2013

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Mycologia, 105(5), 2013, pp. 1190–1201. DOI: 10.3852/12-368

DNA fingerprinting and anastomosis grouping reveal similar genetic diversity in *Rhizoctonia species* infecting turfgrasses in the transition zone of USA

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Abstract: *Rhizoctonia* blight is a common and serious disease of many turfgrass species. The most widespread causal agent, *Thanatephorus cucumeris* (anamorph: *R. solani*), consists of several genetically different subpopulations. In addition, *Waitea circinata* varieties *zeae*, *oryzae* and *circinata* (anamorph: *Rhizoctonia* spp.) also can cause the disease. Accurate identification of the causal pathogen is important for effective management of the disease. It is challenging to distinguish the specific causal pathogen based on disease symptoms or macroscopic and microscopic morphology. Traditional methods such as anastomosis reactions with tester isolates are time consuming and sometimes difficult to interpret. In the present study universally primed PCR (UP-PCR) fingerprinting was used to assess genetic diversity of *Rhizoctonia* spp. infecting turfgrasses. Eighty-four *Rhizoctonia* isolates were sampled from diseased turfgrass leaves from seven distinct geographic areas in Virginia and Maryland. *Rhizoctonia* isolates were characterized by ribosomal DNA internal transcribed spacer (rDNA-ITS) region and UP-PCR. The isolates formed seven clusters based on ITS sequences analysis and unweighted pair group method with arithmetic mean (UPGMA) clustering of UP-PCR markers, which corresponded well with anastomosis groups (AGs) of the isolates. Isolates of *R. solani* AG 1-IB (n = 18), AG 2-2IIIB (n = 30) and AG 5 (n = 1) clustered separately. *Waitea circinata* var. *zeae* (n = 9) and var. *circinata* (n = 4) grouped separately. A cluster of six isolates of *Waitea* (UWC) did not fall into any known *Waitea* variety. The binucleate *Rhizoctonia*-like fungi (BNR) (n = 16) clustered into two groups. *Rhizoctonia solani* AG 2-2IIIB was the most dominant pathogen in this study, followed by AG 1-IB. There was no relationship between the geographic origin of the isolates and clustering of isolates based on the genetic associations. To our knowledge this is the first time UP-PCR was used to characterize *Rhizoctonia*, *Waitea* and *Ceratobasidium* isolates to their infra-species level.

Key words: binucleate *Rhizoctonia*, rDNA-ITS, *Rhizoctonia solani*, turfgrass, UP-PCR, *Waitea circinata*

Introduction

Multiple *Rhizoctonia* species are responsible for causing leaf blight on turfgrasses. Both warm-season and cool-season turfgrasses are susceptible to *Rhizoctonia* blight with the warm humid and warm tropical climatic zones highly favoring the disease (Burpee and Martin 1992).

In the United States, cool-season turfgrasses dominate northern states while warm-season turfgrasses are adapted to the warm-humid or warm-arid conditions of the southern states. The central part of the country that is at the northern range of adaptation of the warm-season grasses and at the southern range of adaptation of the cool-season grasses is known as the transition zone. Although none of the turfgrass types are well adapted to the transition zone, both are routinely grown and managed in this region. *Rhizoctonia* blight poses a major threat to successful growth and maintenance of several important turfgrass species in both southern and transition zones (Burpee and Martin 1992). The most common *Rhizoctonia* species, *R. solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk), is responsible for causing brown patch on cool-season grasses and large patch on warm-season grasses. Both *R. zeae* Voorhees (teleomorph: *Waitea circinata* var. *zeae* Warcup and Talbot) and *R. oryzae* Ryker and Gooch (teleomorph: *W. circinata* var. *oryzae* Warcup and Talbot) are responsible for leaf and sheath spot disease. Unlike other *Rhizoctonia* diseases, yellow patch caused by *R. cerealis* van der Hoeven (teleomorph: *Ceratobasidium cereale* Murray and Burpee; anastomosis group: AG-D) favors cool and wet weather (Smiley et al. 2005, de la Cerda et al. 2007). Another recently identified causal agent, *Waitea circinata* var. *circinata*, is responsible for brown ring patch in golf courses (de la Cerda et al. 2007). All

Submitted 2 Nov 2012; accepted for publication 8 Apr 2013.
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aforementioned species have multinucleate cells except *R. cerealis*, which is a binucleate species. There are reports of other binucleate *Rhizoctonia*-like fungi (BNR) being isolated from turfgrass swards or soils (Hurd and Grisham 1983, Burpee and Goulty 1984, Burpee and Martin 1992). Those isolates differ from *R. cerealis* in physiological characters and anastomosis reactions. In general, diseased turfgrasses develop circular areas of blighted brown leaves. Therefore, reliance on field symptoms to identify *Rhizoctonia* causal agents can be misleading. Microscopically all *Rhizoctonia* species look similar in the asexual state and typically characterized by nonsporulating mycelia with 90 degree branches having dolipore septa (Smiley et al. 2005). The sexual stage of the causal pathogens is rare in nature and difficult to induce in vitro.

Rhizoctonia species and anastomosis groups (AGs) vary in sensitivity to commonly applied fungicides (Martin et al. 1984a, b; Carling et al. 1990; Kataria et al. 1991; Kataria and Gisi 1996; Campion et al. 2003). In addition, different *Rhizoctonia* species have different temperature ranges most conducive for causing disease. Therefore accurate identification of the causal pathogen is important to predict disease progression and make future disease management decisions. Moreover, knowledge on the prevalence and distribution of pathogenic *Rhizoctonia* isolates is important for selecting appropriate turfgrass germplasm with resistance to *Rhizoctonia* blight.

The classical method of grouping and identification of *Rhizoctonia* isolates is based on anastomosis, which is the hyphal fusion reactions of vegetatively compatible isolates, with tester isolates. Anastomosis groups also represent genetic isolation (Ogoshi 1987, Carling 1996). Research has reported six AGs of *R. solani* capable of infecting turfgrasses (Burpee and Martin 1992, Zhang and Dernoeden 1995). However, anastomosis reactions can be difficult to interpret and are time consuming. Some AGs have been divided further into subgroups to reflect differences observed in the culture appearance, morphology, host range, pathogenicity, thiamine requirements and hyphal fusion frequency. These subgroups within a single AG exhibit compatible anastomosis reactions and hence are not distinguishable (Carling 1996). Under these circumstances, AGs may not provide sufficient information on genetic variation and taxonomic relationships among isolates (Carling 1996, Lübeck 2004). Therefore, additional identification methods are needed to supplement anastomosis reactions.

Since the mid-1990s, many studies of *Rhizoctonia* species have used primers specific to ribosomal DNA internal transcribed spacer (rDNA-ITS) regions for evaluating genetic diversity among and within anastomosis groups (Boysen et al. 1996, Kuninaga et al. 1997, Kuninaga et al. 2000, Gonzalez et al. 2001, Hsiang and Dean 2001, Pannecoucque and Höfte 2009). Although analysis of the ITS region is a well tested method for identifying *Rhizoctonia* species, it can be cost prohibitive for investigating a large number of samples. Some isolates are difficult to sequence without cloning, which adds time and cost to the analysis. The advancement of technology and automated sequencing has made the analysis of DNA fingerprinting profiles significantly less expensive than ITS sequencing. Various PCR-based DNA fingerprinting methods have been used for identification and classification of *Rhizoctonia* species (Lübeck and Poulsen 2001, Sharon et al. 2006). Some fingerprinting techniques are more suited for characterizing individuals within an AG rather than among different AGs (Cubeta and Vilgalys 1997, Sharon et al. 2006). The universally primed PCR (UP-PCR) is a fingerprinting technique that generates multiple DNA fragments from an organism without prior knowledge of its DNA sequence. The main difference between UP-PCR and the well known random amplified polymorphic DNA (RAPD) technique is the length of the primers used. Universal primers (UP) are longer (15–21 nt) than conventional RAPD primers (typically 10 nt) and designed to anneal under more stringent conditions (52–60 C) ensuring higher reproducibility of banding profiles (Trigiano et al. 2004, Lübeck and Lübeck 2005). Lübeck and Poulsen (2001) found considerable variation in UP-PCR banding profiles among isolates of *R. solani* from AGs and AG subgroups. However they did not include *Waitea* or *Ceratobasidum* species in their study.

Only a few studies have investigated the genetic diversity of *Rhizoctonia* species causing disease on cool-season turfgrasses in Virginia and Maryland, located within the transition zone of the United States. The primary objectives of this study were to investigate the genetic diversity of *Rhizoctonia* isolates causing brown patch in VA and MD and determine whether UP-PCR fingerprinting simultaneously can characterize isolates by their appropriate AG. Furthermore, we hypothesized a correlation between geographic location within the transition zone and the genetic relationships of the isolates.

MATERIALS AND METHODS

Sample collection and pathogen isolation.—Isolates of *Rhizoctonia* were collected from diseased turfgrasses during summer months of 2008 and 2009 (APHIS permit No. P526P-09-02509). They were collected from less intensively managed tall fescue lawns and more intensively managed golf courses in VA and MD (TABLE I, SUPPLEMENTARY FIG. 1). Fungal isolates collected per site were 9–20. Diseased leaf pieces were rinsed in distilled water and placed onto 2% water agar plates. *Rhizoctonia*-like cultures were verified based on microscopic observations and

transferred to Petri plates with quarter-strength potato dextrose agar (¼× PDA) (Difco Laboratories, Detroit, Michigan) for obtaining pure cultures. Vials of sterile wheat seeds were inoculated with *Rhizoctonia* cultures for long term preservation. Once *Rhizoctonia* isolates had fully colonized the substrate, 15% glycerol was added to the vials, which were stored at −75 C.

A total of 84 randomly selected *Rhizoctonia* isolates from six sites were used (TABLE I). *Waitea* species first were identified from the sample, based on colony morphology (i.e. formation of salmon pink to orange sclerotia) when cultured on PDA plates. Isolates that did not belong to *Waitea* species were grouped into binucleates and multinucleates by staining with 4′, 6-diamidino-2-phenylindole (DAPI) and observing under a fluorescence microscope for number of nuclei present per fungal cell.

Anastomosis grouping.—Where possible isolates of *Rhizoctonia* were grouped into their respective AGs using tester isolates of *R. solani*, *Waitea circinata* varieties and binucleate *R. cerealis*. Isolate pairing was done on large Petri plates having a thin layer of 1.5% water agar. A mycelial plug of the tester strain was placed at the center and paired with three plugs of the field isolate placed near the edge of the plate at equal distance from each other. An inverted phase contrast microscope was used to observe hyphal pairings. Hyphal fusion reactions observed with one field plug and the center tester isolate were considered one replicate and therefore one plate consisted of three replicates of hyphal fusion reactions. Grouping of unknown isolates into their respective AGs were done in accordance to the method described by Carling et al. (2002) and Macnish and Carling (1997).

DNA extraction and assembling and displaying relevant ITS sequences.—The QIAGEN DNeasy plant mini kit (QIAGEN Inc., Valencia, California) was used for DNA extraction from isolates according to the manufacturer's instructions. The ITS region of fungal DNA from the isolates collected in this study was amplified with the primer set of ITS1 (5′–TCCGTAGGTGAACCTGCGG–3′) and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) (White et al. 1990) with the method of Hsiang and Dean (2001) and Pannecoucque and Höfte (2009). Purified ITS templates were amplified with the Big Dye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to the manufacturer's protocol. The amplified products were sequenced by an Applied Biosystems 3730 Genetic Analyzer (Applied Biosystems). Published ITS sequences of relevant *Rhizoctonia* AGs along with species and varieties of *Waitea* and *Ceratobasidium* ITS sequences were downloaded from GenBank and aligned with our sequences. Consensus sequences of each isolate were obtained by assembling the forward and reverse sequences with the Lasergene SeqMan suite (DNASTAR, Madison, Wisconsin). The sequence data of all isolates were aligned by the Clustal W algorithm in MEGA 5.0 (Tamura et al. 2011). The alignments were manually adjusted to optimize the alignment. A phylogenetic tree was created from distance matrix values by the neighbor-joining method (Saitou and Nei 1987) with MEGA 5. The clades of the tree were midpoint rooted and branch support was calculated by bootstrap analysis based on 500 replicate heuristic searches.

UP-PCR product generation.—A UP-PCR reaction mixture of 25 μL containing 1× standard Taq polymerase reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 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) were used for each reaction. Four UP primers initially were tested with 10 *Rhizoctonia* isolates to determine which primers generate multiple fragments. Successful primers were tagged with fluorescent dye 6-FAM to facilitate data collection on the Applied Biosystems 3730 Genetic Analyzer. The primers L45 (5′–GTAAAACGACGGCCAGT–3′), L15/AS19 (5′–GAGGG TGGCGGCTAG–3′), AS15 (5′–GGCTAAGCGGTCGTTAC–3′), and L21(5′–GGATCCGAGGGTGGCGGTTCT–3′) (Cumagun et al. 2000, Lübeck and Lübeck 2005) were tested. The UP-PCR was performed in a PTC-200 thermo-cycler (MJ Research, Ramsey, Minnesota). The initial denaturation was done at 94 C for 2 min, followed by 35 cycles of DNA denaturation at 94 C for 1 min, primer annealing at 52 C for 1 min and primer extension at 72 C for 1 min. A final extension step was performed at 72 C for 10 min. Based on the number and clarity of peaks, L21 and L15/AS19 primers were selected. These primers were also tested for reproducibility by repeating UP-PCR reaction two times with the same set of *Rhizoctonia* isolates. Because the banding profiles were highly uniform, these primers were used on the larger collection of isolates (see TABLE I). Five tester isolates of *Rhizoctonia*, namely AVGCAV (*W. circinata* var. *zeae*, origin: annual bluegrass, USA), BSCCST (*W. circinata* var. *circinata*, origin: annual bluegrass, USA), 87NEP (*W. circinata* var. *oryzae*, origin: rice, Japan), Rh 146 (*R. solani* AG 2-2IIIB, origin: creeping bentgrass, USA) and Rh 102/T (*R. solani* AG 5, origin: unknown) also were used in the analysis. The first two isolates were obtained from Frank Wong, University of California, Riverside. The third isolate, originally sampled by Takeshi Toda of Japan, was obtained from Lane Tredway, North Carolina State University, Raleigh. The fourth and fifth isolates were provided by Lee Burpee, University of Georgia, Athens.

Analysis of UP-PCR data.—PCR products were prepared for analysis by mixing 0.75 μL each sample with 10 μL Hi-Di Formamide (Applied Biosystems) and 0.4 μL the internal marker GeneScan™ 1200 LIZ® (Applied Biosystems). The samples were denatured for 5 min at 94 C and chilled immediately for several minutes on ice before being loaded on the Applied Biosystems 3730 Genetic Analyzer. The GeneScan™ 1200 LIZ® has the ability to size fragments of 20–1200 bp, however amplified bands smaller than 50 bases were not scored. GeneMapper® 3.7 (Applied Biosystems) was used to analyze the data files obtained from the Applied Biosystems 3730 Genetic Analyzer. UP-PCR profiles are binomially scored as zeros and ones by this software. A zero represents no fragment and a one indicates presence of a band of a particular length. The binary table was processed by converting it to a distance matrix based on Nei and Li (1979) distance method with the RESTDIST option of PHYLIP 3.68 (http://evolution.genetics.washington.edu/phylip.

TABLE I. Geographic origin, host, management type, species, anastomosis group and GenBank accession number of isolates used in this study[a]

Isolate type	Origin	Host	Management acronym	Species group	Anastomosis	GenBank accession no.
ANP 202B	Annapolis, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631193
ANP 205A	Annapolis, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631194
ANP 205B2	Annapolis, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631195
ANP 309A	Annapolis, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631196
ANP 301B	Annapolis, MD	Tall fescue	Lawn	Rs	AG 1-IB	JX631170
ANP 306B	Annapolis, MD	Tall fescue	Lawn	Rs	AG 1-IB	JX631171
ANP 107	Annapolis, MD	Tall fescue	Lawn	BNR	unknown	JX631214
ANP 109B	Annapolis, MD	Tall fescue	Lawn	UWC	WAG	JX631224
ANP 304	Annapolis, MD	Tall fescue	Lawn	UWC	WAG	JX631225
BELT 114	Beltsville, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631189
BELT 150	Beltsville, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631190
BELT 262	Beltsville, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631191
BELT 26	Beltsville, MD	Tall fescue	Lawn	Rs	AG 1-IB	JX631156
BELT 2	Beltsville, MD	Tall fescue	Lawn	Rs	AG 1-IB	JX631157
BELT 122	Beltsville, MD	Tall fescue	Lawn	BNR	unknown	JX631205
BELT 17	Beltsville, MD	Tall fescue	Lawn	BNR	unknown	JX631220
BELT 5	Beltsville, MD	Tall fescue	Lawn	Wcz	WAG-Z	JX631239
BELT 159	Beltsville, MD	Tall fescue	Lawn	Wcz	WAG-Z	JX631237
BELT 228	Beltsville, MD	Tall fescue	Lawn	UWC	WAG	JX631221
BLBG 6	Blacksburg, VA	CBG/ABG	Golf green	Rs	AG 2-2IIIB	JX631186
BLBG 13	Blacksburg, VA	CBG/ABG	Golf green	Rs	AG 2-2IIIB	JX631185
BLBG 20C	Blacksburg, VA	CBG/ABG	Golf green	Rs	AG 2-2IIIB	JX631180
BLBG 22C	Blacksburg, VA	CBG/ABG	Golf green	Rs	AG 2-2IIIB	JX631181
BLBG 32C	Blacksburg, VA	CBG/ABG	Golf green	Rs	AG 2-2IIIB	JX631182
BLBG 320	Blacksburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631162
BLBG 510	Blacksburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631165
BLBG 430	Blacksburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631164
BLBG 350	Blacksburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631163
BLBG 211	Blacksburg, VA	CBG/ABG	Golf green	Wcc	WAG	JX631228
BLBG 216	Blacksburg, VA	CBG/ABG	Golf green	Wcc	WAG	JX631229
BLBG 202	Blacksburg, VA	CBG/ABG	Golf green	Wcc	WAG	JX631230
BLBG 8	Blacksburg, VA	CBG/ABG	Golf green	Wcc	WAG	JX631227
HDN 102	Herndon, VA	CBG/ABG	Golf green	Rs	AG 2-2IIIB	JX631201
HDN 208By	Herndon, VA	Tall fescue	Golf rough	Rs	AG 2-2IIIB	JX631202
HDN 225	Herndon, VA	Tall fescue	Golf rough	Rs	AG 2-2IIIB	JX631203
HDN 111A	Herndon, VA	Tall fescue	Golf rough	Rs	AG 1-IB	JX631166
HDN 122A	Herndon, VA	Tall fescue	Golf rough	Rs	AG 1-IB	JX631167
HDN 302	Herndon, VA	Tall fescue	Golf rough	Rs	AG 1-IB	JX631168
HDN 209	Herndon, VA	Tall fescue	Golf rough	BNR	unknown	JX631213
HDN 221	Herndon, VA	Tall fescue	Golf rough	BNR	unknown	JX631216
HDN 324A	Herndon, VA	CBG/ABG	Golf green	BNR	unknown	JX631217
HDN 325B	Herndon, VA	CBG/ABG	Golf green	BNR	unknown	JX631215
HDN 115A	Herndon, VA	Tall fescue	Golf rough	Wcz	WAG-Z	JX631235
HDN 211	Herndon, VA	Tall fescue	Golf rough	Wcz	WAG-Z	JX631236
HDN 222A	Herndon, VA	Tall fescue	Golf rough	UWC	WAG	JX631226
LB 312	Leesburg, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631192
LB 317	Leesburg, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631183
LB 325	Leesburg, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631184
LB 4118B	Leesburg, VA	Tall fescue	Golf rough	Rs	AG 2-2IIIB	JX631197
LB 4303	Leesburg, VA	Tall fescue	Golf rough	Rs	AG 2-2IIIB	JX631198
LB 4316	Leesburg, VA	CBG/ABG	Golf green	Rs	AG 2-2IIIB	JX631199
LB 4319	Leesburg, VA	Tall fescue	Golf rough	Rs	AG 2-2IIIB	JX631200
LB 123	Leesburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631158
LB 124	Leesburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631159

TABLE I. Continued

Isolate type	Origin	Host	Management acronym	Species group	Anastomosis	GenBank accession no.
LB 127	Leesburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631160
LB 234	Leesburg, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631161
LB 4217	Leesburg, VA	Tall fescue	Golf rough	Rs	AG 1-IB	JX631169
LB 204	Leesburg, VA	Tall fescue	Lawn	Rs	AG 5	JX631204
LB 226	Leesburg, VA	Tall fescue	Lawn	BNR	unknown	JX631206
LB 4202A	Leesburg, VA	Tall fescue	Golf rough	BNR	unknown	JX631219
LB 4214A	Leesburg, VA	Tall fescue	Golf rough	BNR	unknown	JX631218
LB 319	Leesburg, VA	Tall fescue	Lawn	Wcz	WAG-Z	JX631233
LB 228	Leesburg, VA	Tall fescue	Lawn	Wcz	WAG-Z	JX631234
LB 4116	Leesburg, VA	Tall fescue	Golf rough	Wcz	WAG-Z	JX631238
LB 4220	Leesburg, VA	CBG/ABG	Golf green	UWC	WAG	JX631222
BSF 69	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631176
BSF 50	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631175
BSF 42	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631174
BSF 90	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631177
BSF 207	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631178
BSF 209	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631179
BSF 214	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631188
BSF 127	Richmond, VA	Tall fescue	Lawn	Rs	AG 2-2IIIB	JX631187
BSF 224	Richmond, VA	Tall fescue	Lawn	BNR	unknown	JX631212
BSF 13	Richmond, VA	Tall fescue	Lawn	UWC	WAG	JX631223
PW 326	Woodbridge, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631173
PW 353	Woodbridge, VA	Tall fescue	Lawn	Rs	AG 1-IB	JX631172
PW 154	Woodbridge, VA	Tall fescue	Lawn	BNR	unknown	JX631207
PW 205	Woodbridge, VA	Tall fescue	Lawn	BNR	unknown	JX631208
PW 216	Woodbridge, VA	Tall fescue	Lawn	BNR	unknown	JX631209
PW 249	Woodbridge, VA	Tall fescue	Lawn	BNR	unknown	JX631210
PW 341	Woodbridge, VA	Tall fescue	Lawn	BNR	unknown	JX631211
PW 220	Woodbridge, VA	Tall fescue	Lawn	Wcz	WAG-Z	JX631232
PW 119	Woodbridge, VA	Tall fescue	Lawn	Wcz	WAG-Z	JX631231

[a] ABG = annual bluegrass; CBG = creeping bentgrass; Rs = *R. solani*; BNR = binucleate *Rhizoctonia*-like fungi; Wcz = W. *circinata* var. *zeae;* Wcc = *W. circinata* var. *circinata*; UWC = unidentified *W. circinata* species.

html). The distance matrix was imported into MEGA 5 for dendrogram constructing based on the unweighted pair group method with arithmetic mean (UPGMA) method (Sneath and Sokal 1973).

RESULTS

Isolate composition.—Isolate identification by molecular techniques indicated that *R. solani* AG 2-2IIIB is the most common pathogen causing *Rhizoctonia* blight in the surveyed areas. Isolates of AG 2-2IIIB made up 36% (30/84) of the total sample, followed by isolates of AG 1-IB representing 21% (18/84) of the sample. Isolates of AG 2-2IIIB were collected from all sites except Woodbridge while isolates of AG 1-IB were present in all surveyed areas expect Richmond (TABLE I). *Rhizoctonia solani* AG 5 was the least common pathogen among sampled turfgrasses in this study; a single isolate was collected from Leesburg, Virginia. *Waitea circinata* var. *zeae* (Wcz) was the most dominant *Waitea* species present in the surveyed areas representing 11% (9/84) of the isolates. Isolates of Wcz were present in low numbers in all locations east of Richmond (SUPPLEMENTARY FIG. 1, TABLE I). There were four isolates of *W. circinata* var. *circinata* (Wcc) and all of these isolates were collected from Blacksburg, Virginia. Six unknown *W. circinata* (UWC) isolates also were collected from Herndon, Leesburg, Richmond, Beltsville and Annapolis (TABLE I). Isolates of BNR were identified in most locations and represented 16% (16/84) of the total sample. All isolates of *R. solani* (n = 7) from creeping bentgrass/annual bluegrass (*Agrostis stolonifera* L./*Poa annua* L.) (CBG/ABG) putting greens belonged to AG 2-2IIIB (TABLE I). Putting green samples also gave four isolates of Wcc, two isolates of BNR and a single isolate of UWC. We summarized the *Rhizoctonia* isolates collected from different host species (TABLE II).

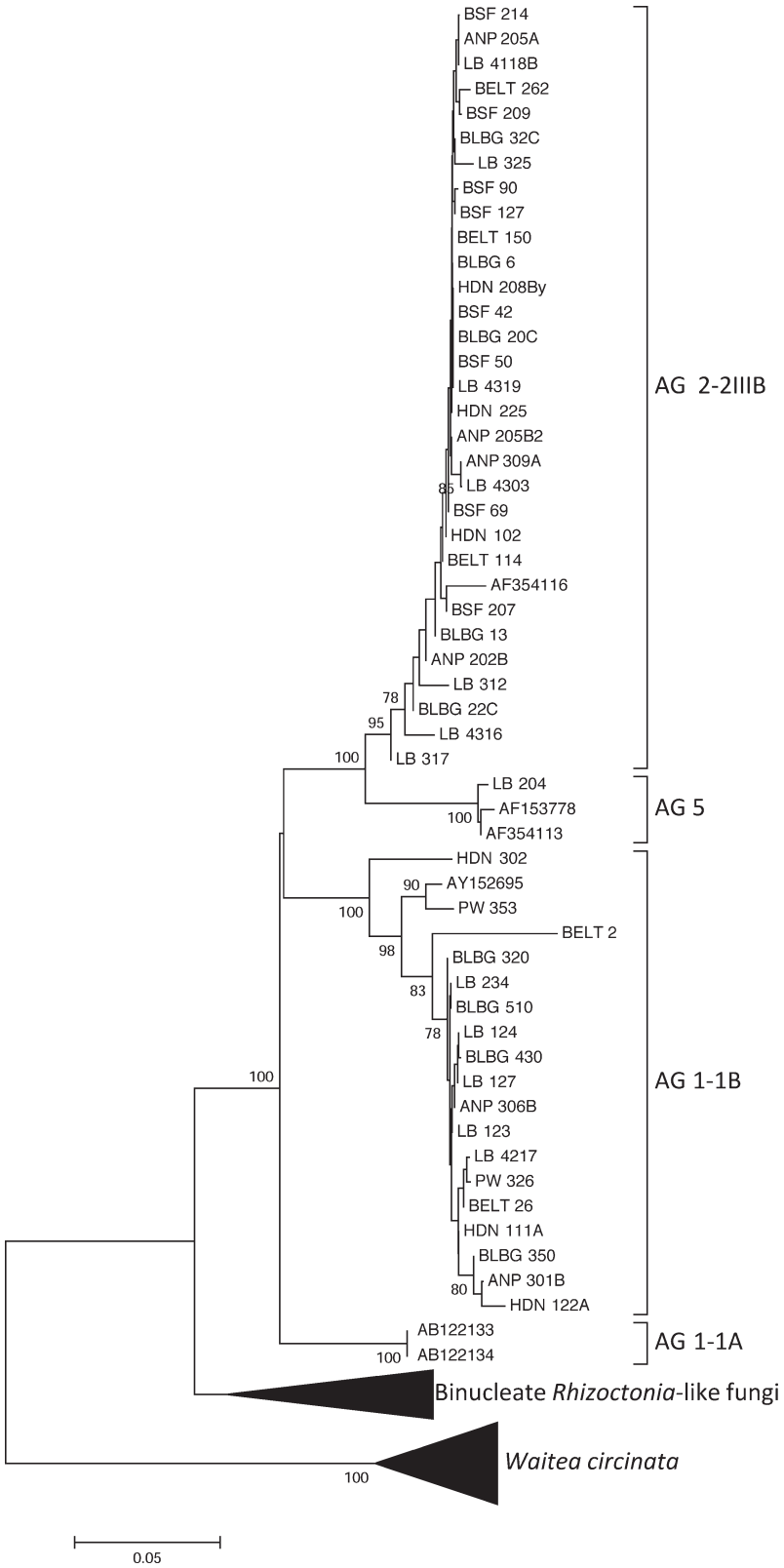

FIG. 1. The neighbor-joining tree of *Rhizoctonia* isolates based on ITS region sequences. Bootstrap values greater than 75% assessed by 500 replications are shown next to the branches. Clades having isolates of binucleate *Rhizoctonia*-like fungi (BNR) and *Waitea circinata* have been collapsed to show the entire phylogenetic tree. The tree is midpoint rooted. AF354116, AF153778, AF354113, AY152695, AB122133 and AB122134 are GenBank accession numbers of *R solani* ITS sequences. The anastomosis groups AG 1-IA, 1-IB, 2-2IIIB and 5 are indicated in the tree.

TABLE II. *Rhizoctonia* isolates based on host species[a]

Rhizoctonia group	No. of isolates from tall fescue	No. of isolates from CBG/ABG
R. solani AG 1-IB	18	—
AG 2-2IIIB	23	7
AG 5	1	—
W. circinata var. *zeae*	9	—
W. circinata var. *circinata*	—	4
Unidentified *W. circinata*	5	1
Binucleate *Rhizoctonia*-like fungi	14	2
Total	70	14

[a] CBG = creeping bentgrass; ABG = annual bluegrass.

Anastomosis grouping.—Anastomosis reactions made it possible to group *R. solani* isolates into aforementioned three AGs, AG 1 (n = 18), AG 2 (n =30) and AG 5 (n = 1). Anastomosis reactions could not resolve AG subgroups. For example, perfect hyphal fusion reactions were observed for both AG 1-IA and AG 1-IB testers when paired with suspected isolates of AG 1. Subsequent molecular identification indicated *R. solani* AG 1 and AG 2 isolates belonged to subgroups IB and 2IIIB respectively. Isolates of BNR (n = 16) did not anastomose with any tester isolates including binucleate AG-D (*R. cerealis*). Field isolates having characters of *Waitea* species (orange to pink, spherical to irregular sclerotia on ¼× PDA plates) anastomosed with all three *W. circinata* testers but did not pair with testers of *R. solani* or *R. cerealis*.

ITS analysis.—The amplified ITS region for the investigated *Rhizoctonia* isolates produced DNA fragments between 550 and 700 bp. The aligned sequences consisted of 529 nucleotide positions including gaps. Phylogenetic analysis of ITS sequences revealed well supported clades for *R. solani*, *W. circinata* and BNR isolates (FIG. 1). *Rhizoctonia solani* isolates grouped into three clusters with bootstrap values of >94% (FIG. 1), and they correspond well with previously identified AGs or AG subgroups (i.e. AG 1-IB, AG 2-2IIIB, AG 5). ITS sequences obtained from GenBank grouped with corresponding clusters of field isolates (FIG. 1). ITS sequences of *W. circinata* grouped into four clades (FIG. 2). Two clades corresponded with Wcz and Wcc. The third clade included six UWC isolates and out of those five isolates grouped closely with *W. circinata* var. *agrostis* GenBank sequences (AB213572, AB213578) and the remaining isolate (BSF 13) grouped closely with *W. circinata* var. *prodigus* isolated from Florida golf courses (accession numbers HM597146, HM597147) (Kammerer et al. 2011). None of the studied isolates grouped with *W. circinata* var. *oryzae* accessions (AB213588, AB213589, AJ000195). Two isolates (BELT 05, BELT 159) with macroscopic features of *W. circinata* var. *zeae* grouped outside all *W. circinata* clades described above.

Out of 16 BNR isolates, 15 grouped together while one isolate (BELT 17) grouped separately (FIG. 2). Based on the sequence information, this isolate is more similar to AG-D (GenBank accessions numbers AB198693, AB198714) than other BNR isolates. There was no relationship between geographic origin of isolates and their genetic profiles because *Rhizoctonia* isolates within a clade represent most of the sampling sites. However, isolates of Wcc were found only from Blacksburg, Virginia, while isolates of Wcz were not identified from Blacksburg or Richmond, Virginia.

UP-PCR analysis.—UP-PCR analysis included all isolates (TABLE I) except HDN 209. Isolate HDN 209 was removed from the analysis because the UP-PCR fingerprint was difficult to resolve. Primer L21 produced a total of 373 amplified products for the total sample set including tester isolates. Primer L15/AS19 gave 380 amplified products across all isolates. The dendrogram derived from the UP-PCR fingerprint data (FIG. 3) corresponds well with the phylogram based on ITS sequences. The BNR isolates BELT 17 and PW 249 clustered separately from the rest of the BNR group (FIG. 3). In contrast to ITS analysis, UP-PCR clustered BELT 5 and BELT 159 within Wcz group (FIG. 3).

The primer combinations L21 and L15/AS19 generated 233 amplified products for the AG 2-2IIIB group consisting of 30 isolates (SUPPLEMENTARY FIG. 2). Only two of the amplified products were monomorphic across all isolates. The AG 1-IB group consisting of 18 isolates produced 228 amplified fragments, two of which were monomorphic. The lowest number of amplified products was observed in the Wcc group consisting of four isolates. This group produced 81 polymorphic and eight monomorphic amplified products for both primers combined.

DISCUSSION

To our knowledge this is the first report of the UP-PCR technique applied successfully to simultaneously resolve AGs of *R. solani*, *Waitea circinata* and Binucleate *Rhizoctonia*. UP-PCR analysis is relatively inexpensive compared to other techniques and is an accurate and efficient method to characterize a large number of unknown *Rhizoctonia* isolates into their respective AG groups and subgroups. The clusters formed by this method largely agreed with the results

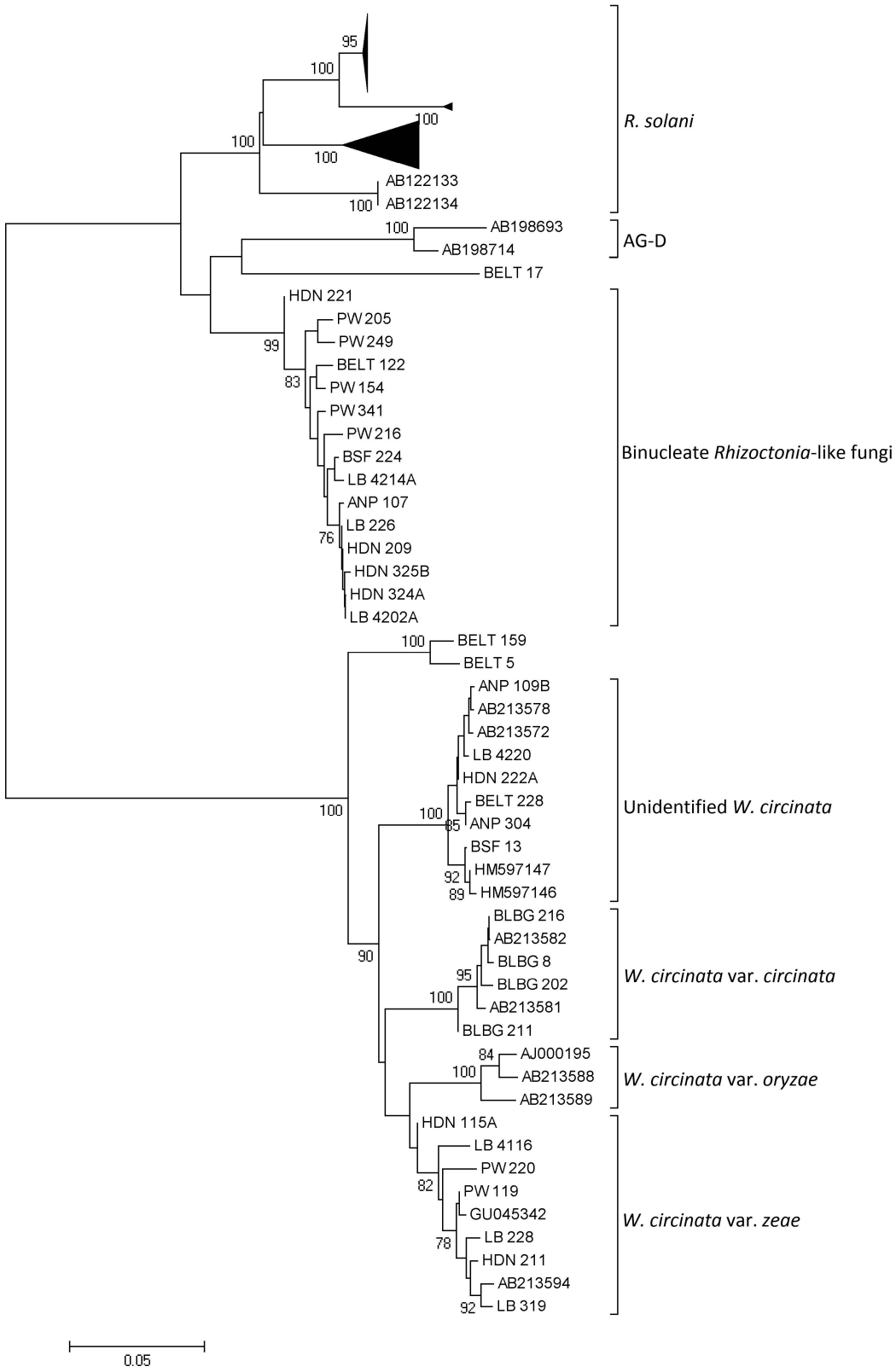

FIG. 2. Expanded clades of *Waitea circinata* and binucleate *Rhizoctonia*-like fungi from the neighbor-joining phylogram (FIG. 1). The *W. circinata* clade consists of *W. circinata* var. *circinata*, *W. circinata* var. *oryzae*, *W. circinata* var. *zeae* and unidentified *W. circinata* groups. Bootstrap values of greater than 75% calculated by replicating the dataset 500 times are shown next to the branches. Taxons starting with AB, AJ, GU and HM are GenBank accessions. Isolates of *Rhizoctonia cerealis* are represented by the cluster AG-D.

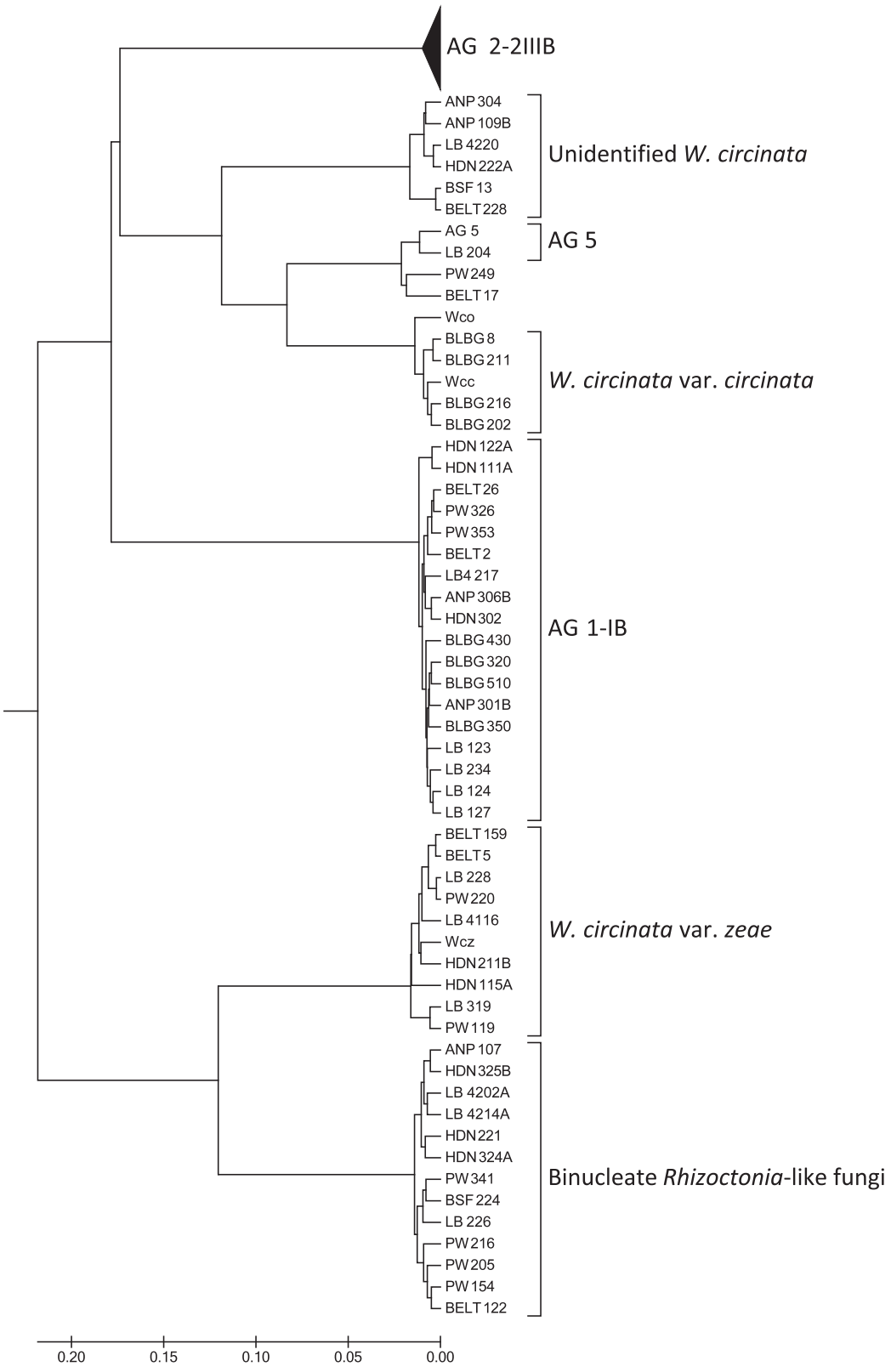

FIG. 3. The UPGMA dendrogram for *Rhizoctonia* isolates derived by universally primed PCR markers. Isolates of *R. solani* anastomosis groups AG 1-IB, 2-2IIIB and 5 grouped separately and are indicated in the tree. More detailed relationships among isolates of the AG 2-2IIIB cluster are illustrated (SUPPLEMENTARY FIG. 2). Isolates of *Waitea circinata* grouped into three clades (viz. *W. circinata* var. *zeae*, *W. circinata* var. *circinata* and unidentified *W. circinata*). Most of the Binucleate *Rhizoctonia*-like fungi grouped into one cluster. The taxons AG 5, Wco, Wcz and Wcc are tester isolates.

of well documented ITS sequence analysis (Kuninaga et al. 1997, Gonzalez et al. 2001, Hsiang and Dean 2001, Toda et al. 2005, de la Cerda et al. 2007).

R. solani AG 2-2IIIB is the most dominant pathogen followed by AG 1-IB, in the surveyed areas of the transition zone, according to our findings. All *R. solani* isolates from CBG/ABG putting greens were AG 2-2IIIB, which agrees with a similar study conducted in Maryland by Zhang and Dernoeden (1997). They also identified isolates of *R. solani* AG 1-IA and AG 2-2IIIB from tall fescue and Kentucky bluegrass (*Poa pratensis* L.). In contrast to Zhang's and Dernoeden's findings, our present analysis did not reveal isolates of AG 1-IA from tall fescue; instead all of our isolates collected from tall fescue belong to the AG 1 subgroup IB. However, they identified subgroups of isolates of *Rhizoctonia* using anastomosis and colony morphology and did not employ any molecular techniques. It is possible that isolates identified as AG 1-IA by Zhang and Dernoeden (1997) would have been identified as AG 1-IB with our molecular techniques. Several molecular identification studies on *R. solani* have reported AG 1-IB isolates from turfgrass. For instance, ITS sequence analysis performed by Kuninaga et al. (1997) in Japan and Hsiang and Dean (2001) in Canada identified *R. solani* AG 1-IB isolates from turfgrass.

In Virginia, isolates of Wcc on turfgrasses were first reported in 2008 from golf courses in Reston (Kammerer et al. 2009). However, in the present study, infected turfgrasses sampled close to Reston (i.e. Herndon, Leesburg) did not reveal any isolates of Wcc. UWC isolates (n = 6) indicate that there may be different, previously undescribed, varieties of *W. circinata* species infecting turfgrasses. Our findings indicate that isolates of BNR are common on infected turfgrasses, especially on lawns with less intensive management. Pathogenicity tests have revealed BNR to be weakly virulent on turfgrasses (Hurd and Grisham 1983, Burpee and Goulty 1984, Burpee and Martin 1992). In comparison to isolates of *R. solani*, hyphal anastomosis reactions of BNR from turf have not been characterized well (Burpee and Martin 1992). We did not find any binucleate isolates of *R. cerealis* responsible for causing yellow patch in the sample. *Rhizoctonia cerealis* is a cool-weather pathogen and therefore it would be unlikely for this pathogen to infect turfgrasses during the summer when our samples were collected.

Lübeck and Poulsen (2001) used visual banding profiles and cross hybridization of PCR products from a single UP primer for identifying different *R. solani* AGs. Visual comparison of banding profiles is not practical for characterizing a large number of unknown isolates. In addition, PCR product cross hybridization is labor intensive and the strength of the hybridization signal falls into several subjective categories such as weak, significant and strong. UP-PCR analysis described here is more objective and has the ability to pool banding profiles of several UP primers to derive more accurate results. The universal primers originally were designed to target intergenic, phylogenetically less conserved genomic regions, and some highly polymorphic amplified fragments have the ability to detect infraspecific variation (Bulat et al. 1998). Another advantage of UP-PCR is its high reproducibility without compromising the cost or labor intensity as opposed to the commonly used amplified fragment length polymorphism (AFLP) technique. In contrast to phylograms generated by ITS sequence analysis, the dendrograms constructed by UP-PCR fingerprinting are not phylogenetic but are phenetic (Ceresini et al. 2002, Lübeck and Lübeck 2005). Therefore, the adjacent clusters of these trees do not necessarily represent evolutionarily closer species or isolates.

Although our results demonstrated clear separation of isolates of *Rhizoctonia* into their AG subgroups, there were a few isolates clustering outside their taxonomic groups. For example, BELT 17 was distinct from the rest of the BNR isolates (FIG. 2) and may represent a different AG or AG subgroup from the other BNR isolates. The ITS sequence analysis clustered *W. circinata* isolates BELT 159 and BELT 5 separately from the rest of *Waitea* groups (FIG. 2). However, UP-PCR grouped these isolates within the Wcz cluster (FIG. 3). Sclerotial morphology of these two isolates on PDA was also similar to the Wcz isolates. These two isolates had a high degree of sequence dissimilarity at 8.5% when compared to other Wcz isolates (percentage sequence similarity of *Rhizoctonia* groups not shown), which might be explained by polymorphisms of rDNA-ITS region within these isolates. Pannecoucque and Höfte (2009) reported detection of ITS polymorphisms in *R. solani* AG 2-1 isolates and observed that many ITS sequences derived from clones of the same isolate did not cluster together. The UP-PCR analysis can be used to overcome the variation observed in ITS because it tests multiple loci simultaneously.

There was no correlation between the sampling site and the genetic relationships of the isolates investigated since the isolates of a single group represented several geographic locations within our sample. However, it seems apparent that different turfgrass management programs may have influenced the diversity of *Rhizoctonia* isolates. For example, all *R. solani* isolates from putting greens with creeping bentgrass and annual bluegrass (CBG/ABG) were designated as AG 2-2IIIB while all AG 1-IB isolates

were from tall fescue lawns or golf course roughs (TABLES I and II). Unlike lawns or roughs, putting greens are mown short with intensively managed routine fungicide spray programs. However we do not have specific fungicide management history, and other cultural management programs such as mowing, fertilization and irrigation for each location surveyed. Our sample contained 14 isolates collected from putting greens. Therefore, it would be more meaningful to characterize additional *Rhizoctonia* isolates from CBG/ABG putting greens to test the dominance of AG 2-2IIIB isolates.

Future research may use molecular techniques performed in this study to understand how *Rhizoctonia* populations change over time at a single location. It also is important to find activity of pathogens adapted to different climatic conditions such as *R. cerealis* (cool-weather pathogen) and *R. solani* (warm-weather pathogen) by surveying the same site at short intervals from mid- spring to early autumn to develop an effective disease management plan.

ACKNOWLEDGMENTS

This research was financially supported by the United States Golf Association. We also appreciate the training and suggestions provided by Dr Bruce Martin, Clemson University, for anastomosis grouping, and the assistance provided by David McCall, Virginia Tech, in making this research a success. The authors also greatly appreciate all the support and help given to them by colleagues and co-workers at Beltsville Agricultural Research Center (BARC), Beltsville, Maryland. Especially Dr Keenan Amundsen's advice and assistance greatly helped in analyzing molecular data.

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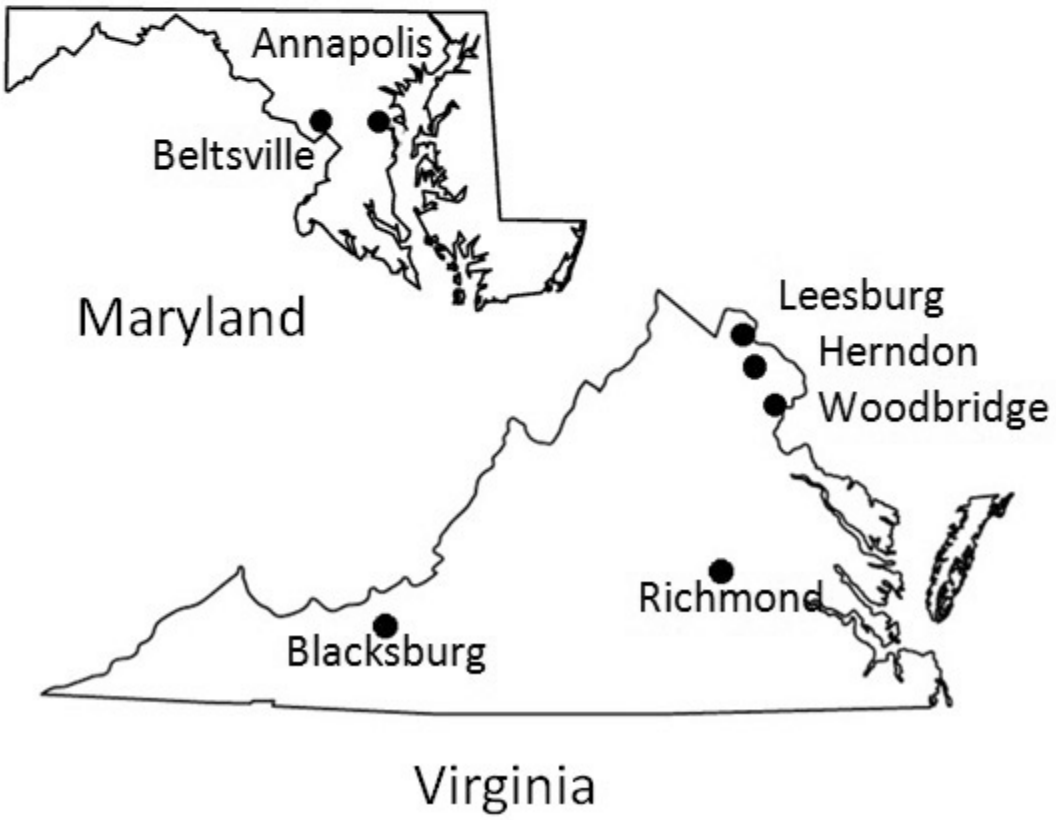
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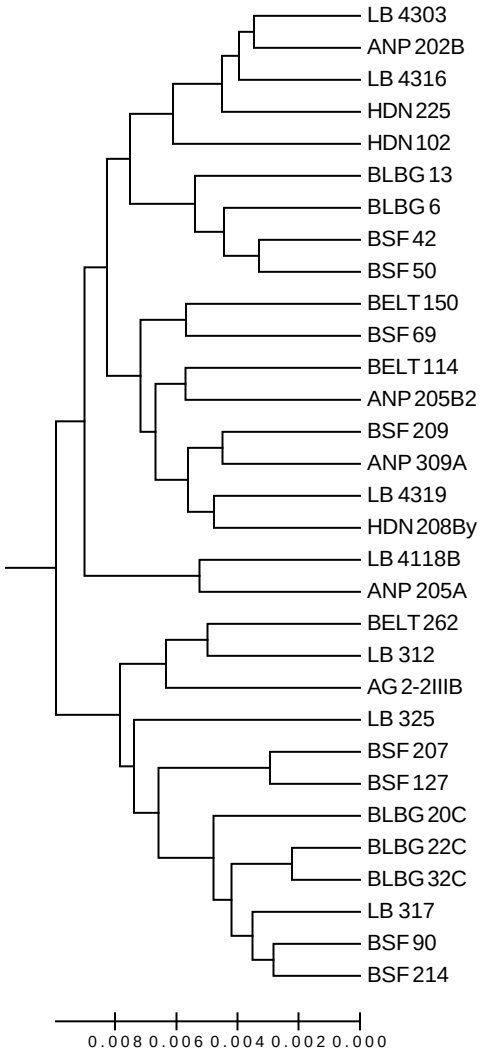
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ANP 205B2
BSF 209
ANP 309A
LB 4319
HDN 208By
LB 4118B
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BELT 262
LB 312
AG 2-2IIIB
LB 325
BSF 207
BSF 127
BLBG 20C
BLBG 22C
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LB 317
BSF 90
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