http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 24(1): 3-9, 2023
ISSN 2528-9691
DOI: 10.23902/trkjnat.1144980

Research Article

CHARACTERIZATION OF *cox3* AND *rnl* GENES ENCODED IN MITOCHONDRIA OF *Fusarium graminearum* Schwabe

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Cite this article as:

Gazdağlı A. & Albayrak G. 2023. Characterization of *cox3* and *rnl* genes encoded in mitochondria of *Fusarium graminearum* Schwabe. *Trakya Univ J Nat Sci*, 24(1): 3-9, DOI: 10.23902/trkjnat.1144980

Received: 18 July 2022, Accepted: 26 October 2022, Online First: 11 November 2022, Published: 15 April 2023

Edited by:
İskender Karaltı

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Key words:
Fusarium graminaerum
Mitochondrial genes
Polymerase chain reaction (PCR)
Sequencing
Phylogenetic relationship

Abstract: In this study, the phylogenetic relationship among *Fusarium graminearum* Schwabe isolates was established for the first time based on mitochondrial *cox3* and *rnl* gene variations. The genes were amplified from 45 isolates purified from Türkiye and Iran together with 2 Korean strains by polymerase chain reaction. The amplicons were sequenced and nucleotide polymorphisms were detected by alignment. The phylogenetic relationship was constructed by using PAUP 4.0a with the maximum parsimony method. Fragments with 477 bp length, belonging to *cox3*, were obtained from 46 samples; 1547 bp-amplicons of *rnl* were produced from 45 samples. Sequence similarities were calculated as 30-100 % and 17-94 % for *cox3* and *rnl*, respectively. Nucleotide variations within the *rnl* was found higher than within *cox3*. It was shown that SNPs and *in-dels*, found in coding regions, cause a codon change and may alter more than one codon by causing frame shift without affect gene functions. Bootstrap values belonging to *cox3* and *rnl* dataset was found ranging from 57 to 84 %, and 54 to 100 %, respectively. Parsimony analysis revealed that Korean isolates were in monophyletic relationship with Turkish and Iranian isolates. It is proposed that the methodology can be applied to other fungal species because the phylogenetic relationships at the intraspecific level are able to establish among *Fusarium* species based on mitochondrial gene variation.

Özet: Bu çalışmada, mitokondriyal *cox3* ve *rnl* genlerinin varyasyonlarına dayalı olarak *Fusarium graminearum* izolatları arasındaki filogenetik ilişki ilk kez ortaya kondu. *cox3* ve *rnl* genleri polimeraz zincir reaksiyonu ile 2 Kore suşu ile Türkiye ve İran'dan saflaştırılan 45 izolattan çoğaltıldı. Amplikonlar dizilendi ve hizalama analizleri ile nükleotid polimorfizmleri tespit edildi. Filogenetik ilişki, maksimum tutumluluk yöntemiyle PAUP 4.0a programı kullanılarak oluşturuldu. 46 örnekten *cox3*'e ait 477 bp uzunluğunda fragmentler çoğaltılırken; *rnl* genine ait 1547 bp-amplikon 45 örnekten çoğaltıldı. *cox3* ve *rnl* için dizi benzerlikleri sırasıyla %30-100 ve %17-94 aralığında hesaplandı. *rnl* gen bölgesinde, *cox3*'e kıyasla nukleotid varyasyonlarının daha yüksek oranda taşındığı belirlendi. Kodlama bölgelerinde bulunan SNP'lerin ve in-del'lerin gen fonksiyonlarını etkilemeden çerçeve kaymasına neden olduğu ve bu varyasyonların birden fazla kodonu değiştirebildiği belirlendi. Filogenetik analizlerle hesaplanan *cox3* ve *rnl* veri setine ait bootstrap değerleri sırasıyla %57 ile %84 ve %54 ile %100 arasında hesaplandı. Parsimoni analizi, Kore izolatlarının Türk ve İran izolatları ile monofiletik ilişki içinde olduğunu ortaya koydu. *Fusarium*'da tür içi filogenetik ilişkilerin mitokondriyal gen varyasyonuna dayalı olarak belirlenebilmesi nedeniyle yöntemin diğer mantar türlerine de uygulanabileceği önerilmektedir.

Introduction

Fusarium graminearum Schwabe is one of the homothallic filamentous phytopathogenic fungal species. This species has haploid genome in the most of life cycle (Goswami & Kistler 2004). The mitochondrial DNA (mtDNA) of the phytopathogen, carried as multiple copies per organelle as in other eukaryotic organisms, is composed of only one compact double stranded circular DNA. The size of mtDNAs of *F. graminearum* is larger compared to other species of the genus and the lengths of the genomes of different species are variable due to presence or absence of intronic regions (Brankovics *et al.* 2018, Yang *et al.* 2020). Approximately one fifth of the mtDNA (20.9 %) is responsible for coding two rRNAs, about twenty-eight tRNAs and fourteen proteins involved in oxidative phosphorylation (Al-Reedy *et al.* 2012). However, the GenBank data revealed that more open reading frames (ORFs) including intronic and unidentified ones participated in the gene expression. Group I introns are located in the interrupted gene structure of the mitochondrial genomes. Such intervening

elements are known as ribozymes because they catalyse their excision from precursor transcripts (Hausner 2003). The mobility of group I introns is carried out through the products of their intron-encoded ORFs. The horizontal gene transfer and number and mobility of the introns are mainly responsible for size differences of mitochondrial genomes. Direct and indirect repeats are located in mostly intragenic regions of mtDNA. Among them, nine direct repeats located in intergenic regions may be found in the terminal regions of the mitochondrial genes and can participate in their ORFs (Al-Reedy *et al.* 2012). Recombination between repetitive sequences are also responsible for size differences of mitochondrial genomes.

Only a few genes in the mtDNA are shared by all eukaryotic cells (Gray 1999, Bietenhader *et al.* 2012*)*. The *cox3* and *rnl* genes are present in all mitochondrial genomes. *cox3* is responsible for encoding the cytochrome c oxidase subunit III (COIII). In spite of nearly invariant, COIII is not essential for proton pump but it is structural stabilizer of the cytochrome c oxidase (Haltia *et al.* 1991). In *F. graminearum*, the gene is composed of three exon and two intron regions. After cutting of all introns in the lengths of 1054 and 1433 bps coding the proteins with endonucleolytic activity, remaining sequences, composed of three exons with 3742 bp (exon1 219 bp; exon2 114 bp; exon3 477 bp), do constitute the ORF of the gene. A total of 5661 bp length of *rnl* gene codes the large subunit rRNA which is found in the structure of ribosomes. The gene contains only one intron (1992 bp in length). Since the 482 bp region of this intervening sequence encodes ribosomal protein 3, it was named as *rps3* gene. As a result, the *rnl* gene bears the *rps3* that overlaps with itself. The *atp9* gene, found in the mitochondria of *F. graminearum*, is responsible for coding the ATP synthase protein subunit c. This gene is 1298 bp in length and consists of two exons of 180 and 44 bps in length (Hensgens *et al.* 1979, Déquard-Chablet *et al.* 2011).

Analysis of mtDNA, which has a rapid rate of evolution, presents valuable data for phylogenetic and lineage studies and provide proper data for understanding the evolutionary history of fungi (Láday *et al.* 2004, Kulik *et al.* 2020). Variations of mtDNAs in different *Fusarium* spp. were screened on both total DNA and mtDNA through RFLP marker analysis (Láday *et al.* 2004, Kistler 2008). Nucleotide variations found in mitochondrial genes made possible to accurately measure genetic diversity, to identify isolates at sub-species level, and to determine phylogenetic relationships among isolates (Avise & Sep 1989, Láday *et al.* 2004). Intragenic diversity of genes enabled not only to be revealed the plasticity of mitochondrial genes but also to be determined the phylogenetic relationships. In this study, diversity of mitochondrial genes in *F. graminearum* isolates purified from different geographical regions of Türkiye and Iran were investigated for the first time. Phylogenetic relationships among the isolates were constructed through evaluation of specific mitochondrial genes variations.

Materials and Methods

Pathogen Growth Conditions and mtDNA Isolation

Forty-seven *F. graminearum* isolates were provided from Department of Plant Protection, Agricultural Faculty, Ondokuz Mayis University (Samsun, Türkiye), from Department of Plant Protection, College of Agriculture, Isfahan University of Technology (Isfehan, Iran) and from Department of Agro-Food Safety and Crop Protection, National Institute of Agricultural Sciences (Seoul, Korea). Agro-ecological locations and the hosts of the isolates are listed in Table 1.

Table 1. *Fusarium graminearum* isolates, their agro-ecological locations, chemotypes and mating types.

Code	Host	Region	Chemotype	MAT1/ MAT2
FgF5	Wheat	Sakarya	15ADON	+
FgF6	Wheat	Sakarya	15ADON	+
FgF7	Wheat	Sakarya	15ADON	+
FgF8	Wheat	Sakarya	15ADON	+
FgF9	Wheat	Balıkesir	15ADON	+
Fg15F	Wheat	Sakarya	15ADON	+
Fg3F	Corn	Samsun	15ADON	+
Fg5F	Corn	Samsun	15ADON	+
Fg6F	Corn	Samsun	15ADON	+
Fg7F	Corn	Samsun	15ADON	+
Fg14F	Wheat	Kastamonu	3ADON	+
Fg1F	Wheat	Bolu	NIV	+
Fg2F	Wheat	Çankırı	15ADON	+
Fg4F	Barley	Bolu	15ADON	+
FgM1	Wheat	Mazandaran	NIV	+
FgM3	Wheat	Mazandaran	NIV	+
FgM5	Wheat	Mazandaran	NIV	+
FgM6	Wheat	Mazandaran	NIV	+
FgM7	Wheat	Mazandaran	NIV	+
FgM9	Wheat	Mazandaran	NIV	+
FgM10	Wheat	Mazandaran	NIV	+
FgT2	Wheat	Mazandaran	NIV	+
FgT3	Wheat	Mazandaran	NIV	+
FgT7	Wheat	Mazandaran	NIV	+
FgT9	Wheat	Mazandaran	NIV	+
FgT10	Wheat	Mazandaran	NIV	+
FgT11	Wheat	Mazandaran	NIV	+
FgT12	Wheat	Mazandaran	NIV	+
FgT16	Wheat	Mazandaran	NIV	+
Fgsh1	Wheat	Mazandaran	DON	+
Fgsh4	Wheat	Mazandaran	NIV	+
Fgsh5	Wheat	Mazandaran	NIV	+
Fgsh7	Wheat	Mazandaran	DON	+
Fgsh10	Wheat	Mazandaran	NIV	+
Fg4	Wheat	Mazandaran	NIV	+
Fg18	Wheat	Moghon	NIV	+
Fg56	Wheat	Gorgan	NIV	+
Fg49	Wheat	Moghon	DON	+
Fg170	Wheat	Gorgan	NIV	+
Fg174	Wheat	Gorgan	NIV	+
Fg5	Wheat	Sari	NIV	+
Fg165	Wheat	Kordkooy	NIV	+
Fgsh13	Wheat	Unknown	15ADON	+
Fgsh14	Wheat	Unknown	DON	+
Fgsh15	Wheat	Unknown	DON	+
H-11	Corn	Korean	15ADON	+
88-1	Barley	Korean	NIV	+

Fusarium isolates were grown on potato dextrose agar (PDA) plates at 25ºC and 50 % humidity for six days in a growth chamber. mtDNA was isolated from 20 mg fresh tissue using Mitochondrial DNA Isolation Kit (Biovision, USA) according to the manufacturer's protocol.

Amplification And Sequencing of mt Genes

Complete mt genomes with the accession numbers DQ_364632.1 and NC_009493.1 for *F. graminearum*, JX10420.1 for *F. fujikuroi* Nirenberg and AY94258.1 for *F. oxysporum* Schltdl. were monitored from the National Center for Biotechnology Information (NCBI). Gene sequences of *cox3* and *rnl* were screened on mtDNAs and each sequence were aligned via CLUSTALW 2.1 program. The primer pairs for each gene were designed for identical nucleotides found in the fragments contained consensus sequences (Table 2). Amplification of *cox3* and *rnl* by PCR was carried out in 25 µl reaction mixtures containing 1 × buffer, 25 ng of genomic DNA, 0.5 µM of primer, 0.2 mM of dNTPs, 2.5 mM $MgCl_2$ and 1 U of *Taq* polymerase. PCRs were performed under the following conditions: 35 cycles of 94ºC for 1 min, 55-62ºC for 1 min and 72ºC for 2 min. Pre-denaturation (at 94ºC for 5 min) and final extension (at 72ºC for 10 min) steps were also carried out. Amplification products were separated by electrophoresis,purified with a commercial kit (Roche, Switzerland) and were sequenced as bidirectional by using the Sanger method (ABI PRISM 3100 Genetic Analyser, USA). ABI chromatograms of each gene sequences were exported as FASTA format and separately aligned with *cox3*, *rnl* and *atp9* sequences of NC_009493.1 reference genome through CLUSTALW 2.1 (Larkin *et al.* 2007).

Data Mining and Phylogenetic Analysis

Similarity among *F. graminearum* isolates were calculated using NJ algorithm (Jeanmougin *et al.* 1998). In order to construct the phylogenetic relationships, unweighted maximum parsimony (MP) analysis of the isolates and combined datasets were conducted with PAUP version 4.0a (Swofford 2004). The most parsimonious trees were searched by using a heuristic search with 1000 random replicates and tree-bisection with reconnection branch swapping. Obtained final datasets were used to determine putative alterations in open reading frames (ORF) via NCBI-ORF FINDER. Amino acid sequences belonging to putative ORF regions and their variations were determined by BLASTP aligning.

Results

Detection of cox3 and rnl Genes and Their Variation Analysis

Specific primers designed for amplifying *cox3* and *rnl* genes in 47 *F. graminearum* isolates are listed in Table 2.

Each gene was separately amplified through PCR (Fig. 1). Amplicons with 477 bp length belonging to exon 3 of *cox3* was obtained from all samples except for Fg170 (Fig. 1a).

Similarity coefficients among isolates ranged from 30 to 100 %. Two groups were found to have identical sequences. One group included Fg15F, Fg6F, Fg14F, Fg1F and FgM9 isolates and the other one included Fg2F, FgT11, FgFg56 and Fgsh15 isolates. The highest variation was calculated between the isolates FgM3 and Fg56 as 70 %. According to BLASTn analysis, the nucleotide sequences of *cox3* belonging to FgT7, FgT10 and Fg174 isolates were significantly different from the reference genome and from each other. The *cox3* gene region amplified in the current study is responsible for coding 38 amino acid length residues in the reference genome (NC_009493.1). Amplified regions from remaining isolates were compatible with the region of the reference genome. However, transversion- and transition-type mutations and *in-dels* were found to be in the gene sequences. BLASTp analysis displayed that these types of variations in most of the isolates resulted in silent and missense mutations within the same ORF. In addition, it was also revealed that some of the variations caused shortening of ORFs via nonsense mutations in a few isolates. No frame shift mutations were detected within the partial sequence of *cox3*.

Table 2. Oligonucleotide primer sequences used in this study

Gene	Primer	Primer Sequence 5'-3'	Product Size (bp)
cox3	Forward	GTGCATTAACACCTACTGTAGAA	477
	Reverse	CTAACTTCCTCAGTAATACAT	
rnl	Forward	ACGTAGTTAAACCGAGCGTTA	1547
	Reverse	TCCTAAACCAATTCATTCATA	

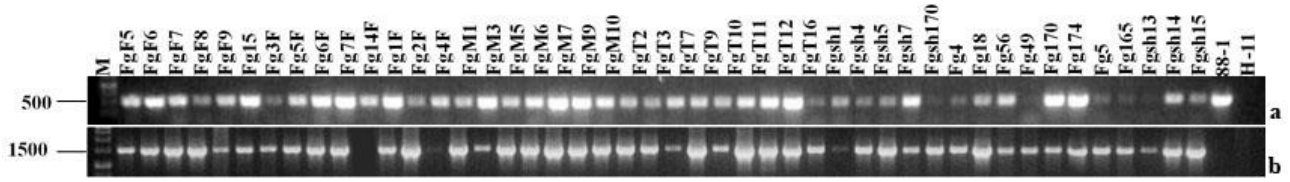

Fig. 1. Agarose gel electrophoresis of PCR products of *F. graminearum* isolates. **a.** 477 bp for *cox3* gene, **b.** 1527 bp for rnl gene. M: 1 kb DNA ladder (Thermo, ABD), NT: No Template

The partial region of *rnl* gene with the length of 1547 bp were produced from 45 isolates. But, primer pair designed for exon 1 sequences did not anneal to the gene in Fg14F and FgH11 isolates (Fig. 1b). In sequenced region of *rnl*, while the highest similarity was calculated between FgM6 and Fg18 as 94 %, the highest variation was detected between FgF7 and Fg5 as 83 %. When sequenced nucleotide fragments of *rnl* gene were aligned, deletions ranging from 1 to 13 nucleotides and insertions ranging from 1 to 22 nucleotides were found to be present in addition to single nucleotide polymorphisms (SNPs).

Phylogenetic Analysis of cox3 and rnl

Aligned partial DNA sequences of *cox3* and *rnl* genes were analysed by MP in order to estimate the phylogenetic relationships of *F. graminearum* isolates obtained from different geographic origins. Bootstrap values belonging to *cox3* dataset were found to range from 57 to 84 %. Two isolates, FgF8 and Fg3F, were located in monophyly out group of the generated cladogram with a bootstrap value of 70 %. Fgsh13, FgM3 and Fg165 produced a monophyly subgroup (MP-BS=57%). Five isolates (Fg7F, FgF49, Fgsh10, FgM7, Fg5) displayed polyphyletic branching with 84 % bootstrap value (Fig. 2). The remaining 30 isolates were placed in monophyletic cladogram together with other isolates.

The MP-BS values of *rnl* gene dataset was found range from 54 to 100 %. F5 and Fg174 formed monophyly outgroup (MP-BS= 61 %). It was determined that the 23 isolates placed into cladogram to constitute two monophyletic sub groups. The Fgsh5 and Fg5 isolates were monophyletic sisters of the two sub phenons (MP-BS= 100 %). Six isolates together with the reference (NC_009493.1), added to the study from NCBI database, were present into sub group I (MP-BS=83 %). FgF6 and Fg7F showed monophyletic relationship in this sub group (MP-BS= 82 %). Sub group II (MP-BS=52 %) consisted of remaining isolates, included Korean sample (88-1). Isolates Fg5F/FgT12 and Fg6F/FgT2 ranked as couple monophyly subgroups with 100 % bootstrap value (Fig 3). It was determined that Fg4/Fg15F, FgF7/FgF8, FgM1/FgM10 were genetically associated with bootstrap values of 93, 91, 58 %, respectively. The most varied nucleotide sequences were detected in Fg5 isolate. Similarity between Fg5 and sh5 were calculated as 62 %.

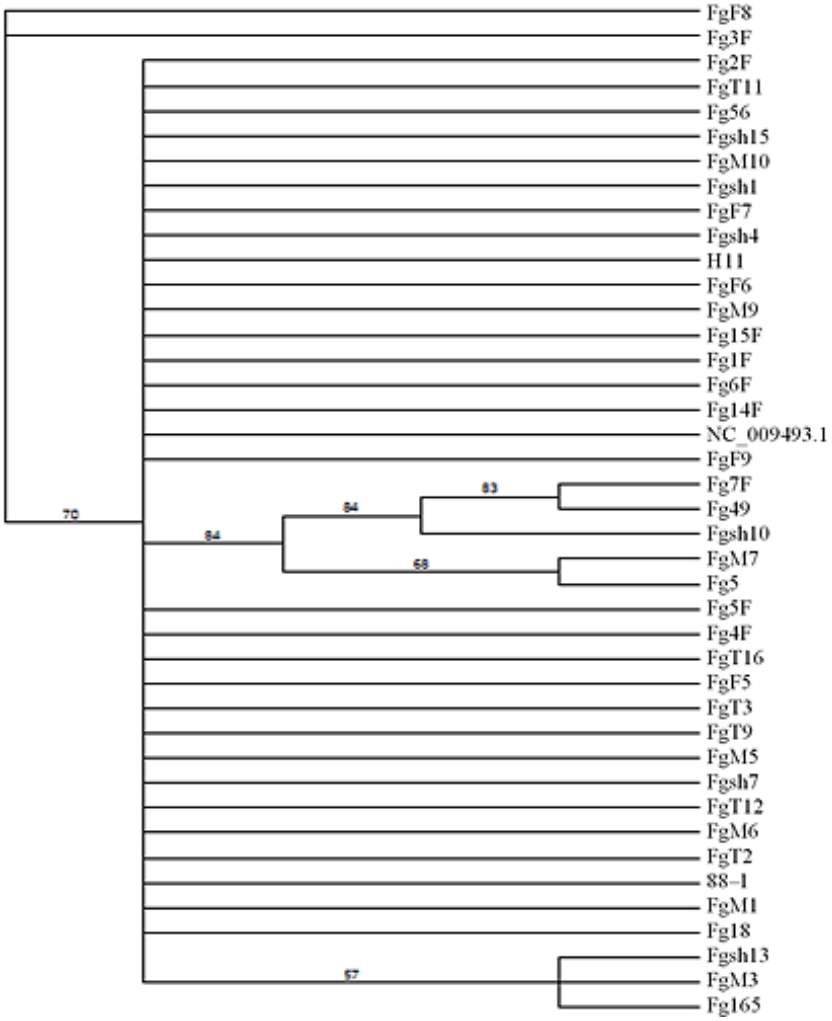

Fig. 2. Most parsimonious tree obtained from *cox3* gene dataset.

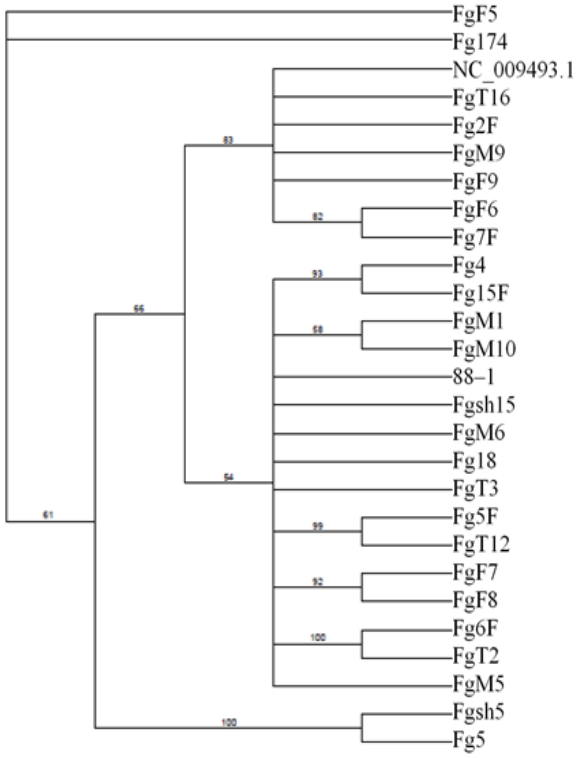

Fig. 3. Most parsimonious tree obtained from *rnl* gene dataset.

Discussion

The mtDNA analysis not only enables the detection of genetic exchange among populations, it also makes it possible to distinguish subpopulations without being related to geographic origins and genetic characteristics (Kistler *et al.*1987). The mtDNA studies fundamentally reveal the phylogenetic relationships of genomes belonging to distinct organisms (Abboud *et al.* 2018, Funk *et al.* 2018). Because of broad spread of *Fusarium* spp. all over the world, determination of species and construction of their phylogenetic relationships are important to differentiate haplotypes (Kim *et al.* 1993). Comparison of genetic diversity and divergence among these species have been mostly performed by the multiple genotyping assays of nuclear DNA (nDNA) (Taylor *et al.* 2000, Yli-Mattila *et al.* 2009, Sarver *et al.* 2011). In the present study, the phylogenetic relationship among *F. graminearum* isolates was investigated based on the analysis of *cox3* and *rnl* genes encoded in mitochondria. Molecular characterization of the two genes were carried out in 45 *F. graminearum* isolates from Türkiye and Iran together with two isolates originated from Korea which were B trichothecene-producing fusaria.

The developments in recombinant DNA technologies made it possible to determine and characterize the complete nucleotide sequences of mtDNA in various species. The mtDNAs of *Fusarium* spp. have various in size due to the numbers of introns and the recombination between repetitive sequences (Al-Reedy *et al.* 2012). mtDNA accumulates more mutation compared to nDNA, and thus it evolves ten time faster than nDNA (Brown *et al.* 1979). Moreover, both dynamic characteristics of mitochondria, which are constantly carried out the fusions and fissions processes, and existence of heterokaryosis, which occur in intra- and interspecific cross between two species cause diversify of mtDNA nucleotide sequences (Westermann 2002, Wiebe 2003, Westermann & Prokisch 2012). It is also seen that repair systems in mtDNA are not effective exactly (Alexeyev *et al.* 2013).Therefore, unrepaired damages in mtDNA have come to exist as mutations, and have come by positive selection pressure until today. Hence, all these mechanisms mediated continuity of mtDNA variations in *Fusarium* populations (Al-Reedy *et al.* 2012).

In this study, polymorphisms found in *cox3* and *rnl* genes were investigated in *F. graminearum* isolates, and the phylogenetic relationship was constructed among the isolates. The partial region of *cox3* coding COIII, one of the three core subunit of aa3 type Cytochrome c oxidase, was amplified with 477 bp length from mitochondrial genomes all isolates except for Fg170. BLASTN analysis revealed that nucleotide sequences of three isolates (FgT7, FgT10 and Fg174) were totally different from that of the reference genome. Single and double in-dels, transversion and transition type mutations were determined in remaining mtDNAs. These findings suggest that these variations in *cox3* originated from the dynamic structure of mitochondria. Whereas nucleotide variations do not change ORF in 23 isolates, frame shift mutations were determined in 16 isolates. Despite determination of frame shift mutations in 16 isolates, it was detected that nucleotide sequence variations

belonging to only FgM2-Fg165 (similarity < 30%) can affect the function of COIII, when nucleotide sequences in partial region were compared as duplex. At the same time, the comparison analysis revealed that one and/or more than one functional domain in *cox3* partial region were shared by 13 isolates. Findings confirm that *cox3* encoding non-functional COIII at any redox centre was selected as a suitable gene for both phylogenetic analysis and assessment of diversity.

Similar to *cox3*, partial *rnl* coding sequences with the length of 1547 bp were amplified from 45 isolates, except for Fg14F and FgH11. But, efficient amplification products were not obtained from mtDNAs of 12 isolates in experimental repetitions. These findings belonging to only Iranian isolates suggest that *rnl* is affected by mycelial heterokaryosis unlike *cox3*. Also, findings revealed that isolates involve relevant sequences (similarity coefficient= 30-50 %). Moreover, it was showed through the analysis of binary combination that Fg5 carried the most varied nucleotide sequences compared to other isolates. The fact that Fg5 was located in the same monophyletic sister group with Fgsh5 in the constructed cladogram supported this finding.

Although the coding regions found in the mitochondrial genomes were reported to highly conservative (Brankovics *et al.* 2018), base substitutions and in-dels were detected in both of the genes sequenced in the present study. Dynamic relationships between nuclear and mitochondrial genomes can be the reason of the variations occurred in the genes in evolutionary processes. At the same time, horizontal gene transfers occurred in different strains of field isolates may contribute to the genetic variation of mitochondrial genes (Brankovics *et al.* 2018). Phylogenetic trees were constructed based on nucleotide variations found in *cox3* and *rnl* genes by using of maximum parsimony algorithm. It was determined that the nucleotide variations detected by BLASTN analysis were in agreement with the distances between isolates in phylogenetic trees established. Isolates found in external branches represent modern-day forms that evolutionary branched earlier than others. Also, it was shown that distances between isolates did not correlate with geographical origins of isolates. The results support the idea that Türkiye and Iran populations might have descended from the same lineage, and that mitochondrial genes (*cox3* and *rnl*) were reliable regions for evaluating the phylogenetic relationships.

In previous studies, phylogenetic relationship among *Fusarium* species was constructed with only RFLP marker variations obtained from mtDNA and rRNA genes (Kistler *et al.* 1987, Kim 1992, Láday *et al.* 2004). In addition, these studies provided the selection of different haplotypes, the discrimination of pathotypes and the determination of genetic divergence. These genealogical concordance studies revealed that *F. graminearum* is a species complex including the isolate collections composed of at least 16 phylogenetically distinct lineages (Starkey *et al.* 2007). Lee *et al.* (2016) reported that they could topologically distinguish the isolates belonging to species complex. It is known that isolates belonging to the Aisan clade, one of the member of species complex, spread from Anatolia to far-east countries (Yli-Mattila *et al.* 2009, Lee *et al.* 2016, Hao *et al.* 2017). In this study, parsimony analysis of both *cox3* and *rnl* genes revealed that Korean isolates were to be at the same monophyly group together with isolates which are causative agents in Türkiye and Iran. Findings confirmed that the isolates used in the present study were found in the Asian clade. The results suggest that these two genes are reliable targets to generate the phylogenetic relationship among *Fusarium* isolates, and the methodology used was shown to be applicable to other phytopathogenic fungus.

Acknowledgement

The authors thank to Prof. Dr. Berna Tunali (Department of Plant Protection, Agricultural Faculty, Ondokuz Mayıs University, Samsun, Türkiye), Prof. Dr. Bahram Sharifnabi (Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan, Iran) and Dr. Theresa Lee (Department of Agro-Food Safety and Crop Protection, National Institute of Agricultural Sciences, Wanju, South Korea) for providing the fungal material.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study.

Author Contributions: Concept: G.A., Design: G.A., Execution: A.G., G.A., Material supplying: G.A., Data acquisition: A.G., G.A., Data analysis/interpretation: E A.G., G.A., Writing: E A.G., G.A., Critical review: G.A.

Conflict of Interest: The authors have no conflicts of interest to declare.

Funding: The study was supported by the Scientific Research Projects Coordination Unit of İstanbul University with project number 43153.

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