Macroarray Detection of Solanaceous Plant Pathogens in the *Fusarium solani* Species Complex

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ABSTRACT

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Members of the *Fusarium solani* species complex (FSSC), which are morphologically similar but have more than 45 distinct lineages, were chosen as targets for the development of a macroarray detection system that would be broadly adaptable. Thirty-three oligonucleotides (17 to 27 mers) were designed from the internal transcribed spacer (ITS) of the ribosomal RNA genes of 17 FSSC isolates, which belong to 12 phylogenetically closely related species. Of the 33 oligonucleotides on the array, 21 were useful in discriminating all 12 species, some of which had only a single nucleotide difference among them. The high specificity of this method was achieved by optimizing the hybridization temperature and oligo probe length, which had a more substantial effect on the array performance than the melting temperature and the DNA G+C content (G/C%) of the probes. The array was validated by testing inoculated greenhouse samples and diseased field plant samples.

Morphologically defined fungal species tend to comprise multiple phylogenetic and biological species, which better represent the biological units that are associated with plant diseases (9). Extensive molecular phylogenetic analyses currently are underway in a number of important plant-pathogenic genera, uncovering complexes of morphologically cryptic phylogenetic species (6,21). DNA sequencing used to define species also can be used for rapid and accurate identification and detection of pathogens in culture and, eventually, in the field and greenhouse. Oligonucleotide arrays have been chosen by many researchers to meet this need because the thousands of target-specific DNA oligomers can be spotted on a microarray slide, permitting a very high rate of throughput. Macroarrays are membrane-based oligonucleotide arrays which have fewer probes per array than microarrays. However, their capacity is still several orders of magnitude larger than other detection methods such as regular and real-time polymerase chain reaction (PCR) and serological detection (4,18). Moreover, macroarrays are less expensive than microarrays. Therefore, they are an appropriate choice for parallel detection of a moderate number of different targets (13,16).

The main technical demands when developing a multispecies detection array are high specificity, sensitivity, and robustness (16). The ultimate specificity in molecular detection technology is the ability to discriminate single-nucleotide polymorphisms (SNPs) (1). In fungal molecular diagnostics, the most commonly used target is the nuclear ribosomal DNA (rDNA) because it has been well studied for fungal molecular phylogeny and, therefore, a large number of rDNA sequences are available. The internal transcribed spacer region (ITS) within the rDNA (including ITS1, 5.8S rDNA, and ITS2) is commonly used in fungal diagnostic macroarrays because its sequence variability usually enables discrimination of closely related species (16,26). Thus, a single pair of conserved ITS primers can be used to amplify the region from any sample and the resulting amplicon hybridized with an oligo array, enabling the detection and identification of fungal (or oomycete) pathogens in the sample (12,13). In addition to specificity, it is critical for a macroarray to have ample sensitivity for detection. There are two definitions of sensitivity for microbial diagnostic arrays. One commonly used definition is the lowest detectable target amount (15); the other is the lowest relative abundance of the target group detectable within the analyzed community (1), which usually is adopted by environmental microbiologists (5). In this study, both methods are used and compared. The robustness of an array can be evaluated by repeating experiments multiple times and by testing the array against multiple targets and closely related organisms (13).

Ultimately, the goal of this project is to produce a macroarray that can detect many fungal and oomycete pathogens of solanaceous crops. Toward that goal, we first focused on the *Fusarium solani* species complex (FSSC) because these will be the most difficult organisms to differentiate due to their high phylogenetic similarities (31). Members of FSSC are ubiquitous saprophytes in soil, pathogens of various plants, and opportunistic animal pathogens (2). Although members of the complex are morphologically similar, a three-gene phylogeny revealed that they comprise more than 45 phylogenetic species (lineages) distributed among three major clades (21,29). Clade 3 comprises 36 phylogenetic species with worldwide distribution. Some of them cause wilt or rot diseases of solanaceous crops and numerous other plants and are among the most commonly isolated species from soil and debris. To date, 18 species of clade 3 are known to cause superficial or systemic infections in immunosuppressed or immunocompromised human patients (31). Members of FSSC that cause solanaceous plant diseases are known to belong only to clade 3. Clade 1 and 2 contain nine species with relatively limited geographical distribution and host range compared with clade 3 (21).

In this article, we describe the development of a multispecies detection macroarray that contains 33 oligonucleotide probes complementary to the ITS sequences of 17 closely related FSSC clade 3 isolates which represent 12 phylogenetic species, including solanaceous plant pathogens and the most commonly encountered strains in the environment. The objectives of this study were threefold: (i) to develop a technique by which solanaceous plant pathogens and most commonly encountered species of FSSC can be quickly and easily identified, (ii) to test parameters that influence array specificity and sensitivity in order to optimize the array detection technique and facilitate future array design, and (iii) to evaluate the array by testing it with field and greenhouse samples.

MATERIALS AND METHODS

Fungal isolates. In this study, 17 FSSC isolates were selected (Table 1; the first 17 items). They represented 12 phylogenetic species of clade 3 with an emphasis on plant pathogens, but belong to the same morphological species: *F. solani* sensu

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W.C. Snyder & H.N. Hansen (2,27). Each of the 17 isolates has a distinct ITS sequence (Table 1). In addition, 11 phylogenetically closely related species were used as references for testing probe specificity, including *F. cuneirostrum* (FSSC clade 2), *F. equiseti*, *F. graminearum* (teleomorph *Gibberella zeae*), *F. illudens* (FSSC clade 1), *F. oxysporum*, *F. sambucinum* (teleomorph *G. pulicaris*), *F. sporotrichioides*, *F. virguliforme* (FSSC clade 2), *Nectria borneensis* (FSSC clade 3), *N. plagianthi* (FSSC clade 1), and *Trichoderma harzianum* (teleomorph *Hypocrea lixii*) (Table 1).

DNA extraction and target amplification and purification. Genomic DNA was extracted from 1- to 2-week-old fungal cultures, plant tissues, or soil samples using the UltraClean Plant DNA Isolation Kit (MoBio, CA) or UltraClean Soil DNA Isolation Kit, following the manufacturer's protocols. The extracted genomic DNA concentration ranged from 15 to 120 ng/μl. ITS was amplified with primers ITS5 and ITS4 (8,30). PCR was carried out with 1× ThermoPol reaction buffer (New England BioLabs, Beverly, MA), 1 mM dNTPs, 0.2 μM each primer, 1 unit of *Taq* DNA polymerase (New England BioLabs), and 15 ng of genomic DNA in a 50-μl reaction. The

following PCR cycling conditions were used: 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).

Detector probe design. ITS sequences of the 17 FSSC isolates were aligned with the CLUSTAL X program (29) and the polymorphic sites were identified visually. Isolate-specific oligonucleotides were designed and screened with two criteria in order to achieve uniform performance in array hybridization. The first criterion was that the length of the oligomer fall between 17 and 27 nucleotides (nt). The second was that the estimated melting temperature (T_m) range from 50 to 60°C. The T_m was estimated using the following formula: $T_m = 64.9 + 41 \times (y + z - 16.4) / (w + x + y + z)$, where w , x , y , and z are the number of the bases A, T, G, and C in the sequence, respectively (11). Additionally, the Primer3 program (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi>, version 0.2; 3,24,25) and the nearest neighbor formula (<http://www.basic.northwestern.edu/biotools/oligocalc.html>, version 3.10; 3,28) were used in further analyses. Thirty-

three probes were generated for the detection of the 17 FSSC isolates (Table 2).

Array production. The detector oligomer probes (20 fmol) were spotted onto Hybond N+ nylon membranes (Amersham Biosciences, NJ) using a 96-pin replicator (model 250520; Nalge Nunc International Corp., IL) in quadruplicate. Three types of controls also were spotted. The positive controls included three fungal universal primers, ITS2, ITS4, and ITS5 (8,30; Table 2) and ConservITS, a 70-mer based on 5.8S rDNA sequence conserved across Kingdom Fungi. Four internal controls (ITS2_1 to ITS2_5) that differed from primer ITS2 at one or two bases also were spotted on the membrane (Table 2). Negative controls were sterile water and spotting buffer (4 μM sodium carbonate buffer, pH 8.4, 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.01% N-lauroyl sarcosine, and 0.004% bromophenol blue). All oligomers were spotted at a concentration of 50 μM diluted in spotting buffer, except the 70-mer, which was spotted at 25 μM. Spotted membranes were air dried for 10 min and then fixed by UV exposure at 240 mJ/cm². After incubation in a 0.5% sodium dodecyl sulfate solution at 60°C for an hour, membranes were

Table 1. Isolates of the *Fusarium solani* species complex and reference species used in this study, including the host or substrate from which the culture was originally isolated, country of origin, and GenBank accession numbers of internal transcribed spacer region (ITS) sequences^a

Isolate name ^b	Host or substrate	Country of origin	ITS GenBank number ^c
S-176	<i>Solanum tuberosum</i>	Canada	DQ094689
S-189	<i>Piper nigrum</i>	Malaysia	DQ094678
S-223	<i>S. tuberosum</i>	Peru	DQ094761
S-560	<i>Pinus</i> sp.	United States	DQ094705
S-564	<i>Ipomoea batatas</i>	United States	DQ094706
S-661	<i>S. tuberosum</i>	Palestine	DQ094762
S-719	<i>S. tuberosum</i>	Egypt	DQ094699
S-781	Soil	Australia	DQ094731
S-850	Soil	Thailand	DQ094720
S-974	<i>Medicago sativa</i>	United States	DQ094770
S-1223	<i>Sphyrna tiburo</i>	United States	DQ094767
S-1338	<i>Passiflora</i> sp.	New Caledonia	DQ094658
S-1341	<i>Cucurbita</i> sp.	Japan	DQ094660
S-1558	<i>Allium</i> sp.	Philippines	DQ094675
S-1592	<i>Theobroma cacao</i>	Ecuador	DQ094746
CA109	<i>Lycopersicon esculentum</i>	United States	DQ315563
CA113	<i>L. esculentum</i>	United States	DQ315564
CA101	<i>L. esculentum</i>	United States	EF055900
0552	<i>Capsicum annuum</i>	United States	EF050587
0627	<i>C. annuum</i>	United States	NA
0628	<i>L. esculentum</i>	United States	NA
<i>F. cuneirostrum</i> NRRL22158 (FSSC Clade 2)	<i>Phaseolus vulgaris</i>	United States	AF178396
<i>F. equiseti</i> 0594	<i>L. esculentum</i>	United States	EF464164
<i>F. graminearum</i> 05131 (<i>Gibberella zeae</i>)	<i>S. tuberosum</i>	United States	EF464165
<i>F. illudens</i> NRRL22090 (FSSC Clade 1)	<i>Beilschmiedia tawa</i>	New Zealand	AF178393
<i>F. oxysporum</i> 0531	<i>L. esculentum</i>	United States	EF464166
<i>F. sambucinum</i> NRRL13504 (<i>Gibberella pulicaris</i>)	<i>S. tuberosum</i>	Canada	X65481
<i>F. sporotrichioides</i> 05-15500	<i>Cucurbita</i> sp.	United States	EF464167
<i>F. sporotrichioides</i> 05-15755C1	<i>Chrysanthemum</i> sp.	United States	EF464168
<i>F. virguliforme</i> NRRL22825 (FSSC Clade 2)	<i>Glycine max</i>	United States	AF178419
<i>Nectria borneensis</i> NRRL22579 (FSSC Clade 3)	NA	Indonesia	AF178415
<i>N. plagianthi</i> NRRL22632 (FSSC Clade 1)	<i>Hoheria galbrata</i>	New Zealand	AF178417
<i>Trichoderma harzianum</i> T-22 (<i>Hypocrea lixii</i>)	NA	United States	EF464169

^a NA = not available.

^b Isolates of *F. solani* are listed only by number, other species are indicated.

^c The sequences were generated in previous studies except for CA109, CA113, CA101, 0552, 0594, 05131, 0531, 05-15500, 05-15755C1, and *T. harzianum* T-22, which were generated in this study.

rinsed in 100 mM Tris (pH 8.0) for 5 min and kept moist at 4°C until use.

Hybridization optimization. The ITS amplicons from FSSC isolates were labeled and hybridized using the Gene Images Alk-Phos Direct Labeling and Detection System with CDP-Star (Amersham Biosciences) following the manufacturer's protocol. Chemiluminescence was detected using Kodak Biomax Light film. Developed films were scanned with a Hewlett-Packard 5300C ScanJet and read by ImageJ 1.33u (National Institutes of Health, MD). The signal intensity for hybridization was measured as the average gray value for the quadruplicate spots for each detection probe on the array. Because the film background of each experiment varied, albeit slightly, background values were removed from each spot. In the rest of this article, the signal intensity of hybridization refers to the gray value difference between the hybridization dots and the background, with higher values indicating stronger signal intensity.

To achieve the best possible array specificity, experimental conditions were optimized with two isolates, S-974 and S-1338. Hybridization temperatures were tested at 45, 50, 52, 55, and 60°C. Film exposure times ranged from 10 min to overnight. Other conditions did not change. Based on the results of this pilot experiment, the selected experimental conditions were as follows: prehybridization at 55°C for 15 min, hybridization at 55°C for 2 h, two primary washes at 50°C for 10 min, and two secondary washes at room temperature (22°C) for 5 min, followed by 30 min of film exposure after 1 h of reaction with the detection reagent.

Once optimal hybridization conditions had been determined, the ITS amplicons from each of the 17 FSSC isolates were labeled and used to hybridize with the macroarray containing the 33 FSSC-specific and 9 control oligomers. Each experiment was conducted at least twice.

Detector probe hybridization analyses. Under the optimized conditions, 12 probes that did not hybridize with their targets were compared with 24 probes that produced detectable signals in terms of the probe length, DNA G+C content (G/C%), and T_m. The statistical functions, including average, standard deviation, and *t* test in the Microsoft Excel x for Mac, were used in comparing the oligonucleotide characteristics.

Array sensitivity tests. Two sets of experiments were performed to evaluate the detection limit of the PCR-coupled macroarray method. In the first experiment, genomic DNA from four FSSC isolates (S-564, S-719, S-1338, and S-1592) was serially diluted prior to use in a standard ITS PCR amplification. In the second, ITS amplicons of two isolates (S-719 and S-1338) were mixed in a range of ratios, simulating a community where the fungus of interest would contribute 1, 5, 10, 20, 80, 90, 95, and 99% of the total DNA.

Table 2. Sequences and properties of oligonucleotide probes on the array

Oligo name ^a	Sequence (5'-3')	Length	T _m (°C) ^b	Target isolate
ITS2	GCTGCGTTCTTCATCGATGC	20	54	Fungi (30)
ITS2_1	GCTGCGTTGTTTCATCGATGC	20	54	Fungi, with a central mismatch
ITS2_2	GCTGCGTTCATCATCGATGC	20	54	Fungi, with a central mismatch
ITS2_3	CCTGCGTTCTTCATCGATGC	20	54	Fungi, with an end mismatch
ITS2_4	GCTGCGTTGATCATCGATGC	20	54	Fungi, with two central mismatches
ITS2_5	CGTGCGTTCTTCATCGATGC	20	54	Fungi, with two end mismatches
ITS4	TCCTCCGCTTATTGATATGC	20	51	Fungi (30)
ITS5	GGAAGTAAAAGTCGTAACAAGG	22	50	Fungi (30)
ConservITS	CGATGAAGAACGCAGCGAAATG	70	71	Fungi
	CGATAAGTAATGTGAATTGCAGA			
	ATTACAGTGAATCATCGAATCTTTGA			
Fs1	TAGACGGCCCTGTAAAACGG	20	54	S-1341
Fs2	GAGGACTCTTAACTCTGTTTCTTA	24	52	S-1341
Fs3	CGCCGTAAAACACCCCAACTCTT	23	57	S-1341
Fs4	TCGCGTAGTAGCTAACACCTCGC	23	59	S-1341,S-560,S-974
Fs5	CGCCGTAAAACACCCCAACTCTTCT	24	57	S-560,S-974
Fs6	CCTGTGAACATACCTAAACGTTG	23	53	S-1592,S-1338,S-974
Fs7	CCTAACTCTGTTGCTATATGTATC	24	52	S-661,CA109,CA113
Fs8	CCTAATTCTGTTGCTATATGTATCTTC	27	54	S-719
Fs9	TCTAACAACATCATCAACCCTGTGA	24	54	S-661,S-719
Fs10	CAGACGGCCCTGTAAACACG	20	56	S-1558,S-176
Fs11	CCTGTGGGCACACGCCGT	18	57	S-1558,S-176
Fs12	CCCTAACTCTGTCTTTATAATGTT	24	51	S-176
Fs13	TTATACAACATCATCAACCCTGTGA	24	52	S-1558,S-176,S-1223,S-189,S-781, S-1592,S-1338,S-223,S-564
Fs14	CAGACGGCCCTGAAACGATG	20	56	S-564
Fs15	GCAGCTTCCAATGCCGTAGTAGCTA	24	57	S-564
Fs16	CAATGCGTAGTAGCTAACACCT	22	53	S-564
Fs17	CGCCGTAAAACACCCCAACTTTAG	24	56	S-564
Fs18	CCTGTGGGCACACGCCGT	19	58	S-189
Fs19	ACATACCTAAAACGTTGCCTCGG	23	55	S-189,S-223
Fs20	ACATACCTATAACGTTGCCTCGG	23	55	S-781
Fs21	CCTGCGGGCACAACGCCGT	19	60	S-781
Fs22	TCTGCGGGCACAACGCCGT	19	60	S-1592
Fs23	CGCCGTAAAACACCCCAACTTCT	22	57	S-1592
Fs24	CCGCGGGCACAACGCCGT	17	59	S-1338
Fs25	GCAGCTTCCATTGCGTAGTAGCCA	24	59	S-1338
Fs26	TTGCGTAGTAGCCAACACCTCGCA	24	59	S-1338
Fs27	CGCCGTGAAACACCCCAACTTCT	22	57	S-1338
Fs28	CCTGCGGGCACAACGCCGT	18	59	S-1223
Fs29	ACTTACCTATAACGTTGCTTCGGC	24	56	S-850
Fs30	CCGAGTCTAAACAACATCATCAAC	23	53	CA109,CA113
Fs31	CCGAGTCTAAACAACATCATCAA	22	51	CA109,CA113
Fs32	CCGTAAGACGGGCCGCC	18	59	CA113
Fs33	CTGTTGCTATATGTATCTTCTGAG	24	52	S-661,S-719,CA109,CA113

^a ITS = internal transcribed spacer region.

^b Melting temperature (T_m) was based on the basic formula (see text).

Although the percentage of each isolate varied, the total amount of DNA used for hybridization was kept constant. Hybridization for both experiments was conducted at 55°C as described above. The results were recorded as positive if the isolate-specific detector produced a detectable signal.

Validation of the FSSC DNA array.

Two types of testing were performed. Two pepper plants (sample numbers 0552 and 0627) and a tomato plant (sample number 0628) with stem discoloration symptoms collected from three different fields in New York State were used. Genomic DNA was extracted from the surface-disinfested sample with UltraClean Plant DNA Isolation Kit (MoBio). PCR amplification and hybridization to the macroarray were conducted as described above. The disinfested tissues also were plated on the Nash-Snyder medium (19), a selective medium for *Fusarium* spp. ITS regions of the fungi isolated from the plant tissues were sequenced as described above. The isolated fungi also were identified by microscopic observation.

In addition to the field samples, inoculated greenhouse materials were used to test the array. Seven-day-old tomato seedlings were inoculated with one of three tomato-infecting isolates (CA101, CA109 and CA113) by directly pouring 10 ml of conidial suspension (10^7 cells/ml) into the potting soil, or soaking the trimmed root in the suspension for 10 min before repotting. Negative controls were treated with water. Three weeks after inoculation, soil and surface-sterilized tomato plant root and stem tissues were collected. DNA extraction, ITS-PCR amplification, hybridization, and sequencing of the ITS region of isolated fungi from the samples were conducted as described above.

RESULTS

Hybridization optimization. In the pilot experiment with isolates S-974 and S-1338, a range of hybridization temperatures between 45 and 60°C was tested. There were 11 oligomer probes that perfectly matched the two targets and 14 probes that had a single mismatch with the targets. The effect of different hybridization temperatures on signal intensity is shown in Figure 1. For both perfect-match (upper panel) and single-mismatch (lower panel) oligomers, the signal intensities decreased with increasing hybridization temperature. At 45°C, all the oligomers had hybridization signals with gray values higher than 20. At 52°C, the level of specificity was still too low because all but one single-mismatch oligomer produced a hybridization signal. When the hybridization temperature was increased to 55°C, only two single-mismatch oligomers produced a hybridization signal. Additionally, 7 of 11 perfect-match oligomers still hybridized with signal gray values higher

than 10. At 60°C, no signal could be detected with any of the mismatch oligomers, nor with 7 of the 11 perfect-match oligomers. The signal intensities produced by the four remaining oligomers were all weak. Based on these results, a hybridization temperature of 55°C was chosen to achieve both high array specificity and reasonable signal intensity. Hybridization at a lower temperature (52°C) produced a stronger signal, but nonspecific hybridization could complicate the results dramatically.

Array specificity. Under the optimized conditions, the hybridization between the FSSC DNA array and the ITS of each of the 17 FSSC isolates produced 16 different patterns (Table 3). Each of the 12 FSSC phylogenetic species was discriminated by the array. Eight oligo probes were identified that were lineage or isolate specific

(Fig. 2) and worked well under the optimized conditions. Every isolate corresponded to a distinct hybridization pattern except for isolates CA109 and CA113, which belong to the same species. Several probes (such as Fs32 for CA113) were designed as lineage or isolate specific but did not produce signals under the relatively stringent conditions. In three cases, even isolates within a phylogenetic species were able to be distinguished, such as S-661 and S-719 (Fig. 2). There was only one nucleotide transition between the ITS sequences of these two conspecific isolates. The oligo probe Fs8 contained the polymorphic position in the middle of its sequence and was able to identify the SNP unambiguously under the optimized hybridization conditions described above (Table 3; Fig. 2).

The positive control ConservITS is a 70-mer probe. As predicted, it produced

Fig. 1. Effects of hybridization temperature on signal intensities of perfect-match (upper panel) and single-mismatch (lower panel) oligonucleotides. Each line represents an oligo probe on the array.

stronger signals than the other 20-mer positive controls (Table 2; Figs. 2 and 3). In all, 21 of the 33 probes hybridized to DNA from the target FSSC isolates (Table 3). The five internal controls ITS2_1 to ITS2_5 were designed to have one or two nucleotide mismatches from the universal fungal primer ITS2. In this study, mismatches that are located within four nucleotide positions from the end of oligomers are defined as end mismatches. Those that are located over four nucleotide positions from the end are central mismatches. The three internal controls that contained central mismatches (Table 2) did not produce a hybridization signal. The other two internal controls that contained end-position mismatches produced weak signals, which were used as a threshold

when scoring hybridization results. Spots that had gray values higher than those of the internal controls were recorded as positive. In our study, 10 FSSC-specific probes had cross-hybridization with nontarget isolates (Table 3). However, all cross-hybridizations were due to end mismatches or indel central mismatches except for one probe, Fs24. There was a central non-indel mismatch between Fs24 and the corresponding locus in the ITS of isolates S-661, S-719, CA109, CA113, and S-850. All other SNPs were discriminated as long as the mismatch was non-indel and located in a central position.

As expected, none of 11 hypocrealean reference species produced hybridization patterns identical to any of the 17 target FSSC clade 3 isolates tested in this study

(Table 3). Of the 11 reference species, 7 did not hybridize with any of the strain-specific probes on the array and only hybridized to the positive controls (ITS2, ITS4, ITS5, and ConservITS; Table 2). Probe Fs4 produced signals with four FSSC isolates (two from clade 1 and two from clade 2) due to the presence of an end mismatch. Additionally, probe Fs24 produced a signal with each of the two FSSC clade 1 isolates, because the sequences were a perfect match.

Detector probe hybridization analyses. One-third (12 of 36) of the probes tested did not hybridize with their targets under the experimentally identified hybridization conditions used in this study (Table 3). To analyze the possible reasons for the negative results, the two probe

Table 3. Hybridization results of internal transcribed spacer region amplicons of 17 *Fusarium solani* species complex clade 3 isolates and 11 reference species with 33 probes and 9 controls under the optimized experimental conditions (hybridization at 55°C)

		Isolates																											
Probe	Position on array	S-1341	S-560	S-974	S-661	S-719	CA109	CA113	S-1223	S-1558	S-176	S-189	S-781	S-223	S-1592	S-1338	S-564	S-850	<i>F. cuneirostrum</i>	<i>F. equiseti</i>	<i>F. graminearum</i>	<i>F. illudens</i>	<i>F. oxysporum</i>	<i>F. sambucinum</i>	<i>F. sporotrichioides</i>	<i>F. virguliforme</i>	<i>N. borneensis</i>	<i>N. plagianthi</i>	<i>T. harzianum</i>
ITS2 ^a	A1,G6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
ITS2_1	B1,H6																												
ITS2_2	C1,A7																												
ITS2_3	D1,B7																												
ITS2_4	E1, C7																												
ITS2_5	F1, D7																												
ITS4	H5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
ITS5	A6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
ConservITS	G1, E7	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Fs1	A2	o																											
Fs2	B2	x																											
Fs3	C2	x	y	y																									
Fs4	D2	x	x	x	y	y	y	y	y	y	y	y	y	y	y		y	y	y			y				y		y	
Fs5	E2	y	x	x	y	y			y										y										
Fs6	F2			x												x	x												
Fs7	G2				x		x	x																					
Fs8	H2					x																							
Fs9	A3				x	x	y	y	y		y	y	y	y	y	y	y												
Fs10	B3									o	o																		
Fs11	C3									o	o																		
Fs12	D3									o																			
Fs13	E3				y	y	y	y	x	x	x	x	x	x	x	x	x	x	y										
Fs14	F3																	o											
Fs15	G3																	x											
Fs16	H3																	o											
Fs17	A4				y	y			y		y							x	y										
Fs18	B4										x																		
Fs19	C4											x			x														
Fs20	D4												o																
Fs21	E4												o	o															
Fs22	F4														o														
Fs23	G4														x														
Fs24	H4				y	y	y	y		y	y	y	y	y		x		y				x							
Fs25	A5				y	y										x												x	
Fs26	B5															x													
Fs27	C5															x													
Fs28	D5								o																				
Fs29	E5																	x											
Fs30	B6				y	y	x	x																					
Fs31	C6				y	y	x	x																					
Fs32	D6							o																					
Fs33	E6				o	o	o	o																					

^a An “x” indicates a signal from a perfect match probe, a “y” indicates a false positive, and an “o” indicates a false negative.

groups (hybridizing and nonhybridizing) were compared in terms of their length, G/C percentage, and Tm (Table 4). Tm did not vary between the two groups ($P > 0.39$), regardless of the three formulas used for Tm calculation. The hybridizing probe group had an average G/C percentage 10.7% higher than that of the nonhybridizing group, close to statistically significant ($P > 0.052$). The average probe length of the hybridizing group was 2.4 nt longer than that of the nonhybridizing group ($P > 0.007$).

Array sensitivity tests. Two tests were used to determine the detection limit of the FSSC array; the results are summarized in Table 5. In the first sensitivity assay, the lowest detectable amount of genomic DNA was 2.4 pg (10^{-12}) for isolate S-1338. For isolates S-564, S-1592, and S-719, the detection limits were 35, 45, and 230 pg, respectively. In the second detection limit study, ITS amplicons of S-1338 and S-719 were mixed together to simulate a community where the fungus of interest contributed 1 to 99% of the total DNA. For S-1338, levels as low as 1% were still detectable. For S-719, the detection limit was 5%.

Validation of the FSSC DNA array. Both field and greenhouse samples were used to validate the FSSC DNA array. From the field pepper sample 0552, the pathogen was detected and identified from both symptomatic stem tissue and root tissue without visible symptoms. The hybridization pattern matched to that of S-1558, with the symptomatic stem tissue (Fig. 3) producing a stronger hybridization signal than the root tissue (*data not shown*). The ITS region of the *F. solani* culture isolated from the stem of sample 0552 was sequenced and found to be identical to that of S-1558 (GenBank accession number EF050587). Morphologically, the fungus was identified as *F. solani* sensu W.C. Snyder & H.N. Hansen (27). However, *F. solani* was not isolated from the root tissue of 0552. Stem and root tissues of pepper sample 0627 from a different field in New York State also produced the same hybridization pattern as that of S-1558. Both *F. solani* and *F. oxysporum* were observed in the cultures isolated from the stem and root tissues of sample 0627. The tomato sample 0628, however, had a different hybridization pattern (it reacted only with the probe Fs4 and the positive controls), which did not match any of those shown in Table 3. *F. solani*, *F. oxysporum*, and *F. equiseti* were observed from the cultures isolated from the stem and root tissues.

Disease symptoms were not observed on greenhouse-grown tomato plants at 3 weeks after inoculation with *F. solani*, or on mock-inoculated control plants. The FSSC DNA array successfully detected the pathogen in the inoculated greenhouse soil samples, but not from the plant tissues. These results were corroborated by the selective plating and DNA sequencing

results, in which the pathogen was detected only in inoculated soil and not in tomato plant tissue. The hybridization patterns from the three soil samples inoculated with CA101, CA109, and CA113 matched with that of S-1558, CA109, and CA113, respectively. Morphology and ITS sequences of the isolated fungal cultures

from the inoculated soil samples further confirmed the identities of these isolates (GenBank accession numbers EF055900 to EF055902).

DISCUSSION

In the past a few years, oligonucleotide array technology has become a popular

Fig. 2. A, One of 21 most-parsimonious trees based on internal transcribed spacer region sequences of 17 *Fusarium solani* species complex isolates in 12 species. Isolates in half brackets belong to the same phylogenetic species. Numbers on branches are bootstrap values higher than 50%. The lineage or isolate-specific probes are labeled on the corresponding branches with their positions on the array written in gray dots. B, Hybridization patterns of S-661 (top) and S-719 (bottom) are shown on the right. Positive controls are circled. The arrow designates the S-719-specific probe Fs8.

Fig. 3. Hybridization pattern of the field pepper sample 0552 (stem) on the left and that of the isolate S-1558 on the right. Positive controls are circled. In addition to the positive controls, the sample produced positive signals with probes Fs4, Fs13, and Fs24 at positions D2, E3, and H4.

method for microbe detection and disease diagnostics (1,12–14,16). It is the only technology available that allows parallel detection of a large number of microbial species (targets).

Most diagnostic arrays designed to date are for detecting and discriminating microbes in certain habitats, such as vascular wilt pathogens of tomato (13). The target organisms usually belong to different genera or even different kingdoms and can be easily discriminated (7). Very few studies on diagnostic arrays have addressed questions on how to discriminate phylogenetically closely related taxa. The 17 FSSC isolates chosen here are morphologically indistinguishable and traditionally have been treated as a single species (2,21). The highest divergence in ITS sequences between any of the isolates is 6%, averaging 12 mismatches among isolate pairs, and ranging from 1 to 30 mismatches across the approximately 500-nt length of the amplicon. The low levels of polymorphism made it difficult to find taxon-specific probes. Genetically, they also are very similar and some differ only by a single nucleotide in the ITS sequence (Fig. 2A). The protocols and results described in this study add to the knowledge base for the design of future high-capacity diagnostic arrays.

Using the macroarray described here, 16 of the 17 FSSC clade 3 isolates could be discriminated (Table 3). In three cases, even isolates within a phylogenetic species were able to be distinguished. In order to discriminate phylogenetically closely related taxa, it is necessary to optimize experimental conditions to achieve high array specificity (23). Using four plant-pathogenic species (*F. oxysporum*, *Verticillium dahliae*, *Phytophthora nicotiana*, and *Pythium ultimum*), a recent study demonstrated higher array specificity when short amplicons at a fairly low concentration were used in hybridization (15). Our data show that the hybridization temperature also plays a critical role in array specificity when discriminating SNPs (Fig. 1). A decrease of only 3°C from the optimal hybridization temperature resulted in nonspecific hybridization in the majority of the probes (Fig. 1). A higher hybridization temperature eliminated more nonspecific

hybridization; however, it was accompanied by an unacceptable decrease in hybridization signal. Using a microarray to detect several different *Fusarium* spp. on cereal grains, Nicolaisen et al. (20) also suggested increasing hybridization temperature to achieve higher specificity. Detector probes in that study had four or more mismatches with the nontargets (20). Our results show a much more dramatic impact of the hybridization temperature on array specificity when discriminating single mismatched targets and probes (Table 4).

In addition to the hybridization conditions, array performance also is determined largely by the oligonucleotide probe properties. Oligonucleotide length and T_m usually are the main parameters to consider in probe design (10,17,23). In this study, we selected 17 to 27 mers and T_m of 50 to 60°C according to previously published array design parameters (13,14,23). It was difficult if not impossible to further narrow the ranges because of lack of DNA sequence differences among these closely related taxa. Together with the three 20-mer positive controls, a total of 36 oligomers falling in those ranges were tested on the array. In Table 4, we compared the oligomers that did not hybridize with their complements (12/36) and those that produced detectable signals (24/36). The only statistically significant difference identified between the two groups was the probe length. The results indicated that oligo probe length may have a more substantial effect on array performance than T_m, G/C%, or secondary structure under the relatively stringent hybridization conditions used in this study. Therefore, to achieve a more uniform array hybridization performance under these conditions, narrowing the oligo probe length should be the first consideration. However, the sample size of the study is not large enough to be conclusive and more experiments are needed to test our findings.

It was reported that discrimination of SNPs was difficult to achieve using oligo probes 20 to 25 nt in length (1). The high array specificity demonstrated here may be attributed to optimizing the hybridization temperature and designing probes such

that SNPs were centrally located. The latter strategy was widely utilized because it previously has been shown that mismatches in the central positions of a probe are more easily detected than those near the ends of the oligo (1). Due to a limited number of differential nucleotides among the closely related FSSC taxa, a small portion of the probes designed for this study had only end mismatches with nontarget species. The potential cross-hybridization was corrected by using the end-mismatch controls (ITS2_3 and ITS2_5; Table 2) on the array. Only signals higher than these end-mismatch controls were considered positive results.

Sequencing errors and nonorthologous sequences may lead to false negative or false positive results in hybridization-based assays. Although nonorthologous ITS2 types are common in the *G. fujikuroi* species complex and some other fungi, no evidence for nonorthology of ITS has been observed in the FSSC (22). In a previous phylogenetic study of the *F. solani* species complex, the ITS of several hundred *F. solani* isolates from various sources were sequenced and nonorthologous ITS types were not found (31). For the 17 selected *F. solani* isolates included in this work, we have amplified and sequenced the ITS at least twice in both forward and reverse directions in order to eliminate sequencing errors. Therefore, sequencing errors are very unlikely to be a reason for false negatives or false positives.

The detection limits in this study ranged from 2.4 pg (S-1338) to 230 pg (S-719) of DNA (Table 5). The large variation in detection levels might be explained by variation in ITS-PCR amplification between isolates. It is possible that PCR amplification could be improved by using a higher-efficiency *Taq* DNA polymerase (15) or by more efficient removal of PCR inhibitors using different DNA extraction methods. Several previous studies using the PCR-coupled macroarray method to detect fungal pathogens reported a 0.25- to 2.5-pg detection limit (13,14). These studies utilized amino-modified oligonucleotide probes and a slightly lower hybridization temperature (54°C). Amino-modified oligomers have been reported to increase array detection levels (23). Because the array is clearly quite sensitive, because the pathogen could be detected in nonsymp-

Table 4. Comparison of perfect match probes that hybridized with their complements and those that did not hybridize with their complements under the experimental conditions^a

Parameters ^b	Hybridized with complement	Did not hybridize with complement	P value ^c
Number of probes tested	24	12	
Probe length (nt)	22.8 (0.4)	20.4 (0.7)	0.007
G/C%	48.9 (2.2)	59.6 (4.6)	0.052
T _m (basic, °C)	55.1 (0.6)	56.0 (0.9)	0.402
T _m (Primer3, °C)	62.4 (1.2)	64.6 (2.2)	0.398
T _m (nearest neighbor, °C)	72.5 (0.7)	72.8 (0.9)	0.833

^a Data shown are averages; numbers in parentheses = standard error.

^b nt = Nucleotides and T_m = melting temperature.

^c P values of *t* tests.

Table 5. Array sensitivity tests results^a

Isolate	Dilution	DNA amount (pg)	Target (%)
S-1338	10 ⁵	2.4	1
S-719	10 ³	230	5
S-564	10 ⁴	45	NT
S-1592	10 ⁴	35	NT

^a The lowest detectable amount of template DNA for four isolates and the lowest detectable percent composition in the community S-1338 and S-719 are shown. NT = not tested.

tomato plant root tissues (field samples 0552, 0627, and 0628), we did not attempt to increase sensitivity by using amino-modified oligomers. The second sensitivity test results indicate that this array can detect a target even when it constitutes a small proportion of the DNA in a given sample (Table 5).

Eleven reference species that are close relatives of *F. solani* or are common Solanaceae pathogens or soil saprophytes were chosen to test the specificity of the array (Table 3). No cross-hybridization was detected between target FSSC clade 3-specific probes on the array and ITS DNA from seven of the reference species. ITS DNA from four isolates hybridized to one probe (Fs4) due to an end mismatch, and two of those four (both FSSC clade 1) also hybridized with a perfect match to Fs24. Neither probe Fs4 nor Fs24 was critical for the identification of the 17 target isolates. Additionally, none of the reference species produced the same hybridization pattern as any of the 17 target FSSC isolates tested in this study (Table 3). These results are indicative of the high specificity of this array.

Additional tests with three diseased field plants and three inoculated greenhouse samples provided further support for the specificity and accuracy of the assay. The presence of *F. solani* in each of the six samples was confirmed by culturing and microscopic observation. We were able to sequence ITS of *F. solani* from four samples, and their sequences corroborated their hybridization pattern on the array. The *F. solani* cultures from two of the six samples (0627 and 0628) were not purified and, therefore, we did not get usable sequencing results from them. The fact that a new hybridization pattern was observed with one of the field samples is intriguing. The sample was quite decayed when received, and there were no other plants in that tomato field with disease symptoms. Thus, we could not test additional plants for this unique pattern.

On a practical level, pathogen detection methods based on culture and microscopic observation are time consuming and require extensive taxonomic knowledge. For morphologically very similar groups like FSSC, the traditional methods cannot identify isolates to species or lower taxonomic levels (21). The macroarray detection method also has many advantages over other methods, such as the ITS PCR restriction fragment length polymorphism method. Most remarkably, it can detect target DNA from a mixture of the host and other pathogens. In addition, a single nucleotide polymorphism is able to be differentiated using a macroarray by designing specific oligomers; however, finding the appropriate endonuclease might not be as easy (32). The validation results with field and

greenhouse materials indicated that the array detection technique is fast (within 12 h) and reliable for complex biological samples. It has great potential to be applied in routine pathogen diagnostic analyses (13). Future experiments will focus on expanding the macroarray to detect all fungal and oomycete pathogens of solanaceous crops.

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