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ORIGINAL ARTICLE

Phylogenetic relationship of *Fusarium* species isolated from keratitis using *TEF1* and *RPB2* gene sequences

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ABSTRACT

Background and Objectives: *Fusarium* species are known to be one of the common causes of keratitis. This study was conducted to identify *Fusarium* spp. causing keratitis and to investigate their genetic diversity using *TEF1* and *RPB2* gene sequences.

Materials and Methods: Twenty-four clinical isolates of *Fusarium* were isolated from the patient with keratitis. Phylogenetic analysis of two-locus of the 24 clinical isolates and three reference strains was carried out using the maximum parsimony and RAxML methods.

Results: Based on gene sequences of the 24 clinical isolates, 17, 4, and 3 isolates were identified as *Fusarium solani* species complex (FSSC), *Fusarium fujikuroi* species complex (FFSC), and *Fusarium oxysporum*, respectively. FFSC include *F. proliferatum* (n=1), *F. globosum* (n=1), *F. verticillioides* (n=1), and *F. brevicatenulatum* (n=1), respectively.

Conclusion: Given that sequence of a sole gene can be challenging and on the other hand, due to the high resistance to antifungal drugs, identification of *Fusarium* species is of substantial significance. In this study, by designing a novel set of primers for the *RPB2* area and using *TEF1* primer, we were able to differentiate 24 *Fusarium* spp. isolated from patients with keratitis.

Keywords: Keratitis; *Fusarium oxysporum*; *Fusarium solani* species complex; *Fusarium fujikuroi* species complex

INTRODUCTION

Fungal keratitis is a corneal infection that occurs mostly in tropical and subtropical regions as well as in temperate climates following the fungal contaminants among farmers, corneal trauma, chronic ocular surface disease, and the use of contact lenses, respectively (1). A broad spectrum of filamentous fungi and yeasts causes fungal keratitis (2, 3). *Fusarium* species (spp.) are known as the most common cause

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of it in tropical and subtropical regions, particularly in developing countries, and it can be caused by its different members (4-7). Due to the resistance to a wide range of antifungal drugs, Keratitis caused by *Fusarium* spp. is of great importance, and eye loss is expected if such a disease is not treated appropriately (8, 9).

Recently, multilocus molecular phylogenetic studies have shown that *Fusarium solani* species complex (FSSC) and *Fusarium oxysporum* species complex (FOSC) are the common causes of fungal keratitis (8, 10-12). A new taxonomy has been done by Sandoval-Denis et al. that the FSSC updates under the genus name Neocosmospora (13). The particular conundrum between FSSC and Neocosmospora is still undergoing. This name change is still disputed by O'Donnell and Graser. Therefore, in this study, the FSSC is used. The FSSC consists of over 60 phylogenetic species that are morphologically homogenous and genetically heterogeneous and only 13 species are able to cause disease in humans (14-17).

In previous studies, the sequencing of Translation elongation factor 1-alpha (*TEF1*), Beta-tubulin (*TUB2*), RNA polymerase II (*RPB2*) genes, the Internal Transcribed Spacer (*ITS*), and the Ribosomal Intergenic Spacer (*IGS*) was performed to identify the *Fusarium* species (15-20). It seems that solo gen fails to identify the *Fusarium* spp. and the use of multiple genes is required (8, 5, 18, 21). Accordingly, the goal of the present study was accurate molecular identification of *Fusarium* spp. isolated from patients with keratitis based on two loci *RPB2* and *TEF1*.

Table 1. List of *Fusarium* isolates used in this study

	Isolate	
FSSC	F1	*F. solani*
	F4	*F. solani*
	F7	*F. solani*
	F9	*F. solani*
	F10	*F. solani*
	F11	*F. solani*
	F12	*F. solani*
	F24	*F. solani*
	F31	*F. solani*
	F32	*F. solani*
	F33	*F. solani*
	F42	*F. solani*
	F44	*F. solani*
	F45	*F. solani*
	F46	*F. solani*
	F50	*F. solani*
	F62	*F. solani*
	PTCC 5284	*F. solani*
FFSC	F3	*F. globosum*
	F13	*F. proliferatum*
	F25	*F. verticillioides*
	F49	*F. brevicatenulatum*
	PFCC 48-125	*F. proliferatum*
FOSC	F15	*F. oxysporum*
	F16	*F. oxysporum*
	F54	*F. oxysporum*
	PTCC 5115	*F. oxysporum*

MATERIALS AND METHODS

Clinical isolates. The present study was conducted on 24 patients with keratitis over a period of one year at Farabi Hospital in Tehran (Table 1). Following the microscopic examination with Gram staining and observation of hyaline hyphae, the samples were cultured on Blood agar and Sabouraud Dextrose Agar (SDA) (Merck, Germany). The samples were inoculated at three points on each plate and incubated at 25°C for 5 days (22). Also, reference strains of *F. solani* complex (PTCC 5284), *F. oxysporum* (PTCC 5115), and *F. proliferatum* (PFCC 48-125) were used in sequence analysis.

DNA extraction. The genomic DNA was extracted from all 24 isolates and three reference strains according to Salehi et al. (23). Sections from the *TEF1* and *RPB2* genes were chosen for sequencing. To amplify these regions, a novel set of primers for the RPB2 area was designed using Lasergene 7 software (Table 2). PCR assays exist in 50 µl reaction, including 25 µl of Premix, 3 µl of DNA extract, 0.8 µM of each primer, and water. PCRs were performed in a thermocycler (Eppendorf, Germany) under the following conditions: 35 cycles of 30 seconds at 94°C, annealing at changing temperatures for 40 seconds (Table 2), 45 seconds at 72°C, and a terminal extension step of 72°C for 7 minutes (24).

Sequencing and gene diversity. PCR products were purified and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. Forward and the reverse DNA sequence were

Table 2. Sequencing primers used in the two loci *RPB2* and *TEF1*

Locus	Primers	Sequence (5` --- 3`)	Annealing temp (°C)	Reference
TEF-1α	Forward Reverse	ATGGGTAAGGAGGACAAGAC GGAAGTACCAGTGATCATGTT	56	21
RPB2	Forward Reverse	CGGTAGCTGGGTTGGTGTTCA TTGGCCCGTTTCTCTTCTTC	62	Manual design

edited, aligned, and analyzed using MEGA7.0.21 software. Subsequently, the nucleotide polymorphic sites were identified. The species were identified and compared using two databases, namely, the CBS (http://www.cbs.knaw.nl/fusarium/) and BLASTn (http://www.ncbi.nlm.nih.gov/genbank/). All isolates were verified to the species level by the *TEF1* sequence.

Phylogenetic analysis. The maximum parsimony (MP) analysis was performed using MEGA7.0.21 software. Maximum likelihood (ML) analysis (25) was performed using the RAxML (26). Optimization in RAxML was carried out using the GTRCAT option. Bootstrap values for maximum likelihood were 1000 replicates, with one search replicate per bootstrap replicate. *Fusarium graminearum* was used as an outgroup to root the dendrogram. The discriminatory power was calculated according to Hunter's formula (27).

RESULTS

Clinical isolates. Totally 24 clinical isolates and three reference strains grow on the SDA. In the event that the fungal species grow on both plates (Blood agar and SDA) and they are the same at all three inoculation points, the fungal species is identified as a cause of keratitis.

Molecular identification. Although it is easy to use the databases to identify the unknown sequences, the interpretation of the corresponding results is challenging. Identification of strains was performed using databases of the CBS and NCBI. As Table 1 represents, the most common isolates belonged to FSSC (n = 17, 70.8%), followed by *Fusarium fujikuroi* species complex (FFSC) (n=4, 16.6%), and *F. oxysporum* (n=3, 12.5%). All 17 identified isolates of the complex were *F. solani*. FFSC, including *F. proliferatum* (n=1), *F. globosum* (n=1), *F. verticillioides* (n=1), and *F. brevicatenulatum* (n=1), respectively. Only *F. oxysporum* was observed in the FOSC members.

Phylogenetic analyses. Phylogenetic analyses and species limits of 27 isolates were performed using the two loci *RPB2* and *TEF1*. In general, 1523 bp of aligned nucleotide sequence was studied. The highest and lowest sequences belonged to FFSC for the *TEF1* gene, *F. oxysporum*, and FFSC for the *RPB2* region, respectively.

The discriminatory power of the two loci *RPB2* and *TEF1* for *F. solani, F. oxysporum*, and FFSC was estimated to be 1.

MP analyses of *TEF1* and *RPB2* genes revealed that the highest informative sites are present in *F. solani* species. Overall, the most informative sites were seen in the *TEF1* gene.

The phylogenetic tree drawn using the RaxML phylogenetic analyses of the combined data set is shown in Fig. 1. Also, The FSSC clade received the highest possible support. FFSC clade is phylogenetically and most closely related to *F. oxysporum*. Also, in the consensus tree constructed, the *F. globosum* of the FFSC was closer to *F. solani* (FSSC) and the *F. verticillioides* were closer to the *F. oxysporum.* The *F. oxysporum* clade takes a basal position to the aforementioned clades.

DISCUSSION

Since most of the *Fusarium* spp. are seen in the complex form and sequence of a sole gene can be challenging, therefore in the current study, a scheme was developed from two genes, namely *TEF1* and RPB2. Sequencing was used to examine the molecular diversity among the *Fusarium* spp. isolated from patients with keratitis. According to the results ob-

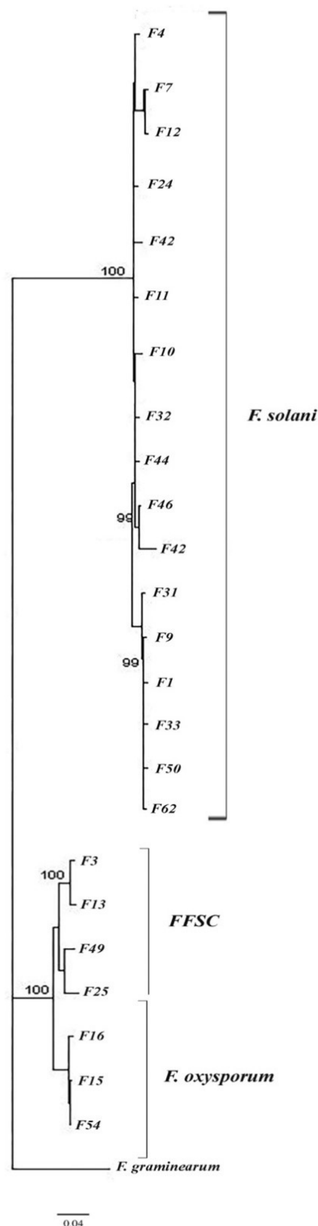

Fig. 1. The consensus tree constructed by the Maximum likelihood analysis from the combined two-locus *TEF1* and *RPB2* of the 24 clinical isolates and three reference strains. Bootstrap branch supports above 95% are shown. *F. graminearum* was used as an outgroup to root the dendrogram.

tained in this study, the dominant species of keratitis infection was identified to be *F. solani* from FSSC (70.8%), which is supported by da Rosa et al. (8) and Sun et al. (28). The results of da Rosa et al. (8) study were different only in that *F. solani* was followed by FOSC, while in the present study and Sun et al. study (28), *F. solani* was followed by FFSC as the dominant species. The results may be attributable to the low number of samples.

In this study, to increase the accuracy concerning the results obtained from the sequence in forward and reverse, as well as to shorten the length of the RPB2 area examined, a new primer was designed. A comparison of the discriminatory power obtained in this study with that of the previous studies (21, 29) shows this primer is sufficiently efficient.

The results of the genotypes *TEF1* of FSSC showed that almost every isolate has an exclusive sequence (single genetically).

The resulting phylogenetic trees showed a high degree of genetic similarity between FFSC which is recognizable from other complexes. Also, a comparison of the dendrogram obtained from the combination of two genes with the dendrogram derived from only individual *TEF1* tree and individual *RPB2* tree (results were not shown) showed high levels of similarity.

Sequencing of the *TEF1* and *RPB2* genes manifested that such genes can differentiate between the FSSC members as well it does with the FGSC members. The study of Sun et al. (28) confirms the afore-mentioned result.

The MP analysis showed no parsimony-informative for *F. oxysporum* in *TEF1* and *RPB2* genes. The most parsimony informative sites were observed in FSSC, in which, the *TEF1* gene had the most informative site (Table 3). However, the most informative gene was *TEF1*. In a similar vein, the study conducted by O'Donnell et al. (10, 20) confirms our results.

CONCLUSION

Given that sequence of a sole gene can be challenging and on the other hand, due to the high resistance to antifungal drugs, identification of *Fusarium* species is of substantial significance. Furthermore, accurate identification of species is important when it comes to evolution of taxonomy, the discovery of new fungal species associated with clinical infections, and a better understanding of their epidemiology and diagnosis. Therefore, in this study, by designing a novel set of primers for the *RPB2* area and using *TEF1* primer, we were able to differentiate 24 *Fusarium* spp. isolated from patients with keratitis. The results of this study showed a very high diversity of *Fusarium* spp. using the designed primers.

Table 3. Characteristics obtained from the MP analysis and phylogenetic analysis of the five housekeeping loci studied

Locus	Fusarium isolates	Amplicon size (bp)	SNPs (No.)	Allele types	PIC (No.)	PIC %
TEF1	FSSC	741	73	17	31	4.1
	FFSC	717	85	1	22	3
	F. oxysporum	712	13	3	0	0
RPB2	FSSC	457	92	17	23	4.4
	FFSC	447	8	1	0	0
	F. oxysporum	447	6	3	0	0

SNP, single-nucleotide polymorphism; PIC; parsimony-informative character

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