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Comparison of *Fusarium oxysporum* fsp *lycopersici* races 1, 2 and 3, and f.sp *radicis lycopersici* based on the sequences of fragments of the ribosomal DNA intergenic spacer region

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Abstract

Sequence analysis of genomic fragments from the intergenic spacer region from three isolates of *Fusarium oxysporum* fsp *lycoperisci* and fsp *radicis lycopersici* was carried out using the big dye terminator sequencing procedure. Two conditions of the DNA templates were also evaluated for their influence on the outcome of the terminator reaction. Results showed that sequencing using the PCR products of M13 primer reaction with either direct *E. coli* colony, (condition 1) or purified plasmid DNA as templates (condition 2), were successful and the sequences of the cloned IGS fragments were the same indicating that time and cost could be minimized by excluding the plasmid purification steps. Based on the sequence analysis of the IGS fragment of race 1 (kis-1a) (ca. 638 bp including the forward and reverse primers sequences) it is observed that there is at least 95% similarity between the *F. oxysporum* races 1, 2, 3, and rly. Using the BioEdit sequence analysis program, there are 14 conserved regions with the longest continuous consensus segment being between nucleotide position number 1 and 129. Region 2 has 18 segment length (164-181), while region 3 is the shortest region with 15 segment length (183-197).

Keywords: *Fusarium oxysporum,* big dye terminator, plasmid DNA-PCR, colony–PCR, sequencing reaction

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INTRODUCTION

The species of *Fusarium* have traditionally been differentiated by their morphological characteristics on selective media[1,2]. It is almost impossible, however, to identify pathogenic types, or forma speciales and races of *Fusarium oxysporum* using morphological features. An inoculation assay using tester plants has been a popular approach of identification of forma speciales. However, this is a time-consuming approach[3]; thus necessitating development of other methods.

Arie *et al*.[4,5] have proposed immunoassys as alternative methods while recently, molecular markers have become popular for identifying species and subspecies in fungi. Some of the techniques that have been reported include amplified fragment length polymorphisms (AFLP)[6], random amplified polymorphic DNA (RAPD)[7], restriction fragment length polymorphisms (RFLP)[8]; direct amplification of length polymorphism[9] among others.

The sequence of DNA encodes the necessary information for living things to survive and reproduce and different organisms are known to have different arrangement of the nucleotides of their DNA. As noted by a contributor in the free on-line encyclopedia -Wikipedia[10] because of the key nature of DNA to living things, knowledge of DNA sequence should come in useful in practically any biological research. Differentiation of the *Fusarium* species/subspecies based on comparison of DNA sequences of the ribosomal DNA (rDNA) and internal transcribed spacer (ITS) regions have been reported[11].

More recently, Hirano and Arie[12] have reported differentiation of *Fusarium oxysporum* f.sp *lycopersici* and f .sp *radicis lycopersici* by a polymerase chain reaction (PCR)-based method using specific primer sets developed from the knowledge of the partial nucleotide sequences of the *endo* (*pg1*) and *exo* (*pgx4*) polygalacturonases genes of the fungi.

Based on the sequences of the rDNA intergenic spacer region, endo polygalacturonase gene (pg1) and the mating type genes (MAT1-1-1 and MAT1-2-1), Kawabe *et al*[13] constructed phylogenetic trees for *Fusarium oxysporum* f.sp *lycospersici* isolates. They found out that although there was no correlation between races and phylogeny based on rDNA-IGS, pg1 and mating type genes world wide, there was correlation among Japanese isolates.

In this study, sequencing the aforementioned 4 fungal isolates is part of basic research to compare and contrast them. It was also aimed at evaluating the influence of condition of DNA template on the sequencing terminator reaction and eventual sequence analysis.

MATERIALS AND METHODS

Evaluation of the effect of different conditions of DNA templates on big dye terminator sequencing reaction

Sequencing reactions were carried out using the big dye terminator sequencing procedure, which is an alternative to the Sanger chain terminator sequencing. The principle behind this procedure is that each of the dideoxynucleotide chain-terminators is labeled with a separate fluorescent dye, which fluoresces at a different wave length[10].

Recombinant plasmid DNAs were initially generated by separately cloning freshly derived IGS- PCR fragments of the 4 fungal isolates into pGEM –Easy vector and transforming them in competent *E. coli* cells using the standard procedure[14]. The IGS fragments that were later sequenced were then derived either directly from the unpurified transformed *E. coli* colony (plasmid + IgS fragment +*E. coli* cells) or from the purified recombined plasmid DNAs (Plasmid DNA+ IGS fragment).

In both cases, the final sequencing reaction templates were generated from PCR procedures in which the M13 forward and reverse primers were used together and the two aforementioned sources of IGS fragments were the DNA templates respectively. In the *E. coli* colony PCR, the template was applied by using sterile toothpick to directly pick just a little portion from the desired colony and then shaking it briefly inside 10µl of the PCR reaction mixture that had already been pipetted into the PCR tubes accordingly.

The annealing condition for the colony PCR differed a little bit from the normal condition that applied when purified recombinant plasmid DNA was used as template. The *E. coli* colony PCR, was performed at 25 cycles of 94°C- 30s, 50°C- 30s, and 72 °C- 1 min instead of the 25 cycles of 94°C- 30s, 58°C- 30s, and 72 °C used for the other PCR.

Treatment of PCR products with Exo-SAP nuclease

The PCR products obtained from both direct bacterial colony PCR and plasmid DNA PCR were subjected to treatment with Exo-SAP (Exonuclease-Shrimp Alkaline Phosphatase conjugate). To 5 µl of PCR product, 2µl of ExoSap was added and the mixture incubated in a thermal cycler at 37°C for 15 min and 80 °C for 15 min. This was to help remove as much impurity as possible from the products before they were eventually used as templates for the sequencing reactions proper.

Other Sequencing PCR conditions

Under the first and second conditions, each 20 µl mixture contained 2 µl of Exo-sap- treated M13 PCR products as templates, 3.0 µl of Big Dye buffer; 1.0 µl of either of 0.8 pmol M13 primer forward or reverse sequence; 1.0 µl of Big dye terminator and 13.0µl of MilliQ H_2O. The PCR temperature cycling condition in all cases was 1 cycle of 96 °C for 1 min, 25 cycles of 96 °C- 10s; 50 °C -5 s; 60 °C- 4 min, and holding at 4 °C. The reaction was carried out with the Gene Amp thermal cycler. A small portion each of the PCR products was analyzed on 2% agarose gel to confirm success or failure of sequencing reaction while the rest were subjected to purification prior being run in the genetic analyzer machine.

Purification of sequencing PCR products

Ten (10) µl PCR products obtained as described above were transferred to 1.5 ml Eppendorf tubes. To 10µl of the product, 1µl of 3M sodium acetate and 30µl 99.5% Ethanol were added and gently mixed. The mixture was left standing at room temperature for 15 min before centrifugation at 15000 rpm for 20 min. The supernatant was decanted and to the pellet, which contained the desired product, 150µl of 70 % ethanol was added. The mixture was centrifuged for 20 min at 15000 rpm. The supernatant was decanted carefully while the DNA pellet (which is invisible) was vacumn-dried for 10 min using the EYELA evaporator. Ten µl of Hi-Di formamide was added and the mixture vigorously vortexed using the tuple mixer (Iwaki Glass Co. Ltd Japan) to re-suspend the DNA fragments. The products were thereafter kept at 20 °C overnight.

Sequencing run and analysis

Just before sequencing analysis using the Applied Biosystem 3130x genetic analyzer system, the purified sequencing reaction product was heated for 5 min in boiling water bath to linearize the DNA fragments and transferred immediately on ice to ensure that they remained linear until analyzed. The reaction mixtures, 10 µl in quantity, were transferred to lanes of the MicroAmp optical wells. At the end of the sequencing run, the results in the form of electrophoregrams and deduced textual sequences, were copied out and transferred to a PC where the Genetyx Mac program or BIOEDIT Sequence Analysis program for Windows were used to analyze the sequences based on homology and complementary searches with the Primers sequences and Alignments.

RESULTS AND DISCUSSION

Plate 1 shows the agarose gel electrophoretic analysis of the IGS-PCR fragments that were cloned into plasmid pGEM -Easy vector prior to transforming in *E.coli* cells. It shows that they banded around 650 bp.

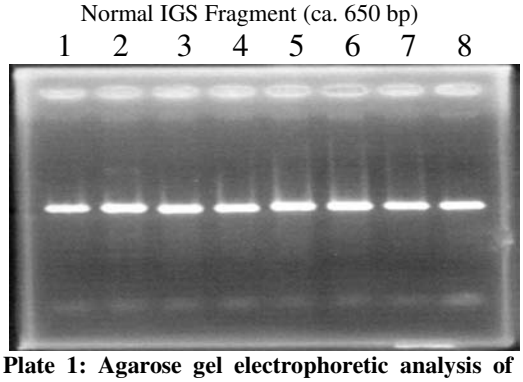

Plate 1: Agarose gel electrophoretic analysis of the PCR products of amplification with the FIGS primers of four *Fusarium* DNA templates. The freshly generated products were cloned in Plasmid pGEM –Easy vector and transformed in *E.coli.*
Lanes 1, 2=Race 1; lanes 3, 4= race 2; lanes 5,6= Race 3; lanes 7,8= rly.

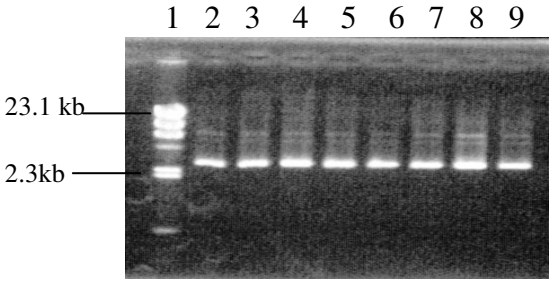

Plate 2: 1% Agarose gel electrophoresis analysis of the purified recombinant plasmid PGEM –T vector DNAs.

Lane 1: λ Hind III DNA marker. Lanes 2-9 are from 8 samples i.e. F.ol Races 1,2, 3, and rly respectively. i.e. lanes 2, 3= races 1, lanes 4, 5= race 2; lanes 6,7= race 3 and lanes 7,9= f.sp *radicis lycopersici*. The desirable bands are the lowermost bold ones (above the 2.3 kb band).

Plate 2 compares the appearance on the gel of the colony PCR products and plasmid DNA PCR products when they were subjected to the same cycling conditions.

The important point here is that both contained the target M13 fragments, which encompasses the desired cloned IGS fragment, hence the banding at a position around 900bp.

The additional information obtained here was that plasmid DNA template could be amplified at annealing temperature as low as 50°C. Ordinarily, the annealing temperature is 58°C.

Plates 3 and 4 show that the desired fragments were amplified in both the direct transformed bacterial colony DNA and purified plasmid DNA-templated reactions.

The bands in Plate 3 are produced with M13 forward and reverse primers used together in the PCR reaction, while those in Plate 4 are produced by either of M13 forward or reverse primers respectively.

This confirmation was necessary to ensure that the PCR products to be purified actually contained the desired amplicons.

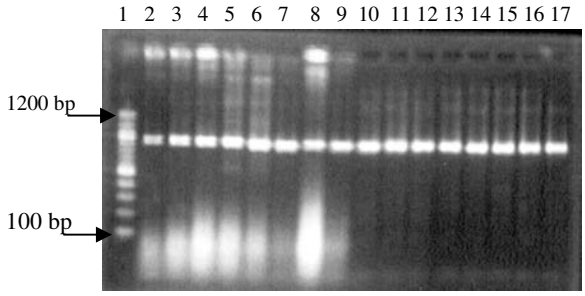

Plate 3: Comparative appearance on 2% agarose gel of M13 PCR products from direct transformed *E. coli* colony and purified plasmid DNA templates. The M13 primers (F and R sequences together) were used. Lane 1: 100bp Marker; 1-9= colony PCR products; 10-17: purified Plasmid PCR products. The PCR was performed at 25 cycles of 94°C- 30s, 50°C- 30s, and 72 °C- 1 min instead of the 25 cycles of 94°C- 30s, 58°C- 30s, and 72 °C normally used for plasmid DNA -templated PCR.

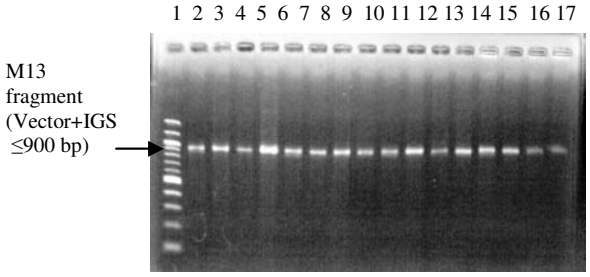

Plate 4: Agarose gel electrophoresis analysis of sequencing PCR products.
Lane 1: 100 bp DNA marker. Lane 2, 3 = rly F and R, 4,5= race 3 (F&R respectively); 6,7 = race 2 (F&R respectively); 8, 9 =Race 1(F&R respectively) from direct colony PCR product template. Lanes 10 to 17 are from plasmid DNA product templates. The PCR temperature cycling condition in all cases was 1 cycle at 96 °C for 1 min, 25 cycles of 96 °C- 10s; 50 °C- 5 s; 60 °C- 4 min, and holding at 4 °C. The reaction was carried out with the Gene Amp thermal cycler.

Electrophoregram results showed that sequencing run using the PCR products of M13 primer PCR with either direct *E. coli* colony (condition 1) or purified plasmid DNA as templates (condition 2), were successful and the sequences of the cloned IGS fragments encompassed in them were the same (Fig 1). The implication of this is that the use of direct colony -PCR product as template can cut down on both the time and expenses required to purify the plasmid DNA beforehand when direct colony PCR is employed to generate the PCR product needed for the sequencing reaction.

Fig 1: Nested trace windows showing typical sequence electrophoregram of the M13 Fragment of recombinant pGEM-IGS of *Fusarium oxysporum* DNA.
This particular one was from F.o.l Kis-1 (Race 1) from the reverse primer as analyzed by the Applied Biosystem Genetic Analyzer. The non decipherable sequences in the edges, are actually vector (M13) sequences. The original window is a single continuous frame nested here for ease of viewing.

Tables 1 to 4 show alphabetical translations (Fasta file format) of the fungal isolates. Races 1, 2, and *radicis lycopersici* have 638 base pairs each while race 3 had 623 bp. Optimal sequence alignment analysis, and consensus or conserved region search was carried out using the BIOEDIT sequence analysis program for the four isolates.

Table 1: The F.ol- kis-1 IGS sequence based on the forward and reverse primers sequences (638bp)

gtaagccgtccttcgcctcgATTTCCCCAATGGGTTCTCC
GGATTTCTGGAGACTTGTAGGGGTTGTGGGAT
TTTTGATGTGTCGTCTCCGGACGGGCGGTGCA
GGGTAGTCGAGTTAGACTTGGTGGAGTTCCGT
CGATAGGAGTTCCGTCGAGTCTGGTCAGCTGT
GTGTTGGACGGTGTAGGGTAGGCTGCTTGGAC
ATGGTCGGTTCGAGGATCGATTCGAGGGCCGG
CCTGTCGATGATGTGTGATGTATGCGGTCTAG
GGTAGGTTGGTTTGTCTTGGTTCGATTTGATGT
CGGCTCCCGTGCAGGCCAGAGTGAGGGGGGT
CCAGGGTAGGTGCAGGGTAGGCAGCTTAGAT
TTGGTCGATCTGGAGGTCGATTCTCCGGCTGG
CGGATCTGACACTGTCGAAACGAGATGCGAG
GGGTGTAGGGTAGGCTAGTTTCGTACTTGCCA
GGTTGCGATTTGGACGAGATATGTGGTTTAGG
GTAGGCTCTAGGGTAAGTAGAATTCGAGTTTC
GTCGCCGATAGTTTTCTGTGGGTGTATGGTAG
GTACAGGGTAGGCAAATCTCTCTCCGGCCAGT
ACTTGTCTCGTGGTCGTGAGTCGATTTTTTTGT
TTTgccatactattgaattttgc

The lower case letters are the sequences complementary to the primer sequences.

Source: Big dye terminator Sequencing analysis November 2006

Table 2: The IGS fragment sequence in F.ol 888601-1a (Race 2

gtaagccgtccttcgcctcgATTTCCCCAATGGGTTCTCC
GGATTTCTGGAGACTTGTAGGGGTTGTGGGAT
TTTTGATGTGTCGTCTCCGGACGGGCGGTGCA
GGGTAGTCGAGTTAGACTTGGTGGAATTCCGT
CGATAGGAGTTCCGTCGAGTCTGGTCAGCTGT
GTGTTGGACGGTGCAGGGTAGGCTGCTTGAAC
ATGGTCGGTTCGAGGATCGATTCGAGGGCCGG
CCCGTCGATGATATGTGATGTATGCGGTCTAG
GGTAGGCTGGTTTGTCTTGGTCCAATTTGATG
TAGGCTCCCGTGCAGGCCAGAGTGAAGGGGG
TCCAGGGTAAGTCCAGGGTAGGCAGCTTAGAT
TTGGTCGATCTGGAGGTCGATTCTCCGGCTGG
CGGATCTGACACTGTCGAAACGAGATGCGAG
CGGTGTAGGGTAGGCTAGTTTCGTCCTCGCCA
GGTTGCGATTTGGACGAGATATGTGGTTTAGG
GTAGGCTATAGGGTAAGTAGAATTCGAGTTTC
GTCGCCGACAGTTTTCTGTGGGTGTATGGTAG
GTACAGGGTAGGCAAATCTCTCTCCGGCCAGT
ACTTGTCTGGTGGTCGTGAGTCGATTTTTTTGT
TTTgccatactattgaattttgc

Source: Big dye terminator Sequencing analysis November 2006

Table 3: The IGS sequence of F.ol- tomino-1c (Race 3)

gtaagccgtccttcgcctcgATTTCCCCAATGGGTTCTCCG
GATTTCTGGAGACTTGTAGGGGTTGTGGGATTTT
TGATGTGTCGTCTCCGGACGGGCGGTGCAGGGT
AGTCGAGTTAGACTTGGTGGAGTTCCGTCGAGT
CTGGTCGGCTGTGTGTTGGACGGTGCAGGGTAG
GCTGCTTGGAGATGGTCGGTTCGAGGATCGATT
CGAGGGCCGGCCTGTCGATGGTGTGTGATGTAT
GCGGTCTAGGGTAGGCTGGTTTGTCTTGGTTCAA
TTTGATGTCGCCTCCCGTGCAGGCCAGAGTGAG
GAGGGTCCAGGGTAGGTACAGGGTAGGCAGCTT
AGATTTGGTTGATCTGGAGGTCGATTCTCCGGCT
GGCGGATCTGACACTGTCGAAACGAGATGCGAG
CGGTGTAGGGTAGGCTAGTTTCGTCCTCGCCAG
GTTGCGATTTGGACGAGATGTGTGGTTTAGGGT
AGGCTCTAGGGTAAGTAGAACTCGAGTTTCGTC
GCCGACAGTTTTCTGTGGGTGTATGGTAGGTAC
AGGGTAGGCAAATCTCTCTCCGGCCAGTACTTG
TCTGGTGGTCGTGAGTCGATTTTTTTGTTTTgccata
ctattgaattttgc

Source: Big dye terminator Sequencing analysis November 2006

Table 4: The IGS sequence of *Fusarium oxysporum* fsp *radicis lycopersici*

gtaagccgtccttcgcctcgATTTCCCCAATGGGTTCTCCG
GATTTCTGGAGACTTGTAGGGGTTGTGGGATTTT
TGATGTGTCGTCTCCGGACGGGCGGTGCAGGGT
AGTCGAGTTAGACTTGGTGGAATTCCGTCGATA
GGAGTTCCGTCGAGTCTGGTCGGCTGTGTGTTGG
ACGGTGTAGGGTAGGCTGCTTGGACATGGTCGG
TTCGAGGATCGATTCGAGGGCCGGCCTGTCGAT
GATGTGTGATGTATGCGGTCTAGGGTAGGTTGG
TTTGTCTTGGTTCGATTTGATGTCGGCTCCCGTG
CAGGCCAGAGTGAGGGGGGTCCAGGGTAGGTG
CAGGGTAGGCAGCTTAGATTTGGTCGATCTGGA
GGTCGATTCTCCGGCTGGCGGATCTGACACTGTC
GAAACGAGATGCGAGGGGTGTAGGGTAGGCTA
GTTTCGTACTTGCCAGGTTGCGATTTGGACGAGA
TATGTGGTTTAGGGTAGGCTCTAGGGTAAGTAG
AATTCGAGTTTCGTCGCCGATAGTTTTCTGTGGG
TGTATGGTAGGTACAGGGTAGGCAAATCTCTCT
CCGGCCAGTACTTGTCTCGTGGTCGTGAGTCGAT
TTTTTTGTTTTgccatactattgaattttgc

Source: Big dye terminator Sequencing analysis November 2006

Based on the sequence analysis of IGS of the *F.ol* race 1, it is observed that there is at least 95% similarity between the *F. oxysporum* races 1, 2, 3, and rly. The graphical alignment representation is shown in Fig 2. As shown in Table 5, there are 14 conserved regions with the longest continuous consensus segment being between nucleotide position number 1 and 129. Region 2 has 18 segment length (164-181), while region 3 is the shortest region with 15 segment length (183-197).

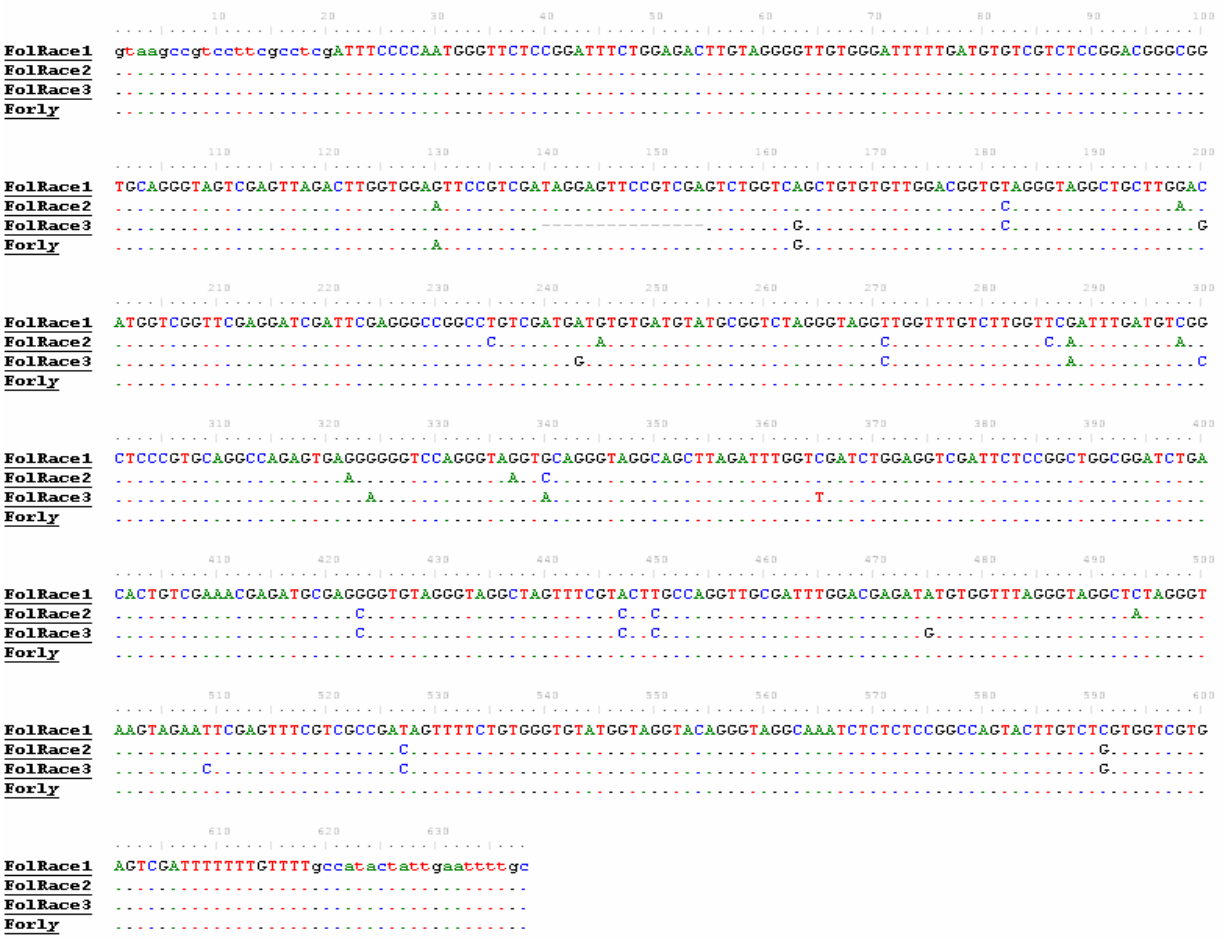

Fig 2: A graphical view of the similarity alignment of the IGS sequences of the four sequenced fungal isolates. Dots represent similar sequences with Race 1

Based on all the facts presented, the ribosomal IGS region was considered representative enough to be used for diagnostic purposes especially in the development of DNA probes that were successfully used in Southern blot analysis to detect the four *Fusarium oxysporum* DNAs sample preparations (Data not shown here). In fact, based on the sequences of this same IGS, MAT1 and pg1 regions, Kawabe *et al.*[13] had constructed an evolutionary lineage tree of this same tomato wilt pathogen showing that there are 3 three evolutionary lineages, which are each composed of a single mating type and a single or closely related vegetative compatibility group.

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