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# Molecular Characterization of Uzbekistan Isolates of *Fusarium oxysporum* f. sp. *vasinfectum*

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## Abstract

A collection of isolates of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) from cotton in Uzbekistan was characterized based on candidate gene sequencing approach. As a first step, cotton seedlings were artificially re-infected with randomly selected 8 unknown FOV isolates from the collection, FOV strains were re-isolated, and monospore cultures were obtained for genomic DNA preparation. Candidate genes such as elongation factor (EF-1α), beta-tubulin (BT), and ribosomal DNA (rDNA) were sequenced from the genomic DNAs of these unknown Uzbekistan FOV isolates and a set of 13 known races of FOV (races 1, 2, 3, 4, 6, 7, 8, and Australian VCG1112), collected from world research centers. A parsimony based phylogenetic analysis of known races of FOV together with unknown FOV isolates from the Uzbekistan collection clearly suggested that Uzbekistan FOV genotypes can be classified as race 1, 2 &6, race 3, and race 4 or 7. Based on EF-1α pair-wise genetic distances, no difference was observed between races 4 and 7 of FOV. Results from our research provided for the first time a comprehensive study on the identification of FOV races collected from different regions of Uzbekistan. In addition, this study will help breeders to develop resistance management strategies to combat the *Fusarium* wilt disease by breeding resistant cotton lines in Uzbekistan.

**Keywords:** FOV, cotton, wilting disease, Fusiarum, race identification

## Introduction

*Fusarium* wilt of cotton, caused by *Fusarium oxysporum* Schlect. f. sp. *vasinfectum* (FOV) (Atk.) Snyd & Hans., is a serious threat to cotton (*Gossypium* spp.) in many production regions in the world [1-6]. FOV is a soil-inhabiting fungus that can survive for long periods in the absence of a host, making it impractical to eradicate the fungus from infested fields. Although some progress has been accomplished in managing the disease, host plant resistance remains the most sustainable way to confront *Fusarium* wilt of cotton [6].

FOV is comprised of different genotypes, sometimes called races. At least eight races (race 1 to race 8) and several undesignated genotypes have been described throughout the world [7,8]. Races were originally classified based on pathogenicity tests on different cotton species including *Gossypium hirsutum* L, *G. barbadense* L. and *G. arboreum* L. [9-11], and by their pathogenicity on alfalfa (*Medicago sativa* L.), soybean (*Glycine max* L.), and tobacco (*Nicotiana tabacum* L.) [11,12]. However, results of pathogenicity tests on hosts other than cotton were difficult to reproduce and were not appropriate for defining races [1,3,6]. More recently, FOV genotypes have been defined based on differences in DNA sequences, *eg.*, sequences of the translational elongation factor (EF-1α), the phosphate permase (PHO) and the beta-tubulin (BT) genes, and intergenic spacer (IGS) regions using restriction enzymes [3,8,13]. Worldwide, genotypes of FOV can be classified into five major lineages such as race 1, 3, 4, 8, and Australian race [3,7,8].

In the U.S.A., the FOV disease was first reported in 1892 in Alabama cotton fields [14], and since then it has been reported in all of the major cotton producing regions in the world. It was also reported that the disease is more severe in the presence of the root-knot nematode (RKN), *Meloidogyne incognita* (Kofoid & White) Chitwood [14]. In cotton, little is known about the genetic basis for resistance to FOV genotypes, or how these geneotypes are affected by environmental factors and interactions with other pathogens (*eg.,* RKN and reniform nematodes, Verticillium wilt caused by *Verticillium dahliae*, or black root rot caused by *Thielaviopsis basicola*) [6]. The postulated pathogenicity mechanisms and the inheritance of FOV resistance significantly differ among FOV genotypes for cotton cultivars [2,15]. Phenotypic analyses indicated that resistance to FOV was determined by one or two major genes with complete to incomplete dominance, and possibly additional minor genes [4,6,16,17].

In Uzbekistan, during 1960 to 1970, cotton production suffered with significant losses in yield and quality because

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**Table 1. Strains of *Fusariuum oxysporium* f. sp. *vasinfectum* of known races and from Uzbekistan collected from symptomatic cotton plants used in the DNA phylogenetic analyses.**

| # | Isolate | Origin/Year collected | Source/Region isolated | GenBank accessions | ID used in this study | Previously known race information |
|---|---|---|---|---|---|---|
| 1 | Race_1_Tulare_CA | USA | R.M. Davis | - | FOV2 | Race 1 (USA) |
| 2 | Race_2_ATCC_16611 | USA | G.M. Armstrong | - | FOV 16611 | Race 2 (USA) |
| 3 | Race_3_Tulare_CA | USA | R.M. Davis | - | FOV3 | Race 3 (USA) |
| 4 | Race_4_Fresno_CA | USA | R.M. Davis | - | FOV 66 | Race 4 (USA) |
| 5 | Race_4_AY714101 | USA | R.M. Davis [3] | AY714101 | | |
| 6 | Race_6_ATCC_36198 | Brazil | G.M. Armstrong | - | FOV36198 | Race 6 (USA) |
| 7 | Race_7_China | China | T. Zhang | - | FOV (China) | Race 7 (China) |
| 8 | Race_7_AF362156 | China | [8] | AF362156 | - | - |
| 9 | Race_8_CA-1_AY714098 | Tulare, CA, USA | S.N. Smith, J.E. DeVay | AY714098 | - | - |
| 10 | LA_110_Group_A | Lousiana, USA | R.M. Davis | - | FOV 110 | Group A (USA) |
| 11 | LA_112_Group_C | Lousiana, USA | R.M. Davis | - | FOV112 | Group C (USA) |
| 12 | FOV_AUSTRALIA-19_AY714097 | Boggabilla, Australia | S. Bentley [3] | AY714097 | - | |
| 13 | FOV_AUSTRALIA_VCG_1112 | Boggabilla, Australia | R.M. Davis, S. Bentley [3] | - | FOV 19 | Aust VCG 1112 (USA) |
| 14 | FOV_347_UZBEK_Orig (316) | Uzb/2010 | *IGPEB/Sirdarya | - | 347 | Unknown |
| 15 | FOV_316_UZBEK_1 | Uzb/2008 | IGPEB/Tashkent | - | 316 (R-1) | Unknown |
| 16 | FOV_316_UZBEK_2 | Uzb/2008 | IGPEB/Tashkent | - | 316 (R-2) | Unknown |
| 17 | FOV_316_UZBEK_3 | Uzb/2008 | IGPEB/Tashkent | - | 316 (R-3) | Unknown |
| 18 | FOV_328_UZBEK_1 | Uzb/2010 | IGPEB/Bukhara | - | 328 (R-1) | Unknown |
| 19 | FOV_444_UZBEK | Uzb/2002 | IGPEB/Tashkent | - | 444 (125) | Unknown |
| 20 | FOV_375_UZBEK | Uzb/2001 | IGPEB/Tashkent | - | 375 (120) | Unknown |
| 21 | FOV_319_UZBEK | Uzb/2010 | IGPEB/Namangan | - | 319 (R-1) | Unknown |

* - IGPEB - Institute of Genetics and Plant Experimental Biology collection; Uzb - Uzbekistan. Uzbekistan has 12 province including Andijan, Fergana, Namangan, Tashkent, Sirdayra, Jizzakh, Samarkand, Navoiy, Bukhara, Qashqadaryo, and Surkhondaryo as well as autonomous Republic of Karakalpakstan (listed by closeness to Tashkent).

of Verticillium and *Fusarium* fungi epidemics, which were efficiently minimized with the development of more resistant Upland cotton varieties [18]. *Fusarium* genotypes historically affected Pima cottons (*G. barbadense*), but in the last few years, researchers reported that Upland cultivars (*G. hirsutum*) have also been affected by *Fusarium* in different cotton growing regions of Uzbekistan [19]. Since 2002 to present day, the domi-nant reported pathogen causing wilt has been *Fusarium*, in particular for the long staple Pima -derived upland cotton variety Bukhara-6. Reports from 2007 to 2011 demonstrated that several other Upland cotton varieties also became highly susceptible to *Fusarium* in Uzbekistan [20].

A unique phytopathogen collection exists in Uzbekistan that was created in 1993 by the initiative of scientists of the Institute of Genetics and Plant Experimental Biology, Academy of Uzbekistan Sciences, within the frame of several governmental and international research projects. The collection includes more than 500 phytopathogen strains belonging to 72 genera and has been recorded and updated through an electronic database [21]. This collection includes many isolates for *Fusarium* fungi collected from different regions of Uzbekistan without any designation of FOV races. Although previous studies explored a FOV isolate from Uzbekistan and reported that it belonged to race 4 of FOV [7,22], the collected site of the Uzbekistan FOV isolate remained unknown. A comprehensive survey was needed to determine the known distribution of races causing *Fusarium* wilt in Uzbekistan cotton. Without a proper identification of races, the development of a resistance strategy to breed resistant cultivars for different gene-specificity of FOV races remains elusive. The specific objective of this research was to identify the races of *F. oxysporum* f. sp. *vasinfectum* isolates collected from cotton plants infected with *Fusarium* wilt of different geographical regions of Uzbekistan and compare them with a known set of FOV isolates of USA and Australia using candidate genes sequence of some specific genomic regions.

## Methods

### *Fusarium* isolates

The *Fusarium* isolates with their origin and source used in this study are presented in **Table 1**. Briefly, a total of eight samples for *Fusarium* isolates with unknown race identity from the Republic of Uzbekistan collection were used to isolate genomic DNA for sequencing comparison with known races of FOV (races 1, 2, 3, 4, 6, 7, 8, and Australian VCG1112). Among Uzbekistan samples, isolate no. 316 (with original number 347) is widely

used in many biological experiments, and greenhouse and field evaluations of FOV infection on cultivars and germplasm of Uzbekistan. Isolate no. 316 represented several biological replications (R1, R2 and R3) and was re-isolated from different infected cotton plants under laboratory conditions. For DNA sequence comparisons, 13 genomic DNA samples of known FOV races were used to place the Uzbekistan's isolates into a specific group or FOV race. Known FOV races were provided by researchers and/or DNA sequence was obtained through the NCBI GeneBank (blast.ncbi.nlm.nih.gov) (**Table 1**).

## Isolation of fungal strains

Infected cotton plants with signs of wilt disease were collected from cotton fields in different regions of Uzbekistan. Diseased plants were washed for 2 hours in tap water and cut on the border of the affected and relatively intact tissues. A piece of cut tissue, after treatment in 0.002% solution of Tween – 80 for 2 minutes and surface sterilization in 70% ethanol and 5% NaOCI for 30 seconds, was placed on petri dishes with potato dextrose/sucrose/glucose agar supplemented with streptomycin sulfate 0.3 g per liter and incubated in an environment-control growth chamber (photoperiod 12:00 at 24-25$^0$C, the period of darkness 12:00 at 18°C). Isolation of cultures was carried out by classical methods following Booth [**23**] and Bilay [**24**] protocols. Starting from the second day of incubation and during the whole vegetation period, petri dishes with fungi were subjected to microscopy and isolation. Single spore cultures were used for identification of microscopic fungi. Conidia suspension was prepared in the test tubes with a fresh culture of the fungus with a series of consecutive 10-time dilutions. The resulting dilution of conidia was introduced into 2% agar growth medium and dispensed on sterile petri dishes and incubated at 24-25$^0$C for 5-7 days [**24**]. Petri dishes only containing single (dense) sporulating colonies were selected for identification of FOV races. Conidial suspension was prepared in sterile water. The concentration of spores was counted under a microscope at low magnification (100-150 ×) and adjusted to a concentration of 10-30 spores in a single drop. On the surface of the agar medium with growth limiter (Triton X-100), a drop of suspension was added to make 10-20 spores. Separately growing colonies were collected 3-5 days later. Confirmation of *Fusarium* isolates was performed according to Booth [**23**], and Gerlach & Nirenberg [**25**].

## Propagation of fungal strains through host plant and pathogenicity test of *Fusarium*

Sterile glass tubes were filled with one of the following nutrition medium containing potato dextrose/sucrose/glucose agar slant (PDAS; PGAS; or PSAS) up to 10 cm scale mark. In 2-3 days fungal strains were placed on to agar surface and incubated in the environmental chamber (12 h of day-light at 24-26°C followed by 12 h in dark at 20-22°C) for 14 days. After incubation the mycelium was covered by warm (45°C) Knopp (limited salts) medium up to 3 cm.

Acid delinted and surface sterilized seeds of commercial *G. hirsutum* L. cotton varieties (AN-Boyovutt -2, Namangan – 77, Navbahor, Gulbahor) were placed on Knopp medium. Each tube containing single cotton seed with the fungal strain was placed into a growth chamber for 8-10 days at 22°C. After incubation the diseased seedlings were subjected to mycological testing and further re-isolation of fungal strain. For the pathogenicity test, acid delinted and surface sterilized seeds of AN-Bayaut -2, Namangan – 77, Navbahor, Gulbahor cultivars were grown in petri dishes on filter paper in sterile conditions at 22°C. After germination, only intact and healthy seedlings were used for further pathogenicity testing. Fungal cultures were incubated on potato glucose agar slant (PGAS) petri dishes in the environmental chamber (12 h of day-light at 24-26°C followed by 12 h in dark at 20-22°C) for 14 days. Ready growth mycelium with conidia was washed by 5-7 ml sterile water and was passed through sterile cheesecloth into tubes. The volume of suspension for each strain was brought up to 50 ml. The conidia suspension was adjusted to 1 x $10^6$ con/ml concentration, calculated by using *hemocytometer*. Pathogenicity test was performed using seedlings of the above cotton cultivars placed on petri dishes with conidia suspension (a more comprehensive pathogenicity study with these and additional genotypes will be published elsewhere).

## DNA isolation and sequencing

Monospore isolates grown on petri dishes were used for DNA isolation. For each sample 200 mg of mycelium was ground into powder in liquid nitrogen. DNA was extracted following previously published protocol [**23**] and stored at -20$^0$C. Gene-specific primers were used to amplify genomic regions on selected FOV isolates (**Table 2**). Primer combinations used were EF1α/EF2α, BT3/BT5, rDNA 18s/rDNA 28S that produced 500-600 bp PCR amplicons. Amplification was carried out in 25-µl reactions containing 1.5 µl 10 ng/µl of DNA, 2.5 µl of 10 x PCR buffer containing 100 mM Tris-HCl, pH 8.3 at 25°C, 500 mM KCl and 15 mM $MgCl_2$, 0.2 µl of 25mM/µl of dNTPs, 0.5 µl of 1 mg/ml BSA, 0.37 µl (5µM) of each primer, 1 unit of Taq polymerase (Applied Biosystems, Foster City, CA) and pure (milli-Q) water using a thermocycler (Gene Amp, PCR System 9700), following PCR-conditions and protocols described by previous studies (**Table 2**). A negative control (no template DNA) was included in each PCR reaction. Amplicons were visualized in 2 % agarose gel by staining with ethidium bromide. The products were purified with polyethylene glycol (PEG) solution (containing 26% PEG 8000, 6.5 mM $MgCl_2$ and 0.6mM sodium acetate pH 5.2) to remove remaining primers. The purified PCR-products were then directly sequenced in both directions using 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA).

## Data analysis

Sequences were analyzed with the Lasergene V 9.1 computer program (DNASTAR, Madison, WI, USA). After each DNA

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**Table 2. Primers used for FOV race identification.**

| Primer name | Forward 5' to 3' | Reverse 5' to 3' | Amplicon size, bp |
|---|---|---|---|
| | Ribosomal DNA (rDNA) (1) | | |
| 18S | TTTCCGTAGGTGAACCTGCGGAAGG | - | 600 |
| 28S | - | CGCTTATTGATATGCTTAAACTCA | |
| | Translation elongation factor (EF-1α), (27) | | |
| EF-1 | ATGGGTAAGGAAGACAAGAC | - | 600 |
| EF-2 | - | GGAAGTACCAGTGATCATGTT | |
| | β-tubulin (BT), (18) | | |
| BT-3 | CGTCTAGAGGTACCCATACCGGCA | - | 500 |
| BT-5 | - | GCTCTAGACTGCTTTCTGGCAGACC | |

sequence from each selected genotype was proofed and trimmed using the software from the ABI DNA Genetic Analyzer (Applied Biosystems, Foster City, CA), sequence data were imported into the Lasergene V 9.1 computer program (DNASTAR, Madison, WI) for further analysis. EditSeq (DNASTAR, Madison, WI) was used as a sequence editor and import or export tool. Sequence identities were determined using BLAST from the National Center for Biotechnology Information (NCBI) (blast.ncbi.nlm.nih.gov). MegAlign (DNASTAR, Madison, WI) was used to generate pairwise and multiple sequence alignments of DNA and reports and percentage identity tables showing the numerical data underlying the comparisons. The Hein [**26**] and ClustalW [**27**] methods were used for aligning all sequences in the work-table. Phylogenetic trees were created using Phylogenetic Analysis Using Parsimony (PAUP) software [**28**] ver. 4.0 beta with parsimony option selected for analysis. To statistically support the clades 1000-times bootstrapping analysis [**29**] were performed using the PAUP software. Trees presented are depicted using tree figure drawing tool 'FigTree' ver.1.31. (http://tree.bio.ed.ac.uk/) from output file of PAUP.

## Results and discussions

### Fungal strains and host plant infection

Isolates of *Fusarium* from the Uzbekistan collection included in this study caused at least mild disease symptoms. Inoculated plants or seedlings showed wilt symptoms such as epinasty and slight dwarfing and chlorotic leaves. Brown to black spots were determined across most vascular tissues in root-stem cross section of infected plants. Similar wilt symptoms have been reported in different FOV studies [**1**,**3**-**6**]. The identity of most of all the isolates recovered from inoculated plants from Uzbekistan was confirmed by EF-1α, BT, and rDNA marker-gene sequences as described below.

### Phylogenetic analyses

We collected genomic DNA for most known races of FOV worldwide to identify *Fusarium* isolates from the Uzbekistan phytopathogen collection. We sequenced fragments of specific marker-genes such as elongation factor (EF-1α), beta-tubulin (BT), and ribosomal DNA (rDNA) using primer-pairs recently used to identify the races of the FOV pathogen (**Table 2**). DNA sequences were compared to published FOV DNA marker-gene sequences using BLAST from the NCBI (blast.ncbi.nlm.nih.gov) to confirm similarity to FOV. Except for two unknown Uzbek isolates (FOV_319 and FOV_444), DNA sequences for all gene fragments of remaining samples used in our study (**Table 1**) were confirmed to belong to *Fusarium oxysporum f. sp. vasinfectum*. BLAST results of EF-1α sequences indicated that isolate FOV_319 and FOV_444 more likely belongs to other *Fusarium* species (data not shown); therefore, they were removed from further analyses with EF-1α sequences.

Phylogenetic analysis based on individual marker-gene sequence data statistically clustered all FOV samples into distinct groups. Although sequence data from all genes used in our study revealed similar phylogenetic groups and tree topology, herein, we first present our results only using EF-1α marker-gene to explain and view the generated phylogram. We removed the biological replications for some samples (*eg.,* for isolate no 316 or one of the highly similar FOV sequences for Australia isolate) from the final tree (**Figure 1**). The phylogenetic tree demonstrated that all unknown *Fusarium* isolates of Uzbekistan clustered into 3 major clades with known races of FOV (**Figure 1**). FOV strain from Australia (FOV_AUSTRALIA_19_AY714097) and isolate Group C (LA_112_Group_C) were placed as out groups. The first clade included the known race 3 of FOV [**10**]. One of the unknown Uzbekistan FOV isolate (no. 316) was included with the race 3 FOV clade. The second clade included the known races 4 and 7 of FOV [**3**,**8**] with the unknown Uzbekistan FOV isolate no. 328, which also clustered within the FOV strain Group A (LA_110 Goup A). The third clade included known races 1, 2 &6 and one of unknown Uzbekistan FOV isolate no. 375. Phylogenetic clades 2 and 3 were closer to each other and rooted from one of the known FOV genotype - race 8 (**Table 1** and **Figure 1**).

These phylogenetic groups based on EF-1α gene sequence can also be clearly demonstrated with pairwise genetic distance shown in **Table 3**. Out-group FOV samples were distantly related and three major clades share greater genetic similarity within each clade (**Table 3** and **Figure 1**). Even though FOV races

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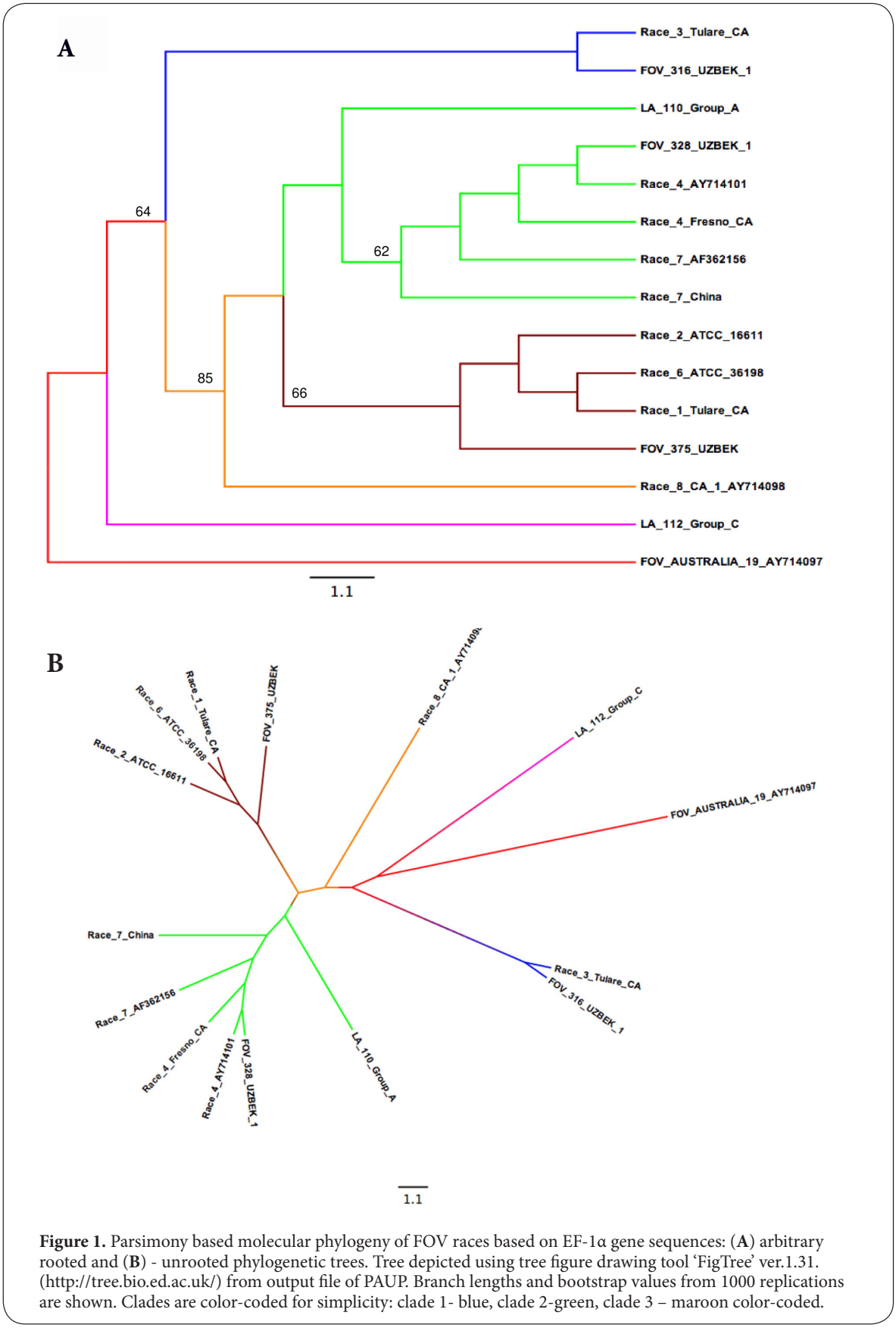


**Figure 1.** Parsimony based molecular phylogeny of FOV races based on EF-1α gene sequences: (**A**) arbitrary rooted and (**B**) - unrooted phylogenetic trees. Tree depicted using tree figure drawing tool 'FigTree' ver.1.31. (http://tree.bio.ed.ac.uk/) from output file of PAUP. Branch lengths and bootstrap values from 1000 replications are shown. Clades are color-coded for simplicity: clade 1- blue, clade 2-green, clade 3 – maroon color-coded.

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**Table 3. Pairwise genetic distance estimates based on Elongation Factor (EF-1α) gene sequence that were generated using MegAlign (DNASTAR).**

| Fusarium Wilt | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Race_1_Tulare_CA | *** | 99.8 | 96 | 99.5 | 99.5 | 100 | 99.5 | 98.7 | 97.1 | 97.4 | 99.5 | 99.8 | 99.5 | 99.4 | 96.1 |
| Race_2_ATCC_16611 | 0.2 | *** | 96.1 | 99.7 | 99.7 | 99.8 | 99.7 | 98.9 | 97.2 | 97.6 | 99.7 | 99.7 | 99.7 | 99.5 | 96.3 |
| Race_3_Tulare_CA | 1.3 | 1.1 | *** | 96.1 | 96.1 | 96 | 96.1 | 97.1 | 97.9 | 98.5 | 96.1 | 95.8 | 96.1 | 96 | 93.5 |
| Race_4_Fresno_CA | 0.5 | 0.3 | 1.1 | *** | 100 | 99.5 | 100 | 98.9 | 97.2 | 97.6 | 100 | 99.4 | 100 | 99.5 | 96.1 |
| Race_4_AY714101 | 0.5 | 0.3 | 1.1 | 0 | *** | 99.5 | 100 | 98.9 | 97.2 | 97.6 | 100 | 99.4 | 100 | 99.5 | 96.1 |
| Race_6_ATCC_36198 | 0 | 0.2 | 1.3 | 0.5 | 0.5 | *** | 99.5 | 98.7 | 97.1 | 97.4 | 99.5 | 99.8 | 99.5 | 99.4 | 96.1 |
| Race_7_China | 0.5 | 0.3 | 1.1 | 0 | 0 | 0.5 | *** | 98.9 | 97.2 | 97.6 | 100 | 99.4 | 100 | 99.5 | 96.1 |
| LA_110_Group_A | 0.5 | 0.3 | 1.1 | 0.3 | 0.3 | 0.5 | 0.3 | *** | 98.1 | 98.4 | 98.9 | 98.5 | 98.9 | 98.7 | 95.3 |
| LA_112_Group_C | 1.3 | 1.1 | 1.3 | 1.1 | 1.1 | 1.3 | 1.1 | 1.1 | *** | 99 | 97.2 | 96.9 | 97.2 | 97.1 | 94.4 |
| FOV_316_UZBEK_1 | 1 | 0.8 | 0.7 | 0.8 | 0.8 | 1 | 0.8 | 0.8 | 1 | *** | 97.6 | 97.2 | 97.6 | 97.4 | 94.7 |
| FOV_328_UZBEK_1 | 0.5 | 0.3 | 1.1 | 0 | 0 | 0.5 | 0 | 0.3 | 1.1 | 0.8 | *** | 99.4 | 100 | 99.5 | 96.1 |
| FOV_375_UZBEK | 0.2 | 0.3 | 1.5 | 0.7 | 0.7 | 0.2 | 0.7 | 0.7 | 1.5 | 1.1 | 0.7 | *** | 99.4 | 99.2 | 96 |
| Race_7_AF362156 | 0.5 | 0.3 | 1.1 | 0 | 0 | 0.5 | 0 | 0.3 | 1.1 | 0.8 | 0 | 0.7 | *** | 99.5 | 96.1 |
| Race_8_CA-1_AY7141098 | 0.7 | 0.5 | 1.3 | 0.5 | 0.5 | 0.7 | 0.5 | 0.5 | 1.3 | 1 | 0.5 | 0.8 | 0.5 | *** | 96.1 |
| FOV_AUSTRALIA-19_AY714097 | 1.8 | 1.6 | 1.8 | 1.6 | 1.6 | 1.8 | 1.6 | 1.6 | 1.8 | 1.5 | 1.6 | 2 | 1.6 | 1.8 | *** |

Percent of Similarity in upper triangle.
Percent of Divergence in lower triangle.

can be classified with gene-markers, certain FOV strains still are difficult to classify and place in specific lineages [30]. The phylogenetic results of this study suggest that in the Uzbekistan phytopathogen collection, FOV_375_Uzbek isolate belongs to the races 1, 2, and 6 of FOV group based on a 99.7 genetic distance (GD); FOV_316 Uzbek isolate belongs to race 3 of FOV (GD = 98.5); and FOV_328 Uzbek isolate belongs to the races 4 and 7 group (GD= 100). In a previous study [31], races 4 and 7 were indistinguishable genetically using marker-genes EF-1α and rDNA sequence data. However, pathogenicity tests have shown race 7 FOV from China to be different from race 4 FOV of India. Race 4 was more pathogenic to Pima cottons than race 7, while race 7 was more pathogenic to Upland cottons than race 4 [9]. Additional research is needed to resolve the conflict between pathogenicity tests and genetic relatedness studies for distinguishing races 4 and 7, and/or other conflicts between races.

To get additional confirmation of our FOV race findings, we combined sequence data for all three used genes (EF-1α, BT, and rDNA) in our study and reanalyzed the phylogenetic groupings as shown in **Figure 2**. Results were highly similar to what we previously observed with the EF-1α gene sequence analysis. However, one unknown FOV isolate from the Uzbekistan collection (FOV_444_Uzbek) grouped within the clade of FOV races 1, 2 & 6, revealing the race identity of this isolate. However, the other unknown isolate FOV_319_Uzbek remained very distant and was an out group for the tree presented in **Figure 2**. Based on NCBI blast results, isolate FOV_319_Uzbek might belong to *F. solani* (data not shown). These results showed that a combination of several gene sequences was useful to resolve differences in inter- and intraspecies level.

Similar phylogentic relationships have been reported for the classification of the known races of FOV using EF-1α and BT marker-genes [3,31,32]. For example, races 1 and 6 were reported to be similar with clades bootstrapping numbers of 86 and 65 [3,32] which were similar to our bootstrapping numbers (**Figures 1,2**). In addition, race 4 of FOV grouped closer to LA_110_Group_A [32], and in our study, the phylogenetic clade of races 4 and 7 was rooted to the LA_110_Group A FOV strain (**Figure 1**).

The marker-genes demonstrated a reliability of race identity for unknown FOV isolates from Uzbekistan using a comparative sequencing approach. This is a significant step forward because it allows us to understand FOV disease symptoms and its spread, and to correctly design and judge biological and genetic experiments. The *F. oxysporum* f. sp. *vasinfectum* pathogenicity mechanisms and the inheritance of FOV resistance-genes significantly differ among FOV races for cotton genotypes [6,15]. In recent inheritance and quantitative trait (QTL) studies [6,17,33], resistance to races 1, 7 and the Australian *Fov* races was reported to be inherited by gene interactions detected in more than one chromosome. Recently, a major dominant gene conferred resistance to race 4 FOV in Pima-S6 [6,15]. Collectively the results from these studies suggest a different gene-specificity of FOV resistance in cotton (*Fov1* (race 1) - chromosome 16, *Fov4* (race 4) – chromosome 14, $FW^R$ (race 7) - chromosome 17, and – Australian race of *Fov* – chromosomes 6, 22, and 25) [6,15,17,33].

## Conclusions

In this study, we explored the FOV isolates collected from Uzbekistan farmer's land and identified several FOV races,

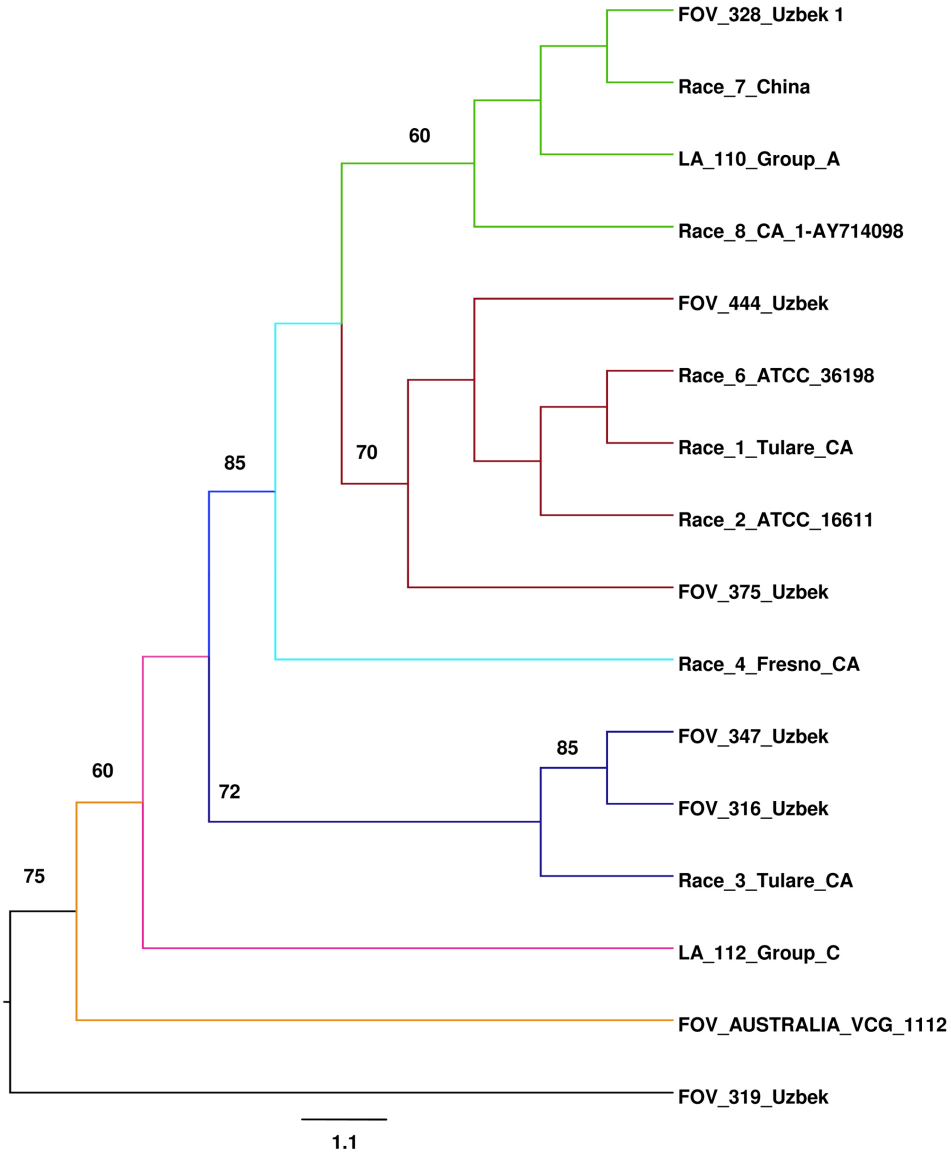


**Figure 2.** Phylogeny of FOV races based on combined analysis of EF-1α, β-tubulin, and rDNA gene sequences. Tree depicted using tree figure drawing tool 'FigTree' ver.1.31. (http://tree.bio.ed.ac.uk/) from output file of PAUP. Branch lengths and bootstrap values from 1000 replications are shown. Clades are color-coded for simplicity. Note: Due to unavailability of all gene sequences (EF-1α, β-tubulin, and rDNA) in the NCBI site. Race_7_AF362156, Race_4_AY714101, and FOV_AUSTRALIA_19_AY714097 sequences were not included in the combined analysis.

which add knowledge about FOV genotypes spread in Uzbekistan, helping to understand the genetic mechanisms of FOV resistance. Results should be useful for phytosanitary services in Uzbekistan to quickly diagnose and monitor the FOV in Uzbekistan as well as for running an efficient breeding programs to breed the resistant cultivars based on FOV genotypes attacking the cotton.

## Competing interests
The authors declare that they have no competing interests.

## Authors' contributions
SSE, MU, SS, JNJ and IYA designed the experiment, performed data collection and data analysis as well as drafted the manuscript. SSE, IBS, AA, ATA, and BES performed pathogenicity analysis, DNA isolation, sequencing of candidate genes and data analysis. LAG provided FOV isolates from the collection and prepared fungal cultures for the study. MU, SS, JNJ and IYA monitored the experimental work and critically read and edited the manuscript. All authors read and approved the final manuscript.

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