# Genetic Charaterization of Isolates of *Fusarium oxysporum* f. sp. *lycopersici* as Determined by ARDRA and RAPD Analysis

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## Abstract

Twelve isolates of *Fusarium oxysporum* f. sp. *lycopersici* from Thailand, the causal organism of Fusarium wilt of tomato, were examined for ITS1-5.8S-ITS2 region by amplified ribosomal DNA restriction analysis (ARDRA) and genomic DNA by random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). All isolates could be distinguished from each other by ARDRA analysis, and clustered into four groups (A, B, C and D) using unweighted pair group method with arithmetic average (UPGMA) analysis. The isolates were grouped into two groups (E and F) by UPGMA analysis based on RAPD-PCR data. There was similarity between the UPGMA dendrograms of ARDRA and RAPD-PCR analysis, all of the pathogenic isolates were in group A and E, respectively. Pathogenic isolates have been clearly distinguished from non-pathogenic ones by RAPD-PCR. This study showed that, at the DNA level pathogenic isolates are more closely related, whereas non-pathogenic isolates are more diverse.

**Keywords**: ARDRA, *Fusarium oxysporum* f. sp. *lycopersici,* RAPD-PCR

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# Introduction

*Fuasrium oxysporum* Schlechtend. Fr. f. sp. *lycopersici* (Fol) (Sacc.) W. C. Snyder & H. N. Hansen, is an economically important soilborne plant pathogen. The fungus is imperfect fungi in the class Hyphomycetes. It causes Fusarium wilt specifically in tomato. Fol penetrates directly through the roots of tomato plant and can infect the host plant at all growth stages. The fungus spreads throughout the vascular bundles and inhibits water flow causing wilting, finally death of plant. This disease was first described by G.E. Masse in England in 1895. It is a devastating disease that occurs in major tomato - growing regions of the world, and has been reported in at least 32 countries (Cai et al., 2003). In severe cases it may cause up to 80 % loss in tomato production (Malhotra et al., 1993).

Forma specialis (f. sp.) is a very useful grouping of this particular plant pathogen. The forma specialis, *lycopersici*, was classified on the high specific host species, and its only host is tomato (*Lycopersicon esculentum*. Mill.). Three physiological races (1, 2 and 3) of Fol have been reported. Their races are defined by a characteristic pattern of vilulence on differential tomato cultivars that contain a single resistance gene (McGrath et al., 1987; Stall, 1961). Identification of *Fusarium oxysporum* to forma speciales and race levels based on host - pathogen interaction (i.e., virulence) is difficult, because there are many variables, such as, temperature, host age and method of inoculation (Correll, 1991, Banyatratchata et al., 2005). Studies on genetic relationship and phylogeny have been useful for characterization of fungal diversity. Vegetative compatibility and molecular marker analysis have helped to elucidate the evolutionary relationship among races within several formae speciales of *Fusarium oxysporum* (Kistler, 1987; Manicom et al., 1987; Kistler et al., 1991; Woo et al., 1996 and Dobinson, K, 2000). Fol was characterized by pathogenicity, race and vegetative compatibility group (VCG) in California after Fol race 3 was first observed (Cai et al., 2003). Three races of *F. oxysporum* f. sp. *lycopersici* (race 1, 2 and 3) are associated with four VCGs, 0030 to 0033. Race 1 and race 2 occur in VCGs 0030 to 0032 and race 3 in VCGs 0030 and 0033 (Elias and Schneider, 1991). In addition, RFLP analysis of total genomic DNA was used to examine the relationship between race 1 and race 2 isolates of *F. oxysporum* f. sp. *lycopersici* representing several VCGs. The result was concluded that different races may have evolved from within several of the VCGs within this forma specialis (Elias et al., 1993). Several researchers reported on the use of a random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique for rapid identification within several forma speciales of *F. oxysporum* (Grajal-Martin et al.,1993;

Assigbetse et al., 1994; Yli-Mattila et al., 1996, Chiocchetti et al., 1999 and Jimenez-Gasco, 2003)

In Thailand, little is known about genetic complexity of Fol populations. Race 1 and race 2 were first identified by pathogenic reaction on standard differential host (Bunyatratchata et al., 2005). Subsequently, molecular biology method for race identification of Fol was developed by RAPD analysis (Chayphad et al., 2005). The study of Fol should do continuously in order to detect and monitor. The objective of this study was to determine the genetic characterization within the 18SrDNA gene of isolates of *F. oxysporum* f. sp. *lycopercisi* by means of Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Random Amplified Polymorphic DNA (RAPD) polymerase chain reaction. DNA polymorphism were used to evaluate levels of phylogenetic relationships among isolates of *F. oxysporum* f. sp. *lycopercisi.*

## Materials and Methods

**Fungal isolates** : Seven field isolates of Fol ( KK1, KK2, KK3,KK4, KK5, KK6 and KS) were recovered from wilt symptomatic tomatoes in northeast region of Thailand. Three isolates (CM1, CM2 and CM3) were obtained from Chiang Mai University and one isolate (PP1) was taken from Department of Agriculture, Ministry of Agriculture and Cooperatives; Thailand. The standard cultures of Fol (Fol 004, race 1; Fol 007, race 2 and Fol 029, race 3) were kindly provided by Prof. Dr. B. J. C. Cornelissen ( Amsterdam, the Netherlands). Fol 030 (race 3) was kindly provided by Prof. Dr. S.C. Bost (Tennesee, United State). All isolates were tested for pathogenicity on Sida variety previously by Bunyatratchata et al. (2005).

### DNA extraction

Total genomic DNA was extracted from fresh mycelium using the CTAB and a modified procedure from Doyle and Doyle (1987). The fungal culture were grown in potato dextrose broth (PDB) for 7 days at room temperature. About 0.2 gram of fresh mycelium was ground in 700 µl of CTAB buffer (20 mM EDTA, 100 mM Tris – base, 1.4 M NaCl, 2 % w/v CTAB, 1.0 % PVP – 40) mixed with 1 µl of β – mercaptoethanol. Ground tissue and buffer were mixed well and incubated at 60° C for 1 h. The homogenate was extracted with equal volumn of a mixture of chloroform and isoamyl alcohol (24 : 1 vol / vol). The extraction liquid was slowly mixed by inversion and centrifuged at 13,000 rpm, 10° C for 10 min. The supernatant was collected and extracted two more times with chloroform and isoamyl alcohol. Total DNA in the aqueous phase was precipitated by 280 µl of cooled isopropanol, incubated at –20° C for 20 min and centrifuged for 10 min. The precipitated DNA was washed three times in 76 % ethyl alcohol with 10 mM

ammonium acetate. The pellet was air – dried for 2 h and resuspended in 40 µl TE buffer (pH 8.0) and incubated at room temperature until fully dissolved. The DNA was treated with RNase for 30 min at 37 0C. DNA was stored at –20 °C until used. The concentration and purity of DNA were estimated by measuring absorbances at $A_{260}$ and $A_{280}$ nm. DNA sample were diluted to a working concentration of 20 ng/µl.

## PCR amplification of ITS1–5.8S–ITS2 of rRNA gene

The internal transcribed spacer (ITS) region (ITS1–5.8S–ITS2) of rDNA was amplified by PCR using the primers ITS18S (5′–TGAACCTGCGGAAGGATCATT–3′ and ITS28S (5′–CCTCCGCTTATTGATATGCTTAA–3′) as described by Paavanen – huhtala et al. (1999). Polymerase chain reactions (PCRs) were carried out in 25 µl volume. Each reaction tube contained 20 mM Tris – HCl (pH 8.8), 10 mM $(NH_4)_2 SO_4$, 0.6 mM of each dNTPs, 4 mM $MgCl_2$, 40 ng of primers (Protigo LLC, USA), 2 – 3 units of Tsp (*Thermus* species) DNA polymerase and 6 ng of genomic DNA. Amplification was performed in a programmable thermal cycler (Hybaid, USA) under the following conditions: initial denaturation at 90° C for 90s, 30 cycles of 92° C for 30s, 55° C for 60s, 68 ° C for 30s followed by a final extension at 68 ° C for 3 min. A negative control (no DNA template) was always included. Amplification product was subjected to 1.5 % agarose gel electrophoresis. The sizes of the amplified ITS bands were estimated by comparison with a 100 bp DNA ladder (New England, BioLabs Inc., N3231S).

## Amplified Ribosomal DNA Restriction (ARDRA) analysis

Amplified rDNA was digested with seven restriction enzymes (*Hind* III, *EcoR* I, *BamH* I, *Hpa* I, *Pst* I, *Taq* I and *Msp* I) according to the manufacturer's recommendation. The digested DNA were separated by electrophoresis on 1.5 % agarose in Tris–borate–EDTA (TBE) buffer for 90 min at 50 V in a gel electrophoresis apparatus (GelMate 2000, TOYOBO), stained with ethidium bromide and visualized with UV transilluminator (Vilber Lourmat, Serial No. VOZ 6090).

## Random Amplified Polymorphic DNA (RAPD) Analysis

Genomic DNA from 15 isolates of Fol was amplified by RAPD–PCR using 12 random primers of E kit from Operon Technology : E01(5′–CCCAAGGTCC–3′), E02 (5′–GGTGCGGGAA–3′), E03 (5′–CCAGATGCAC–3′), E04 (5′–GTGACATGCC–3′), E05(5′–TCAGGGAGGT–3′), E06 (5′–AAGACCCCTC–3′), E07 (5′–AGATGCAGCC–3′), E08(5′–TCACCACGGT–3′), E09 (5′–CTTCACCCGA–3′), E10 (5′–CACCAGGTGA–3′), E11(5′–GAGTCTCAGG–3′), E12 (5′–TTATCGCCCC–3′).

Each 25 µl of RAPD reaction contained 0.2 unit of *Taq* polymerase, 1 x reaction buffer, 0.25 mM of each dNTPs, 8 µM of primer, 2 mM of $MgCl_2$ and 20 ng of genomic DNA. The reactions were amplified in a thermocycler (Hybaid, USA). The amplification conditions consisted of initial denaturation at 94 ° C for 1 min, followed by 39 cycles of 94 ° C for 1 min, 36 ° C for 1 min and 72 ° C for 1 min. The final extension step was at 72 ° C for 2 min and then the reactions were held at 4 ° C until gel electrophoresis. Amplified products were separated on a 2 % agarose gel in 0.5x TBE buffer at 100 v for 1 h. The sizes of separated bands including DNA molecular standards and negative controls (PCR reagent without target DNA) were detemined in each gel run. Amplification was repeated twice for each sample. Gels were stained with ethidium bromide to visualize the DNA.

### Data analysis

The DNA bands produced by the ARDRA and the RAPD-PCR of each isolate were scored based on their presence (1) or absence (0) of bands. Cluster analysis was done by the unweighted paired group method using arithmetic averages (UPGMA). All calculations were performed with NTSYSpc version 2.

## Result

### PCR amplification of ribosomal DNA regions

The ITS regions (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) of each isolate of Fol were amplified by PCR using the fungal primers (ITS18S/ITS28S). The size of amplified rDNA fragments was approximately 550 bp in all Fol isolates studied (Fig. 1).

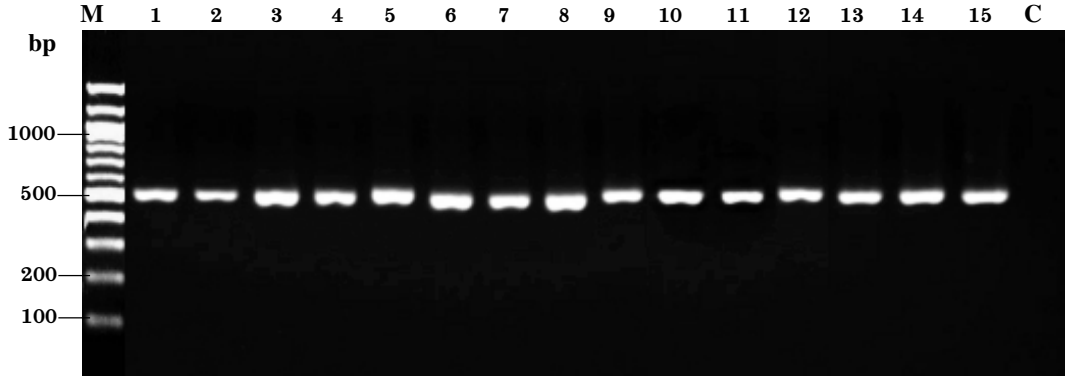


**Fig. 1 Amplified rDNA fragments (ITS1 - 5.8S - ITS2 region) of *Fusarium oxysporum* f.sp. *lycopersici* (Fol) determined by primers ITS 18S and ITS 28S. Size of the molecular weight fragments was approximately 550 bp.**

**lane M = 100 bp DNA ladder**

**lane 1-15 = Fol isolates KK1, KK2, KK3, KK4, KK5, KK6, KS, CM1, CM2, CM3, PP, Fol 004 (race 1) , Fol 007 (race 2), Fol 029 (race 3) and Fol 030 (race 3), respectively.**

**lane C = Control (PCR reagent without target DNA)**

## ARDRA analysis

Amplified rDNA from 15 isolates was digested with seven restriction enzymes (*Hind* III, *EcoR* I, *BamH* I, *Hpa* I, *Pst* I, *Taq* I and *Msp* I). DNA fingerprint after digestion with *Msp* I, *Taq* I and *Pst* I produced five (457, 387, 263, 216 and 173 bp; Fig. 2), three (252, 214, and 168 bp; result not shown) and two (539 and 424 bp; result not shown) distinct fragments respectively. Digestion with *Hind* III, *EcoR* I, *BamH* I and *Hpa* I, did not produce polymorphism within this ITS1-5.8S-ITS2 of rRNA gene (result not shown). The RFLP profile was scored based on the presence (1) or absence (0) of each DNA fragment. Similarity indices were used for cluster analysis. The dendrogram (Fig. 3) consists of four groups (A, B, C and D), group A contained 10 isolates of pathogenic strains (KK1, KK2, KK3, KK4, KK6, CM2, Fol 004, Fol 007, Fol 029 and Fol 030) and 2 isolates of non-pathogenic strains (CM3 and PP), each of groups B, C and D contained one isolate of of non - pathogenic strain (KS, CM1and KK5, respectively).

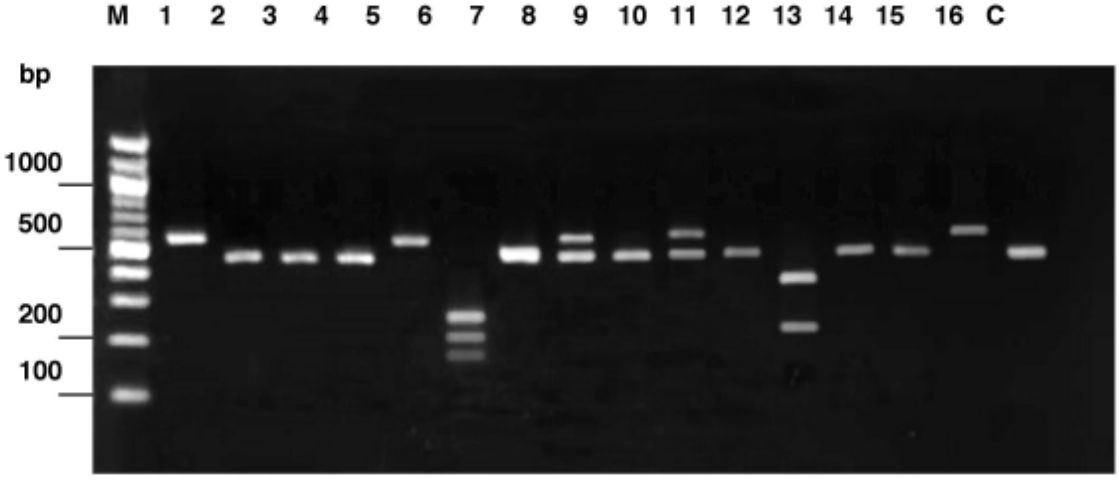


**Fig. 2 Restriction fragment length polymorphism patterns in ribosomal DNA gene of isolates of *Fusarium oxysporum* f. sp. *lycopersici*. The ITS1 - 5.8S - ITS2 region of rDNA was amplified with primer ITS 28S / ITS 18S and digested with *Msp* I. (lane M is 100 bp DNA ladder, lane 1 is uncut rDNA, lane 2 - 16 are *Fusarium oxysporum* f.sp. *lycopersici* ; 2 : KK1, 3 : KK2, 4 : KK3, 5 : PP1, 6 : KK5, 7 : KK6, 8 : CM3, 9 : KK4, 10 : CM1, 11 : CM2, 12 : KS, 13 : Fol 004 (race1), 14 : Fol 007 (race 2), 15 : Fol 029 (race 3), 16 : Fol 030 (race 3) and C is negative control, respectively.)**

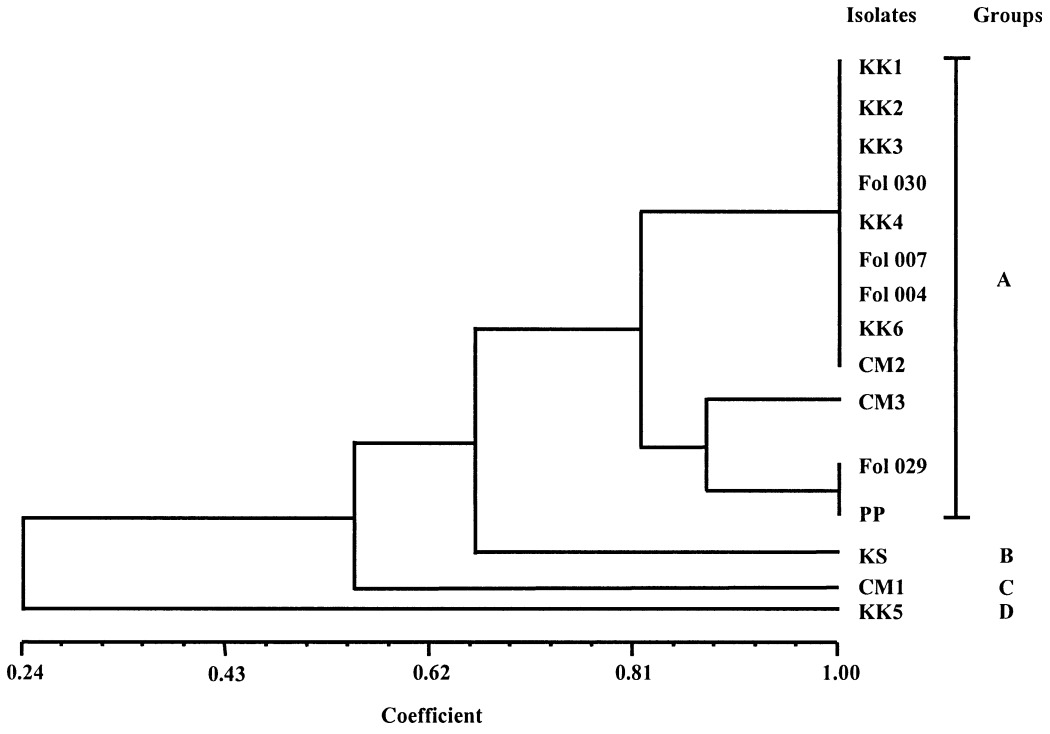


**Fig. 3 Cluster analysis of *Fusarium oxysporum* f. sp. *lycopersici* by Amplified Ribosomal DNA Restriction Analysis (ARDRA). Restriction fragment length polymorphism was compared by the unweighted pair grouping method with arithmetic averages (UPGMA)**

## RAPD – PCR analysis

Twelve RAPD primers (OPE1 – OPE12) were used in this experiment. From RAPD profile illustrated that each primer generated different banding patterns, for instance, OPE01, OPE04, OPE05, OPE06, OPE08, OPE09, OPE10 and OPE11 produced six, eight, nine, six, five, seven, three and five DNA patterns, respectively (see example in Fig. 4). Race 1 (Fol 004, lane 1) and race 2 (Fol 007, lane 2) showed the same DNA patterns consisted of ten amplified DNA fragments of approximately 3000, 2250, 1750, 1400, 1200, 1050, 800, 700, 610, and 375 bp, respectively by OPE05 (Fig. 4A). The banding patterns characteristic of race 1 and race 2 differed from race 3 (Fol 029, lane 3) which created nine DNA bands, approximately 3000, 2250, 1750, 1400, 1200, 990, 700, 610 and 375 bp, respectively. Seven banding patterns were formed by OPE 09 (Fig. 4B). Three race of Fol which attained from the Netherlands such as race 1 (Fol 004, lane 1), race 2 (Fol 007, lane 2) and race 3 (Fol 029, lane 3), exhibited the like DNA patterns in OPE09 that comprised of six DNA bands about 1750, 1400, 950, 800,580 and 500 bp but varied from race 3 (Fol 030), obtained from United States produced eight DNA bands, approximately 1750, 1200, 1000,

950, 800, 580, 500 and 300 bp, respectively.

Nine primers (OPE01,OPE04, OPE05, OPE06, OPE08, OPE09, OPE10, OPE11 and OPE12) were selected for cluster analysis, an example of typical RAPD pattern with two primers (OPE05 and OPE09) is shown Fig. 4. The sizes of the scored bands ranged from 300 bp to 3000 bp. Fifteen isolates of Fol could be separated into 2 clusters (E and F) by using a UPGMA analysis of RAPD-PCR patterns (Fig. 5). Group E contained 10 isolates of pathogenic strains (KK1, KK2, KK3, KK4, KK6, CM2, Fol 004, Fol 007, Fol 029 and Fol 030) and one isolate of non - pathogenic strain CM3, and group F contained four isolates of non - pathogenic strain (KK5, KS, CM1 and PP).

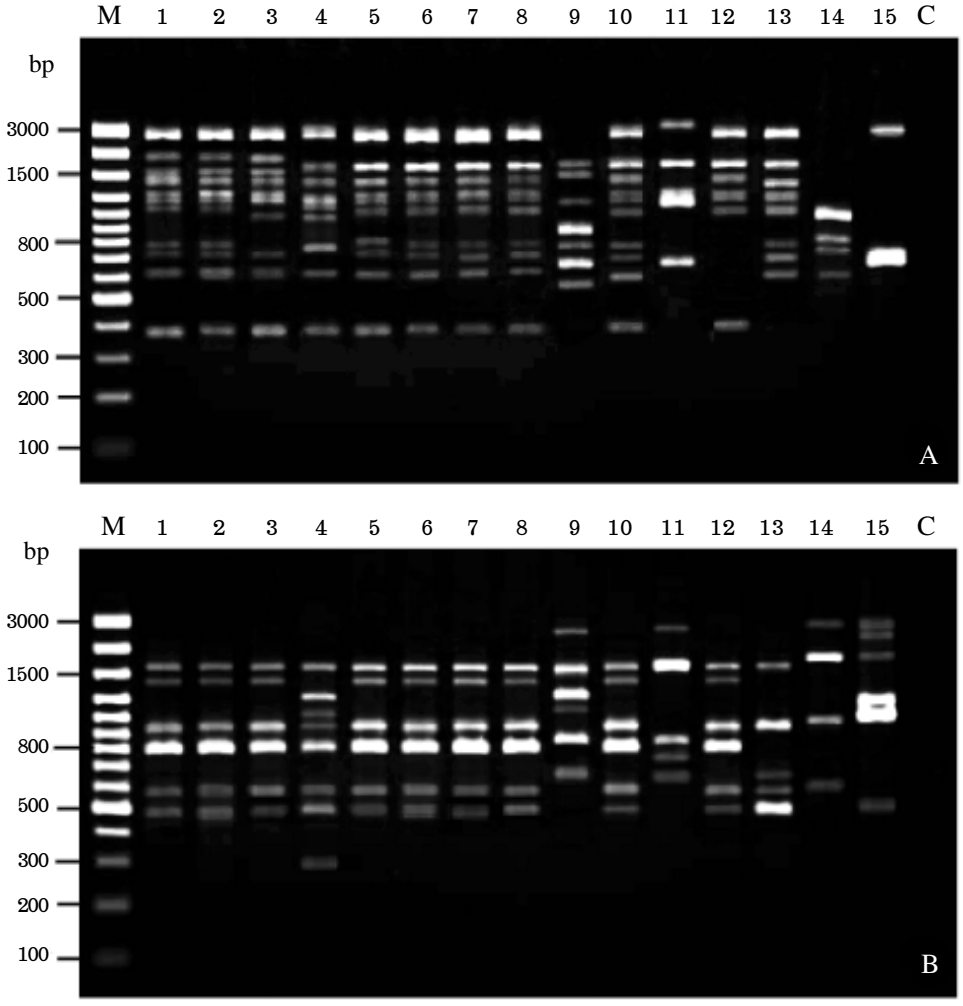


**Fig. 4 DNA fingerprints of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) amplified with primer OPE05 (A) and OPE09 (B) (lane M is 100 bp DNA ladder, lane 1 - 15 are Fol 004 (race1), Fol 007 (race 2), Fol 029 (race 3), Fol 030 (race 3), KK1, KK2, KK3, KK4, KK5, KK6, CM1,CM2, CM3, KS and PP respectively and lane C is negative control**

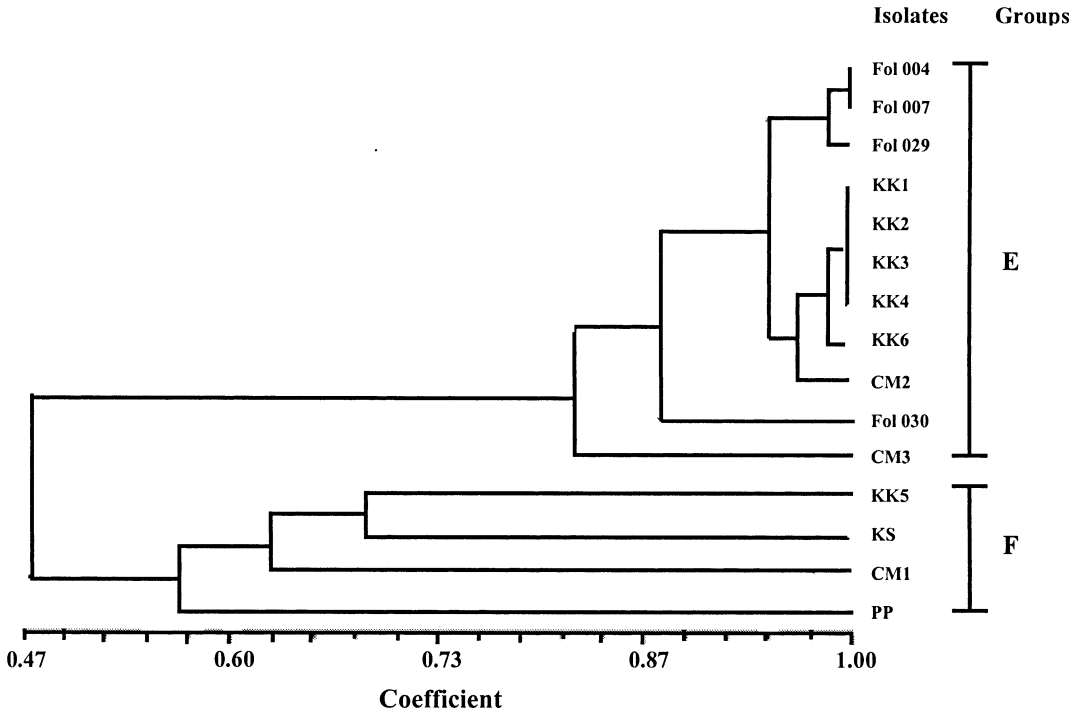


**Fig. 5 Cluster analysis of *Fusarium oxysporum* f. sp. *lycopersici* by random amplified polymorphic DNA (RAPD). DNA fingerprint, obtained from primer kit E (OPE01, OPE04, OPE05, OPE06, OPE08, OPE09, OPE10, OPE11and OPE12), was compared by the unweighted pair grouping method with arithmetic averages (UPGMA).**

## Discussion

Analysis of rDNA gene is very useful in identification and taxonomic studies. The isolates of *Fusarium oxysporum* f. sp. *lycopercisi* (Fol) were characterized using ITS1-5.8S-ITS2 rDNA regions with a view to determine the relationship between a genetic characterization and pathogenicity. ITS1-5.8S-ITS2 regions was amplified using ITS 28S and ITS 18S primers, the amplified product was digested using restriction enzymes (*Hind* III, *EcoR* I, *BamH* I, *Hpa* I, *PST* I, *Taq* I and *Msp* I) and then separated on 1.5 % agarose gel. DNA polymorphisms were shown in the restriction digests with enzymes *Msp* I, *Pst* I and *Taq* I. The banding patterns were analysed, at least four groups (A, B, C and D) were identified (Fig 3). Group A contained twelve isolates, ten isolates were pathogenic strains and two isolates were non-pathogenic strains. The result indicated that pathogenic strains and some of non-pathogenic strains were in the same group (Group A). This study corresponded to several studies which showed that non-pathogenic isolates could be vegetatively

compatible with pathogenic isolates (Cai et al., 2003, Distler, 1997). This could indicate that mutation in pathogenic isolates may occur. Group B, C and D each contained an isolate of non pathogenic strain. The results showed that non – pathogenic isolates of Fol had higher diversity in the population. The results from this study corresponded with Bao et al, 2002 which showed that, at the DNA level, pathogenic isolates are more closely related, whereas non – pathogenic isolates are more diverse. The RAPD-PCR analysis showed that fifteen isolates of Fol were devided into two groups (E and F; Fig. 5). There were similarity between the UPGMA dendrograms of ARDRA and RAPD-PCR analysis. All of ten pathogenic isolates were closely related and clustered in group A (based on ARDRA) and E (based on RAPD-PCR) and the other groups (B, C, D based on ARDRA and F based on RAPD – PCR) each contained non-pathogenic isolates. We concluded that *Fusarium oxysporum* isolates could be separated into two clusters, pathogenic isolates and non-pathogenic isolates. This is in agreement with Assigbetse et al., 1994, Woo et al., 1996 and Chayphad et al., 2005. They found that pathogenic and non-pathogenic *Fusarium oxysporum* isolates have been clearly distinguished from each other by RAPD – PCR.

The genetic diversity among the fifteen isolates was supported by the variable banding patterns observed in RAPD profiles. An example of nine and seven banding patterns operated by two primers, OPE05 and OPE09, is shown in Fig. 4A and 4B, respectively. There is a problem in the interpretation of DNA polymorphism since some DNA bands were not clearly. The results found that race 1 (Fol 004, lane 1) and race 2 (Fol 007, lane 2) created the same banding patterns showed ten DNA bands varied from race 3 (Fol 029, lane 3) which exhibited nine DNA bands by OPE05 (Fig. 4A). We suggested that OPE05 gave a polymorphism between race 1, race 2 and race 3; amplified DNA fragments of approximately 1050 and 800 bp present in race 1 race 2 was not found in race 3; reciprocally, a fragment of approximately 990 bp was present in race 3 but not in race 1 and race 2. Also, Chyphad et al. (2005) reported that race identification by RAPD analysis, the primers OPE03 and OPH20 can be used to discriminate clearly between Fol race 3 and group of race 1 and race 2. The primer OPE03 created six DNA bands, approximately 3000, 1800, 1400, 1300, 900, 350 bp in Fol 004 (race 1) and Fol 007 (race 2) differed from Fol 029 (race 3) produced five DNA bands, approximately 3000, 1800, 1400, 1300 and 900 bp. The OPH20 created five DNA bands, approximately 1500, 1100, 800, 540 and 400 bp in Fol 004 (race 1) and Fol 007 (race 2) varied from race 3 produced four DNA bands, approximately 1500, 1100, 800 and 400 bp. They indicated that OPE03 and OPH20 gave polymorphisms between race

1, race 2 and race 3; both of amplified DNA fragments of approximately 350 bp (from OPE03) and 540 bp (from OPH20) present in Fol race 1, race 2 but was not found in Fol race 3. These data, we concluded that RAPD analysis is an effective way to identify isolates of *F. oxysporum* f. sp. *lycopersici* race 3 and distinguished them from nonpathogenic isolates. In addition, at OPE09, Fol 029 from the Netherlands did not have DNA pattern like to Fol 030 from United States, although they belong to the same race (race 3) (Fig. 4B) suggested that there is genetic variation within this forma specialis. This is a correspondence with the results of Woo et al. (1996), reported that Fop1 (from South Carolina) and Fop11 (from Italy) were race 1 of *Fusarium oxysporum* f. sp. *phaseoli* but they had different RAPD patterns since these two isolates maybe have diverse genetic origins and their pathogenic similarity may have developed independently. Pathogenicity test is the only means of determining the pathological classification of fungal strains present in diseased plants. In future studies, molecular techniques should be used in providing markers for identification of race. These techniques can reduce the time and greenhouse space needed for pathological classification.

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