Research Article   **Open Access**

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

Bimal S. Amaradasa[1]*, Dilip Lakshman[2] and Keenan Amundsen[3]

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

Abstract

Patch diseases caused by *Thanatephorus 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 pathogen 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.

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 *Thanatephorus 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

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 genetic diversity of many organisms. AFLP is a multilocus marker and does not need prior knowledge of sequence information to generate polymorphic markers. Technology advancements have made AFLP technique a relatively cheap, easy, fast and reliable method to generate hundreds of informative genetic markers [19]. No detailed studies have been done to ascertain whether AFLP on *Rhizoctonia* should be performed at genus level, species level or infra-species level (i.e. at AG and AG subgroup level or *W. circinata* variety). The objective of this study was to determine if AFLP is capable of revealing AG, AG 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

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*

Isolate	Origin	Host	Management Type	Species acronym	Anastomosis group	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 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 267	Beltsville, MD	Tall fescue	Lawn	Rs	AG 2-2IIIB	
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 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 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 4114	Leesburg, VA	Tall fescue	Golf rough	Rs	AG 2-2IIIB	-
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
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 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
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 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 220	Woodbridge, VA	Tall fescue	Lawn	Wcz	WAG-Z	JX631232
PW 119	Woodbridge, VA	Tall fescue	Lawn	Wcz	WAG-Z	JX631231
VABCH 8	Virginia Beach, VA	Tall fescue	Lawn	Wcz	WAG-Z	-
VABCH 10	Virginia Beach, VA	Tall fescue	Lawn	Wcz	WAG-Z	-

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

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

Isolate	Species Acronym*	AG	Host‡	Location	Donor†
EDHGED 2-1	Wcc	Not assigned	ABG	California, USA	FW
BSCCST 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

*Rs: *R. solani*; Wcc: *W. circinata* var. *circinata*; Wcz: *W. circinata* var. *zeae*
‡ABG: Annual Bluegrass
†FW: Frank Wong, University of California Riverside, USA (currently Bayer Crop Science, USA). LB: Lee Burpee, University of Georgia, USA. MC: Marc Cubeta, North Carolina State University, USA.

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* (Wcc) 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 *Eco*RI (New England BioLabs, Beverly, MA) and *Mse*I (New England BioLabs). Thereafter, digested products were ligated with *Eco*RI Double Stranded (ds) adapter (EA1: 5′-CTCGTAGACTGCGTACC-3′ and EA2 3′-CATCTGACGCATGGTTAA-5′) and *Mse*I ds adapter (MA1: 5′-GACGATGAGTCCTGAG-3′ and MA2: 3′-TACTCAGGACTCAT-5-3′). Both digestion and ligation reactions were done in one step by preparing a reaction mixture of 20 µl having 2 U of each restriction enzyme, 1.2 U of T4 DNA ligase (New England BioLabs), 0.1 µM of *Eco*RI adapter, 1 µM of *Mse*I adapter and 100 ng of DNA template. The reaction mixture also included 1× *Eco*RI buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl$_2$, 0.025% Triton X-100), 1× *Mse*I buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM DTT), 1× T4 ligase buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 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: *Eco*RI primer + *A* (5′-GACTGCGTACCAATTC*A*-3′) and *Mse*I primer + *C* (5′-GATGAGTCCTGAGTAA*C*-3′). Each sample of 25 µl included 5µl of digestion and ligation reaction from the previous step, 0.5 µM each of *Eco*RI and *Mse*I primers, 1× Taq polymerase reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$), 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 at 72°C. PCR products were diluted ten-fold with TE buffer and used as the template DNA for selective amplification using four *Eco*RI and *Mse*I primer pairs with three selective nucleotides (*Eco*RI primer + *ACA* and *Mse*I primer + *CAA*, *Eco*RI primer + *AAA* and *Mse*I primer + *CTA*, *Eco*RI primer + *AAC* and *Mse*I primer + *CAC*, *Eco*RI primer + *AGT* and *Mse*I primer + *CTG*). *Eco*RI and *Mse*I primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and IDT (Coralville, IA), respectively. All *Eco*RI 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 *Eco*RI and *Mse*I 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 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 products 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 through 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 sizes. A Size Standard Curve is generated for each sample and 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 (Φ_{PT}) which is an analogue of Wright's F_{ST}. Normally, F_{ST} and its analogues are greater than zero but rarely exceed 0.5. An F_{ST} 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 dendrograms 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 *Eco*RI-*AAC* and *Mse*I-*CAC*, *Eco*RI-*AGT* and *Mse*I-*CTG*, *Eco*RI-*AAA* and *Mse*I-*CTA*, and *Eco*RI-*ACA* and *Mse*I-*CAA* produced 230, 234, 265, and 213 alleles, respectively for the isolates analyzed. Allele 7 produced by *Eco*RI-*AAA* and *Mse*I-*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 dendrograms produced by different similarity indices to the results of ITS sequence analysis in a previous study having all but BELT 267 and LB 4114 isolates (Supplemental Figure 1) [20]. Though all the similarity indices tested largely grouped *R. solani* 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., *Eco*RI-*AAC* and *Mse*I-*CAC*, *Eco*RI-*AGT* and *Mse*I-*CTG*, *Eco*RI-*AAA* and *Mse*I-

CTA, and *Eco*RI-*ACA* and *Mse*I-*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 Wzc, 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
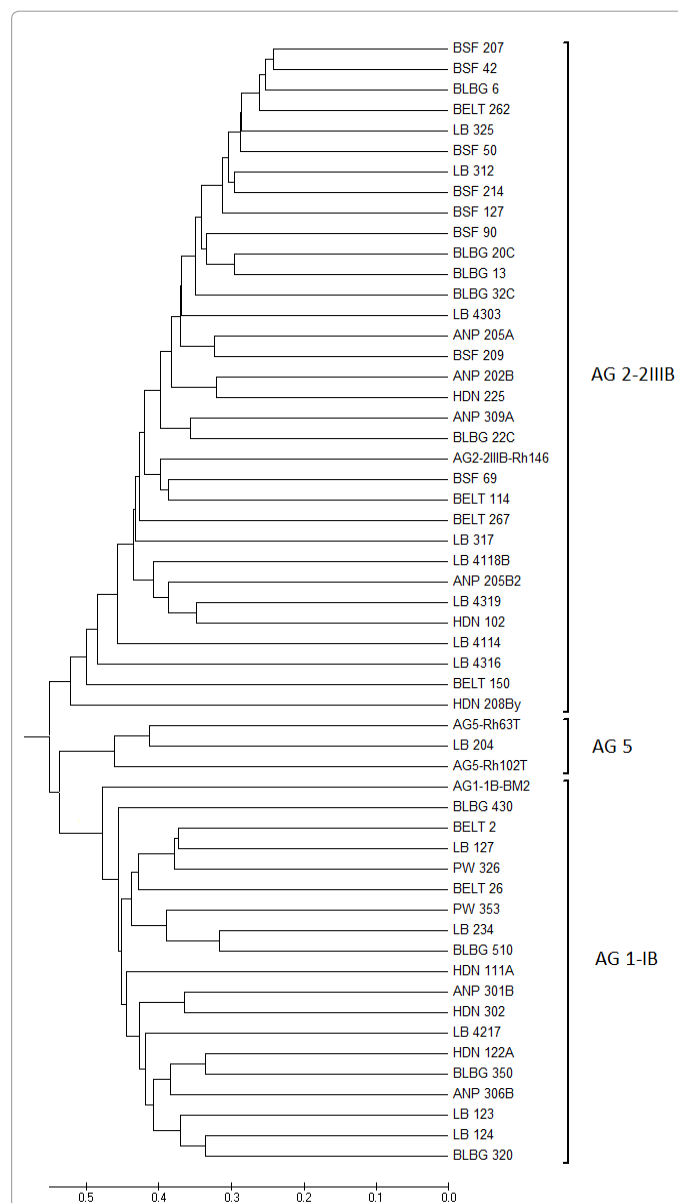

Figure 1: UPGMA dendrogram for *R. solani* isolates derived from Dice's genetic distance matrix. Isolates of *R. solani* anastomosis groups AG 1-IB, 2-2IIIB, and 5 grouped separately and are indicated in the tree.
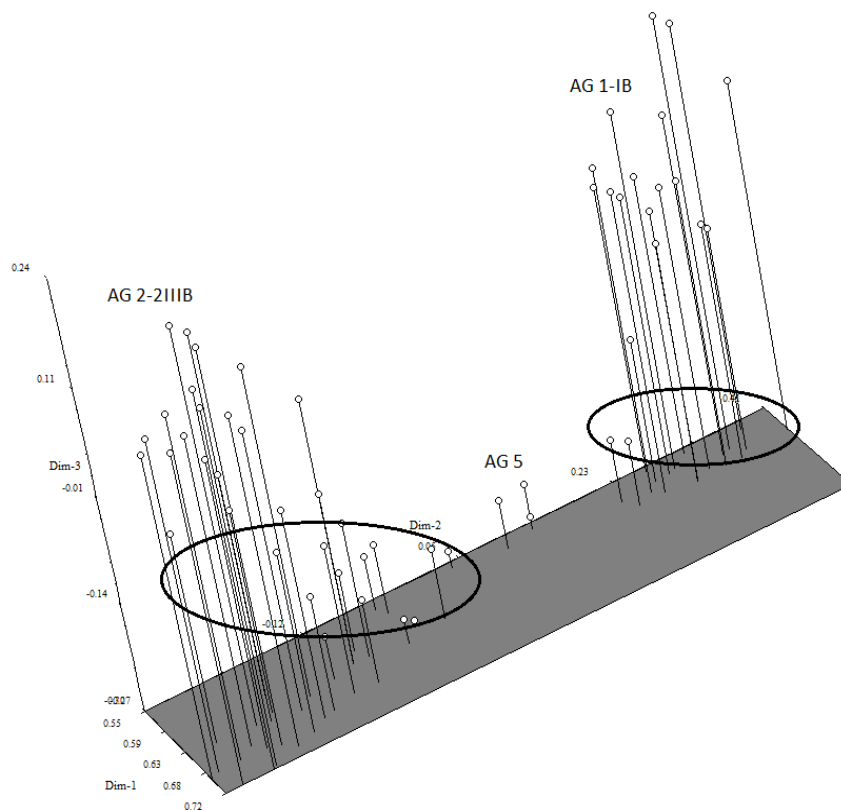
Figure 2: Placement of *R. solani* isolates on a three dimensional plot using PCA based on Dice's genetic distance matrix.

Group	Source of variation	df	SS	% Variation	Φ_{PT}	P value
R. solani[a]	Among populations	2	982	14	0.142	0.001
	Within populations	52	7605	86	0.858	0.001
	Total	54	8587			
W. circinata[b]	Among populations	2	348	12	0.120	0.0001
	Within populations	21	1840	86	0.880	0.0001
	Total	23	2188			

P value for randomization test for ΦPT is based on 999 permutations across the full data set.
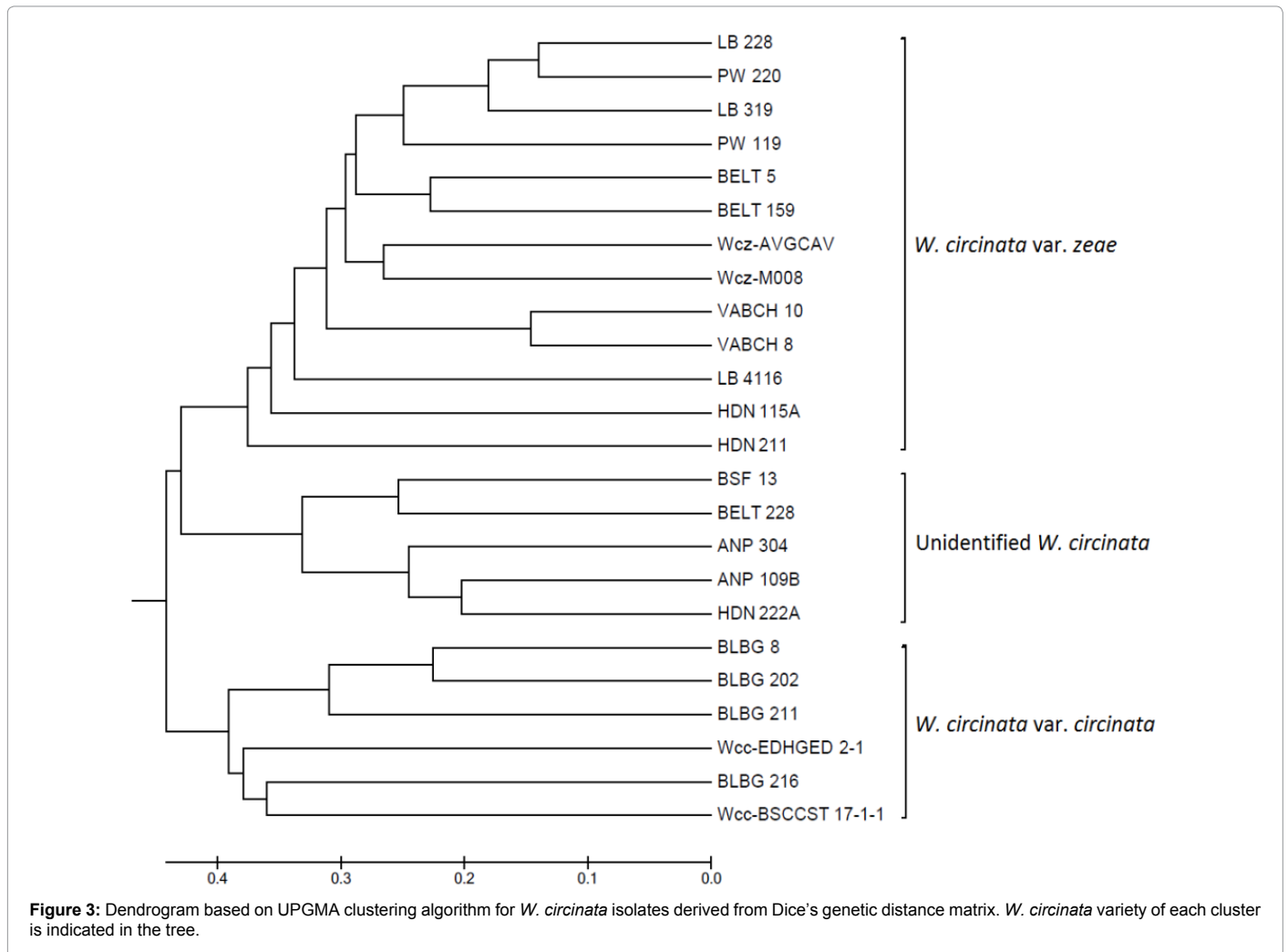*R. solani* group consisted of three subpopulations namely, AG 1-IB, AG 2-2IIIB, and AG 5.
W. circinata group consisted of varieties zeae, circinata and an unidentified subgroup.

Table 3: Analysis of molecular variance (AMOVA) of *R. solani* and *W. circinata* isolates based on four AFLP primer data.

dendrogram, they resolved differently thereafter. UWC isolate BSF 13 grouped close to *W. circinata* var. *prodigus* in the ITS tree (GenBank accessions HM597147, HM597146, and HQ850254), while UWC isolate ANP 109B grouped with *W. circinata* var. *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 *agrostis*, *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 *W. circinata* isolates to their subgroups Wzc, Wcc and UWC along dimension 2 and 3 (Figure 5), which was corroborated with an AMOVA that showed significant difference among *W. circinata* subgroups (p=0.0001), representing 12.0% of the total genetic variance (Table 3).

Discussion

The present study investigated the applicability of the AFLP technique for grouping *R. solani* and *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 *W. circinata* and *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 *Rhizoctonia* and *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.
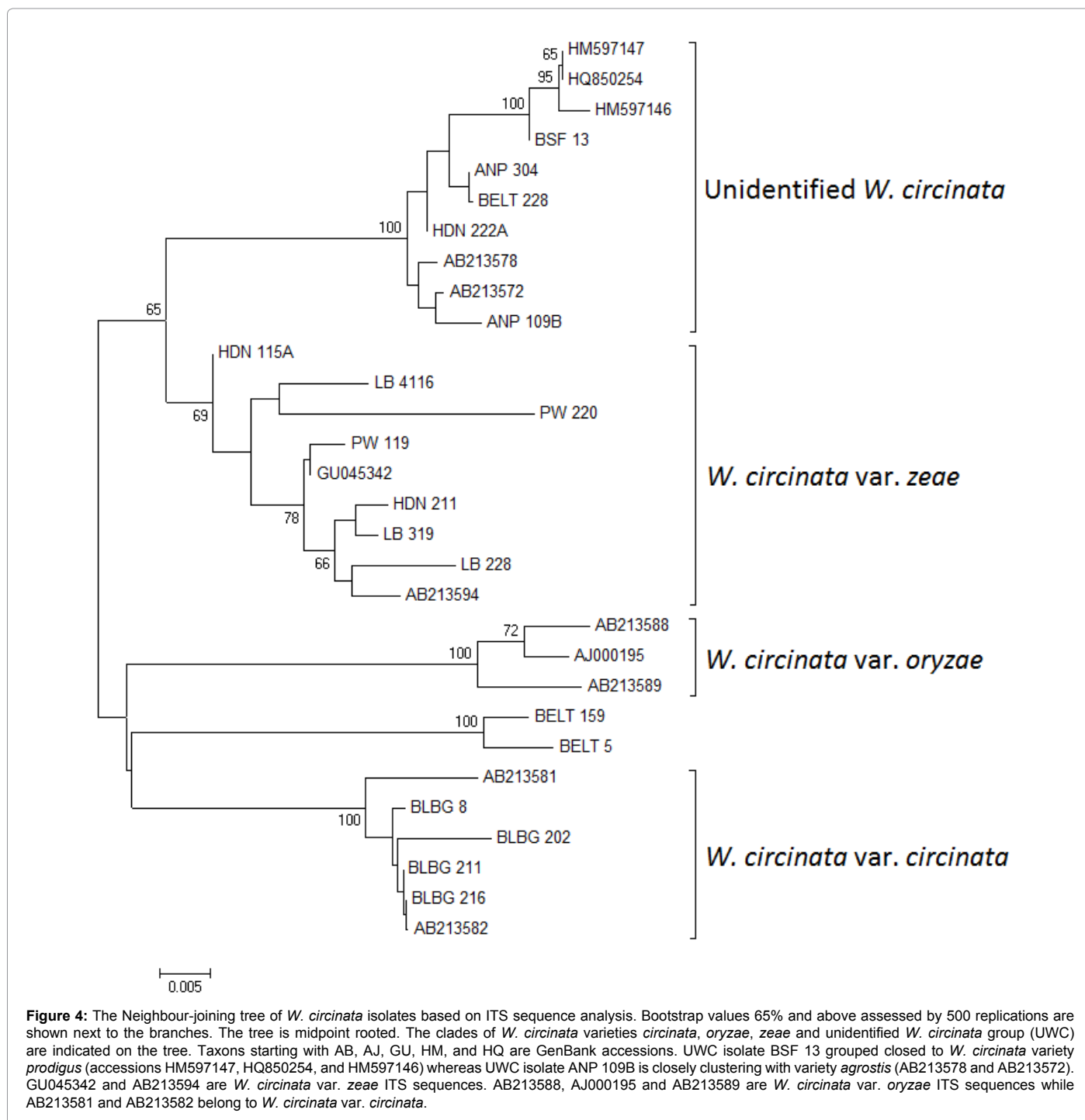
Therefore, AFLP is not useful to make phylogenetic inferences among higher taxonomic levels but is more suitable for deriving relationships among closely related lineages.

The AFLP markers used in our analysis was able to accurately resolve *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 *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 *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 *Rhizoctonia* and *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 *R. solani* AG4 isolates obtained from the rhizosphere of six vineyards in Mexico was reported by Meza-Moller et al. [38]. They analysed 41 *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 *W. circinata* varieties. Therefore, we feel our analysis is unique since we used a large number of *R. solani* and *W. circinata* isolates to test applicability of AFLP in resolving isolates to AG and AG subgroup level.

Figure 4: The Neighbour-joining tree of *W. circinata* isolates based on ITS sequence analysis. Bootstrap values 65% and above assessed by 500 replications are shown next to the branches. The tree is midpoint rooted. The clades of *W. circinata* varieties *circinata*, *oryzae*, *zeae* and unidentified *W. circinata* group (UWC) are indicated on the tree. Taxons starting with AB, AJ, GU, HM, and HQ are GenBank accessions. UWC isolate BSF 13 grouped closed to *W. circinata* variety *prodigus* (accessions HM597147, HQ850254, and HM597146) whereas UWC isolate ANP 109B is closely clustering with variety *agrostis* (AB213578 and AB213572). GU045342 and AB213594 are *W. circinata* var. *zeae* ITS sequences. AB213588, AJ000195 and AB213589 are *W. circinata* var. *oryzae* ITS sequences while AB213581 and AB213582 belong to *W. circinata* var. *circinata*.

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 *W. 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
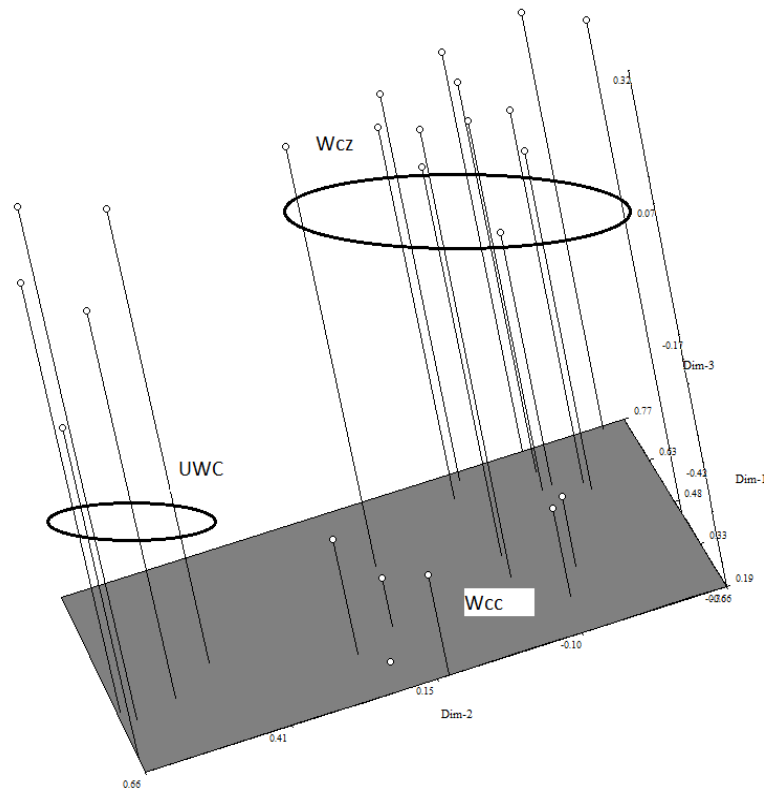
Figure 5: Three dimensional PCA scatter diagram of studied *Waitea circinata isolates* along first three principal components based on Y genetic distance matrix.

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 makers 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.

Acknowledgements

References

1. Burpee L, Martin B (1992) Biology of Rhizoctonia species associated with turfgrass. Plant Disease 76: 112-117.

2. De La Cerda KA, Douhan GW, Wong FP (2007) Discovery and characterization of Waitea circinata var. circinata affecting annual bluegrass from the western United States. Plant Disease 91: 791-797.

3. Kammerer SJ, Burpee LL, Harmon PF (2011) Identification of a new Waitea circinata variety causing basal leaf blight of seashore paspalum. Plant Disease 95: 515-522.

4. Toda T, Hayakawa T, Mghalu JM, Yaguchi S, Hyakumachi M (2007) A new Rhizoctonia sp. closely related to Waitea circinata causes a new disease of creeping bentgrass. Journal of General Plant Pathology 73: 379-387.

5. Lübeck M, Poulsen H (2001) UP-PCR cross blot hybridization as a tool for identification of anastomosis groups in the Rhizoctonia solani complex. FEMS Microbiol Lett 201: 83-89.

6. Sharon M, Kuninaga S, Hyakumachi M, Sneh B (2006) The advancing identification and classification of Rhizoctonia spp. using molecular and biotechnological methods compared with the classical anastomosis grouping. Mycoscience 47: 299-316.

7. Zhang M, Dernoeden PH (1995) Facilitating anastomosis grouping of Rhizoctonia solani isolates from cool-season turfgrasses. HortScience 30: 1260-1262.

8. Smiley RW, Dernoeden PH, Clarke BB (2005) Compendium of Turfgrass Diseases. (3rdedn), American Phytopathological Society, St. Paul, MN.

9. Martin SB, Campbell CL, Lucas LT (1984) Response of Rhizoctonia blights of tall fescue to selected fungicides in the greenhouse. Phytopathology 74: 782-785.

10. Martin SB, Lucas LT, Campbell CL (1984) Comparative sensitivity of Rhizoctonia solani and Rhizoctonia-like fungi to selected fungicides in vitro. Phytopathology 74: 778-781.

11. Carling DE, Helm DJ, Leiner RH (1990) In vitro sensitivity of Rhizoctonia solani and other multinucleate and binucleate Rhizoctonia to selected fungicides. Plant Disease 74: 860-863.

12. Kataria HR, Hugelshofer U, Gisi U (1991) Sensitivity of Rhizoctonia species to different fungicides. Plant Pathology 40: 203-211.

13. Ogoshi A (1987) Ecology and pathogenicity of anastomosis and intraspecific groups of Rhizoctonia solani Kühn. Annual Review of Phytopathology, 25: 125-142.

14. Carling DE (1996) Grouping in Rhizoctonia solani by hyphal anastomosis reaction. In B Sneh, S Jabaji-Hare, S Neate, G Dijst eds Rhizoctonia species:

Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Kluwer Academic Publishers, Dordrecht.

15. Boysen M, Borja M, del Moral C, Salazar O, Rubio V (1996) Identification at strain level of Rhizoctonia solani AG4 isolates by direct sequence of asymmetric PCR products of the ITS regions. Curr Genet 29: 174-181.

16. Strausbaugh CA, Eujayl IA, Panella LW, Hanson LE (2011) Virulence, distribution and diversity of Rhizoctonia solani from sugar beet in Idaho and Oregon. Canadian Journal of Plant Pathology 33: 210-226.

17. Grosch R, Schneider JH, Peth A, Waschke A, Franken P, et al. (2007) Development of a specific PCR assay for the detection of Rhizoctonia solani AG 1-IB using SCAR primers. J Appl Microbiol 102: 806-819.

18. Pannecoucque J, Höfte M (2009) Detection of rDNA ITS polymorphism in Rhizoctonia solani AG 2-1 isolates. Mycologia 101: 26-33.

19. Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. Trends Ecol Evol 14: 389-394.

20. Amaradasa BS, Horvath BJ, Lakshman DK, Warnke SE (2013) DNA fingerprinting and anastomosis grouping reveal similar genetic diversity in Rhizoctonia species infecting turfgrasses in the transition zone of USA. Mycologia 105: 1190-1201.

21. Ceresini PC, Shew HD, Vilgalys RJ, Cubeta MA (2002) Genetic diversity of Rhizoctonia solani AG-3 from potato and tobacco in North Carolina. Mycologia 94: 437-449.

22. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, et al. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407-4414.

23. Rohlf FJ (2005) NTSYS-pc: numerical taxonomy and multivariate analysis system. Version 2.2. Setauket, New York: Exeter Software.

24. Dice LR (1945) Measures of the amount of ecologic association between species. Ecology 26: 297-302.

25. Sneath PHA, Sokal RR (1973) Numerical taxonomy. W.H. Freeman and Co., San Francisco, CA.

26. Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. University of Kansas Science Bulletin 38: 1409-1438.

27. Sokal RR, Sneath PHA (1963) Principles of numeric taxonomy. W.H. Freeman, San Francisco, pp. 359.

28. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739.

29. Rohlf FJ, Sokal RR (1981) Comparing numerical taxonomic studies. Systematic Zoology 30: 459-490.

30. Hotelling H (1933) Analysis of a complex of statistical variables into principal components. Journal of Educational Psychology 24: 417-520.

31. Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491.

32. Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28: 2537-2539.

33. Wright S (1978) Evolution and the Genetics of Populations. Variability within and among natural populations. Vol 4. The University of Chicago Press, Chicago.

34. Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. Mol Ecol 11: 155-165.

35. Lübeck M (2004) Molecular characterization of Rhizoctonia solani. In DK Arora, GG Khachatourians eds Applied mycology and biotechnology Volume 4: Fungal genomics. Elsevier B.V., Amsterdam.

36. Majer D, Mithen R, Lewis BG, Vos P, Oliver RP (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 100: 1107-1111.

37. Cubeta MA, Vilgalys R (1997) Population Biology of the Rhizoctonia solani Complex. Phytopathology 87: 480-484.

38. Meza-Moller A, Esqueda M, Sanchez-Teyer F, Vargas-Rosales G, Gardea AA, et al. (2011) Genetic Variability in Rhizoctonia solani Isolated from Vitis vinifera based on Amplified Fragment Length Polymorphism. American Journal of Agricultural Biology 6: 317-323.

39. López-Olmos K, Hernandez-Delgado S, Mayek-Perez N (2005) AFLP fingerprinting for identification of anastomosis groups of Rhizoctonia solani isolates from common bean (Phaseolus vulgaris L.) in Mexico. Revista Mexicana de Fitopatologia 23: 147-151.