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Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of *Fusarium* species, the causal agent associated with mulberry root rot disease in Northeastern Thailand

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Thirty isolates of *Fusarium* species associated with mulberry root rot disease were characterized by using morphological, pathogenicity and molecular characteristics. All isolates were identified into eleven species including *F. solani*, *F. oxysporum*, *F. phaseoli*, *F. culmorum*, *F. moniliforme*, *F. graminearum*, *F.scirpi*, *F. anthophilum*, *F. dlamini*, *F. dimerum* and *F. beomiforme*. They were tested for their pathogenicity on mulberry cutting in plastic glass, 30 days after inoculation showed that *F. solani* was the most pathogenic, causing root rot at 90% disease incidence. The present study showed that all isolates can recovered from fibrous root, lateral root and basal of the cutting trees at 38 – 96%, 22 - 94 % and 16.04 – 48.02%, respectively. This has been the first pathogenic proven since 1954 that *F. solani* is the causal agent of mulberry root rot. Amplified Ribosomal DNA Restriction Analysis (ARDRA) was used to analyze the ITS1 - 5.8S rDNA - ITS2 region, amplified with primer ITS1 and ITS4. The amplified products (590 bp) were digested with the restriction enzymes *Alu*I, *Eco*RI, *Hae*III, *Hinf*I and *Msp*I. The dendrograms generated by combination all five enzymes enabled the differentiation of *F. solani* the causal agent of mulberry root rot from other species.

Key words : mulberry root rot, *Fusarium*, ARDRA

Introduction

Mulberry belongs to the genus *Morus* of the family *Moraceae*, is widely distributed around the worlds (Yoshida *et al*., 2002). Root rot is a major disease

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of mulberry in the North-east of Thailand. The disease can be found wherever the plant is grown and direct losses on the crop due to the disease can reach 100% (Sanoamuang and Saksirirat, 1984). The disease was first report in 1954, since then its etiology has been extensively studied (Sanoamuang and Saksirirat, 1984; Yamakawa *et al*., 1991). Symptoms of the disease appear as sudden leaf withering starting from the bottom of the branch upwards, followed by defoliation in isolated patches in the plantation. The bark of the roots peels off easily with a particular odor and the plants suddenly die. Through irrigation water, soil and garden implements, the disease spreads quickly to nearby mulberry trees both in the same row and the adjacent rows where the root zones are in overlapping proximity (Sanoamuang and Saksirirat, 1984). Sanoamuang *et al*. (1986) reported that *Fusarium solani* f.sp. *mori* was always found associated with the mulberry roots in both healthy and diseased trees collected from all sericulture experiment stations in the northeast region of Thailand. Consistently, Philip *et al.* (1997) suggested that the occurrence of *F. solani*, *F. oxysporum*, *Lasiodiplodia*, *Rhizoctonia solani* and *Rosellinia necatrix*, as serious pathogens causing root rot in mulberry infected. In addition, Kazempour and Kamran (2005) reported that severe root rot of mulberry trees were associated with *F. solani*, *F. oxysporum*, *L. theobromae*, *R. solani* and *R. necatrix.*

Fusarium species are ubiquitous soil-borne fungi commonly found in all natural and cultivated soil and in the rhizosphere of plants. They are responsible for severe diseases on a variety of important crops (Nelson *et al*., 1994). For example, Kolattukudy and Gamble (1995) have reported that at least 111 plant species belonging to 87 different genera of agronomic, horticultural or forestry importance were susceptible of *F. solani* attacks. Summerell *et al.* (2003) reported that a utilitarian approach to *Fusarium* identification including morphological, biological and phylogenetic species concepts. In general, morphological species concepts are based on the similarity of observable morphological characters, e.g. spore size and shape. Biological species concepts required that members of the same species are sexually cross-fertile and that the progeny of the crosses are both viable and fertile. Finally, DNA sequences have been used to generate characters that usually are treated cladistically to form phylogenies.

The ribosomal DNA (rDNA) regions have often been chosen for taxonomic and phylogenetic studies, because sequence data are available and contain both variable and conserved regions, despite the discrimination at the genus, species or intraspecific levels. The rDNA repeat includes both highly conserved genes and more variable spacer regions. The spacer regions, including the internal transcribed spacer (ITS) and the intergenic spacer (IGS).

The rDNA region analyses have been used successfully to differentiate *Fusarium* species (Nagarajan *et al*., 2004). Amplified rDNA restriction analysis (ARDRA) has been used for the identification of *Fusarium* species. Restriction analysis with *Hae*III of an amplified 18S rDNA fragment was also shown useful to differentiate *F. solani* f.sp. *phaseoli* from *F. solani* f.sp. *glycines* (Oliveira *et al*., 2002). The potential and power of ARDRA to identify member of *Fusarium* species was already put forward, but was never worked in detail for a large member of species. In this study, we investigated the value of ARDRA to identify *Fusarium* species, the causal agent associated with mulberry root rot disease in Northeastern Thailand.

Materials and methods

Origin, identification and morphological characterization of isolates

Isolates associated with mulberry root rot were collected, together with information regarding geographical origin (Table 1). All isolates were identified after Booth (1977); Nelson *et al*. (1994); Seifert (1996) and Aoki *et al.* (2003). After the isolates were grown on PDA for 10 days, colony growth and spore morphology and size were characterized, with measurements taken from 50 microconidia and 50 macroconidia per isolate.

Pathogenicity test on mulberry cutting

Pathogenicity tests were conducted using Koch's Postulate to confirm the *Fusarium* species as the causal agent of root rot of mulberry. The plants used were 4 months old and showed no disease symptoms.

The *Fusarium* isolates used were the representative of isolates, which was successfully isolated and identified from root of mulberry. To obtain inoculum, *Fusarium* spp. was grown on pieces of mulberry twig that cut into 0.5 mm and autoclave twice. The plugs of *Fusarium* spp. were added into the pieces and incubated at 28 ºC for 14 days.

Mulberry cutting cultivar Noi were washed and surface sterilized before grown in 10 cm diameter plastic glasses using sterile peat moss and *Fusarium* spp. inoculum (10^7 cfu/ml) were placed on the bottom before planting. The experiment was conducted in a completely Randomized Block Design (RCBD) with five replicates for each *Fusarium* species and control. Disease incidence were observed.

Table 1. Origin and identity of *Fusarium* isolates from mulberry root rot used in this study.

Isolates	Species assignment	Location
KE01-2-3	*F. solani*	BanKee, Cheangyuen district, Mahasarakham province
KE05-1-2	*F. solani*	BanKee, Cheangyuen district, Mahasarakham province
KE05-1-3	*F. solani*	BanKee, Cheangyuen district, Mahasarakham province
KKU01-1-1	*F. solani*	Khon-Kaen University
KKU03-1-3	*F. solani*	Khon-Kaen University
KW01-1-2	*F. solani*	BanKwao-Yai, Kantarawichai district, Mahasarakham province
ROI-ET-03-1-5	*F. solani*	BanWan-fai, Art-Sahmaht district, Roi-et province
SIC01-1-1	*F. solani*	BanWan-fai, Art-Sahmaht district, Roi-et province
KE03-2-4	*F. oxysporum*	Silk innovation center, Mahasarakham university
		BanKee, Cheangyuen district, Mahasarakham province
KW-SH-05	*F. oxysporum*	BanKwao-Yai, Kantarawichai district, Mahasarakham province
SIC-SD-02	*F. oxysporum*	
KE04-2-3	*F. phaseoli*	Silk innovation center, Mahasarakham university
ROI-ET-07-1-4	*F. phaseoli*	BanKee, Cheangyuen district, Mahasarakham province
KE02-2-1	*F. culmorum*	BanWan-fai, Art-Sahmaht district, Roi-et province
ROT-ET-10-1-4	*F. culmorum*	BanKee, Cheangyuen district, Mahasarakham province
ROI-ET-07-1-1	*F. moniliforme*	BanWan-fai, Art-Sahmaht district, Roi-et province
ROI-ET-SD-04	*F. moniliforme*	BanWan-fai, Art-Sahmaht district, Roi-et province
ROI-ET-SD-07	*F. moniliforme*	BanWan-fai, Art-Sahmaht district, Roi-et province
KKU03-2-1	*F. dlamini*	BanWan-fai, Art-Sahmaht district, Roi-et province
KKU09-2-1	*F. dlamini*	Khon-Kaen University
KW04-2-2	*F. dlamini*	Khon-Kaen University
KKU06-2-1	*F. dimearum*	BanKwao-Yai, Kantarawichai district, Mahasarakham province
KKU10-2-3	*F. graminearum*	
KKU-SD-02	*F. anthophilum*	Khon-Kaen University
KW-SH-10	*F. scirpi*	Khon-Kaen University
ROI-ET-10-1-7	*F. beomiform*	Khon-Kaen University
		BanKwao-Yai, Kantarawichai district, Mahasarakham province
		BanWan-fai, Art-Sahmaht district, Roi-et province

Molecular characterization

Genomic DNA extraction

Isolates were grown in potato dextrose broth (PDB) for 7 days at 28 °C on a shaker at 120 rpm. Mycelia were harvested by filtration through filter paper (Whatman No.1) and freeze-dried for 24 hr. Total DNA extraction was prepared by a modified method of Zhang *et al.* (2005). Approximately 50 mg of freeze-dried mycelium was ground in liquid nitrogen. The resulting powder was suspended in 0.7 ml of lysis buffer (400 mM Tris-HCl (pH 8.0), 60 mM EDTA (pH 8.0), 150 mM NaCl, 1% SDS and 3 µl of 2 – mercaptoethanol. Samples were incubated at 65°C for 30 min and adding 150 µl of potassium acetate (pH 4.8) mixed gently.

This mixture was centrifuged for 5 min at 12,000 rpm. The aqueous phase was collected, extracted with an equal volume of chloroform: isoamyl alcohol (24:1) mixed gently and centrifuged for 5 min at 12,000 rpm, the aqueous phase was collected and DNA was then precipitated by adding with an equal volume

of cold isopropanol, incubated at -20°C for 30 min and pelleted by centrifugation for 5 min at 12,000 rpm.

The DNA pellet was washed with 70% cold ethanol, air dried and resuspended in 50 µl of TE buffer (10 mM Tris-Hcl, 0.1 mM EDTA, pH 8.0). RNA was degraded by treatment with RNase (50 µg/ml) for 30 min at 37°C (Lee *et al*., 2000). DNA concentration and purity were measured using a spectrophotometer at 260 nm and 280 nm.

Amplification of the ITS regions (PCR-ITS)

Genomic DNA from all isolates was amplified using universal primers from fungi designed to amplify ITS regions (White *et al*., 1990). ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGAT ATGC-3′) were the primers used. Amplification was performed in 50 µl of reaction mixture containing 100 ng genomic DNA, 2.0 µM each primers, 0.25 unit *Taq* polymerase, 2.5 mM $MgCl_2$, 0.2 mM each dNTP (dATP, dCTP, dGTP and dTTP), 10 µl of 5X PCR buffer, sterile distilled water to a final volume of 50 µl. Amplifications were performed in a Gradient DNA Thermal Cycler programmed for the following parameter: 95°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final incubation at 72°C for 5 min. Amplification products (5 µl of a 50 µl reaction) were electrophoresed in 2% agarose gels with TBE running buffer, stained with ethidium bromide and either scanned into a computer imaging file or photographed (Sambrook *et al.,* 1989).

Restriction digestion of the amplified ITS regions

The PCR amplification products were digested with 5 different restriction enzymes (Fermentas): *Alu*I, *Eco*RI, *Hae*III, *Hinf*I and *Msp*I. The reactions were prepared following the manufacturer's instructions individuzlly for each enzyme. The digeation reactions took place in water-bath accordingly the instruction of each enzyme. The products (5 µl of a 50 µl reaction) were electrophoresed in 2% agarose gels with TBE running buffer, stained with ethidium bromide and either scanned into a computer imaging file or photographed (Sambrook *et al*., 1989).

The computer program NTSYS-PC (version 2.1) was used to analyze the relationship among the *Fusarium* spp. studied. Similarity matrix was generated using the program Qualitative. This matrix was subjected to the unweighted pair group method with arithmetical averages (UPGMA). Cluster analysis was

performed on the similarity matrix with the SAHN program using UPGMA and dendrogram was produced with the TREE program.

Results

Identification and morphological characterization of isolates

The 26 isolates compared in this study showed considerable variability in appearance and mycelial color on PDA. Four isolates produced sparse aerial mycelia, while 12 showed abundant cottony mycelia. Colony color varied from white to being to purple. Macroconidial and microconidial morphology also showed a considerable degree of variation among the isolates studied. Macroconidial size varied from 24.25 to 37.25 µm. Four distinct maccrocoidial shapes were observed (shapes A,B,C and E according to Seifert, 1996), and four distinct microconidial shapes were observed (shapes, A, B, C and D according to Seifert, 1996) (Table 2).

Pathogenicity test on mulberry cutting

Thirty days after inoculation, disease incidences were observed. All eleven of *Fusarium* species were pathogenic on mulberry cutting. *F. solani* shows the highest infection with a disease incidences of 90.0%, significantly from others species. There were no significant difference of recovery from root let between *F. solani*, *F. anthophilum* and *F. dlamini* showed that 94 – 96% recovery. There were no significant difference of recovery from lateral root between *F. solani*, *F. phaseoli*, *F. anthophilum*, *F. dlamini*, *F. dimerum* and *F. beomiforme* showed that 80 – 94% recovery. There were no significant difference of recovery from basal between *F. solani*, *F. phaseoli*, *F. culmorum*, *F. anthophilum*, *F. dlamini* and *F. beomiforme* showed that 36-48.02% recovery (Table 3).

Molecular characterization

Amplification of the ITS regions (PCR-ITS)

Amplification of fungal genomic DNA, using universal primer ITS1, ITS4 produced a single amplification product of approximately 590 bp in all fungi isolates tested.

Restriction digestion of the amplified ITS regions

All PCR products were digested with each of the five restriction enzymes. Depending on the restriction enzymes and isolates, the PCR products were digested into two to six fragments. Restriction fragments less than 50 bp were not taken into consideration because they were not clearly resolved by electrophoresis in 2% agarose gels. Results of digestion with *Alu*I, *Eco*RI, *Hae*III, *Hinf*I and *Msp*I are shown in Fig. 1. Digestion of the PCR products with *Alu*I showed four banding patterns, giving rise to fragment of 450, 400, 120 and 50 bp. Digestion with *Eco*RI resulted in two banding patterns with one cutting site in this region of DNA. Digestion with *Hae*III showed four banding patterns, giving rise to fragment of 300, 250, 100 and 80 bp. Digestion with *Hinf*I showed five banding patterns, giving rise to fragment of 300, 290, 280, 260 and 50 bp. Digestion with *Msp*I showed six banding patterns, giving rise to fragment of 380, 280, 210, 180, 100 and 50 bp.

We scored 15 bands produced by 5 restriction enzymes that showed polymorphism. The cluster analysis separated the 30 isolates into three major groups (82% similarity). The first group included 12 isolates of *F. solani* (100% similarity), 3 isolates of *F. moniliforme* and 1 isolate of *F. dimerum*. The second group included three isolates each of *F. oxysporum* and *F. dlamini*, one isolate each of *F. anthophilum*, *F. beomiforme* and *F. scirpi*. The third group included two isolates each of *F. phaseoli* and *F. culmorum*, and one isolate of *F. graminearum* (Fig. 2).

Discussion

The genus *Fusarium* contains many species of fungi that are commonly found in soil and of organic substrata and is widely distributed throughout the world (Burgess, 1981). Some species are capable of causing wilts, crown rots, root rots, or fruit rots. Others are opportunists because they colonize plant tissue after some type of stress debilitates the plant. Interestingly, many species of *Fusarium* that abound in the soil are not capable of causing disease. In this study we can isolate eleven species including *F. solani*, *F. oxysporum*, *F. phaseoli*, *F. culmorum*, *F. moniliforme*, *F. graminearum*, *F.scirpi*, *F. anthophilum*, *F. dlamini*, *F. dimerum* and *F. beomiforme*. From pathogenicity test, *F. solani* was the most pathogenic of mulberry root rot. Consistently, The Hindu (2004) reported as *F. solani* and *F. oxysporum* were as the major disease causing pathogens. In addition, symptoms of mulberry root rot disease were similar to soybean sudden death syndrome (SDS) caused by *F. solani* (Yang and Lundeen, 1997). Therefore, it is possible that *F. solani* is the causal agent of mulberry root rot.

Table 3. Disease incidence and percentage *Fusarium* species recovery from mulberry root and basal of trees, 30 days after inoculation.

Species	% Disease incidence	% Recovery		
		Root let	Lateral root	Basal
1. *Fusarium solani*	90.0 [a 1/]	94.0 [a]	88.0 [ab]	40.0 [abc]
2. *Fusarium oxysporum*	20.8 [cd]	66.0 [cd]	72.0 [bc]	24.0 [bcde]
3. *Fusarium phaseoli*	40.6 [b]	78.0 [e]	82.0 [ab]	36.0 [abcd]
4. *Fusarium culmorum*	44.4 [bc]	38.0 [e]	48.2 [d]	44.0 [ab]
5. *Fusarium moniliforme*	26.6 [cd]	42.0 [e]	22.2 [e]	16.04 [fg]
6. *Fusarium graminearum*	34.6 [bc]	56.0 [de]	58.2 [cd]	24.02 [def]
7. *Fusarium scirpi*	20.8 [cd]	52.0 [de]	60.0 [cd]	30.02 [bcde]
8. *Fusarium anthophilum*	40.6 [bc]	96.0 [a]	94.0 [a]	40.02 [abc]
9. *Fusarium dlamini*	1.0 [d]	94.0 [a]	92.0 [a]	48.02 [a]
10. *Fusarium dimerum*	20.8 [cd]	88.0 [ab]	80.0 [ab]	22.0 [de]
11. *Fusarium beomiforme*	20.8 [cd]	68.0 [bcd]	82.0 [ab]	46.0 [ab]
12. Control	1.0 [d]	4.6 [f]	2.8 [f]	0.00 [f]

[1/]Percentage of disease incidence and percentage recovery determined 30 days after inoculation. Means (n=5) followed by the same letter are not significantly different ($P = 0.05$) based on a protected test of least significant difference .

Hunt *et al.* (2004) studied about fungal community structure and diversity in two types of agricultural grassland soil were investigated by amplified 18S ribosomal DNA restriction analysis (ARDRA) and 18S ribosomal DNA sequence analysis. Two primer sets were used in combination to amplify approximately 550 bp of rDNA from three major fungal groups. 18S ARDRA was used to analyze 170 rDNA clines, and three diversity indices were calculated. The results suggest that 18S rDNA based approaches are a useful tool for initial screening of fungal communities and that they represent a more comprehensive picture of the community than plate culturing. In this research, we have shown that the ARDRA technique based on the combination of five restriction enzymes is reliable and valuable for phylogenetic and taxonomic studies. Here, we present our finding with the use of ARDRA for the identification of *Fusarium* species. The method consists of amplification of the 18S rDNA and sequent restriction digestion of the amplicon. The restriction patterns obtained with combination of these patterns into a restriction profile was shown to enable identification of *Fusarium* species. Consistently, Oliveira *et al.* (2002) used molecular methods to characterize diversity among pathogenic and non pathogenic isolates of *Fusarium* spp. and determine genetic relationships among *formae speciales* and suggested that the ARDRA technique, using the enzyme *Hae*III is a promising marker to differentiate the *formae speciales phaseoli* from *glycines* within the *F. solani* complex. This studies showed that ARDRA technique was useful for identifying the *Fusarium* species.

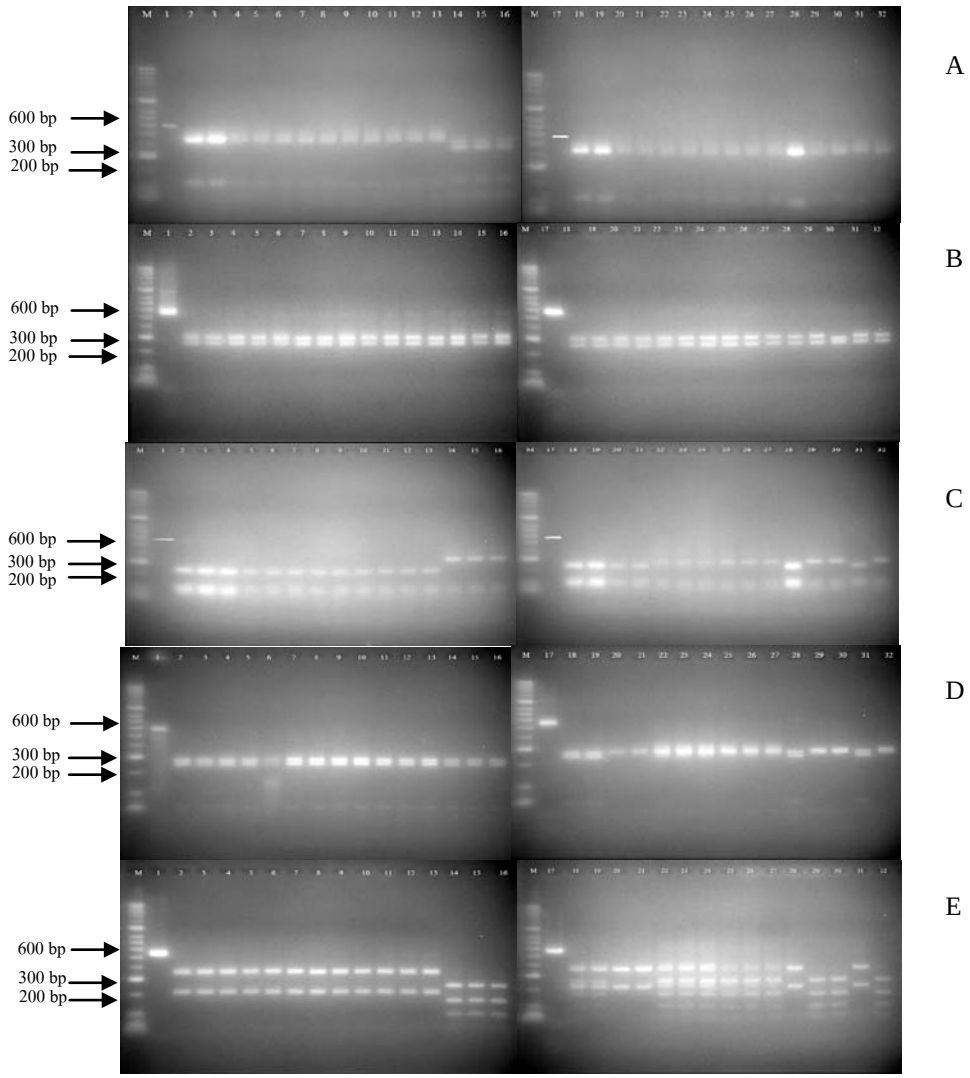

Fig. 1. Agarose gels showing restriction patterns of PCR amplified rDNA digested with *Alu*I (A), *Eco*RI (B), *Hae*III (C), *Hinf*I (D) and *Msp*I (E). M : DNA size marker of HyperLadder II, 1, 17 : amplification of the ITS region, 2-13: *F. solani*, 14-16: *F. oxysporum*, 18-19: *F. phaseoli*, 20-22: *F. culmorum*, 23-24 : *F. moniliforme*, 25-27 : *F. dlamini*, 28: *F. graminearum*, 29: *F. beomiform*, 30: *F. scirpi*, 31: *F. dimerum*, 32: *F. anthophilum*.

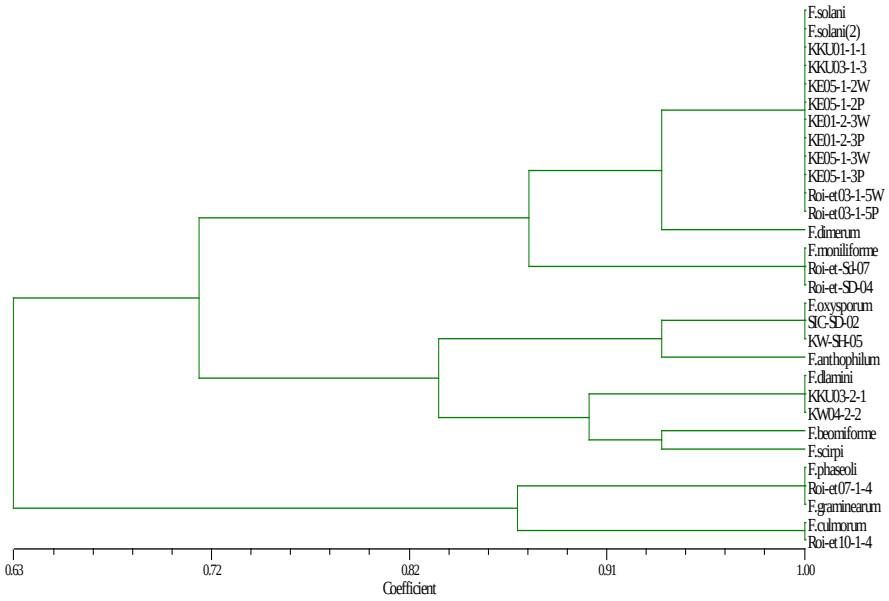

Fig. 2. UPGMA dendrogram showing relationships among the 30 isolates of *Fusarium* based on restriction site data.

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Table 2. Cultural and morphological characteristics of *Fusarium* spp. isolates from mulberry root rot used in this study.

Isolates	Species	Conidial mass colony	Colony reverse color on PDA	Aerial mycelia on PDA	Colony diameter (cm)[a]	Shape	Macroconidia[b]	Septa	Apical cell shape	Basal cell shape	Chlamy-dospores	Micro-conidia[b]
KE01-2-3	*F. solani*	pink	red	moderate	4.0	E	24.70x4.95	3	blunt	blunt	+	A, C
KE05-1-2	*F. solani*	pink	pink	moderate	4.2	E	29.68x5.00	3	blunt	blunt	+	B, C
KE05-1-3	*F. solani*	white	red	sparse	3.5	E	26.00x4.93	3	blunt	blunt	+	C
KKU01-1-1	*F. solani*	cream	orange brown	abundant	7.9	E	24.83x4.93	3	blunt	blunt	+	B, C
KKU03-1-3	*F. solani*	pink	light brown	sparse	8.4	E	26.17x4.83	3	blunt	blunt	+	B, C
KW01-1-2	*F. solani*	pink	red	abundant	3.8	E	24.25x4.95	3	blunt	blunt	+	A, C
ROI-ET-03-1-5	*F. solani*	pink	pink	moderate	4.1	C	28.68x5.00	3	blunt	blunt	+	B, C
SIC01-1-1	*F. solani*	pink	dark red	abundant	3.4	E	28.33x5.00	2	blunt	blunt	+	C
KE03-2-4	*F. oxysporum*	purple	purple	moderate	5.6	C	30.83x4.95	3	hooked	foot shaped	+	A, B
KW-SH-05	*F. oxysporum*	purple	purple	abundant	6.5	C	29.68x4.93	3	hooked	foot shaped	+	B
SIC-SD-02	*F. oxysporum*	purple	purple	moderate	5.7	C	31.17x4.95	3	hooked	foot shaped	+	A, B
KE04-2-3	*F. phaseoli*	white	orange brown	abundant	5.0	E	25.75x4.83	2	hooked	foot shaped	+	B, C
ROI-ET-07-1-4	*F. phaseoli*	white	orange	abundant	4.8	A	34.33x4.95	3	hooked	foot shaped	+	B, C
KE02-2-1	*F. culmorum*	pink	light brown	moderate	3.9	C	34.58x4.80	3	hooked	blunt	+	A
ROT-ET-10-1-4	*F. culmorum*	cream	light brown	abundant	5.5	A	34.93x5.00	3	blunt conical	foot shape	+	_
ROI-ET-07-1-1	*F. moniliforme*	cream	dark brown	abundant	6.8	B	33.93x5.00	5	conical	blunt	+	B, C
ROI-ET-SD-04	*F. moniliforme*	cream	cream	abundant	6.5	B	34.33x4.93	5	conical	blunt	+	B, C
ROI-ET-SD-07	*F. moniliforme*	cream	cream	abundant	7.0	B	33.75x4.95	5	blunt	blunt	+	B, C
KKU03-2-1	*F. dlamini*	purple	light brown	sparse	8.1	C	29.68x4.33	3	blunt	blunt	+	B
KKU09-2-1	*F. dlamini*	cream	light brown	moderate	5.1	C	28.33x4.83	3	blunt	blunt	+	B
KW04-2-2	*F. dlamini*	purple	orange brown	moderate	9.0	C	28.68x4.33	3	blunt	blunt	+	B
KKU06-2-1	*F. dimearum*	cream	light brown	sparse	8.5	E	25.33x5.04	2	blunt	foot shaped	+	B
KKU10-2-3	*F. graminearum*	cream	cream	abundant	4.6	C	30.83x4.33	5	conical	foot shaped	-	-
KKU-SD-02	*F. anthophilum*	purple	light	moderate	7.9	A, D	37.25x4.33	3	hooked	foot shape	-	C, E
KW-SH-10	*F. scirpi*	orange	orange red	moderate	8.3	A, D	35.45x4.83	5	_	foot shape	+	A
ROI-ET-10-1-7	*F. beomiform*	white	white	abundant	6.5	_	_	_		_	+	C, D, E

[a] After 10 days growth on PDA.
[b] Morphological characteristics according to Seifert (1996).