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# MOLECULAR-GENETIC IDENTIFICATION AND TAXONOMIC RELATIONSHIPS OF FUNGI BELONGING TO FUNGI IMPERFECTI

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## ABSTRACT

*To identify phytopathogenic and entomopathogenic fungi, mostly belonging to the classes Ascomycetes and Deuteromycetes we used standard and changed primers to amplify mitochondrial small subunits of rRNA (NMS3, NMS4), nuclear small subunit18S rRNA (NS7, NS8) and the two of internal transcribed spacer regions (ITS1, ITS2), between the genes 18S and 28S rRNA. Using the standard primers NMS1 and NMS2, ITS1 and ITS2 did not lead to the amplification of DNA of the fungi in PCR. It was interesting to note that only when the region of the small subunit 18 S RNA (NS7 and NS8) was used could positive results be obtained amplifying the DNA of both entomopathogenic and phytopathogenic fungi. Using the modified primers provided means for the differentiation of 13 strains of entomopathogenic fungi and 6 strains of Ascomycetes from different geographic zones.*

**KEYWORDS:** *Rrna, Ribosomal DNA, Kanamycin, Phytopathogenic, Tag-Polymerases.*

## INTRODUCTION

Mycelial phytopathogenic and entomopathogenic fungi, mostly belonging to the classes Ascomycetes and Deuteromycetes or fungi imperfecti, express variability, multinucleusness of cells. Reproduction is usually asexual or absent. These features and the need for selective media for identification have conditioned poor genetic studies and problematic taxonomy which is mostly based on their morphological features. Besides these fungi differ in their highly variable physiological and biochemical properties, conditioned on intraspecific genetic differences as well as the influence of environment (particularly the state of the host organism) [1]. The DNA sequence coding for ribosomal DNA is intensively used for studying the taxonomic relationships and the genetic variability of fungi. Ribosomal DNA is present in the nuclear as well as in the mitochondrial genome. The rRNA consists of highly conservative and variable regions, which

include the small subunit 18S, 5.8S and the large subunit 28S rRNA genes [2, 3, 4]. Nuclear and mitochondrial RNAs of fungi exist in genome as tandem (repeated) subunits. There are thousands of copies of these subunits in genome. They are used as molecular probes in detecting species and evolutional variability. The mitochondrial DNA is known to be evolutionarily more variable than the nuclear DNA. Therefore, the mitochondrial DNA possesses more potential to be used for the differentiation of closely related species [5].

To identify the fungi under study we used primers to amplify mitochondrial small subunits of rRNA (NMS1, NMS2), nuclear small subunit18S rRNA (NS7, NS8) and the two internal transcribed or spacer regions, ITS1 and ITS2, between the genes 18S and 28S rRNA. The two ITS regions separate 5.8S from the small subunit and the large subunit rRNA genes. The aim of this work was to find a universal primer for the identification of entomopathogenic and phytopathogenic fungi to prove their taxonomic positions.

## MATERIALS AND METHODS

The strains of entomopathogenic and phytopathogenic fungi were obtained from soil and infected Colorado potato beetles and were kept at the laboratory of microorganism collection of the Academy of Sciences, Uzbekistan. All sporulating cultures were identified microscopically. The strains were grown in enriched medium YPD with agar, containing 10g/l bactopeptone ( Difco), 2 g/l bacto-yeast extract( Difco), 20 g/l glucose monohydrate( Difco), and 20 g/l agar (Difco) for 6-7 days at 27ºC on Petri plates. Then 400 ml of YPD media without agar was inoculated with spores and grown for 1-2 days until the sporulation started. Total DNA of the fungi was isolated using the standard method [6]. The quality and quantity of the DNA were checked using spectrophotometer, then in 1% agarose gel and was further used for the amplification. The PCR reaction was conducted with the volume of 50 µl, containing 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, 10 mM Tris HCL (pH 9.0) , 2.0 mM $MgCl_2$ and 0.1 Triton X-100, 50ng/µl DNA, 0.001 µg/µl from each of the oligonucleotide primers and 1 u of Tag-polymerases (Promega, Madison,Wis); the reaction was conducted on ice. The polymerase chain reaction was conducted in the thermocycler TwinBlock Systems (Ericom Inc. San Diego, Calif.). The cycling parameters were as follows: denaturation at $94^0$C for 10 min; annealing at 50-$55^0$C for 1 min; polymerization at $72^0$C for 1 min; completing the chains at $72^0$C for 5 min. The cycle was repeated 25-30 times.

The PCR product was analyzed by using standard electrophoresis in 1% agarose gel containing 10ng/ml ethidium bromide and visualized under a UV transiluminator. The PCR products, amplified using the NMS3 and NMS4, ITS1 and ITS4, NS7 and NS8, from the fungi were cloned in the plasmid pCR-XL-TOPO using TOPO-XL PCR Cloning kit (Invitrogen Corporation, Calif), containing a kanamycin resistant gene. The PCR products, cloned in the plasmid, were restricted with EcoR I to check the amplified insertions. The sequence of the cloned PCR products at the concentration of 500ng/ml was sequenced with fmol system or using Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Two clones from each of the isolates were completely sequenced to decrease the probability of errors in sequencing. The DNA sequence was analyzed by the program developed in Genetic Computing Group [7]. The control nucleotide sequences were taken from the Gene Bank: *Trichoderma cf.harzianum.cbs*. NMS locus AF3993; *Fusarium solani* NMS locus AF125026; *Aspergillus oryzae* NRRL 506 AF459735 ITS locus; *Aspergillus oryzae* NMS locus NRRL U292 15.

**RESULTS**

The primers, used for the identification of entomopathogenic and phytopathogenic fungi were taken as standard primers [8, 9] (Table 1) [Insert table 1 about here]. Using the standard primers NMS1 and NMS2, ITS1 and ITS2 did not lead to the amplification of DNA of the fungi in PCR. It was interesting to note that only when the region of the small subunit 18 S RNA (NS7 and NS8) was used could positive results be obtained amplifying the DNA of both entomopathogenic and phytopathogenic fungi. However, analysis of the sequence followed by the detection of the homology of the gene 18S rRNA (NS locus) between entomopathogenic and phytopathogenic fungi, and construction of the phylogenetic tree using the Blast program showed that it is not possible to use this gene to determine species belonging and the taxonomic relationships among the fungi under this study [Insert figure 1 about here]. We have changed the primers to use them for genes of the small mitochondrial (NMS 3, NMS4) and the nuclear (ITS1 , ITS4) rRNA subinit. The primers were used in the PCR reaction to amplify the ribosomal genes of the fungi (Table 2) [Insert table 2 about here]. Using the modified primers we could obtain an amplified, approximately 600 bp, region for NMS3 and NMS4 small subunits of the rRNA locus and an approximately 580 bp region for ITS1 and ITS4 locus from 13 strains of entomopathogenic fungi and 6 strains of Ascomycetes from different geographic zones. The phylogenetic trees based on the mitochondrial subunit, ITS1 and ITS4 regions, and NS region are shown in figures 1, 2, 3 respectively [insert 1,2, and 3 about here].

Many authors have used nuclear and mitochondrial RNA to determine taxonomic relations and to identify fungi species [8]. Some of these authors used either nuclear (ITS region, 18S subunit of rRNA), or small mitochondrial (NMS) region, or introns of small subunits of rRNA [10, 11, 12]. However, the identification of species was carried out either only among entomopathogens (Deuteromycetes-*Beauveria bassiana*, *Metarhizium anisopliae, Pacielomyces fumoroseus*) or among phytopathogens (Ascomycetes – *Fusarium spp.*, *Verticillium spp.*, *Aspergillus spp*) [8,9,6,14]. Some strains of *B.bassiana* were identified by PCR – RFLP analysis, using ITS region [12,10,11,13]. Thus in our study we wanted to determine which rRNA genes can be used to reveal taxonomic relationships and to identify species of entomopathogenic and phytopathogenic fungi, belonging to the same class – Fungi Imperfecti.

When the phylogenetic tree using mitochondrial small subunit was analyzed it was noted that strain 14, which was morphologically identified as *P. fumosoroseus* turned out to belong to phytopathogens - *Fusarium* (Fig.2). According to its ITS1 and ITS4 regions strain 14 belonged to entomopathogens –*B.bassiana*, and according to the NS region the strain was found to belong to *A. nidulans*. However, according to its both morphological and microbiological features, as well as the data on the nucleotide sequence and the sequence of ITS1 and ITS4 region strain 14 is most likely to belong to the entomopathogenic fungus *B. bassiana* (Fig. 3). The comparative analysis of the nucleotide sequences of ribosomal genes (rRNA), as it can be seen from many studies and experimental data, proved that these genes can be used to reveal the phylogenetic relationships among different taxonomical groups. Thanks to its comparatively slow evolution the small subunit of rRNA is used to study the level of the relationship among relative organisms, the mitochondrial rRNA on the other hand evolves faster. It is highly susceptible to changes (17).Vertebrates, for example, present 5 to 10 times faster evolution in their mtDNA than in nuclear DNA (16). Although mitochondrial genome is not involved in

entomopathogenity and therefore can be used as a good marker for the examination of genetic diversity it can be used at ordinal or family levels [17, 15]. The internal transcribed spacer region and the intergenic spacer of the nuclear rRNA repeat units evolve fastest and may vary among species within a genus or among populations [8].

**TABLE 1. THE NUCLEOTIDE SEQUENCE OF THE STANDARD PRIMERS USED FOR THE PCR AMPLIFICATION**

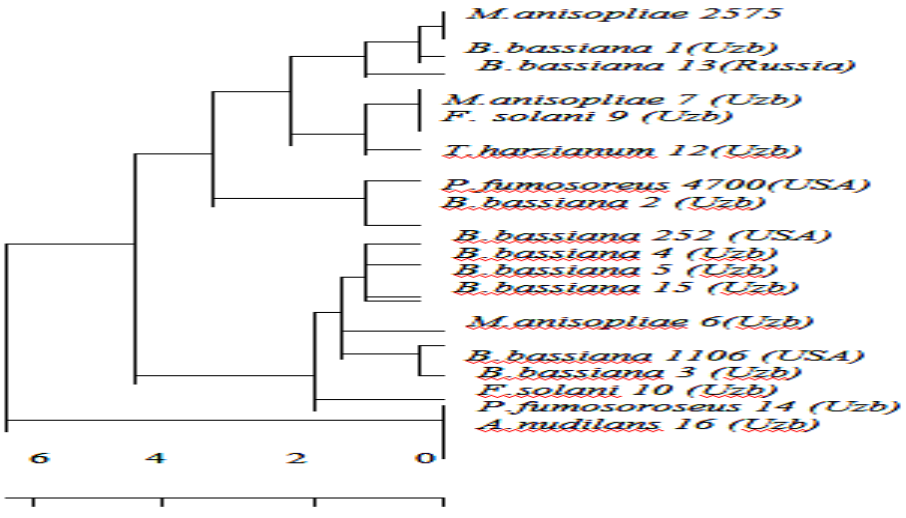
| **Nuclear small subunit 18S rDNA** |
|---|
| NS7- GAG GCA ATA ACA GGT CTG TGA TGC<br>NS8- TCC GCA GGT TCA CCT ACG GA |
| **Nuclear ITS** |
| ITS1- TCC GTA GGT GAA CCT GCG G<br>ITS2- TCC TCC GCT TAT TGA TAT GC |
| **Mitochondrial small subunit** |
| NMS1- CAG CAG TGA GGA ATA TTG GTC AAT G<br>NMS2- GCG GAT CAT CGA ATT AAA TAA CAT |

Thus these changed primers for the small mitochondrial NMS3, NMS4 and nuclear ITS1, ITS4 rRNA subunit can give an access to the mitochondrial small rRNA gene region and the internal transcribed spacer region. They may be useful as specific probes at the generic or species level for identification of both ascomycetes and entomopathogenic fungi.

**TABLE 2. THE NUCLEOTIDE SEQUENCE OF THE CHANGED STANDARD PRIMERS USED FOR THE PCR AMPLIFICATION**

| **Nuclear rRNA** |
|---|
| ITS1- 5` TCC GT(AT) GGT GAA CC(AT)GCG G-3`<br>ITS4- 5` TCC TCC GTC TAT TGA TAT GC-3` |
| **Mitochondrial small rRNA** |
| NMS3-5` CTG AAC TGG CAA CTT GGA GTG-3`<br>NMS4-5` ACT GGT AGA AAC GGT CTA GTG-3` |

**Fig. 1. Phylogenetic tree based on the nuclear small 18S RNA.**



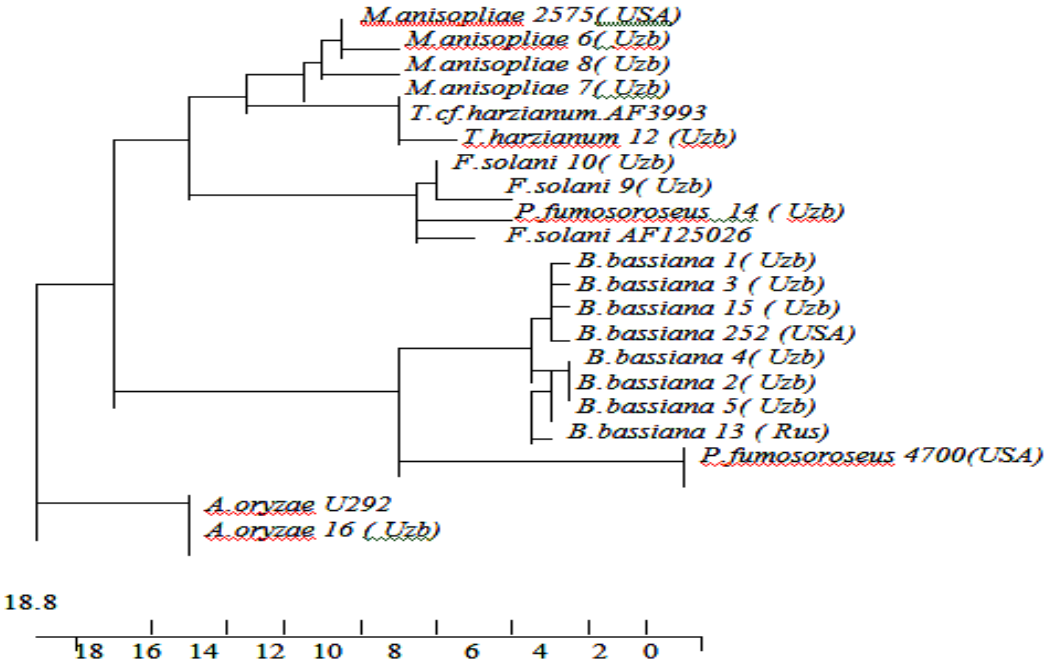



**Fig.2. Phylogenetic tree based on mitochondrial small rRNA.**

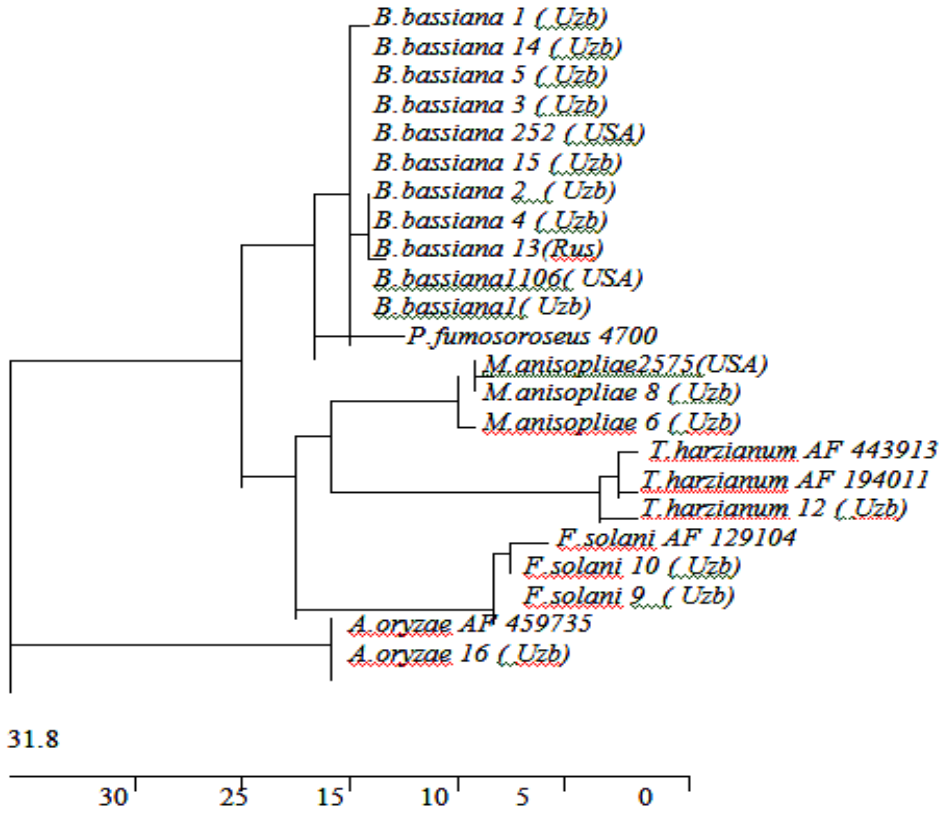



**Fig.3. Phylogenetic tree based on the rRNA ITS1-5,8S - ITS4**

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