GENETIC DIVERSITY OF Waitea circinata var. zeae IN SOUTH CAROLINA REVEALED BY AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

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Rhizoctonia zeae (Waitea circinata var. zeae) is pathogenic to rice (Oniki et al., 1985); corn (Sumner and Bell, 1982); onion (Erper et al., 2006); sugarbeet (Kuznia and Windels, 1994); wheat and barley (Ogoshi et al., 1990) and tall fescue (Martin and Lucas, 1983). Waitea circinata var. zeae also causes foliar lesions on bermuda grass, creeping bentgrass and annual bluegrass (Burpee and Martin, 1992; Hsiang and Dean, 2001) during midsummer. Diseases of turfgrass caused by these pathogens occur most frequently during the warm and humid season, at temperatures between 28 and 36°C, inciting leaf and sheath spot (Burpee and Martin, 1992; Smiley et al., 1992).

Waitea circinata (Warcup and Talbot) classified into three varieties, W. circinata var. circinata, W. circinata var. oryzae and W. circinata var. zeae based on differences in the colony morphology of the vegetative state (Gunnell, 1986). Waitea circinata var. circinata forms orange to dark brown, globose sclerotia up to 2 mm in diameter; W. circinata var. oryzae forms orange to salmon, irregularly shaped sclerotia; and W. circinata var. zeae forms orange to brown, regularly shaped sclerotia up to 1 mm in diameter (Leiner and Carling, 1994). Rhizoctonia zeae was assigned to Waitea anastomosis group WAG-Z (Oniki et al., 1985).

Previous studies have examined genetic variation of these three varieties at molecular level. Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) of rDNA internal transcribed spacer (ITS) region revealed that isolates of W. circinata var. circinata, W. circinata var. oryzae and W. circinata var. zeae separated into individual clusters (Toda et al., 2005). These results confirmed by using sequence analysis of the internal transcribed spacer (ITS) region of rDNA (de la Cerda et al., 2007; Toda et al., 2007).

Amplified fragment length polymorphism (AFLP) is a genetic mapping technique based on selective amplification of a subset of restriction enzyme-digested DNA fragments to create a unique fingerprint for a particular genome (Vos et al., 1995). It is highly reproducible and amenable to a wide range of applications and...
DNA sources. For these reasons, the method has steadily gained popularity in applications, including genetic mapping (Mueller and Wolfenbarger, 1999; Savelkoul et al., 1999), medical diagnostics (Klaassen et al., 2002; Borst et al., 2003; van den Braak et al., 2004), genetic diversity and phylogenetic studies (Tredway et al., 1999; Bakkeren et al., 2000; Doignon-Bourcier et al., 2000; Rademaker et al., 2000; Mougel et al., 2002; Lee et al., 2004) and environmental management studies (Lucchini, 2003).

The similarities within each variety of *W. circinata* were very high, but similarities were significantly lower between varieties by using rDNA-ITS region (Toda et al., 2007). In this study, we used amplified fragment length polymorphism (AFLP) to examine the genetic diversity of field population of *W. circinata* var. *zeae*.

**MATERIALS AND METHODS**

**Isolate collection and maintenance**

Isolates of *W. circinata* var. *zeae* (*Rhizoctonia zeae*) obtained from turfgrass samples exhibiting symptoms of the sheath and leaf spot disease collected from 7 fields scattered in South Carolina, USA. They were obtained by placing a single symptomatic leaf blade onto ¼ strength potato dextrose agar (PDA) in 100 mm petri plates (¼ PDA; 4.95 g potato dextrose agar, 5.63 g granulated agar (Fisher Scientific, Pittsburgh, PA) per 500 ml of deionized water). Plates maintained at room temperature, and 24 to 48 h later, a 1 to 2 mm hyphal tip from colonies typical of *Rhizoctonia* was excised and transferred to a new plate of ¼ PDA. For the duration of the study, isolates maintained on ¼ PDA with transfers to new media every 21 days unless otherwise noted. Isolate characteristics, cultural morphology and anastomosis group with the tester strain of *W. circinata* var. *zeae* were identified in Plant Disease Diagnostics Clinic, Clemson University.

**DNA extraction**

Mycelia of each isolate from 15 isolates which anastomized with *W. circinata* var. *zeae* were cultured in potato dextrose broth (PDB) at 25°C. After 3 to 4 days, the mycelial mat was harvested by filtration, grinded in liquid nitrogen and stored at -80°C. Total genomic DNA extracted from individual isolate using the DNeasy plant mini kit (QIAGEN #69104) according to the manufacturer’s description.

**AFLP assay**

Genomic DNA was digested with the restriction enzymes EcoR1 (New England Biolabs #R0101S) and MseI (New England Biolabs #R0525S), ligated to adapters using T4 DNA Ligase (New England Biolabs #M0202S), and used in a pre-selective amplification step using the AFLP Ligation/Preselective Amplification Module (Applied Biosystems P/N 402273). AFLP fragments generated as in Vos et al. (1995) with minor modifications, as detailed below. MseI and EcoRI digestion of genomic DNA and ligation of
double-stranded adaptors were completed in a one-step reaction (37°C, 2 h) using 0.5-1.0 μg of DNA, 2.2 μL of 5x ligase buffer, 1.1 μL of 0.5 mol/L NaCl, 0.5 μL of 1 mg/mL bovine serum albumin, 1 μL of 50 μmol/L MseI adaptor, 1 μL of 5 μmol/L EcoRI adaptor, 0.25 μL MseI, 0.25 μL EcoRI, and 0.33 μL of T4 DNA ligase, and then adding water to a total volume of 11 μL. The adaptor ligation reaction was then diluted 10-fold for use in the preselective PCR (4.5 μL DNA solution, 1x PCR buffer, 1.5 mmol/L MgCl₂, 1 μmol/L dNTPs, 2.75 μmol/L EcoRI preselective primer (E: 5′-GTAGACTGCGTACCAATTC-3′), 2.75 μmol/L MseI preselective primer (M: 5′-GACGATGAGTCCTGAGTAA-3′), and 3 U of AFLP Amplification Core Mix (Applied Biosystems P/N 402005) in a total volume of 20 μL). PCRs were conducted in a Thermocycler (iCycler, BIO RAD) in a total volume of 20 μL using a concentration of 10 pmol for each primer. The preselective PCR included an initial denaturation of 95°C for 3 min, which was followed by 20 cycles (each) of 94°C for 20 s, 56°C for 30 s (with a decrease of 1°C in each successive cycle), and 72°C for 2 min; then 20 cycles each of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min; with a final extension of 10 min at 72°C. PCR products from preselective amplification were visualized with ethidium bromide staining following electrophoresis in 1.5% (w/v) agarose gel.

**Genetic analyzer electrophoresis**

One microliter of the selective amplification product was mixed with 0.5 μL of the GeneScan 500 ROX size standard (Applied Biosystems P/N 402985) and 8.5 μL of Hi-Di Formamide (Applied Biosystems P/N 4311320). The mixture was denatured and loaded on the 16-capillary system of the Applied Biosystems 3130 Genetic Analyzer. A 36-cm capillary array (Applied Biosystems P/N 4315931) and 3130 POP-7 polymer (Applied Biosystems P/N 4352759) were used. The protocol used the run module Fragment Analysis 36_POP-7 and dye set F.

**Data analysis**

A default AFLP specific analysis method in GeneMapper software v4 was used to recognize and analyze AFLP data. The analysis output can be set up to produce final results in the standard binary format, where 1s (ones) represent the presence and 0s (zeros) represent absence of a
given fragment category. The analyzed results can be exported in a format such as tab-delimited text and used in further analysis.

Neighbor-joining similarity matrix was used to investigate genealogical lineages and population structure, based on pairwise comparisons of the total number of DNA polymorphisms (AFLPs) between individual isolates. Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10 package (Swofford, 2005) used to generate Neighbour-joining tree based on Nei and Li distances. A graphic display of the neighbor-joining tree was developed using TREEVIEW.6.

RESULTS AND DISCUSSION

Genetic characterization of Waitea circinata var. zeae isolates

AFLP analysis was conducted on 15 Waitea circinata var. zeae isolates collected from turfgrass in South Carolina, USA. Using combinations of fluorescently labelled primers, a different number of fragments (ranging in size from 50-500 bp) were visualized as peaks in the electropherograms after selective amplification (Fig. 1). The five primer combinations used in this study generated a total of 721 clearly scorable peaks, of which 715 fragments (99%) were polymorphic and 6 fragments (1%) were monomorphic; 260 fragments (36%) were useful as isolate-specific markers (Tables 1 and 2). The number of peaks generated per primer combination ranged from 100 to 197. Meanwhile, the percentage of polymorphic peaks per primer combination varied between 98% and 100%. Although, EcoRI-AC and MseI-CAC primer pairs generated the largest number of AFLP isolate-specific markers (63 markers) while the least number (39 markers) was generated by EcoRI-TC and MseI-CAA primer pairs. The total number of AFLP specific markers and the percentage varied among the 15 isolates, with the largest proportion (25%) for isolate 45 (65 markers), whilst the isolate 5 had the lowest number (2 markers) and proportion (0.7%).

AFLP analysis is based on the ligation of adapters to genomic restriction fragments followed by a PCR-based amplification with adapter specific primers. As reported by Vos et al. (1995), the cleavage frequency of the restriction endonucleases and the number of selective bases in the primers used might control the number of amplified fragments. This analysis produced a large number of reproducible and unambiguous markers for fingerprinting. In this study, high numbers of fragments were observed on the capillary electrophoresis when compared with polyacrylamide gels and the bands that are smaller than 100 bp were included in the analysis. Using capillary electrophoresis with a proper polymer and standard conditions it is possible to obtain data within the range 50-2000 bp, however, in our experiment we used a genescan-500 size standard and the data analyzed is limited to the range of that standard.
GENETIC DIVERSITY OF *Waitea circinata* var. *zeae*

**Genetic relationships**

The similarity indices among the 15 *Waitea circinata* var. *zeae* isolates based on AFLP (Table 3) were detected by PAUP version 4.0b10 package based on Nei and Li distances. The similarity percentage varied from 2 to 34. The strongest relationship was scored between *Waitea circinata* var. *zeae* isolate 46 and isolate 3 (similarity of 34%) followed by 31% similarity with isolate 13. In contrast, the isolate 3 and isolate 5 also isolate 2 and isolate 60 were shown to be the most genetically distant isolates (similarity indices of 2% and 3%, respectively).

The data obtained by the AFLP assay were statistically analyzed by Neighbour-joining cluster analysis and a dendrogram was produced. Cluster analysis categorized the 15 isolates into 5 distinct groups and 4 subgroups (Fig. 2). The first group consisted of two isolates (23 and 60) each in one subgroup and closed in one lineage clade. Group 2 was made up of the largest number of isolates (10 isolates), but was divided into two subgroups. The first subgroup consisted of 3 isolates (isolate 5, 77 and 49) the two isolates 5 and 77 closed in one lineage clade. The second subgroup made up of 7 isolates (13, 68, 30 and 51 in the first sub-sub group) and (3, 85 and 45 in the second sub-sub group). Of which the two isolates 30 and 51; 13 and 68; 3 and 85 were closed in lineage clade, respectively. These results showed high diversity among *Waitea circinata* var. *zeae* isolates and the three isolates 2, 46 and 79 were most distant. Based on cluster analysis, results showed that isolates collected from the same turfgrass field were associated with one cluster lineage clade. The isolates 2, 46 and 79, which collected from different field, showed one lineage clade for each isolate. AFLP has been used effectively to evaluate the genetic diversity and genetic relatedness in strains and isolates of fungi (de Barros Lopes *et al*., 1999; Mueller *et al*., 1996; Hynes *et al*., 2006; Collado-Romero *et al*., 2008).

Sequence similarities of the rDNA-ITS region between isolates within each variety of *W. circinata* were high, but they were lower among the varieties (Toda *et al*., 2007). We used AFLP analysis to examine the genetic diversity and provide a foundation for examining the genetic structure of variety *zeae* population. The high number of polymorphic fragments and the low level of similarity indices confirm the high genetic diversity among *Waitea circinata* var. *zeae* isolates. Some previous studies confirm the data discovered in the current work based on AFLP. Amplified fragment length polymorphism fingerprinting was used by Ceresini *et al.* (2002) to evaluate the genetic diversity of field populations of *Rhizoctonia solani*. They reported that AFLP analysis has significant potential as a tool for studying the population genetics of *Rhizoctonia* spp. The complex multi-locus fingerprints produced by the AFLP technique are highly reproducible and provide a large number of informative markers derived from loci dispersed throughout the nuclear genome (Ridout and Donini, 1999). The
The fact that AFLP markers are generally distributed across the genome gives the technique some advantages over sequence analysis for closely related isolate. A sequence analysis that relies on data from single DNA regions can give misleading results (Riesberg and Soltis, 1991) and the presence of paralogous DNA regions (Baldwin et al., 1995). AFLP therefore seem a particularly appropriate and efficient method for genetic diversity studies in W. circinata var. zeae in which insufficient variation can be detected through sequence analysis. This study showed the possibility of using AFLP technique on the discrimination between more closely related isolates of W. circinata var. zeae.

**SUMMARY**

_Waitea circinata_ were classified into three varieties, _W. circinata_ var. _circinata_, _W. circinata_ var. _oryzae_, and _W. circinata_ var. _zeae_. Based on rDNA-ITS region sequence, the similarities within each variety were very high, but similarities were significantly lower between varieties. To address this hypothesis, amplified fragment length polymorphism (AFLP) was used to examine the genetic diversity of field population of _W. circinata_ var. _zeae_. Total genomic DNA was extracted from 15 isolates collected from different fields scattered in North Carolina. AFLP analysis was conducted using 5 combinations of fluorescently labeled primers. GenMapper and PAUP softwares were used to analyze the AFLP data.

The results showed that the five primer combinations used in this study generated a total of 721 clearly scorable peaks, of which 715 fragments (99%) were polymorphic; 6 fragments (1%) were monomorphic and 260 fragments (36%) were useful as isolate-specific markers. _EcoRI-AC_ and _MseI-CAC_ primer pairs generated the largest number of AFLP isolate-specific markers (63 markers) while the least number (39 markers) was generated by _EcoRI-TC_ and _MseI-CAA_ primer pairs. The isolate 45 had the largest number of AFLP specific markers (65 markers), while the isolate 5 had the lowest number (2 markers).

Cluster analysis categorized the 15 isolates into 5 distinct groups and 4 subgroups. The isolates 2, 46 and 79, which collected from different field, showed one lineage clade for each isolate. Isolates, which collected from the same turfgrass field, associated with individual lineage clade.

This study concluded that the possibility of using AFLP technique on the discrimination between more closely related isolates of _W. circinata_ var. _zeae_. AFLP therefore seem a particularly appropriate and efficient method for genetic diversity studies in _W. circinata_ var. _zeae_ in which insufficient variation can be detected through sequence analysis.

**REFERENCES**

Bakkeren, G., J. W. Kronstad and C. A. Levesque (2000). Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in


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Table (2): Isolate-specific markers in 15 *Waitea circinata var. zeae* isolates resulting from AFLP analysis.

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Table (3): Similarity indices% calculated by PAUP version 4.0b10 package based on Nei and Li's distances among 15 Waitea circinata var. zeae isolates.

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Fig. (1): Electropherograms of 15 *Waitea circinata* var. *zeae* isolates AFLP profiles using *Eco*RI-TG and *Mse*I-CAA primer pairs.
Fig. (1): Cont'

Isolate 46

Isolate 49

Isolate 51

Isolate 60

Isolate 68

Isolate 77

Isolate 79

Isolate 85