AFLP Fingerprinting for Identification of Infra-Species Groups of *Rhizoctonia solani* and *Waitea circinata*

Bimal S. Amaradasa**, Dilip Lakshman1 and Keenan Amundsen2

1Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA  
2Floral and Nursery Plants Research Unit and the Sustainable Agricultural Systems Lab, Beltsville Agricultural Research Center-West, Beltsville, MD 20705, USA  
3Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE 68583 USA

**Abstract**

Patch diseases caused by *Thanatophorus cucumeris* (Frank) Donk and *Waitea circinata* Warcup and Talbot varieties (anamorphs: *Rhizoctonia* species) pose a serious threat to successful maintenance of several important turfgrass species. Reliance on field symptoms to identify *Rhizoctonia* causal agents can be difficult and misleading. Different *Rhizoctonia* species and Anastomosis Groups (AGs) vary in sensitivity to commonly applied fungicides and they also have different temperature ranges conducive for causing disease. Thus correct identification of the causal pathogens is important to predict disease progression and make future disease management decisions. Grouping *Rhizoctonia* species by anastomosis reactions is difficult and time consuming. Identification of *Rhizoctonia* isolates by sequencing Internal Transcribed Spacer (ITS) region can be cost prohibitive. Some *Rhizoctonia* isolates are difficult to sequence due to polymorphism of the ITS region. Amplified Fragment Length Polymorphism (AFLP) is a reliable and cost effective fingerprinting method for investigating genetic diversity of many organisms. No detailed analyses have been done to determine the suitability of AFLP for inferring infra-species level of *Rhizoctonia* isolates. The objective of the present study was to develop AFLP fingerprinting to identify infra-species level of unknown *R. solani* Kühn and *W. circinata* isolates. Seventy-nine previously characterized *R. solani* (n=55) and *W. circinata* (n=24) isolates were analyzed with AFLP markers generated by four primer pairs. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) correctly grouped *R. solani* and *W. circinata* isolates according to their AG, AG subgroup or *W. circinata* variety. Principle component analysis (PCA) corroborated UPGMA clusters. To our knowledge this is the first time AFLP analysis has been tested as a method to decipher the AG, AG subgroup or *W. circinata* variety across a wide range of *Rhizoctonia* isolates.

**Keywords:** AFLP; *Rhizoctonia solani*; *Waitea circinata*; anastomosis groups; turfgrasses

**Introduction**

Patch diseases caused by multiple *Rhizoctonia* species pose a serious threat to growth and maintenance of several important turfgrass species in the southern and transition zones of the USA [1,2]. The transition zone refers to the central part of the country where climatic conditions are not favorable for either cool-season or warm-season turfgrasses. However, both turfgrass types are routinely grown and managed in this region. The form-genus *Rhizoctonia* includes uninucleate, binucleate, and multinucleate species, and of these, multinucleate *Thanatophorus cucumeris* (Frank) Donk (=*R. solani* Kühn) and *Waitea circinata* Warcup and Talbot varieties agrostis, zeae, oryzae, circinata, and prodigus; and binucleate *Ceratobasidium cereale* Murray and Burpee (=*R. cerealis* Van der Hoeven, AG-D) have been reported from diseased turf lawns and golf greens [1-4]. *Rhizoctonia solani* is a genetically diverse species consisting of many Anastomosis Groups (AGs) [5,6]. Six AGs have been reported to cause blight in turfgrass with AG 1 (–IA and –IB), AG 2 (–2IIIB and –2LP), and AG 4 being more common on infected turfgrasses than other AGs [7,8].

Reliance on field symptoms to identify *Rhizoctonia* causal agents can be difficult and misleading. In general, *Rhizoctonia* affected turfgrasses show circular areas of blighted brown colour leaves. Microscopically, all *Rhizoctonia* species look more or less similar, i.e. nonsporulating mycelia with 90 degree branches having dolipore septa [8]. However, *R. solani* isolates can be distinguished from *W. circinata* varieties by colony morphology on Potato Dextrose Agar (PDA). *Rhizoctonia solani* produces brown to dark brown sclerotia on PDA whereas sclerotia of *W. circinata* are orange to salmon in the formative stages and darkens to brown as cultures age [1,2,8]. Also, sclerotia of *W. circinata* are frequently submerged in the media unlike *R. solani* which are formed on the agar surface. Although, colony morphology on PDA can differentiate *R. solani* from *W. circinata*, colony features are not reliable to distinguish AGs within these species. It is common to isolate multiple *Rhizoctonia* species and AGs from infected turfgrasses. Different *Rhizoctonia* species and AGs vary in sensitivity to commonly applied fungicides [9-12] and they also have different temperature ranges conducive for causing disease [8]. Therefore, correct identification of the causal pathogen and its AG is important to predict the disease progression and make future disease management decisions. For plant breeders, knowledge of the main causal pathogens at different locations is important for selecting appropriate turfgrass germplasm with resistance to *Rhizoctonia* blight.

The classical method of grouping isolates of *Rhizoctonia* is based on anastomosis with tester strains. However this method is sometimes difficult to interpret and may take excessive amounts of time when grouping many isolates. Some isolates which are known as Bridging

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*Corresponding author: Bimal S. Amaradasa, Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA, Tel: +1-402-470-7311; E-mail: baramaradasa2@unl.edu

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Isolates (BI) can anastomose with more than one AG leading to further confusion [13].

It is also important to note that anastomosis reactions cannot be used to distinguish subgroups within an AG because subgroups anastomose with each other [14].

Although analysis of Internal Transcribed Spacer (ITS) region is a well-tested method for identifying *Rhizoctonia* species, it can be cost prohibitive for investigating a large number of samples. Some *Rhizoctonia* isolates are difficult to sequence due to polymorphisms in the ITS region [15-17]. These isolates may require cloning before sequencing, which adds more time and cost to the analysis. ITS sequence polymorphism may make it difficult to group *Rhizoctonia* isolates to their AG subgroups [18].

PCR based fingerprinting method Amplified Fragment Length Polymorphism (AFLP) is a cost effective alternative for assessing isolates to their AG subgroups [18].

**Isolates used in this study and ITS sequence analysis**

*Rhizoctonia* isolates (n=71) used in this study were collected from lawns and golf courses of Virginia and Maryland during summer months of 2007 to 2009 (Table 1). A previous study had identified these isolates to species, AG or AG subgroup level using colony morphology on PDA, anastomosis reactions, and ITS sequence analysis as described by Amaradasa et al. [20]. The present study also included eight tester strains consisting of *R. solani* (AG 1-IB, 2-2IIIB and 5) and *W. circinata* (var. *zeae* and *circinata*) (Table 2). Accordingly, there were 55 *R. solani* species, we analyzed and 16 *W. circinata* subgroup or variety of unknown *R. solani* and *W. circinata* isolates commonly occurring on cool-season turfgrasses in comparison to the conventional ITS sequence analysis. Since no detailed analyses have been done to determine whether AFLP is suitable for grouping multiple species of *Rhizoctonia* together or whether this method is appropriate for deciphering the genetic diversity of isolates within a single *Rhizoctonia* species, we analyzed *R. solani* and *W. circinata* isolates together as well as separately.

### Materials and Methods

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| Isolate | Origin | Host | Management | Species | 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 308B | Annapolis, MD | Tall fescue | Lawn | Rs | AG 1-IB | JX631171 |
| 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 | JX631169 |
| 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 267 | Beltsville, MD | Tall fescue | Lawn | Rs | AG 2-2IIIB | JX631156 |
| BELT 2 | Beltsville, MD | Tall fescue | Lawn | Rs | AG 1-IB | JX631157 |
| 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 208B | Herndon, VA | Tall fescue | Golf rough | Rs | AG 2-2IIIB | JX631202 |
| Isolate   | Species Acronym* | AG    | Host† | Location | Donor† |
|----------|------------------|-------|-------|----------|--------|
| EDHGED 2-1 | Wcc              | Not assigned | ABG     | California, USA | FW |
| BSCST 17-1-1 | Wcc          | Not assigned | ABG     | California, USA | FW |
| AVGCAV | Wcz             | WAG-Z | ABG     | California, USA | FW |
| M008 | Wcz             | WAG-Z | Rice      | Japan     | MC |
| Rh102/T | Rs              | AG 5   | Unknown  | Unknown   | LB |
| Rh 63/T | Rs              | AG 5   | wheat crown | California, USA | LB |
| Rh146 | Rs              | AG 2-2IIIB | Bentgrass | Georgia, USA | LB |
| BM2 | Rs              | AG 1-IB | Unknown  | Unknown   | BM |

*ABG: Annual Bluegrass; CBG: Creeping Bentgrass; Rs: R. solani; Wcc: W. circinata var. circinata; Wcz: W. circinata var. zeae; Wcc: W. circinata var. circinata; UWC: Unidentified W. circinata species.

Table 1: Geographic origin, host, management type, species, and anastomosis group of isolates used in this study*. 

Table 2: Rhizoctonia and Waitea tester isolates used in this study.
isolates including tester strains, which consisted of 33 AG 2-2IIIB, 19 AG 1-IB, and three AG 5. A total of 24 isolates represented *W. circinata* with 13, six and five isolates belonging to *W. circinata* var. *zeae* (Wcz), *var. circinata* (Wc) and an unknown *W. circinata* group (UWC), respectively. In addition to these isolates, ITS phylogram included Genbank deposited ITS sequences of *W. circinata* varieties *agrostis* and *prodigus*.

**Generation of AFLP markers**

DNA was purified using the QIAGEN DNeasy plant mini kit (QIAGEN Inc., Valencia, CA) according to the manufacturer’s instructions. AFLP analysis explained below was based on the method described by Ceresini et al. [21] and Vos et al. [22]. All reaction plates contained the isolate BELT 267 in duplicate to ascertain reproducibility of AFLP fragments. DNA samples were digested with restriction enzymes EcoRI (New England BioLabs, Beverly, MA) and Msel (New England BioLabs). Thereafter, digested products were ligated with EcoRI Double Stranded (ds) adapter (EA1: 5´-CTGTTAGACTGCGTACC-3´ and EA2 3´-CATCTGAGCGATGGTATAA-5´) and Msel ds adapter (MA1: 5´-GAGCATGACTGTGCTAG-3´ and MA2: 3´-TACTCAGGACTCAT-5´). Both digestion and ligation reactions were done in one step by preparing a reaction mixture of 20 µl having 2 µl of each restriction enzyme, 1.2 U of T4 DNA ligase (New England BioLabs), 0.1 µM of EcoRI adapter, 1 µM of Msel adapter and 100 ng of DNA template. The reaction mixture also included 1× EcoRI buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100), 1× Msel buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT), 1× T4 ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP), and 2µg of bovine serum albumin. The reaction mixture was incubated overnight at room temperature to complete digestion and ligation reactions and thereafter, diluted ten-fold by adding sterile TE (Tris-EDTA) buffer and stored at -20°C for later use. The first amplification (pre-amplification) was carried out with one selective nucleotide for each primer: EcoRI primer + A (5´-GACTGCGTACCAATTC-3´) and Msel primer + C (5´-GATGTAGTCTCTCATTCA-3´). Each sample of 25 µl included 5µl of digestion and ligation reaction from the previous step, 0.5 µM each of EcoRI and Msel primers, 1× Taq polymerase reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂), 0.2 mM dNTP and 1 U of Taq polymerase (New England BioLabs). The PCR was performed in a thermocycler (MJ Research PTC-200, Global Medical Instrumentation, Ramsey, MN) with initial denaturation at 94°C for 2 min followed by 20 cycles of 30 s at 94°C, 1 min at 56°C and 1 min 56°C at 72°C. PCR products were diluted ten-fold with TE buffer and used as the template DNA for selective amplification using four EcoRI and Msel primer pairs with three selective nucleotides (EcoRI primer + ACA and Msel primer + CAA, EcoRI primer + AAA and Msel primer + CTA, EcoRI primer + AAC and Msel primer + CAG, EcoRI primer + AGT and Msel primer + CTC). EcoRI and Msel primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and IDT (Coralville, IA), respectively. All EcoRI primers were end labeled with fluorescence dye 6-FAM™ at the 5´ end. Each selective PCR mixture of 20 µl included 4 µl of diluted preselective reaction, 0.5 µM each of EcoRI and Msel primers, 1× standard Taq polymerase reaction buffer, 0.2 mM dNTP and 1 U of Taq polymerase. The PCR reaction was performed for 36 cycles with the following cycle profile. Cycle 1 with 30 s of DNA denaturation step at 94°C, 30 s annealing step at 65°C, and 1 min extension step at 72°C. The same conditions were used in cycle 2-12 as in cycle 1, but included a progressive drop in the annealing temperature of 0.7°C in each cycle. Cycles 13-36 included 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. A final extension of 5 min at 72°C completed the reaction. Presence of AFLP banding profiles were confirmed by electrophoresing 5µl of PCR samples in 1.7% agarose gel for one hour at 100 V and visualizing ethidium bromide stained gels under UV light.

**AFLP data capture and analysis**

We used the size standard GeneScan™ 500LIZ (Applied Biosystems, Foster City, CA) with capillary electrophoresis system ABI 3730 (Applied Biosystems) to capture AFLP fragments. The size standard was added to each PCR sample for automated data analysis and is essential for precise DNA fragment size comparisons between electrophoresis runs. GeneScan 500LIZ has a DNA fragment sizing range of 35-500 bp with 16 single-stranded labeled DNA fragments. Samples amplified with primers were analyzed by loading a denatured cocktail containing 0.5 µl of PCR sample, 9 µl Hi-Di formamide (Applied Biosystems, Warrington) and 0.5 µl of 500LIZ. The GeneMapper software V4.1 (Applied Biosystems) was used to extract and analyze raw data files obtained from the ABI 3730. AFLP products and size standard fragments can be distinguished since FAM dye-labeled AFLP fragments are associated with blue signals/peaks while LIZ dye-labeled size fragments generate orange signals. 500LIZ electropherograms had clear, tall peaks without any missing or extra ones and GeneMapper software correctly detected them. The Analysis Range settings of the software were changed 50 to 500 to limit the allele calling analysis within that range. Thereafter, samples were analyzed using the Advanced peak detector algorithm. DNA fragments smaller than 50 bp were not scored to avoid artefacts of primer-dimer formation. The Advanced mode uses the defined size standard values to select peaks of the size standard on an electropherogram. This is achieved by ratio matching where the software uses relative distances between neighboring peaks to correctly define precise DNA fragment size comparisons between electrophoresis runs. This is used as a reference to accurately compare and capture the size of AFLP amplicons among sample runs. AFLP amplicons of *R. solani* and *W. circinata* isolates were analyzed separately as well as together to determine the suitability of this method in each situation. Initially, we created a bin set by setting the minimum peak intensity to 100 relative fluorescent units (rfu) for peaks generated by FAM labeled AFLP products. A bin set is a set of allele definitions specific to a set of samples with a set of analysis conditions. GeneMapper software is capable of scoring alleles directly from a new set of samples without any bin set or using a previously generated bin set. Better results were obtained by first generating a bin set using a low peak amplitude threshold (ex. 100 rfu) which captures most of the AFLP fragments and then applying that bin set to analyze the same sample set with higher Peak Amplitude Threshold settings in order to filter weak signals and background noise. Once peaks were scored, a binary table was generated of ones and zeros relating to presence absence of alleles. We tested binary tables generated with peak capture thresholds of 500, 1000, 1500, and 2500 rfu. Each scoring table of zeros and ones generated by four AFLP primers was imported to NTSYS version 2.2 [23] and converted to a different similarity indices using Qualitative data tab of Dis/similarity module. The compared indices included Dice, SM, Phi, O, and Y [24-27]. These similarity values were used in MEGA 5 [28] software to construct an UPGMA [25] tree in order to cluster isolates according to their genetic distances. Cophenetic goodness-of-fit tests were also performed as described in NTSYS to ascertain how well the distance matrices are represented by UPGMA dendrograms. For this, COPH module was used to produce a cophenetic value matrix [29] for each UPGMA dendrogram and compared to the relevant distance matrix using the MxCOMP program to compute the correlation between the two matrices. Cophenetic correlation of > 0.9 is a very good fit while 0.8 to 0.9 is a good fit [23]. The genetic distances generated by different
similarity indices were used to compute eigenvalues and eigenvectors in NTSYS using Eigen function in the Ordination module. NTSYS was then used to perform Principal Component Analysis (PCA) [30] by plotting the first three eigenvectors for each similarity index tested.

Genetic variability among subpopulations of *R. solani* and *W. circinata* as shown by UPGMA analysis was determined by analysis of molecular variance (AMOVA) [31] in GenAlEx version 6.5 [32]. Since AFLP markers generate a binary matrix without any information of intra-individual variation (heterozygosity), AMOVA was performed by calculating PhiPT (ΦST), which is an analogue of Wright’s FST. Normally, FST and its analogues are greater than zero but rarely exceed 0.5. An FST value of 0.05 or less is generally considered as reasonably low and may be interpreted to mean that structuring between subpopulations is weak [33,34].

**Results**

All four AFLP selective primers produced a large number of polymorphic alleles for each isolate. For instance, the average number of alleles scored per *R. solani* isolate per selective primer was 64. The two BELT 267 samples in each plate gave similar fingerprinting patterns indicating high reproducibility of the AFLP technique. Binary tables generated with a peak capture threshold of 1000 rfu gave better results than other rfu values in terms of grouping isolates to their correct AGs. None of the UPGMA dendograms constructed with different similarity matrices could correctly group all *R. solani* and *W. circinata* isolates when analyzed together (results not shown). A few isolates of AG 2-2IIIB grouped with *W. circinata* var. *zeae* (Wcz) group while *W. circinata* var. *circinata* (Wcc) cluster consisted of a few Wcz isolates. Therefore, we did not proceed with the analysis of combined AFLP data for *R. solani* and *W. circinata* isolates.

**AFLP analysis of Rhizoctonia solani isolates**

The AFLP primer pairs EcoRI-AAC and Msel-CAC, EcoRI-AGT and Msel-CTG, EcoRI-AAA and Msel-CTA, and EcoRI-ACA and Msel-CAA produced 230, 234, 265, and 213 alleles, respectively for the isolates analyzed. Allele 7 produced by EcoRI-AAA and Msel-CTA primer pair was monomorphic across all *R. solani* isolates. AG 1-IB isolates had a total of three monomorphic alleles while AG 2-2IIIB resulted in two. There were no clones resembling isolates with same DNA fingerprinting pattern among AG 1-IB or AG 2-2IIIB. All the alleles (942 in total) produced by four primer pairs were pooled together to make a single binary matrix for calculating genetic similarity of isolates. We compared the dendograms produced by different similarity indices to the results of ITS sequence analysis in a previous study [20]. Though all the similarity indices tested largely grouped isolates to their correct AG, the Dice coefficient based UPGMA tree generated the highest cophenetic correlation value of 0.8968 (Figure 1) and corresponded very well with ITS analysis (Supplemental Figure 1). The tree consisted of three clusters that represented the correct AG or AG subgroup (i.e. AG 1-IB, AG 2-2IIIB, or AG 5) of each *R. solani* isolate studied (Figure 1). The PCA for *R. solani* isolates clearly separated them into AG or AG subgroup along dimension 2 (Figure 2). This difference was tested using AMOVA, which showed significant difference among AG subgroups (p=0.0001) representing 14.2% of the total genetic variance (Table 3).

**AFLP analysis of Waitea circinata isolates**

The same selective primers mentioned above viz., EcoRI-AAC and Msel-CAC, EcoRI-AGT and Msel-CTG, EcoRI-AAA and Msel-CTA, and EcoRI-ACA and Msel-CAA produced, 97, 122, 91, and 175 alleles, respectively for the 24 *Waitea* isolates. All alleles were polymorphic across the *Waitea* isolates. However, there were one, five and 20 monomorphic alleles among Wcz, Wcc, and UWC isolates, respectively. Similar to *R. solani*, there were no clonal isolates among *W. circinata* isolates. Binary tables generated for each primer set were pooled to produce a single table of 485 alleles and used to calculate genetic similarity values. The Y coefficient gave the best UPGMA tree with the highest cophenetic correlation value of 0.8 (Figure 3). Although UPGMA tree largely corresponded with ITS phylogram (Figure 4), there were few differences. BELT 159 and BELT 5 with colony morphology similar to Wcz on PDA grouped separately in the ITS analysis (Figure 4), whereas AFLP dendrogram had them grouped together with the rest of Wcz isolates (Figure 3). Though the five UWC isolates formed a single large cluster in both ITS phylogram and AFLP dendrogram, they were separated from the other subgroups (Figure 3). This was confirmed by AMOVA which showed significant genetic difference among UWC isolates (p=0.0001) representing 21.5% of the total genetic variance (Table 3).
dendrogram, they resolved differently thereafter. UWC isolate BSF 13 grouped close to \textit{W. circinata} var. \textit{prodigus} in the ITS tree (GenBank accessions HM597147, HM597146, and HQ850254), while UWC isolate ANP 109B grouped with \textit{W. circinata} var. \textit{agrostis} (AB213578 and AB13572) (Figure 4). The rest of the UWC isolates (ANP 304, BELT 228, and HDN222A) grouped in between (Figure 4). The AFLP dendrogram (Figure 3) agreed with the ITS tree clearly by grouping BSF 13 and ANP 109B in two sub-clusters but ANP 304 and BELT 228 did not cluster closely as in ITS phylogram. We did not have AFLP data of varieties \textit{agrostis}, \textit{prodigus} and other GenBank accessions used in the Figure 4 for comparison. Wcc isolates clustered similarly in both AFLP and ITS trees. PCA clearly separated \textit{W. circinata} isolates to their subgroups Wzc, Wcc and UWC along dimension 2 and 3 (Figure 4), which was corroborated with an AMOVA that showed significant difference among \textit{W. circinata} subgroups ($p=0.0001$), representing 12.0% of the total genetic variance (Table 3).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Group} & \textbf{Source of variation} & \textbf{df} & \textbf{SS} & \textbf{\% Variation} & \textbf{\Phi_{PT}} & \textbf{P value} \\
\hline
\textit{R. solani} & Among populations & 2 & 982 & 14 & 0.142 & 0.001 \\
& Within populations & 52 & 7605 & 86 & 0.858 & 0.001 \\
& Total & 54 & 8587 & & & \\
\hline
\textit{W. circinata} & Among populations & 2 & 348 & 12 & 0.120 & 0.0001 \\
& Within populations & 21 & 1840 & 86 & 0.880 & 0.0001 \\
& Total & 23 & 2188 & & & \\
\hline
\end{tabular}
\caption{Analysis of molecular variance (AMOVA) of \textit{R. solani} and \textit{W. circinata} isolates based on four AFLP primer data.}
\end{table}

\textbf{Discussion}

The present study investigated the applicability of the AFLP technique for grouping \textit{R. solani} and \textit{W. circinata} isolates into their infra-species level. In this method, genomic DNA is digested with two restriction endonucleases and two double stranded oligonucleotide adapters are ligated to each fragment. These modified fragments are amplified by two primers recognizing the adapter sequences and adjacent restriction site/s using PCR [22,35]. The resulting banding patterns are highly reproducible and the proportion of the genome analyzed is larger than other DNA fingerprinting techniques such as RAPD [36]. When both \textit{W. circinata} and \textit{R. solani} isolates were analyzed together, AFLP markers did not result in an acceptable dendrogram. It is possible that co-migration of AFLP amplicons generated by genetically distant \textit{Rhizoctonia} and \textit{Waitea} isolates have caused this. High variability of AFLP fingerprinting profiles among distant taxa reduces similarities among them to level of chance [19].
Figure 3: Dendrogram based on UPGMA clustering algorithm for W. circinata isolates derived from Dice's genetic distance matrix. W. circinata variety of each cluster is indicated in the tree.

Therefore, AFLP is not useful to make phylogenetic inferences among higher taxonomic levels but is more suitable for deriving relationships among closely related lineages.

The AFLP markers used in our analysis was able to accurately resolve \textit{R. solani} isolates to AGs and AG subgroups. Clustering of isolates within the UPGMA dendrogram corresponded well with ITS pylogram and was also corroborated by the PCA scree plots. The AMOVA results also showed significant difference between these subgroups. Both PCA and AMOVA results are positive indicators of the confidence of UPGMA clusters.

ITS sequence analysis grouped Wcz isolates BELT 159 and BELT 5 separately from rest of the Wcz cluster. There was high sequence dissimilarity of 8.5\% between above two isolates and other Wcz (sequences dissimilarity not shown). It is possible that ITS region polymorphism of these two isolates have contributed to the discrepancy. Previous studies have reported the ITS region polymorphism within \textit{Rhizoctonia} isolates and how it can compromise accuracy of phylograms [17,18]. Contrary to the results of ITS sequence clustering, AFLP analysis grouped these two isolates within the Wcz cluster. Since AFLP generates multilocus markers, the effect of polymorphism on a single locus is negligible.

Contrary to our findings, previous reviews indicate AFLP and other DNA fingerprinting techniques are more efficacious for studying genetic variation of \textit{Rhizoctonia} at the individual level rather than at the subgroup level within an AG or different AGs [6,37]. However, these hypotheses were based on few studies without proper investigation of analyzing a large number of \textit{Rhizoctonia} and \textit{Waitea} isolates belonging to different AGs. Ceresini et al. [21] employed the AFLP technique to evaluate genetic diversity of isolates within AG3 obtained from potato (PT) and tobacco (TB). AFLP analysis on 32 PT and 36 TB isolates placed them into two distinct groups based on their host. A similar genetic diversity study of \textit{R. solani} AG4 isolates obtained from the rhizosphere of six vineyards in Mexico was reported by Meza-Moller et al. [38]. They analysed 41 \textit{Rhizoctonia} isolates using AFLP markers, which revealed three main groups in the UPGMA dendrogram and six groups from principal component analysis. None of the above studies included different AGs. López-Olmos [39] grouped isolates of AG 2-3, AG BI, and AG 5 from common bean using AFLP. However, each AG was represented by only one or a few isolates, thus limiting applicability of AFLP fingerprinting in deriving AGs of unknown isolates. No peer reviewed documentation is available on performing AFLP on different \textit{W. circinata} varieties. Therefore, we feel our analysis is unique since we used a large number of \textit{R. solani} and \textit{W. circinata} isolates to test applicability of AFLP in resolving isolates to AG and AG subgroup level.
We used the ABI 3730 electrophoresis system to capture AFLP amplicons since it is sensitive enough to differentiate fragments having one base pair difference. This aided in scoring a large number of polymorphic fragments for the four AFLP primers employed (942 and 485 fragments across _R. solani_ and _Waitea circinata_ isolates, respectively). However, size standard used with the ABI 3730 capillary gel electrophoresis system limited the longest fragment size that can be scored to 500 bp. Although, scanning gel images can record longer fragments, this method results in less number of total markers compared to capillary gel electrophoresis system and also requires additional labor. When GeneMapper is used it is important to optimize the peak capture amplitude to get the best results. A peak having low amplitude may be generated from background noise and not represent a true AFLP fragment. Therefore, it is necessary to test with different peak detection levels and choose the best for a particular data set. Best peak height depends on run conditions of the capillary electrophoresis system and AFLP samples. Our data set gave better results when the peak capture threshold was set to 1000 rfu. GeneMapper is also capable of analyzing multiplexed AFLP fragments. Multiplexing refers to the labeling of amplicons of different isolates with different fluorescent labels.
dyes and analyzing as a single sample. This dramatically reduces the cost of AFLP analysis via capillary electrophoresis.

The main disadvantage of AFLP lies on the dominance nature of its markers which make it unable to determine homologous alleles. Due to this, amid all of the benefits of the AFLP technique such as high reliability, ability to generate molecular markers from uncharacterized organisms, etc., it cannot replace co-dominant markers such as RFLP and microsatellites, completely. Nevertheless, our results showed AFLP can be used successfully to determine genetic structure of unknown *R. solani* and *W. circinata* isolates infecting cool-season turfgrasses by identifying to infra-species level. *Rhizoctonia* species infecting turfgrasses are difficult to identify using disease symptoms. Accurate identification of causal pathogens to AG or AG subgroup is important since they have differential sensitivity to fungicides and environmental conditions. Our analysis show AFLP is a good alternative for classical methods to characterize a large number of unknown *R. solani* and *W. circinata* isolates to infra-species level reliably and cost effectively.

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