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DNA Sequencing and UP-PCR Characterization of *Fusarium oxysporum* Isolates from Three Cucurbit Species

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Abstract: *Fusarium oxysporum* is a main agriculture pathogen which is responsible for wilting in cucumber, muskmelon and watermelon. In order to study the genetic relationship and phylogeny of *Fusarium oxysporum*, 36 isolates were obtained from Liaoning province. The genetic relationship of *Fusarium* was determined by using EF-1α and β-tubulin DNA sequencing as well as a universally primed polymerase chain reaction (UP-PCR) analyses. The results show that all 36 isolates were identified as being infected with *F. oxysporum* by using the specific primers FOF1 and FOR1. Based on the combined sequence and UP-PCR data, the 36 isolates and GenBank isolates were classified into three major groups (Lineage1-3). These groups corresponded with the three clades of *F. oxysporum*. Inspection of the sequence data revealed Lineage 1 and Lineage 2 had similar sequences whereas Lineage 3 was significantly different from the other two. And this finding matched the results of UP-PCR analysis. However, the GenBank isolates were classified into Lineage 1 and none of the three techniques applied in this study provided host-related resolution for the pathotype isolates or the geographic origin of isolates. This study confirmed that phylogenetic groups have little connection with virulent groups.

Key words: Formae specialis, EF-1α, β-tubulin, plant pathogen

INTRODUCTION

Fusarium wilt is a worldwide soil-borne disease which caused by *Fusarium oxysporum* Schl. It is the most serious disease in watermelon, muskmelon, cucumber, wax gourd and other types of melon and has been the cause of substantial economic loss, as well as posing a grave threat to crop production (Leslie *et al*., 2001). In China, *Fusarium* wilt is wide-spread almost through the entire country. Annual loss is 10 to 30% by strain rate and even 100% in the most serious cases (Yangxiu and Guanlin, 2002). However, individual pathogenic isolates within the species have a limited host range and a high degree of host resistance. Eight formae speciales have been reported outside of China, whilst four formae speciales have been reported in China.

However, exceptions to these findings concerning formae specialis have been reported McMillan (1986) reported that isolates of *F. oxysporum,* which were obtained from wilted cucumber plants in the Bahamas, were also pathogenic to muskmelon and watermelon. Similar isolates that were found by Kim *et al*. (1993) which reported that some isolates of the formae specialis *niveum* infected some summer squash cultivars. Based on the cross-infectivity of these formae speciales, research about the genetic relationships within and among these formae specialis are needed.

DNA-based techniques have been used as the main method to study the genetic diversity and phylogeny of *Fusarium* species (Baixia *et al*., 2009; Zhang *et al*., 2010). These techniques are vital in the study of melon research, *F. oxysporum* genetic diversity, and the understanding of *Fusarium* wilt component characteristics and evolution. Over the past several years, genetic diversity in *F. oxysporum* has been examined by using various genetic markers, such as RFLP, RAPD, DNA fingerprinting (Benthy *et al*., 1998; Namiki *et al*., 1994) and rDNA sequences. Among these procedures, RFLP and RAPD analysis have the advantage of potentially detecting numerous polymorphisms at the DNA level. The UP-PCR (universally primed PCR) has been applied successfully for the identification of differences among populations of fungal pathogens, including *Fusarium*, *Trichoderma* and *Rhizoctonia solani* (Yli-Mattila *et al*., 2004; Lubeck and Poulsen, 2001; Bulat *et al*., 1998; Elad *et al*., 1983).

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The objective of this study was to examine the diversity of *F. oxysporum* strains isolated from cucumber, muskmelon and watermelon plants. Representing known phylogenetic groups previously characterized in this species complex and isolates from this study were compared. EF-1α, β-tubulin DNA sequence analyses and UP-PCR analyses were used to reveal the relationship among them (Abd-Elsalam *et al*., 2002).

MATERIALS AND METHODS

Isolates: These strains were obtained from plant tissues collected from various locations in China (Table 1). All isolates were single spores and maintained on potato dextrose agar. They were assayed by specific primers FOF1 and FOR1. Two relevant sequences of *Fusarium* sp. (ATCC16416, DQ452427) were also obtained from GenBank and estimated with all isolates (Namiki *et al*., 2001).

DNA extraction and Specific primers analyses: DNA was extracted from isolates using the CTAB method (Liu *et al*., 2000). All 36 isolates tested were identified using *F. oxysporum* specific primers FOF1: 5'-ACATACCACTTGTTGCCTCG-3'; FOR1: 5'-CGCCAATCAATTTGAGGAACG-3'. Two *F. moniliforme* strains and one *F. oxysporum* f.sp. *Lycopeici* were used in the procedure as negative and positive contrast samples (as having been identified by our laboratory). PCR reaction was performed in 20 μL volume of 10×buffer 2 μL, dNTP 0.2 μL (10 mM), Taq 0.2 μL (5U μL^{-1}), primers 1 μL (10 μmol), DNA 1 μL (25 ng) ddH_2o 14.4 μL. (All reagent purchased from Tiangen program, China) The PCR program was for 25 cycles after the first denaturation step at 9°C for 60 sec, the second denaturation step was 94°C for 60 sec, 58°C for 30 sec and, 72°C for 1 min followed by an extension step at 72°C for 7 min.

Sequence analyses: Fragments of the genes encoding EF-1α and β-tubulin were amplified by PCR using primer pairs EF1 (ATGGGTAAGGAAGACAAGAC) (O'Donnell *et al*., 2000) / EF2 (GGAAGTACCAGTGATC ATGTT) and T1 (AACATGCGTGAGATGTAAGT) (O'Donnell *et al*., 1998) / T22 (TCTGGATGTTGTTGGG AATCC). The PCR program ran for 35 cycles after the first denaturation step at 94°C for 2 min, the second denaturation step was 94°C for 1 min, the third at 53°C for 1 min and finally 74°C for 1 min followed by an extension step at 72°C for 4 min. Sequencing results of each sample of bi-directional sequence splicing were exported using SeqMan of DNAstar software. EF-1α and β-tubulin sequences of all the isolates tested and three Gene bank isolates were assembled and aligned using Clustalx 1.81. MEGA4.0 was used to estimate phylogenetic relationships and to test combinability of datasets. The aligned sequences were then corrected where necessary.

UP-PCR analysis: Thirteen UP primers were used in the present study: 0.3-1, 0.3-2, 3-1, 3-2, AA2M2, AS4, AS15, AS15inv, L15, L15/AS19, L21, L45, HE45 (Yli-Mattila *et al*., 2004). UP-PCR was performed in 20 μL volume of 10×buffer 2 μL,$MgCL_2$ 2 μL (25 mmol), dNTP 0.6 μL (10 mM), Taq 0.2 μl(5 U μL^{-1}), primers 1.5 μL (10 μmol), DNA 1 μL (25 ng) ddH_2o 12.7 μl. The cycling parameters were as follows: DNA denaturation at 9°C for 3 min; 92°C for 50 sec, 55°C for 90 sec, 72°C for 60 sec, run for 35 cycles; the final extension step was performed at 72°C for 3 min (Zhao *et al*., 2009). In reference to the UP-PCR amplification map, any significant finding was assigned as "1", otherwise it was assigned a strip value of "0", followed by a clustering analysis using UPMGA method of NTSYS 2.0 software. The isolate clusters were then mapped.

RESULTS

Specific primers analyses: Here in our screen, the results for the primers FOF1/FOR1 PCR showed that the 36 isolates tested with the positive contrast of *F. oxysporum* f. sp. *lycopeici* strains were amplified to a 340 bp (base pairs) target fragment in Table 1 and 2 *F. moniliforme* negative contrast isolates were not amplified bands. Combined morphologic characterization would determine whether the 36 isolates are *F. oxysporum*.

Sequences analysis: The sequence alignment characteristics and scores of the various phylogenetic trees generated are summarized in Table 2. However, only the tree inferred from the combined EF-1α and β-tubulin sequence data is presented (Fig. 1). EF-1α and β-tubulin were analyzed individually and the results showed that both methods separated the test isolates and the GenBank isolates into two groups. However, the lineage of the isolates based on EF-1α did not match that based on β-tubulin. The combined EF-1α and β-tubulin sequence data separated the analyzed taxa into three well-supported lineages (Lineages 1-3, Fig. 1), and every branch showed a high bootstrap value, indicating that this method produced a high credibility phylogenetic tree. In the tree group, each clade contained isolates from the tree cucurbit species. Therefore, there were seven isolates from Muskmelon, three isolates from Cucumber and nine isolates from Watermelon, in Lineage 1. In Lineage 2, there

Table 1: The tested *F. oxysporum* isolates and its geographic origins

Working No.	Strains	Plant origins	Working No.	Strain	Plant origins
1	FFS0801	Muskmelon	19	FXM0821	Watermelon
2	FFS0815	Muskmelon	20	FSY0827	Watermelon
3	FFS0814	Muskmelon	21	FSY0974	Watermelon
4	FFS0805	Muskmelon	22	FSY0822	Watermelon
5	F0302	Muskmelon	23	FSY0976	Watermelon
6	FFS0816	Muskmelon	24	FSY951	Cucumber
7	FFS0808	Muskmelon	25	FSY0950	Cucumber
8	FAS0825	Muskmelon	26	FSY0953	Cucumber
9	FFS0810	Muskmelon	27	FSY0954	Cucumber
10	FFS0809	Muskmelon	28	FSY0955	Cucumber
11	FSY0812	Muskmelon	29	FSY0957	Cucumber
12	F0304	Muskmelon	30	FSY0958	Cucumber
13	FXM0826	Watermelon	31	FAS0972	Cucumber
14	FSY0829	Watermelon	32	FAS0973	Cucumber
15	FXM0830	Watermelon	33	FLY0975	Cucumber
16	FXM0831	Watermelon	34	FSY0935	Cucumber
17	F0418	Watermelon	35	F0311	Cucumber
18	FXM0820	Watermelon	36	F0312	Cucumber

Table 2: Sequence alignment characteristics and phylogenetic tree scores inferred from the individual EF-1α and β-tubulin partitions and the combined datasets

Dataset	Aligned sites[a]	Conserved sites[b]	Pars. info sites[c]	Singleton sites[d]	Tree length[e]	Nodes>50% Bs[f]
EF-1α	750	307	401	30	668	3
β-tubulin	1158	496	612	46	279	7
EF-1α+β-tubulin	1271	504	685	69	792	18

[a]No. of aligned sites including gaps. [b]No. of Conserved sites. [c]No. of parsimony informative sites. [d]Singleton sites. [e]Tree length. [f]No. of nodes with bootstrap values of 50% or more

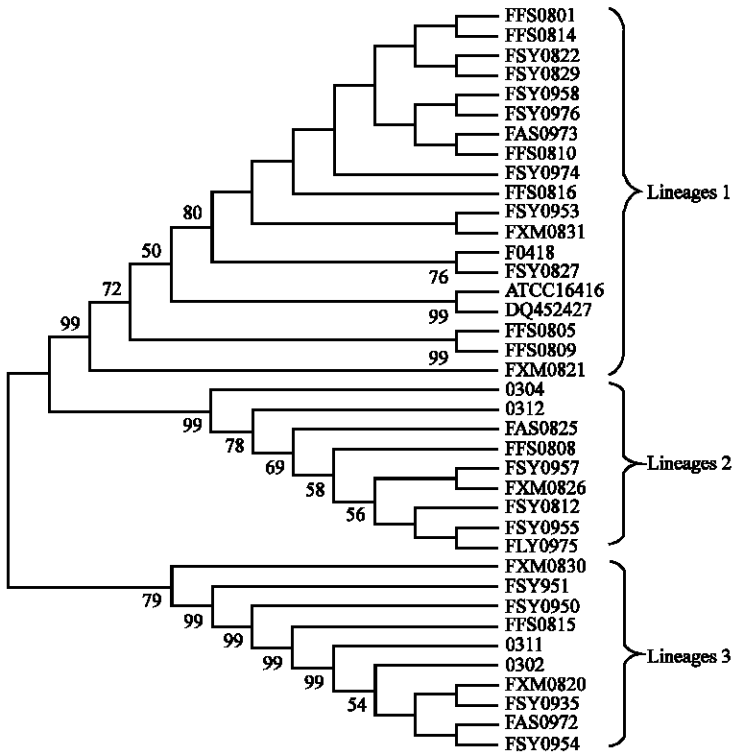

Fig. 1: Parsimonious tree inferred from the combined EF-1α and β-tubulin data set. Bootstrap values of 50% and more are indicated above nodes

were four isolates from Muskmelon, four isolates from Cucumber and two isolates from Watermelon. There were two isolates from Muskmelon, six isolates from Cucumber, two isolates from Watermelon, in Lineage 3. The GenBank isolates were classified into Lineage 1. Inspection of the sequence partitions revealed the presence of β-tubulin

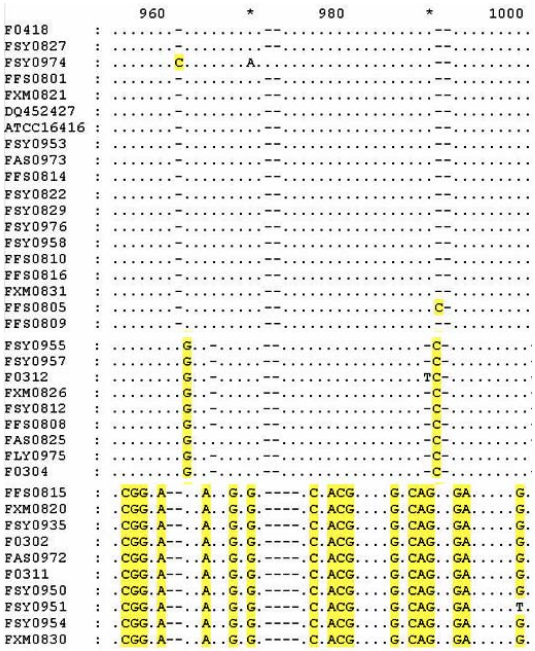

Fig. 2: The combined sequences (5'-3', sites 940-1000) of β-tubulin haplotypes representing the three lineages. The shaded sites show nucleotides that uniquely distinguish particular lineages. The dots indicate nucleotides that are identical to the ones in the first sequence at the respective sites. The numbers at the top of sequences indicate nucleotide sites

Table 3: Screening amplification primers of UP-PCR

Primers	Nucleotide sequence 5'-3'	Scored bands	Polymorphic bands
0.3-2	GTAAAACGAGGCCAGT	10	10
3-1	TAAGGTGGCGGCCAGT	11	10
AS4	TGTGGGCGCTCGACAC	13	8
AA2M2	GGCTAAGCGGTCGTTAC	9	10
AS15	CATTGCTGGCGAATCGG	9	11
AS15inv	GTAAAACGAGGCCAGT	9	13
Total		61	62

sites (Fig. 2), with significant differences between Lineage 3 and the other two lineages. Lineage 3 was genetically distinct in reference to Lineages 1 and 2, whereas only positions 962 and 995 distinguish Lineage 1 from Lineage 2. FSY0951 and FSY0950 differ at sites 962 and 982 compared with the other isolates in Lineage 3.

UP-PCR analysis: UP-PCR amplification of genomic DNA from three *F. oxysporum* isolates of different regions from the 13 universal primers were screened using, a total of six stable polymorphism primers (Table 3), as well as different primer bands between 8 and 13 and 100 bp to 2000 bp amplified DNA fragments were used. The UP-PCR data (Fig. 3) separated the 36 isolates into three major groups, with *F. oxysporum* f.sp. *Lycopeici* as the outgroup. These major UP-PCR-based groups corresponded with those in Lineage 3, which emerged from the sequence data. Lineage 1 was more similar to Lineage 2 than to Lineage 3. This matched the results of the sequence analysis. Only two of the taxa, FSY951 and FSY0950, were unresolved and these were grouped into a different lineage from that indicated by the DNA sequence comparisons. These taxa were grouped into Lineage 2 in the sequence data dendrogram (Fig. 1). The bands of outgroup isolates differed from other strains (Fig. 3). These data were consistent with the sequence data (Fig. 4).

DISCUSSION

In this study, DNA sequencing and UP-PCR were evaluated for their ability to resolve phylogenetic relationships among 36 *F. oxysporum* isolates from melon, muskmelon and cucumber. This data show that DNA sequencing and UP-PCR classified the 36 *F. oxysporum* isolates and GenBank isolates into three lineages. In previous study, Zhao *et al*. (2009) had analyzed the 25 strains of test *Fusarium* isolates and 3 strains of positive control *Fusarium* isolates by UP-PCR. However, the conclusion were not in accordance with us. Here in our results, the lineages that were resultant of the DNA sequences and UP-PCR analyses were also concordant. According to the combined EF-1α and β-tubulin sequence data, Lineage 1 is almost identical to Lineage 2 which was

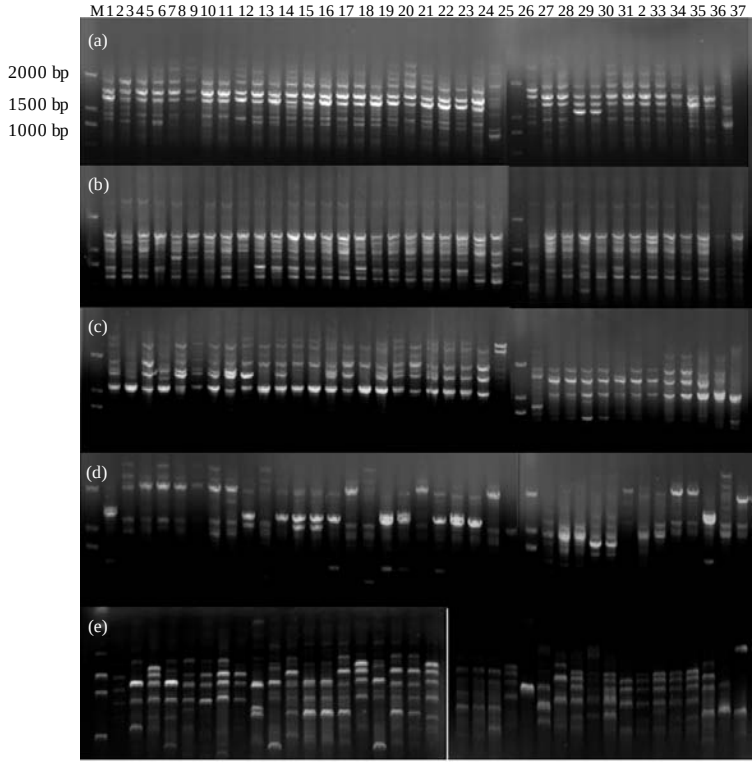

Fig. 3(a-e): UP-PCR banding profiles of the *Fusarium* strains with six UP primers. The profiles are generated with primer 0.3-2, 3-1, AA2M2, AS4, L45a and AS15inv

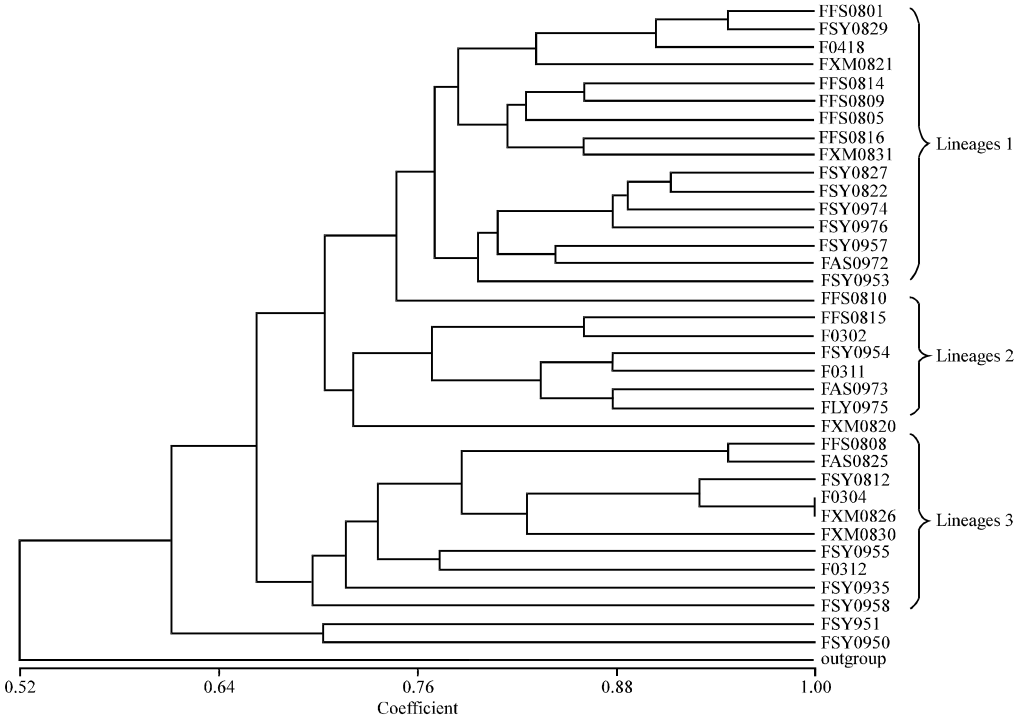

Fig. 4: Digitized UP-PCR patterns and dendrogram for 37 isolates of *Fusarium oxysporum* f. sp. Schl. The dendrograms were constructed with the UPGMA clustering method

also observed in the UP-PCR. The DNA sequences in Lineage 3 were significantly different when compared to Lineages 1 and 2.

Some other studies have reported that phylogenetic groups have little connection with virulence groups (Baayen *et al*., 2000; Brown and Simpson, 1994) because formae speciales are based on phenotypic characteristics (Hammerschmidt *et al*., 2001). These characteristics are influenced by a range of factors and not necessarily linked to phylogenetic placement (Marlatt *et al*., 1996). However, the sequence data shows that most isolates from Muskmelon and Watermelon were separated in Lineage 1 compared to the strains of cucumber. These results indicate that the isolates from Muskmelon and Watermelon have a close phylogenetic relationship. It is also reported that they have phylogenetic relationships by a development of sequence-based DNA markers (Lee *et al*., 2001).

This study indicated that phylogenetic groups have little connection with geographical origin. Previous studies have also reported similar results (Bogale *et al*., 2006). Thus, three clear natural groups were delineated by the DNA sequences and UP-PCR data. The other two isolates found in GenBank belonged to different biotypes of the isolates, which were assigned to the same groups in this study. Sequence alignment also showed that their genes were very similar. This finding indicated that UP-PCR and DNA sequences cannot be used to discriminate formae speciales.

The regional distribution of the isolates this study was not wide enough which mainly distributed in Liaoning Province. Randomness greatly limited the separation and species of *F. oxysporum*. Thus, further determination of their pathogenicity is needed to understand fully the formae speciales of the three groups.

The results show that all 36 isolates were identified as being infected with *F. oxysporum* by using the specific primers FOF1 and FOR1. Based on the combined sequence and UP-PCR data, the 36 isolates and GenBank isolates were classified into three major groups (Lineage1-3). These groups corresponded with the three clades of *F. oxysporum*. This study confirmed that phylogenetic groups have little connection with virulent groups.

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