35.1 ± 16.4	59.3 ± 21.5	0.0 ± 0.0		18.4 ± 10.2	8.1 ± 3.0
Fox1-24 (1;20)	0.0 ± 0.0			19.1 ± 9.5	10.6 ± 1.4	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0

*Results are only shown for those oligonucleotides that displayed hybridization signals.

[†]Mismatch positions are indicated between brackets.

[‡]Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). Values are means ± standard errors ($n = 4$ from two hybridization runs).

ITS, internal transcribed spacer.

by the perfect match oligonucleotides. In addition, in some cases even no signals were observed (data not shown) demonstrating that, under the labeling conditions used, relatively short amplicons are not suitable for assessing pathogen presence in environmental samples.

The results of this study illustrate the high specificity that can be obtained with DNA arrays, even allowing discrimination of single nucleotide differences. As a consequence, when using the appropriate oligonucleotide sequences, closely related microbial species can be easily differentiated by this technology, as also illustrated by other studies (Lévesque *et al.*, 1998; Uehara *et al.*, 1999; Fessehaie *et al.*, 2003; Lievens *et al.*, 2003; Nicolaisen *et al.*, 2005). Furthermore, we demonstrated that center mismatches do not always provide the highest degree of specificity; the discriminatory potential of a single mismatch oligonucleotide depends on the sequence of the oligonucleotide used. As a consequence, in order to differentiate SNPs, multiple oligonucleotides harboring the unique polymorphism at different positions should be screened for specificity when developing an oligonucleotide array. Nevertheless, based on our results hybridization can generally be prevented when the mismatch occurs in the 3′ half of the immobilized oligonucleotide. Apart from this, we showed that hybridization of 10 ng amplicons mL^{-1} hybridization buffer should be an appropriate concentration when pure cultures need to be identified. When disregarding mismatches at the extreme ends, cross-hybridization signals are generally weak at this amplicon concentration and do not interfere with recognition of specific signals. However, to increase assay specificity, multiple detector oligonucleotides can be spotted for a single pathogen. Ultimately, it may be advantageous to include multiple diagnostic regions to improve the overall specificity of the assay.

Overall, DNA array technology has proven to be very efficient for SNP detection. In addition to plant pathogen diagnosis, this study is relevant for any field where DNA arrays are used to detect mutations or polymorphisms,

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ranging from clinical diagnostics to environmental microbiology, making this technology even more appealing for diverse applications.

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