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Development of SCAR markers and UP-PCR cross-hybridization method for specific detection of four major subgroups of *Rhizoctonia* from infected turfgrasses

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Abstract: A rapid identification assay for *Waitea circinata* (anamorph: *Rhizoctonia* spp.) varieties *zeae* and *circinata* causing patch diseases on turfgrasses was developed based on the universally primed PCR (UP-PCR) products cross-blot hybridization. Tester isolates belonging to the two varieties of *W. circinata* were amplified with a single UP primer L21, which generated multiple DNA fragments for each variety. Probes were prepared with UP-PCR products of each tester isolate by labeling with digoxigenin. Field-collected *W. circinata* isolates and representative isolates of different *R. solani* anastomosis groups (AG) and AG subgroups were amplified with L21, immobilized on nylon membrane and cross hybridized with the two probes. Isolates within a *W. circinata* variety cross-hybridized strongly, while non-homologous isolates did not cross-hybridize or did so weakly. Closely related *W. circinata* varieties *zeae* and *circinata* were clearly distinguished with this assay. Sequence-characterized amplified region (SCAR) markers also were developed from UP-PCR products to identify isolates of *Thanatephorus cucumeris* (anamorph: *R. solani*) AG 1-IB and AG 2-2IIIB. These two AGs are commonly isolated from diseased, cool-season turfgrasses. The specific SCAR markers that were developed could differentiate isolates of AG 1-IB or AG 2-2IIIB groups. These SCAR markers did not amplify a product from genomic DNA of nontarget isolates of *Rhizoctonia*. The specificities and sensitivities of the

SCAR primers were tested on total DNA extracted from several field-grown, cool-season turf species having severe brown-patch symptoms. First, the leaf samples from diseased turf species were tested for the anastomosis groups of the causal pathogen, and thereafter the total DNA was amplified with the specific primers. The specific primers were sensitive and unique enough to produce a band from total DNA of diseased turfgrasses infected with either AG 1-IB or AG 2-2IIIB.

Key words: brown patch, cool-season turfgrass, cross-blot hybridization, digoxigenin-labeled probe, *Rhizoctonia solani*, SCAR marker, UP-PCR, *Waitea circinata*

INTRODUCTION

Turfgrass diseases caused by *Rhizoctonia* species (sensu lato) are generally known as *Rhizoctonia* blight. Cool-season turfgrasses, such as tall fescue (*Festuca arundinacea* Schreb.), creeping bentgrass (*Agrostis stolonifera* L.) and perennial ryegrass (*Lolium perenne* L.), are more susceptible to *Rhizoctonia* blight (Martin and Lucas 1984; Burpee and Martin 1992; Zhang and Dernoeden 1997) than warm-season grasses. The disease severity on cool-season turfgrasses is highest during summer when the weather is hot and humid (Smiley et al. 2005). In the southern United States, *Rhizoctonia* blight poses a major threat to successful growth and maintenance of these turfgrasses. Symptoms of *Rhizoctonia* blight can vary depending on the turfgrass species, mowing height, causal pathogen and environmental conditions. In general, diseased turfgrasses have circular areas of blighted brown leaves (Smiley et al. 2005). Out of all *Rhizoctonia* diseases, brown patch caused by *R. solani* Kühn (teleomorph: *Thanatephorus cucumeris* [Frank] Donk) is the most common and widespread. Other *Rhizoctonia* species infecting turfgrasses during hot, humid weather include *R. zeae* and *R. oryzae* (teleomorph: *Waitea circinata* var. *zeae* and var. *oryzae*). *Waitea circinata* var. *circinata* (proposed anamorph: *R. circinata*) is a relatively new addition to *Rhizoctonia* causal pathogens (de la Cerda et al. 2007). Field symptoms of disease caused by *Rhizoctonia* species are similar, and visual diagnosis is not a reliable way to determine the causal pathogen. Furthermore, the main causal agent, *R. solani*, is a species complex

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having several anastomosis groups (AGs) (Cubeta and Vilgalys 1997). Out of them, AG 1, AG 2-IIIB and AG 4 have been identified as occurring commonly on cool-season turfgrasses (Burpee and Martin 1992, Smiley et al. 2005). The traditional methods of identifying *Rhizoctonia* isolates are by cultural morphology and anastomosis reactions. However, these methods are laborious, time consuming and sometimes difficult to interpret (Carling et al. 2002; Lübeck 2004).

Rhizoctonia species and AGs vary in sensitivity to commonly applied fungicides (Martin et al. 1984a, 1984b; Carling et al. 1990; Campion et al. 2003; Kataria et al. 1991; Kataria and Gisi 1996). Different *Rhizoctonia* species have different temperature ranges most conducive to disease (Smiley et al. 2005). Therefore rapid and accurate identification of the causal pathogen is important to predict disease progression and make future disease management decisions. For plant breeders, knowledge of the main causal pathogens at different locations is important in selecting appropriate turfgrass germplasm with resistance to *Rhizoctonia* blight.

Universally primed PCR (UP-PCR) is a DNA fingerprinting technique closely related to the random amplified polymorphic DNA (RAPD) method (Bulat et al. 1998). UP-PCR has the ability to generate numerous bands from primarily intergenic, less conserved regions of the genome (Bulat et al. 1998). UP primers are longer (15–21 nt) than RAPD primers (typically 10 nt) and therefore can anneal under more stringent conditions (52–60 C) ensuring greater reproducibility of banding profiles. Cross-blot hybridization of UP-PCR products generated with a single primer is a potential tool for identifying many *Rhizoctonia* isolates simultaneously (Lübeck and Poulsen 2001). The strength of the hybridization signal is used to determine the relatedness of the reference isolate to the unknown one. Although analysis of UP-PCR banding profiles also can group and may identify unknown isolates, efficient band scoring requires analysis by a genetic analyzer, which can be costly. Cross-blot hybridization eliminates this added expense.

Primers used in DNA fingerprinting are capable of amplifying DNA fragments from any organism and therefore cannot be used to detect a specific species or a strain from soil or infected plant parts (Lübeck and Lübeck 2005). However, by identifying unique molecular markers, the fingerprinting methods including UP-PCR can be used for rapid identification of organisms of interest. The essential feature of a marker in this context is to detect all members of the target group and discriminate all others. When such markers are sequenced they are called sequence-

characterized amplified regions (SCARs) (Lübeck and Lübeck 2005). Studies have employed different molecular techniques for rapid identification of *R. solani*. Salazar et al. (2000) designed specific primers to amplify AG 2 subgroups from rDNA internal transcribed spacer (ITS) sequences. A molecular marker from RAPD banding profile was used by Toda et al. (2004) to identify AG 2-2LP from other *R. solani* AG subgroups.

Research indicated *R. solani* AG 1 and 2 dominate patch diseases on cool-season turfgrasses (Martin and Lucas 1984; Zhang and Dernoeden 1997). A genetic diversity study conducted by the authors of this paper in Maryland and Virginia revealed *R. solani* AG 1-1B and AG 2-IIIB to be the dominant causal agents on tall fescue (Amaradasa et al. 2013). The survey also revealed that AG 2-IIIB dominated creeping bentgrass/annual bluegrass putting greens. In recent years *W. circinata* varieties have been reported frequently to cause disease on golf courses in the eastern and western USA (de la Cerda et al. 2007). These diseases need different cultural practices and chemical applications to manage disease progression. The suitability of UP-PCR products cross-blot hybridization as an identification tool has never been tested on *W. circinata* species.

This study was carried out to test whether UP-PCR products cross-blot hybridization could be used as a rapid method for identifying unknown isolates of *W. circinata* var. *zeae* and var. *circinata*. We also hypothesized that the sequence data of unique UP-PCR fragments can be used to design specific primers for detection of *R. solani* AG 1-1B and AG 2-IIIB.

MATERIALS AND METHODS

Fungal isolates and DNA extraction.—*Rhizoctonia* tester isolates (TABLE I) and field isolates (SUPPLEMENTARY TABLE I) were used for the experiments described below. The field isolates were collected from infected turfgrasses from Virginia and Maryland. Phylogenetic analysis of the internal transcribed spacer (ITS) region of the field isolates was used to identify them to species, AG or AG subgroup (Boysen et al. 1996). The ITS sequence analysis grouped six *W. circinata* isolates separately from the established *W. circinata* varieties. These isolates are described as unknown *W. circinata* (UWC). The isolates were cultured at room temperature on potato dextrose broth (Difco Laboratories, Detroit, Michigan) and DNA extracted after 4 d with the protocol of the Qiagen DNeasy plant mini kit (Qiagen Inc., Valencia, California).

UP-PCR product-cross hybridization.—Tester isolates (TABLE I) and 21 field isolates of *W. circinata* were used for cross hybridization. The field isolates consisted of 11 isolates of *W. circinata* var. *zeae* (Wcz) (PW 119, PW 220, LB 319, LB 228, HDN 115A, HDN 211, BELT 159, LB 4116, BELT 5, VABCH 8, VABCH 10), four isolates of *W. circinata* var.

TABLE I. *Rhizoctonia* and *Waitea* tester isolates used in this study

Isolate	Species ^a	AG	Host	Origin	Obtained from ^b
92'AR Soy1	Rs	AG 1-IA	Soybean	Arkansas, USA	CR
92'AR Soy2	Rs	AG 1-IA	Soybean	Arkansas, USA	CR
92'AR Soy3	Rs	AG 1-IA	Soybean	Arkansas, USA	CR
CR1	Rs	AG 1-IB	Unknown	Arkansas, USA	CR
BM2	Rs	AG 1-IB	Unknown	Unknown	BM
CR2	Rs	AG 1-IC	Unknown	Arkansas, USA	CR
BM3	Rs	AG 1-IC	Unknown	Unknown	BM
BM4	Rs	AG 2-1	Unknown	Unknown	BM
Rh146	Rs	AG 2-IIIB	Bentgrass	Georgia, USA	LB
Rh141	Rs	AG 2-2LP	Zoysiagrass	Illinois, USA	LB
ACC3	Rs	AG 2-2LP	Zoysiagrass	Kansas, USA	MK
CGCF18[2]	Rs	AG 2-2LP	Zoysiagrass	Kansas, USA	MK
SGGC2	Rs	AG 2-2LP	Zoysiagrass	Kansas, USA	MK
1AP	Rs	AG 3	Unknown	Unknown	DL
RS23	Rs	AG 4	Unknown	Unknown	DL
Rh102	Rs	AG 5	Unknown	Unknown	LB
IOEEA	Rs	AG 6	Sweet gum	North Carolina, USA	MC
Rh89/T	Rs	AG 8	Oat root	Scotland	LB
EDHGED	Wcc	Not assigned	ABG ^c	California, USA	FW
AVGCAV	Wcz	WAG-Z	ABG	California, USA	FW
87NEP	Wco	WAG-O	Rice	Japan	MC
C610	Rc	AG-D	Unknown	Unknown	DL
BELT 122	BNR	Unknown	Tall fescue	Maryland, USA	DL

^aRs: *R. solani*, Wcc: *W. circinata* var. *circinata*, Wcz: *W. circinata* var. *zeae*, Wco: *W. circinata* var. *oryzae*, Rc: *R. cerealis*. BNR: binucleate *Rhizoctonia*-like fungus.

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^cABG: annual bluegrass.

circinata (Wcc) (BLBG 8, BLBG 211, BLBG 216, BLBG 202) and six isolates of UWC (BELT 228, LB 4220, BSF 13, ANP 109B, ANP 304, HDN 222A). PCR products for all isolates were generated by L21 primer (5'-GGATCCGAGG-GTGGCGGTTCT-3') (Bulat et al. 2000), which had been reported to produce multiple fragments for *R. solani* isolates (Lübeck and Poulsen 2001). A PCR reaction mixture of 25 µL contained 1× standard Taq polymerase reaction buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl) (New England Biolabs, Ipswich, Massachusetts), 0.2 mM dNTPs, 0.4 µM of primer, 30–50 ng DNA template and 1 U Taq polymerase (New England Biolabs). Product amplification was carried out in a MJ Research PTC-200 thermal-cycler (Global Medical Instrumentation, Ramsey, Minnesota) with these conditions: an initial denaturation at 94 C for 2 min, followed by 35 cycles at 94 C for 30 s, 56 C for 30 s, and 72 C for 1 min, and a final extension at 72 C for 10 min. The resulting banding patterns of the PCR products were resolved on a 1.7% agarose gel in 1× Tris-Borate-EDTA (TBE) buffer by running at 100 V for 1 h. Thereafter the gel was stained with ethidium bromide and viewed under UV light. The UP-PCR products of the tester isolates EDHGED, AVGCAV and 87NEP (TABLE I) were used for probe preparation according to the instructions given in the DIG High Prime DNA Labeling and Detection Kit (Roche

Applied Sciences, Mannheim, Germany). Efficiency of DIG-labeled probes was determined by applying a series of dilutions (1 ng–1 pg) on a nylon membrane and detecting the strength of the signal. Dilutions of DIG-labeled probes were carried out by adding DNA dilution buffer containing 100 µg/mL fish sperm. This test also included manufacturer-provided DIG-labeled positive control and a negative control containing only DNA dilution buffer. The nylon membrane was subjected to immunological detection with an anti-digoxigenin-alkaline phosphatase conjugate and CDP-Star substrate. CDP-Star is an extremely sensitive chemiluminescent substrate for alkaline phosphatase (AP). Light emission from the CDP-Star was recorded on X-ray films and confirmed that the probes produced a significant signal for at least 100 pg blots for the experiment.

The target UP-PCR products of 21 field isolates of *W. circinata* and tester isolates representing different varieties of *W. circinata* and AGs of *Rhizoctonia* infecting turfgrasses were diluted with DNA dilution buffer to obtain uniform concentrations of 25 ng/µL. Diluted UP-PCR solutions were denatured by heating for 10 min at 95 C and rapidly cooling on ice. One microliter of products from each isolate was spotted separately on positively charged Amersham Hybond™-N+ nylon membrane (GE Healthcare UK Ltd.,

Buckinghamshire). After the spots dried, the membrane was placed on a filter paper soaked with 10× saline sodium citrate (SSC) and cross-linked with a Stratalinker® UV cross-linker (Stratagene, La Jolla, California). Approximately 100 000 µJ/cm² energy was applied to fix the DNA to the membrane. After cross-linking, the membrane was washed in sterile distilled water and dried on filter paper. A prehybridization step was carried out by inserting the membrane in a sealed plastic bag with DIG Easy Hyb solution (Roche Applied Sciences) and shaking 30 min in a 39 C water bath. For each 100 cm² membrane, 20 mL preheated prehybridization solution was used. Hybridization was done by introducing denatured DIG-labeled probe to the membrane and incubating 4 h with gentle agitation in a 39 C shaking water bath. The hybridization solution was prepared by mixing probe with 5 mL DIG Easy Hyb to obtain a final probe concentration of 30 ng/mL. Two low stringency washes were done with a 30 mL solution of 2× SSC and 0.1% sodium dodecyl sulfate (SDS) and shaking 5 min at room temperature. The high stringency washes were carried out twice at 70 C for 20 min in a solution of 0.5× SSC/0.1% SDS. All subsequent incubations for immunological detection steps were carried out on a shaker at room temperature (ca. 25 C) with the reagents supplied by Roche Applied Sciences. The membrane was rinsed in 20 mL washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% [v/v] Tween 20) 5 min and incubated 30 min in 100 mL blocking solution (purified fraction of milk powder in maleic acid buffer). The immunological reaction was carried out by incubating the membrane 30 min with 50 mU/mL anti-DIG-AP fragment in 30 mL blocking buffer. After incubation, the membrane was washed twice for 15 min in 100 mL washing buffer and equilibrated 5 min in detection buffer (1 M Tris-HCl, pH 9.5, 1 M NaCl). CDP-Star ready-to-use substrate was deployed for the detection of the hybridization reactions. The membranes were placed on a transparent plastic sheet and 1 mL CDP-Star was added and immediately covered with another plastic sheet and the substrate was spread evenly. After incubating 5 min, excess liquid was squeezed out and the edges sealed. Membranes were exposed to X-ray films for 5 min. The entire experiment of cross-blot hybridization was repeated three times with each probe to determine consistency of results.

Identification of UP-PCR SCAR markers.—Ten isolates of AG 1-IB (BELT 26, BLBG 510, LB 124, LB 234, PW 353, HDN 122A, LB 4217, ANP 306B, CR1, BM2) and 11 isolates of AG-22IIIB (BELT 150, BLBG 13, BLBG 32C, BSF 42, BSF 214, BSF 127, LB 312, LB 4316, ANP 205A, HDN 208By, Rh146) (TABLE I, SUPPLEMENTARY TABLE I) representing different geographies were tested with closely related AG subgroups to detect unique molecular markers. For this purpose UP-PCR products of AG 1-IB were compared with similar products of AG 1-IA and 1-IC isolates (TABLE I), and AG 2-2IIIB products were compared with AG 2-2LP (TABLE I). UP-PCR products of each isolate were resolved on a 1.7% agarose gel in 1× TBE buffer by running at 100 V for 1 h and viewed under UV light after staining with ethidium bromide. To design specific primers, a unique band for AG 2-2IIIB and AG 1-IB were identified by visually inspecting

different banding profiles generated by four primers, namely L21 (5′-GGATCCGAGGGTGGCGGTTCT-3′), L15/AS19 (5′-GAGGGTGGCGGCTAG-3′), L45 (5′-GTAA-AACGACGGCCAGT-3′) and AS15inv (5′-CATTGCTGGC-GAATCGG-3′) (Lübeck and Lübeck 2005). For AG 1-IB, a single PCR fragment amplified by UP primer L21 was selected. For AG 2-2IIIB, a distinct PCR fragment was selected from UP primer L45. The gel bands of selected products were excised and DNA extracted with a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, California). The Invitrogen Topo TA Cloning Kit (Invitrogen Corp., Carlsbad, California) was used to clone DNA products with electrocompetent *Escherichia coli* cells. Eight transformed bacterial colonies were selected per fungal DNA fragment and tested for inserts by PCR. One confirmed transformant per fragment was cultured in 50 mL LB liquid medium (Luria-Bertani broth), and plasmids were purified with a Qiagen Plasmid Midi Kit (Qiagen Inc., Valencia, California). We sequenced the inserts with a BigDye 3.1 sequencing kit (Applied Biosystems, Foster City, California) and analyzed with an ABI 3730 DNA sequencer (Applied Biosystems).

The specific primer pairs L21-F (5′-TCATTGCCAT-CAACTGTGACT-3′), L21-G (5′-CAAGGTTTGTGGAG-ATTGAG-3′) and L45-A (5′-ACCAATGAGTCGGTCTCG-TC-3′), L45-C (5′-CGTCTACGAGAAAACCTTGCAT-3′) for AG subgroups 1-IB and 2-2IIIB respectively were designed from the sequence data obtained with the ABI 3730 (see SUPPLEMENTARY TABLE II for sequence details). All primers were synthesized by Eurofins MWG Operon, Huntsville, Alabama. The primer pair L21-F/G was tested for the ability to amplify a PCR fragment on the tester isolate BM2 and another four AG 1-IB field isolates (BELT 2, BLBG 320, LB 123, PW 326). The AG 2-2IIIB tester isolate Rh 146 and three confirmed AG 2-2IIIB field isolates (BELT 114, BLBG 6, BLBG 32C) were amplified with specific primer pair L45-A/C. These two primer pairs also were tested for specificity on other *R. solani* AGs, *R. cerealis* and *Waitea circinata* varieties. Amplification reactions using specific primer pairs L21-F/G and L45-A/C were performed with 50 ng pure DNA. The remaining components of the PCR-reaction mixture were the same as the reaction mixture used for the UP-PCR technique. To determine the optimum PCR conditions different annealing temperatures, 58–63 C, were used. The thermal-cycler was programmed for 2 min at 94 C followed by 35 cycles of 1 min at an annealing temperature, 30 s at 72 C, 30 s at 94 C, and one cycle of 10 min at 72 C. DNA amplifications were resolved by electrophoresis on 1.7% agarose gels and viewed under UV light as mentioned previously.

Detection of AG 1-IB and AG 2-2IIIB from infected turfgrass leaves with specific primers.—Leaf pieces of field-grown perennial ryegrass, tall fescue and creeping bentgrass with severe brown-patch symptoms were collected from Mead, Nebraska, during summer 2012. The total number of samples included four each of creeping bentgrass and perennial ryegrass and two samples of tall fescue. Leaf samples also were collected from healthy turfgrass outside the disease patches. A few leaf pieces of each diseased

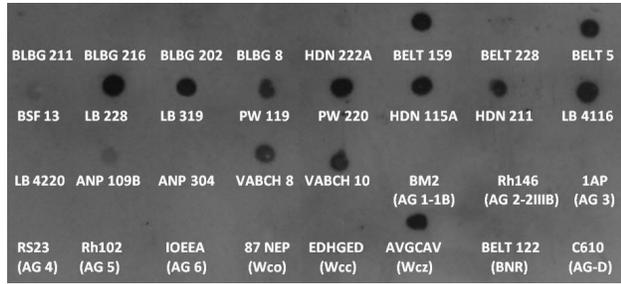


FIG. 1. Cross-blot hybridization of universally primed PCR (UP-PCR) products of *W. circinata* isolates with a probe made with the *W. circinata* var. *zae* (Wcz) tester isolate AVGCAV. The labeled probe was prepared for the total UP-PCR products with UP primer L21. A significant to strong signal was produced only by isolates of Wcz. The isolate name is below each of the blot. Isolates BLBG 211, BLBG 216, BLBG 202 and BLBG 8 belong to *W. circinata* var. *circinata*. HDN 222A, BELT 228, BSF 13, LB 4220, ANP 109B and ANP 304 are isolates of *W. circinata*, which did not fall into any known variety. All others are tester isolates. The anastomosis group (AG) or species name of the tester isolates is printed within brackets (see TABLE I for details).

sample were cultured on 2% water agar plates to isolate the causal pathogen. These isolates were grown on PDA plates and presence of *R. solani* was confirmed on the basis of culture morphology. These cultures were fused with tester isolates to determine the anastomosis group according to Carling et al. (2002). Isolates of *R. solani* from all creeping bentgrass samples belonged to AG 2, while isolates from perennial ryegrass samples were AG 1. Causal pathogen of one sample of tall fescue belonged to *R. solani* AG 1, while the other was an AG 2. The total DNA of diseased and healthy leaves was extracted with the Qiagen DNeasy Plant Mini Kit (Qiagen Inc.). DNA extractions were amplified by the specific primer pairs L21-F/G and L45-A/C to test whether these can detect the brown patch pathogen. Each of the 25 µL PCR reaction mixture contained all the components used for UP-PCR plus bovine serum albumin (0.5 ng/µL), which was added to prevent a PCR inhibitor (Toda et al. 2004). The thermal-cycler was programmed for an initial denaturation at 94 C for 2 min, followed by 35 cycles at 94 C for 30 s, 58 C for 30 s, and 72 C for 1 min, and a final extension at 72 C for 10 min. DNA amplifications were resolved by electrophoresis on 1.7% agarose gels with conditions mentioned previously, stained with ethidium bromide and viewed for DNA fragments under UV light.

RESULTS

Cross-blot hybridization.—All isolates of *Waitea circinata* var. *zae* (Wcz) produced strong to significant signals when hybridized with Wcz probe AVGCAV (FIG. 1). *W. circinata* var. *circinata* (Wcc), unidentified *W. circinata* (UWC) and other *Rhizoctonia* tester isolates either did not produce a signal or produced a weak signal. Wcc probe (EDHGED) produced a

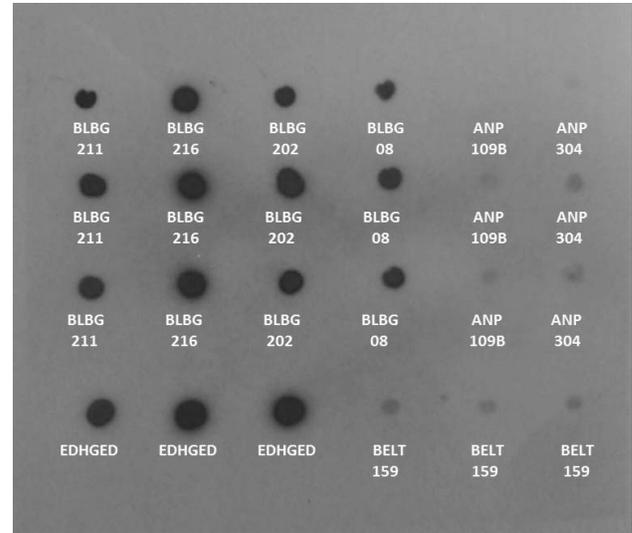


FIG. 2. Cross-blot hybridization of universally primed PCR (UP-PCR) products of *W. circinata* isolates with a probe made with the *W. circinata* var. *circinata* (Wcc) tester isolate EDHGED. The labeled probe was prepared for total UP-PCR products with UP primer L21. A significant to strong signal was given only by isolates of Wcc for all three replicates. Other *W. circinata* (only three isolates are shown) did not produce a signal. Isolate names are on the radiography film below each blot. BLBG 211, BLBG 216, BLBG 202 and BLBG 8 belong to Wcc group. BELT 159 is a confirmed isolate of *W. circinata* var. *zae*. ANP 109B and ANP 304 belong to an unknown *W. circinata* variety.

strong signal for all four tested isolates of Wcc but no signal for all other closely related isolates (FIG. 2). The probe NEP87 (a confirmed isolate of *W. circinata* var. *oryzae*) did not produce a significant signal for any of the tested *W. circinata* isolates or other *Rhizoctonia* tester isolates.

UP-PCR SCAR markers.—UP-PCR carried out with DNA of AG 1-IB isolates from different source locations produced a unique marker for the L21 primer (FIG. 3A), which could differentiate the AG 1-1A and the AG 1-1C isolates from AG 1-1B isolates. Only five of 10 tested isolates of AG 1-1B are illustrated (FIG 3A). UP primer L45 generated a unique marker for AG 2-IIIB isolates distinguishing AG 2-2LP (FIG. 3B) (only five of 11 tested isolates of AG 2-IIIB are shown). Both fragments were approximately 700 bp long and were selected for designing unique primers. One of the objectives of this study was to identify unique fragments that were shorter than 1000 bp to aid sequencing. L15/AS19 and AS15inv primers did not result in unique bands for AG 1-1B or AG 2-IIIB isolates. The AG 1-1B specific marker generated by primer L21 was 639 bp long, while the specific marker for AG 2-IIIB generated by primer L45 was 734 bp. The AG 1-1B specific fragment

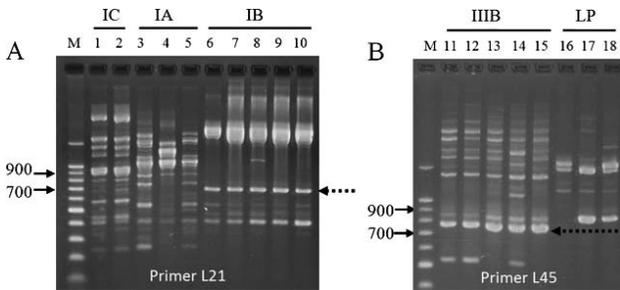


FIG. 3. Universally primed PCR banding patterns for *R. solani* AG 1 and AG 2-2 isolates. A. Banding patterns of AG 1-IA, -IB and -IC isolates amplified with L21 primer. Lanes 1–10, BM3, CR2, 92'AR Soy1, 92'AR Soy2, 92'AR Soy3, BELT 26, BLBG 510, LB 124, PW 353 and BM2 respectively. B. Banding patterns of AG 2-2IIIIB and -2LP isolates amplified with L45 primer. Lanes 11–18, BELT 150, BLBG 13, BLBG 32C, BSF 42, Rh146, Rh141, ACC3 and CGCF18[2] respectively. Lane M, molecular marker. Anas-tomosis groups (AGs) of the isolates are indicated above the lane numbers. The dashed arrows indicate the molecular markers selected for sequencing.

had 78% identity at the nucleotide level and 60% homology at the amino acid level with the sterol regulatory element binding protein cleavage-activating protein of the basidiomycetous fungi *Laccaria bicolor* (GenBank No. XM_001878515.1) and *Piriformospora indica* (GenBank No. CCA66870.1) respectively (data not shown). The AG 2-2IIIIB specific genomic fragment did not reveal significant homology at the nucleotide level with any available fungal genome sequences but revealed only a weak (35%) homology at the amino acid level with the endonu-

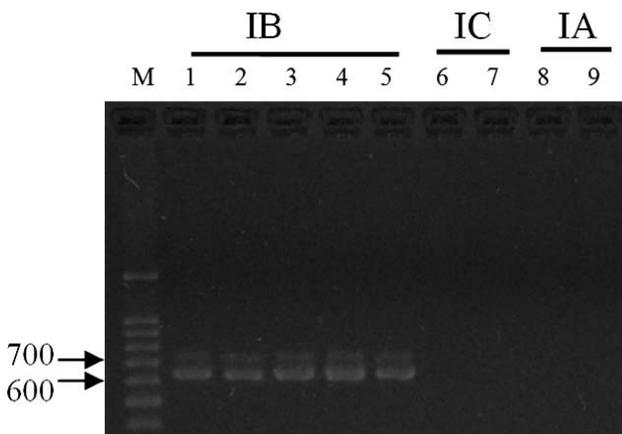


FIG. 4. PCR products generated by primer pair L2-1F/G for *R. solani* AG 1-IA, IB and IC isolates. Lanes 1–5, AG 1-IB isolates BELT 2, BLBG 320, LB 123, PW 326 and BM2 respectively; lanes 6–7, AG 1-IC isolates BM3 and CR2 respectively; and lanes 8–9, AG 1-IA isolates 92'AR Soy1 and 92'AR Soy2 respectively. Lane M is the molecular marker.

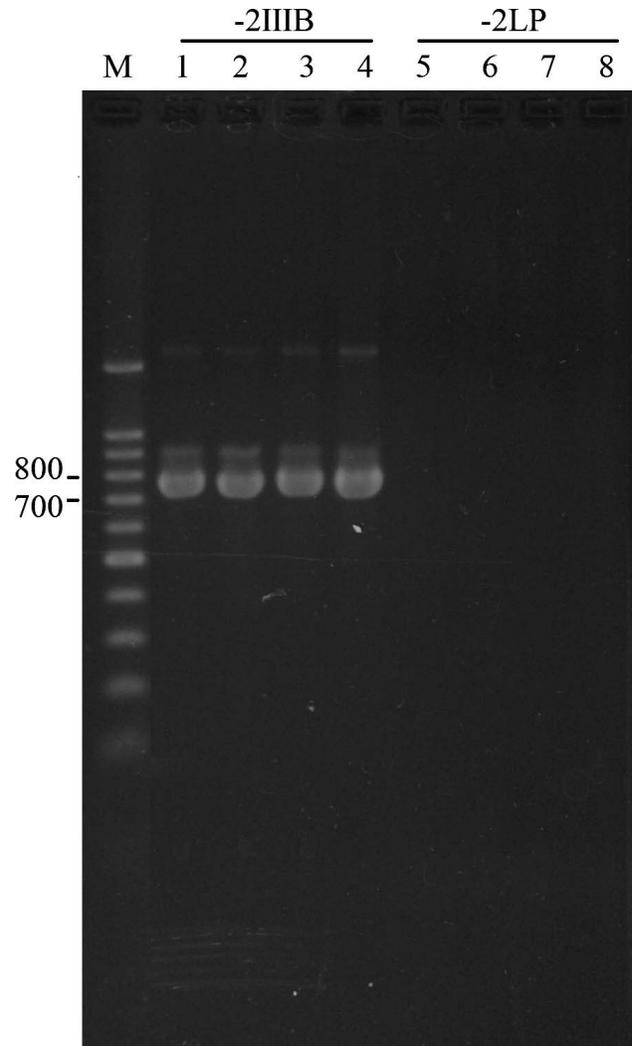


FIG. 5. PCR products generated with primer pair L45-A/C for *R. solani* AG 2-2 isolates. Lanes 1–4, AG 2-2IIIIB isolates BELT 114, BLBG 6, BLBG 32C and Rh146; lanes 5–8, AG 2-2LP isolates Rh141, ACC3, CGCF18[2] and SGGC2. Lane M is the molecular marker.

lease/exonuclease/phosphatase family protein of ascomycetous fungus *Verticillium albo-atrum* (GenBank No. XP_003006791.1) (data not shown).

The sequence data of specific markers of AG 1-IB and AG 2-2IIIIB were used to design specific primer pairs L21-F/G and L45-A/C (see MATERIALS AND METHODS). The primer pairs L21-F/G and L45-A/C produced fragments only for isolates of AG 1-IB (FIG. 4) and AG 2-2IIIIB (FIG. 5) respectively when pure DNA of the causal pathogens and their closely related AGs were amplified by PCR. A wide range of annealing temperatures (58–63 C) produced similar results for both primer pairs by generating bands for either AG 1-IB isolates or AG 2-2IIIIB isolates. No bands were produced for closely related AG

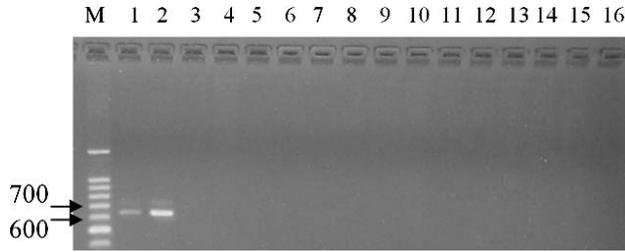


FIG. 6. PCR products derived from primer pair L21-F/G for different *Rhizoctonia* species and AGs. Lanes 1–2, *R. solani* AG 1-IB isolates BELT 2 and BM2 respectively; Lane 3, binucleate *Rhizoctonia*-like fungal isolate BELT 122; Lane 4, *R. cerealis* isolate C610; Lanes 5–8, *W. circinata* isolates AVGCAV, 87NEP, PW 220 and EDHGED respectively; Lanes 9–16, *R. solani* AG 8 (Rh89/T), AG 6 (IOEEA), AG 5 (Rh102), AG 4 (RS23), AG 3 (1AP), AG 2-IIIB (Rh146), AG 2-2LP (Rh141) and AG 2-1 (BM4) respectively. Lane M is the molecular marker.

subgroups of AG 1-IB (i.e. IA, IC) and AG 2-IIIB (i.e. 2-2LP). The specific primer pairs tested on pure DNA of other *R. solani* AGs, *R. cerealis* and varieties of *W. circinata* at 63 C annealing temperature did not yield amplification products (FIGS. 6, 7).

Detection of Rhizoctonia solani from diseased leaves.—The total DNA of all brown-patch samples of perennial ryegrass produced a single band only when amplified with specific PCR primer pair L21-F/G (FIG. 8), indicating all causal pathogens were AG 1-IB. The total DNA of all bentgrass samples with brown-patch symptoms produced a single DNA fragment when amplified with specific PCR primer pair L45-A/C, indicating all *R. solani* causal pathogens were AG 2-IIIB (FIG. 9). These samples did not produce a band for primer pair L21-F/G. The total DNA of one leaf

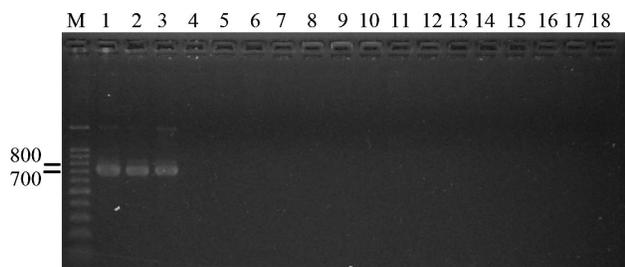


FIG. 7. PCR products derived from primer pair L45-A/C for different *Rhizoctonia* species and AGs. Lanes 1–3, AG 2-IIIB isolates BELT 150, BLBG 6 and Rh146 respectively; Lane 4, AG 2-1 isolate BM4; Lane 5, binucleate *Rhizoctonia*-like fungal isolate BELT 122; Lane 6, *R. cerealis* isolate C610; Lanes 7–10, *W. circinata* isolates AVGCAV, 87NEP, PW 220 and EDHGED respectively; Lanes 11–18, *R. solani* AG 8 (Rh89/T), AG 6 (IOEEA), AG 5 (Rh102), AG 4 (RS23), AG 3 (1AP), AG 1-IC (CR2), AG 1-IB (BM2) and AG 1-IA (92'AR Soy1) respectively. Lane M is the molecular marker.

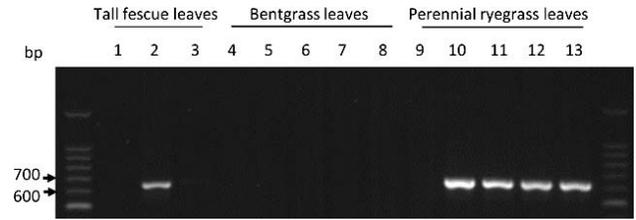


FIG. 8. PCR products derived from *R. solani* AG 1-IB specific primer pair L21-F/G using total DNA of turfgrass leaves with brown patch symptoms. One sample of tall fescue (lane 2) and all samples of perennial ryegrass (lanes 10–13) produced a single fragment. Diseased creeping bentgrass samples (lanes 6–9) did not produced a band. Lanes 1, 4 and 9 are negative controls having DNA from healthy turfgrass leaves.

sample of tall fescue revealing brown-patch symptoms produced a DNA fragment when amplified with specific PCR primer pair L21-F/G (FIG. 8). The other sample produced a DNA fragment for specific PCR primer pair L45-A/C (FIG. 9). The total DNA extracted from healthy turfgrass leaves did not produce a band for either primer pair.

DISCUSSION

Cross-blot hybridization was carried out with UP-PCR products of tester isolates to determine how efficiently isolates of unknown *W. circinata* varieties can be identified. This study revealed that UP-PCR banding profiles of *W. circinata* varieties display sufficient polymorphism and hence cross hybridization of UP-PCR products can be used to detect unknown *Waitea* isolates. Most isolates produce strong hybridization signals for homologous probes and weak to no signals for partially homologous or probes lacking homology. Therefore, UP-PCR product cross-blot hybridization can rapidly identify *W. circinata* isolates. A study by

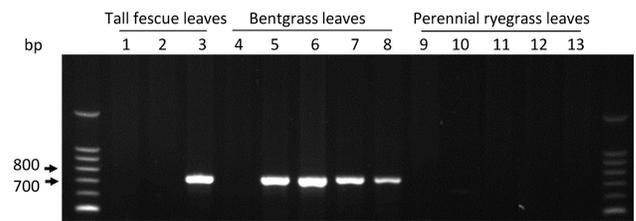


FIG. 9. PCR products derived from *R. solani* AG 2-IIIB specific primer pair L45-A/C using total DNA of turfgrass leaves with brown-patch symptoms. One sample of tall fescue (lane 3) and all samples of creeping bentgrass (lanes 5–8) produced a single fragment. Diseased perennial ryegrass samples (lanes 10–13) did not produced a band. Lanes 1, 4 and 9 are negative controls having DNA from healthy turfgrass leaves.

Lübeck and Poulsen (2001) had reported UP-PCR could differentiate AGs of *R. solani* by cross-blot hybridization. They identified 16 isolates of *R. solani* into their AG subgroup with this technique. In particular, UP-PCR hybridization can be a useful technique to rapidly screen and link a large number of unknown *Rhizoctonia* and *Waitea* isolates to their AGs and AG subgroups. However, the success of this assay heavily depends on the synthesis of efficient probes with high-quality UP-PCR fragments. The UP-PCR products of different tester isolates can be used to make DNA microarrays. These chips with micron-range spots of DNA fragments of reference isolates arrayed on a glass slide can be used to identify numerous unknown isolates simultaneously (Wilgenbus and Lichter 1999).

DNA/DNA cross hybridization between even closely related isolates of fungi is low (Kuninaga 1996). Because different *Rhizoctonia* AGs and AG subgroups have low sequence complementarity, cross hybridization can be used for the designation of isolates into these genetic entities (Vilgalys 1988; Carling and Kuninaga 1990; Kuninaga 1996). DNA hybridization percentages for *Rhizoctonia* isolates falling into different AGs are $\leq 15\%$ while members within an AG mostly have high ($\geq 90\%$) hybridization percentages, according to Kuninaga (1996). Isolates representing different subgroups have less than 60% hybridization percentages, except for AG 2-2 subgroups (2IIIB, 2IV, 2LP) that are about 70%. Lübeck and Poulsen (2001) compared DNA/DNA hybridization values of *R. solani* with UP-PCR cross-hybridization values to estimate the percentage of genomic similarity (expressed as a DNA hybridization value) from the intensity of signal. A strong UP-PCR signal seems to indicate a DNA hybridization value of $> 75\%$ for *R. solani*, according to Lübeck and Poulsen. Because our study focused on *W. circinata*, these percentages may not be directly comparable.

The specific primers L21-F/G and L45-A/C, which were designed to identify AG 1-IB and AG 2-IIIB groups respectively, did not generate any bands for nontarget *Rhizoctonia* species. No PCR products were generated for closely related AG 1 supgroups AG 1-IA and AG 1-IC or AG 2 subgroup AG 2-2LP, even at the low annealing temperature of 58 C. The total DNA of turfgrass with severe brown-patch symptoms produced a single PCR product when amplified with AG 1-IB and AG 2-IIIB specific primers. This indicates the uniqueness of the UP-PCR bands targeted by specific primers L21-F/G and L45-A/C in identifying specific *R. solani* AGs. Therefore, UP-PCR fragments can be used to design specific primers for detection of *R. solani*.

Rhizoctonia species are readily isolated from diseased plants and soils (Ogoshi 1987). They grow

relatively faster in water agar compared to most other fungi and therefore can be easily separated to obtain pure cultures. However, disease symptoms, cultural morphology and the microscopic view of different AGs of *Rhizoctonia* can be similar and therefore diagnosis based on these characters is not reliable. PCR-based amplification of pathogen DNA by specific primers is an alternative method to rapidly and accurately identify AGs and AG subgroups of *Rhizoctonia* isolates. Specific-primer designing was based on identifying a unique band present in all isolates of a target AG type and absent in all other AGs and AG subgroups. Specific primer testing was done with all possible *Rhizoctonia* species and AGs that have been reported to infect turfgrasses to ensure no PCR product was generated from DNA of nontarget organisms. *Rhizoctonia solani* isolates infecting turfgrasses include AG 1–AG 6 (Burpee and Martin 1992; Zhang and Dernoeden 1997; Smiley et al. 2005). *Waitea circinata* var. *zeae*, *oryzae* and *circinata* also are capable of inciting different patch diseases on turf (Smiley et al. 2005; de la Cerda et al. 2007). The main binucleate pathogen to cause *Rhizoctonia* blight is *R. cerealis* (Burpee and Martin 1992). In addition, there are numerous binucleate *Rhizoctonia*-like species isolated from turfgrasses (Hurd and Grisham 1983; Burpee and Gouly 1984; Burpee and Martin 1992).

It is possible to identify different *Rhizoctonia* AGs and AG subgroups by PCR-based techniques. Salazar et al. (2000) developed specific primers based on ITS sequence data that could amplify *R. solani* AG 2-1, AG 2-2 and AG 2-3 and ecological group AG 2-t (isolated from tulip plants). However, these primers were not capable of separating AG 2-2 cultural types AG 2-2IIIB, AG 2-2IV and AG 2-2LP. Toda et al. (2004) designed primers from a unique product of RAPD banding patterns to specifically identify AG 2-2LP isolates from diseased turfgrass and reported that those specific primers could amplify and generate a PCR product when the DNA concentration was higher than 1 ng/ μ L. Bounou et al. (1999) developed SCAR markers for specific identification of *R. solani* AG 3 isolates causing black scurf of potato. Leclerc-Potvin et al. (1999) identified binucleate biocontrol *Rhizoctonia* isolates of AG-G with a similar approach. Brisbane et al. (1995) developed RAPD-generated sequenced tag site (STS) molecular markers to identify *Rhizoctonia solani* AG 4 or AG 8 infecting wheat in South Australia. Johanson et al. (1998) used a PCR-based method to distinguish the three *Rhizoctonia* spp. complex (i.e. *R. solani*, *R. oryzae*, *R. oryzae-sativae*) causing rice-sheath blight. Grosch et al. (2007) identified a distinct molecular marker of 1.54 kb for isolates of AG 1-IB and AG 1-ID with the RAPD technique. Then they used the sequence

information of the 1.54 bp amplicon to design a SCAR primer set specific for AG 1-IB isolates. The specific primer set produced a single fragment of 324 bp only from genomic DNA of isolates of AG 1-IB, according to the authors. Therefore, our discovery of a second AG 1-IB-specific SCAR primer set will be a nice addition for molecular identification purposes.

The specific PCR primers developed in this study to identify AG1-IB and AG2-2IIIB, and the UP-PCR cross-blot hybridization tests to closely differentiate related *W. circinata* varieties *zeae* and *circinata* will be highly useful for future epidemiological investigations of *Rhizoctonia* patch diseases. PCR-based identification of plant pathogens is preferred because primers can amplify even small quantities of target organism DNA. Accordingly, our specific primers revealed the ability to amplify a PCR product from total DNA extracted from brown-patch diseased leaves. These specific primers may be used to develop multiplex assays to identify the presence of different *Rhizoctonia* pathogens in field samples.

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