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

Z. EL FIKY[1], H. FOULY[2] AND B. MARTIN[3]

1. *Genetics Department, Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt*
2. *Plant Pathology Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt*
3. *Department of Entomology, Soils and Plant Sci., Clemson University, Clemson, SC 29634, USA*

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 *Eco*R1 (New England Biolabs #R0101S) and *Mse*I (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. *Mse*I and *Eco*RI 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 *Mse*I adaptor, 1 µL of 5 µmol/L *Eco*RI adaptor, 0.25 µL *Mse*I, 0.25 µL *Eco*RI, 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 $MgCl_2$, 1 µmol/L dNTPs, 2.75 µmol/L *Eco*RI preselective primer (E: 5′-GTAGAC TGCGTACCAATTC-3′), 2.75 µmol/L *Mse*I 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, and 72°C for 2 min, and then a final extension of 10 min at 72°C. The preselective PCR products were diluted 10-fold for use in the selective PCR. An aliquot of the pre-selective amplification reaction was then used in the selective amplification step with primers from the AFLP Selective Amplification Module (Applied Biosystems P/N 4303051). The selective amplification product was amplified using five primer pairs: FAM-*Eco*RI-AC and *Mse*I-CAA, FAM-*Eco*RI-TC and *Mse*I-CAA, FAM-*Eco*RI-TG and *Mse*I-CAA, FAM-*Eco*RI-AC and *Mse*I-CAC and FAM-*Eco*RI-AC and *Mse*I-CAG. The selective PCR included an initial denaturation of 94°C for 3 min, which was followed by 10 cycles (each) of 94°C for 20 s, 66°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, *Eco*RI-AC and *Mse*I-CAC primer pairs generated the largest number of AFLP isolate-specific markers (63 markers) while the least number (39 markers) was generated by *Eco*RI-TC and *Mse*I-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 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

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. *Eco*RI-AC and *Mse*I-CAC primer pairs generated the largest number of AFLP isolate-specific markers (63 markers) while the least number (39 markers) was generated by *Eco*RI-TC and *Mse*I-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

Ustilaginomycetes. Mycologia, 92: 510-521.

Baldwin, B. G., M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell and M. J. Donoghue (1995). The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard., 82: 247-277.

Borst, A., B. Theelen, E. Reinders, T. Boekhout, A. C. Fluit and P. H. M. Savelkoul (2003). Use of amplified fragment length polymorphism analysis to identify medically important *Candida* spp., including *C. dubliniensis*. J. Clin. Microbiol., 41: 1357-1362.

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

Ceresini, P. C., H. D. Shew, R. Vilgalys and M. A. Cubeta (2002). Genetic diversity of *Rhizoctonia solani* AG-3 from potato and tobacco North Carolina. Mycologia, 94: 437-449.

Collado-Romero, M., J. Mercado-Blanco, C. Olivares-Garcia and R. M. Jimenez-Diaz (2008). Phylogenetic analysis of *Verticillium dahliae* vegetative compatibility groups. Phytopathology, 98: 1019-1028.

de Barros Lopes, M., S. Rainieri, P. A. Henschke and P. Langridge (1999). AFLP fingerprinting for analysis of yeast genetic variation. Int. J. Syst. Bacteriol., 49: 915-924.

de la Cerda, K. A., G. W. Douhan and F. P. Wong (2007). Discovery and characterization of *Waitea circinata* var. *circinata* affecting annual bluegrass from the Western United States. Plant Disease, 91: 791-797.

Doignon-Bourcier, F., A. Willems, R. Coopman, G. Laguerre, M. Gillis and P. de Lajudie (2000). Genotypic characterization of *Bradyrhizobium* strains nodulating small Senegalese legumes by 16S-23S rRNA intergenic gene spacers and amplified fragment length polymorphism fingerprint analyses. Appl. Environ. Microbiol., 66: 3987-3997.

Erper, I., G. H. Karaca, M. Turkkan and I. Ozkoc (2006). Characterization and pathogenicity of *Rhizoctonia* spp. from onion in amasya. J. Phytopathol, 154: 75-79.

Gunnell, P. S. (1986). Characterization of the teleomorphs of *Rhizoctonia oryzae-sativae*, *Rhizoctonia oryzae*, and *Rhizoctonia zeae*, and the effect of cultural practices on aggregate sheath spot of rice, caused by *R. oryzaesativae*. PhD. Thesis. University of California, Davis.

Hsiang, T. and J. D. Dean (2001). DNA sequencing for anastomosis grouping of *Rhizoctonia solani* isolates from *Poa annua*. Int. Turfgrass Soc. Res., 9: 674-678.

Hynes, S. S., O. Chaudhry, M. A. Providenti and M. L. Smith (2006). Development of AFLP-derived, functionally specific markers for environmental persistence studies of fungal strains. Can. J. Microbiol., 52: 451-461.

Klaassen, C. H. W., H. A. van Haren and A. M. Horrevorts (2002). Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. J. Clin. Microbiol., 40: 101-104.

Kuznia, R. A. and C. E. Windels (1994). *Rhizoctonia zeae* pathogenic to spring wheat and sugarbeet seedlings. Phytopathology, 84: 1159.

Lee, C. Z., G. Y. Liou and G. F. Yuan (2004). Comparison of *Aspergillus flavus* and *Aspergillus oryzae* by amplified fragment length polymorphism. Bot. Bull. Acad. Sin., 45: 61-68.

Leiner, R. H. and D. E. Carling (1994). Characterization of *Waitea circinata* (*Rhizoctonia*) isolated from agricultural soils in Alaska. Plant Disease, 78: 385-388.

Lucchini, V. (2003). AFLP: a useful tool for biodiversity conservation and management. Comptes Rendus Biologies., 326 (Supplement 1): 43-48.

Martin, S. B. and L. T. Lucas (1983). Pathogenicity of *Rhizoctonia zeae* on tall fescue and other turfgrasses. Plant Disease, 67: 676-678.

Mougel, C., J. Thioulouse, G. Perriere and X. Nesme (2002). A mathematical method for determining genome divergence and species delineation using AFLP. Int. J. Syst. Evol. Microbiol., 52: 573-586.

Mueller, U. G. and L. L. Wolfenbarger (1999). AFLP genotyping and fingerprinting. Trends Ecol. Evol., 14: 389-394.

Mueller, U. G., S. E. Lipari and M. G. Milgroom (1996). Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutes*. Mol. Ecol., 5:119-122.

Ogoshi, A., R. J. Cook and E. N. Bassett (1990). *Rhizoctonia* species and anastomosis groups causing root rot of wheat and barley in the Pacific Northwest. Phytopathology, 80: 784-788.

Oniki, M., A. Ogoshi, T. Araki, R. Sakai and S. Tanaka (1985). The perfect state of *Rhizoctonia oryzae* and *R.*

zeae and anastomosis groups of *Waitea circinata*. Trans. Mycol. Soc. Jpn., 26: 189-198.

Rademaker, J. L. W., B. Hoste, F. J. Louws, K. Kersters, J. Swings, L. Vauterin, P. Vauterin and F. J. de Bruijn (2000). Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. Int. J. Syst. Evol. Microbiol., 50: 665-677.

Ridout, C. J. and P. Donini (1999). Use of AFLP in cereals research. Trends Plant. Sci., 4: 76-79.

Riesberg, L. H. and D. E. Soltis (1991). Phylogenetic consequences of cytoplasmic gene flow in plants. Evol. Trends Plant, 5: 65-84.

Savelkoul, P. H. M., H. J. M. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J. L. W. Rademaker, L. Schouls and J. A. Lenstra (1999). Amplified-fragment length polymorphism analysis: the state of an art. J. Clin. Microbiol., 37: 3083-3091.

Smiley, R. W., P. H. Dernoeden and B. B. Clarke (1992). Compendium of Turfgrass Diseases. American Phytopathological Society, St. Paul., MN.

Sumner, D. R. and Bell D. K. (1982). Root diseases induced in corn by *Rhizoctonia solani* and *Rhizoctonia zeae*. Phytopathology, 72: 86-91.

Swofford, D. L. (2005). PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0b10 for Windows. Sinauer Associates, Sunderland, Massachusetts.

Toda, T., T. Hayakawa, J. M. Mghalu, S. Yagushi and M. Hyakumachi (2007). A new *Rhizoctonia* sp. closely related to *Waitea circinata* causes a new disease of creeping bentgrass. J. Gen. Plant Pathol., 73: 379-387.

Toda, T., T. Mushika, T. Hayakawa, A. Tanaka, T. Tani and M. Hyakumashi (2005). Brown ring patch: a new disease on bentgrass caused by *Waitea circinata* var *circinata*. Plant Disease, 89: 536-542.

Tredway, L. P., J. F. White, B. S. Gaut, P. V. Reddy, M. D. Richardson and B. B. Clarke (1999). Phylogenetic relationships within and between *Epichloe* and *Neotyphodium endophytes* as estimated by AFLP markers and rDNA sequences. Mycol. Res., 103: 1593-1603.

van den Braak, N., G. Simons, R. Gorkink, M. Reijans, K. Eadie, K. Kremers, D. van Soolingen, P. Savelkoul, H. Verbrugh and A. van Belkum (2004). A new high-throughput AFLP approach for identification of new genetic polymorphism in the genome of the clonal microorganism *Mycobacte-*

rium tuberculosis. J. Microbiol., Methods, 56: 49-62.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, 23: 4407-4414.

Table (1): Survey of AFLP fragments in 15 *Waite circinata* var. *zeae* isolates using five primer pairs combinations.

Fragment	*Eco*RI-AC and *Mse*I-CAA	*Eco*RI-TC and *Mse*I-CAA	*Eco*RI-TG and *Mse*I-CAA	*Eco*RI-AC and *Mse*I-CAC	*Eco*RI-AC and *Mse*I-CAG	Total
Total	114	100	154	197	156	721
Monomorphic	0.0	2	2	2	0.0	6
Polymorphic	114	98	152	195	156	715
Isolate-specific marker	58	39	54	63	46	260

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

Primer pairs	Isolate	Isolate-specific marker	Molecular size (bp)
*Eco*RI-AC and *Mse*I-CAA	Isolate 2	7	84, 137, 206, 264, 346, 455, 459
	Isolate 3	3	54, 116, 374
	Isolate 5	0	0
	Isolate 13	3	236, 275, 392
	Isolate 23	0	0
	Isolate 30	7	100, 285, 361, 409, 411, 425, 497
	Isolate 45	15	88, 131, 145, 160, 178, 182, 190, 199, 227, 264, 312, 313, 430, 476, 477
	Isolate 46	2	154, 432
	Isolate 49	2	119, 467
	Isolate 51	0	0
	Isolate 60	0	0
	Isolate 68	1	107
	Isolate 77	17	63, 79, 102, 122, 204, 219, 234, 246, 250, 255, 309, 310, 325, 343, 415, 421, 464
	Isolate 79	1	403
	Isolate 85	0	0
*Eco*RI-TC and *Mse*I-CAA	Isolate 2	3	72, 163, 224
	Isolate 3	1	227
	Isolate 5	0	0
	Isolate 13	4	206, 344, 425, 465
	Isolate 23	1	401
	Isolate 30	7	80, 90, 193, 234, 237, 259, 493
	Isolate 45	9	91, 131, 174, 196, 311, 314, 359, 413, 418
	Isolate 46	3	62, 139, 308
	Isolate 49	2	71, 121
	Isolate 51	2	249, 269
	Isolate 60	0	0
	Isolate 68	1	68
	Isolate 77	3	137, 171, 338
	Isolate 79	0	0
	Isolate 85	3	114, 150, 262
*Eco*RI-TG and *Mse*I-CAA	Isolate 2	2	279, 306
	Isolate 3	2	225, 247
	Isolate 5	0	0
	Isolate 13	5	78, 237, 333, 430, 465
	Isolate 23	5	134, 166, 182, 257, 477
	Isolate 30	7	120, 132, 180, 232, 470, 497, 498
	Isolate 45	16	63, 69, 112, 129, 142, 152, 228, 280, 290, 304, 373, 401, 404, 428, 440, 492
	Isolate 46	1	155
	Isolate 49	1	363
	Isolate 51	2	236, 243
	Isolate 60	2	234, 417
	Isolate 68	2	213, 220
	Isolate 77	5	139, 147, 198, 244, 474
	Isolate 79	2	171, 172
	Isolate 85	2	52, 288

Table (2): Cont'

*Eco*RI-AC and *Mse*I-CAC	Isolate 2	5	175, 222, 271, 312, 345
	Isolate 3	1	295
	Isolate 5	1	137
	Isolate 13	3	301, 302, 322
	Isolate 23	3	112, 323, 489
	Isolate 30	7	82, 119, 316, 336, 387, 454, 488
	Isolate 45	14	58, 94, 95, 116, 124, 188, 280, 282, 283, 292, 342, 400, 461, 481
	Isolate 46	3	144, 382, 427
	Isolate 49	4	234, 287, 419, 479
	Isolate 51	2	84, 215
	Isolate 60	1	134
	Isolate 68	1	495
	Isolate 77	13	110, 126, 132, 140, 182, 308, 318, 325, 350, 351, 392, 444, 483
	Isolate 79	3	202, 245, 265
	Isolate 85	2	198, 276
*Eco*RI-AC and *Mse*I-CAG	Isolate 2	5	175, 201, 241, 346, 370
	Isolate 3	0	0
	Isolate 5	1	249
	Isolate 13	1	469
	Isolate 23	1	368
	Isolate 30	8	112, 124, 139, 140, 151, 161, 299, 339
	Isolate 45	11	99, 180, 189, 197, 202, 211, 245, 265, 298, 314, 446
	Isolate 46	1	437
	Isolate 49	0	0
	Isolate 51	0	0
	Isolate 60	3	145, 247, 376
	Isolate 68	0	0
	Isolate 77	9	206, 290, 327, 350, 363, 404, 419, 489, 499
	Isolate 79	5	108, 156, 191, 239, 344
	Isolate 85	1	106

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.

Isolates	2	3	5	13	23	30	45	46	49	51	60	68	77	79	85
2	100														
3	31	100													
5	30	2	100												
13	28	6	4	100											
23	11	19	26	25	100										
30	10	18	26	19	11	100									
45	16	24	26	26	20	19	100								
46	4	34	26	31	11	13	15	100							
49	6	26	26	24	13	16	19	5	100						
51	7	27	26	24	11	13	19	10	9	100					
60	3	21	26	19	13	9	19	7	9	7	100				
68	4	22	26	15	11	10	19	8	7	8	5	100			
77	15	20	26	22	15	20	29	20	19	11	19	20	100		
79	7	22	26	19	11	10	19	8	9	5	7	10	15	100	
85	8	27	26	20	12	18	20	8	5	6	8	8	20	6	100

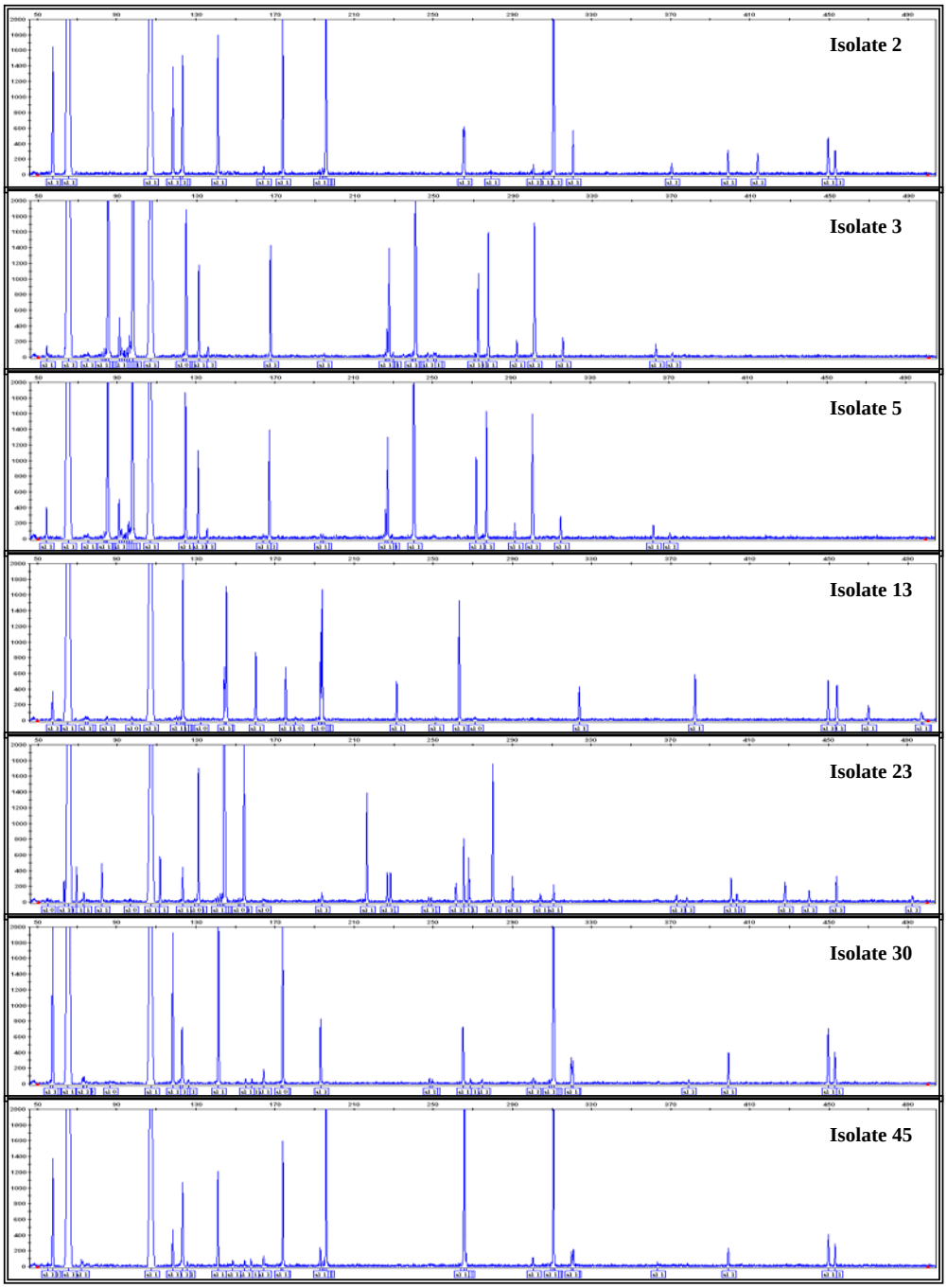
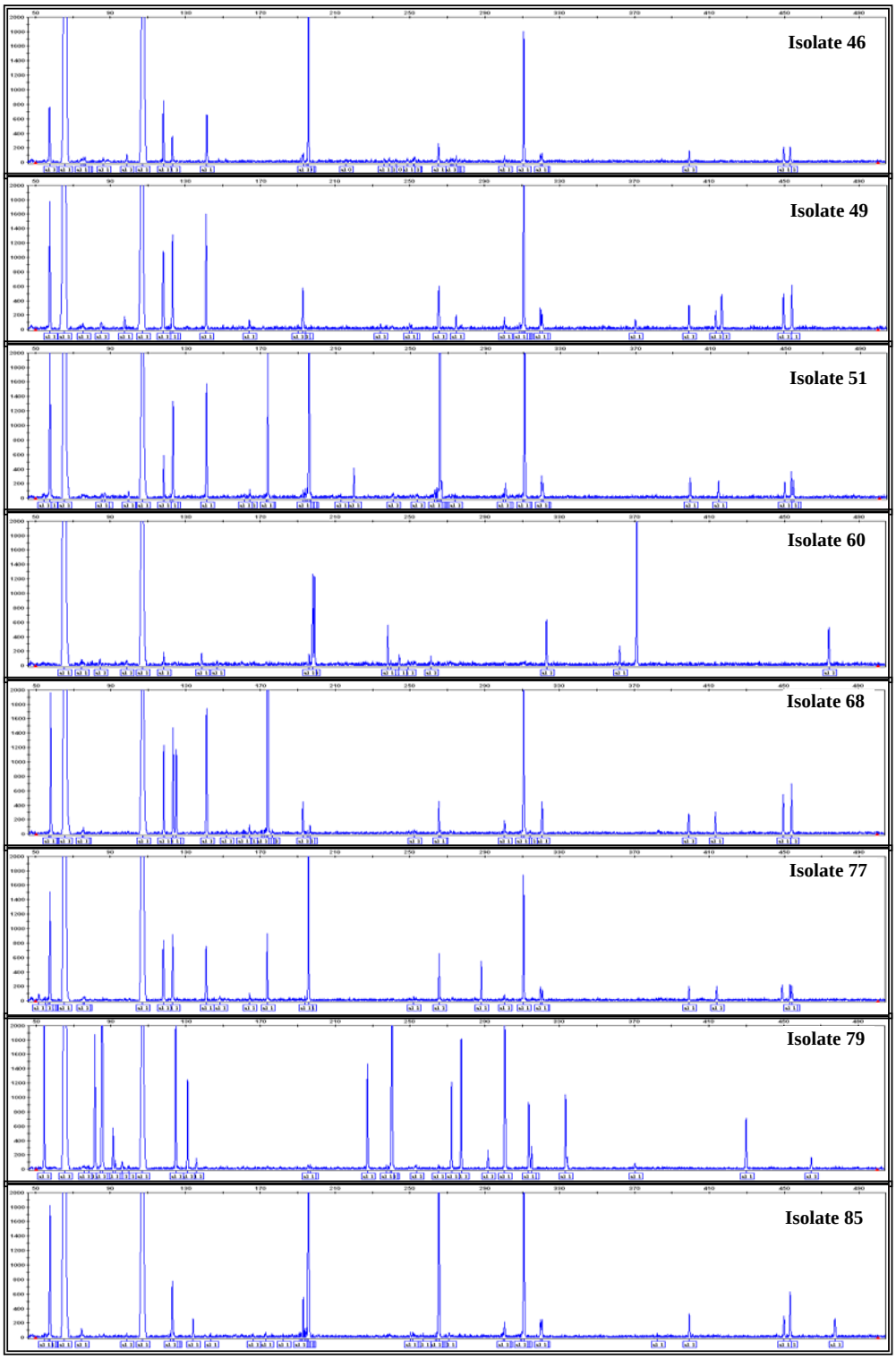


Fig. (1): Electropherograms of 15 *Waitea circinata* var. *zeae* isolates AFLP profiles using *Eco*RI-TG and *Mse*I-CAA primer pairs.

Fig. (1): Cont'


Isolate 46
Isolate 49
Isolate 51
Isolate 60
Isolate 68
Isolate 77
Isolate 79
Isolate 85

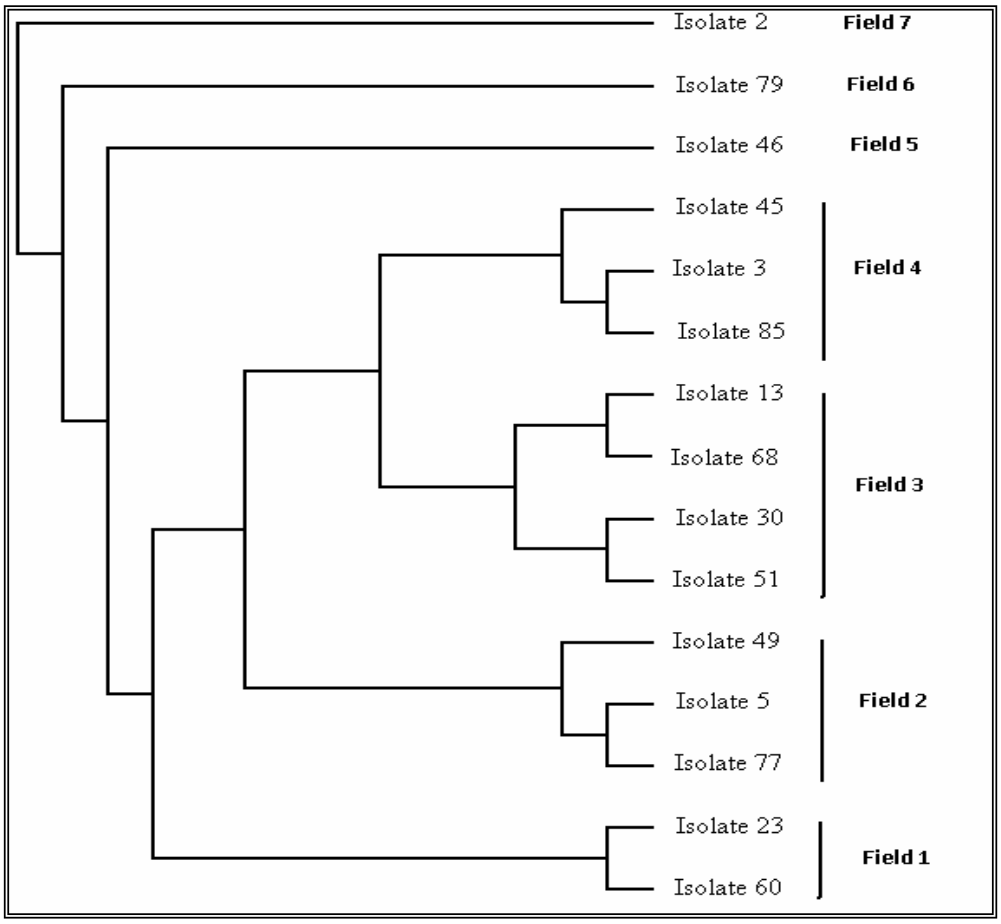


Fig. (2): Neighbour-joining tree derived from AFLP patterns of all 15 *Waitea circinata* var. *zeae* isolates using five primer pairs combinations.