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Molecular Identification and Phylogeny of Some *Hypocreales* Members Isolated from Agricultural Soils

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Abstract: *Hypocreanlean* fungi are include important plant pathogens around the world. Head blight and crown rot disease of cereals caused by these species are responsible for large economic losses due to reduction in seed quality and contamination of grain with their mycotoxins. Although morphological and biochemical tests are still fundamental there is an increasing more towards molecular diagnostics of these fungi. This paper reviews to PCR identification of *Hypocreanlean* fungi isolated from agricultural soil from Eskisehir City. Five *Hypocreanlean* fungi belong to 4 different genera as *Bionectria, Fusarium, Gibberella* and *Nectria* were isolated from 56 soil samples. DNA of these strains were isolated by glass beads and vortexing extraction method and used for PCR amplification with universal fungal specific primers. The internal transcribed spacer (ITS) regions of fungal ribosomal DNA (rDNA) were sequenced by CEQ 8000 Genetic Analysis System. The ITS-5.8S sequences obtained in this study were compared with those deposited in the GenBank Database. Phylogenetic position of investigated closely related *Hypocreanlean* fungi was determined.

Key words: *Hypocreanlean*; PCR; ITS; Phylogeny; Eskisehir

Tarımsal Topraklardan İzole Edilen Bazı *Hypocreales* Üyelerinin Moleküler Teşhisi ve Filogenisi

Öz: *Hypocreanlean* mantarları Dünya'daki önemli bitki patojenleridir. Bu türlerin sebep olduğu baş tahribat ve tahıl hasar hastalığı, tohum kalitesinde azalma ve tahılın mikotoksinler ile bulaşması nedeniyle büyük ekonomik kayıplardan sorumludur. Morfolojik ve biyokimyasal testler hala temel olsa da, bu mantarların moleküler teşhisine yönelim giderek artmaktadır. Bu makale, Eskişehir'deki tarım topraklarından izole edilmiş olan *Hypocreanlean* mantarlarının PCR ile identifikasyonunu incelemektedir. Bionectria, Fusarium, Gibberella ve Nectria olmak üzere 4 farklı cinse ait 5 *Hypocreanlean* mantarı 56 farklı toprak numunesinden izole edilmiştir. Bu suşların DNA'sı, cam boncuklar ve vorteks ekstraksiyon yöntemi ile izole edilmiş ve üniversal fungal spesifik primerler ile PCR amplifikasyonu için kullanılmıştır. Fungal ribozomal DNA'nın (rDNA) iç transkripsiyonlu ayırıcı (ITS) bölgeleri, CEQ 8000 Genetik Analiz Sistemi ile dizilenmiştir. Bu çalışmada elde edilen ITS-5.8S dizileri, GenBank veri tabanında depolanan dizilerle karşılaştırılmıştır. İncelenen birbirine yakın akraba *Hypocreanlean* mantarlarının filogenetik konumu belirlenmiştir.

Anahtar kelimeler: *Hypocreanlean*; PCR; ITS; Filogeni; Eskisehir

Introduction

The best-known *Hypocreanlean* fungi are a broad order that are include members of *Fusarium* and *Acremonium* genera. They are anamorphs of teleomorph genera, such as *Gibberella, Nectria* and *Bionectria* that are mainly seen in agricultural, ecological, or biodiversity studies (Howard, 2002; Stone et al., 2004).

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These genera are widely distributed in soil and on organic substrates and have been isolated from permafrost in the arctic and from the sand of Sahara. They are amongst the fungi most frequently isolated by the plant pathologist. The predominant interest the genus has been and still is in their role as plant pathogens (Booth, 1971; Ismail et al., 2015) as well as there are species which are highly mycotoxigenic, producing a range of toxins affecting wildlife, livestock and humans (Antonissen et al., 2014). The current fungal taxonomic systems have been still identified by macroconidia and microconidia in the asexual stage, morphological character of chlamydospore, host range, and secondary metabolites. However, the plasticity and intergradations of the phenotypic traits offered difficulty in identifying the filamentous fungi (Ismail et al., 2015; Young et al., 2000). In addition, because of their capacity for rapid change, species identification presents certain problems (Booth, 1971; Hsuan et al., 2011). For these reasons, the molecular biological method has been recently introduced in *Hypocreanlean* fungi systematic and the molecular variation at the DNA level has been studied in many works (Young et al., 2000). In addition to DNA sequencing, phylogenetic analyses have been supported strong information about genetic relationship of closely related *Hypocreanlean* fungi (Hsuan et al., 2011). This paper evaluates the use of ITS sequences for identification and phylogenetic analysis of closely related *Hypocreanlean* fungi isolated from agricultural soils in Eskisehir province.

Material and Method

Fungal Strains

All of the strains used in this study were obtained from agricultural soils in Eskisehir province and identified using traditional methods according to the Booth (1971), Gerlach & Nirenberg (1982) and Nelson et al. (1983). Additional information on these and related strains can also be found elsewhere (Demirel et al., 2005). All strains were stored in suitable conditions at the Culture Collections of KUKENS (WDCM101), the Centre for Research and Application of Culture Collections of Microorganisms. Cultures were maintained at 4°C on potato dextrose agar (PDA) for use in the present study.

DNA Extraction, PCR Amplification and Sequencing

Genomic DNA extraction were conducted with strains grown on PDA for 7 days at 25ºC using a modified method of Van Burik et al. (1998). DNA concentration were estimated visually in 1% agarose gels containing 5 µg/mL ethidium bromide by comparing band intensity with known quantities of DNA high range markers and the extracted DNA was stored at −20ºC. To examine the phylogenetic relationship among the test strains of *Fusarium*, the nuclear ribosomal ITS1-5.8S-ITS2 region was amplified with primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White et al., 1990). PCRs were performed using Techne Thermal Cycler (Techgene, Techne, UK) in 25-mL solution containing 1 µL of genomic DNA, 2.5 µL of 2.5-µM forward and reverse primers, 2.5 µL of Taq buffer + KCl-$MgCl_2$ (Fermentas), 2.5 µL of 25-mM $MgCl_2$ (Fermentas), 2 µL of 2.5-mM dNTPmix, 0.25 µL of 5-U/µL Taq DNA polymerase (Fermentas) and 11.75 µL of sterile deionised water. The amplification conditions consisted of initial denaturation at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 1 min, annealing at 55 ºC for 45 s, extension at 72 ºC for 1.30 s and final extension at 72 ºC for 5 min. To confirm the amplification, 5 µl of the PCR product together with marker (GeneRuler DNA Lader 50 bp Fermentas) was resolved by gel electrophoresis on 1% agarose gel containing 5 µg/mL ethidium bromide in 1X TAE buffer. Gel were photographed by Gel Documentation system (Uvitec M02 4611) (Demirel, 2016).

After agarose gel blocks containing DNA fragment were cut out and purified with Promega Wizard® SV Gel and PCR Clean-Up System, Cycle sequencing products were purified with Dynabeads® Sequencing Clean-Up to remove unincorporated dye-labeled nucleotides. Then, all the sequencing reactions were performed using CEQ™ DTCS Quick Start Kit (Beckman Coulter) by CEQ 8000 Genetic Analysis System.

Data Analysis

The ITS sequences were blasted with GenBank sequences (Altschul et al., 1990) to verify their identity; the closest Blast results are reported for each taxon (Table 1). The alignments were performed using the Muscle in MEGA 6.0 software package, together with the other sequences of morphologically and phylogenetically related species that were obtained from NCBI GenBank (Tamura et al., 2013). The aligned data sets were investigated using ML analysis based on the Tamura–Nei model (Tamura and Nei, 1993) as implemented in the MEGA 6.0 with 1000 bootstrap replications. All the positions containing gaps and missing data were eliminated. *Fusarium oxysporum* (KT794176) was used as the out group. The obtained sequence data have been deposited in GenBank with accession numbers.

Results and Discussion

The PCR products (570 bp) were obtained from all of the species by using the universal fungal primers (ITS1/ITS4), Figure 1 shows that the sizes obtained for the full ITS region amplified of all of the strains. The rDNA base sequences belong to investigated strains are presented in Table 1 together with closest Blast results. When each of this sequences were investigated by Blast, identity and coverage values were found between 98-100% and 97-100%, respectively (Table 1). The phylogenetic trees were obtained by comparison to all sequences with Genbank nucleotide sequence database that have ITS1-5.8S rRNA-ITS2 sequences (Figure 2). Figure 2 shows that the members of genera *Bionectria, Fusarium, Gibberella* and *Nectria* have almost identical topology with respect to the ITS locus. A phylogenetic tree based on ITS region was structured at higher divergence levels. For investigated mainly closely related members, identical positions and four sections for specific clades such as *Bionectria, Fusarium, Gibberella* and *Nectria* genera were found.

The genus *Fusarium* is the anamorph stage of *Gibberella* genus (Samuels et al., 2001). The members of these genera are known as main and wide plant pathogens (Howard, 2002; Stone et al., 2004; Dragich and Nelson, 2014; Chehri, 2016). These two genera have been distinguished with especially teleomorph structures of *Gibberella* genus. The complexity about the their morphologic and microscopic identification has been related with varies problems such as depending on the host, loosing of stock cultures, limitations associated with morphological characters (Summerell et al., 2003; Hsuan et al., 2011; Antonissen et al., 2014). The findings of this study demonstrated the efficiency of ITS region and phylogenetic analysis of belong to these two genera. Figure 2 shows that *Fusarium* and *Gibberella* genera have considerably identical topology with the ITS locus. The genus *Gibberella* occurred in two main clades and two clear divergences, namely *Gibberella avenacea* and *Gibberella tricincta*, were noted. Furthermore, investigated members of *Fusarium* and *Gibberella* genera are polyphyletic.

The genus *Nectria* is a big genus with about 650 members and many species of *Nectria* genus are known as plant pathogens, and some of them are toxigenic to animals and humans (Schroers and Samuels, 1997). The genus *Bionectria* is one of the other plant pathogenic *Hypocreanlean* fungi and very similar to *Nectria* member (Schroers, 2001; Samaga et al., 2014; Melo et al., 2014). These two genera have some differences about their morphologic and chemical structure. However, *Bionectria* and *Nectra* genera have very similar morphologic and microscopic properties and distinguish of them has been very problematic for mycologist (Schroers and Samuels, 1997; Schroers, 2001).

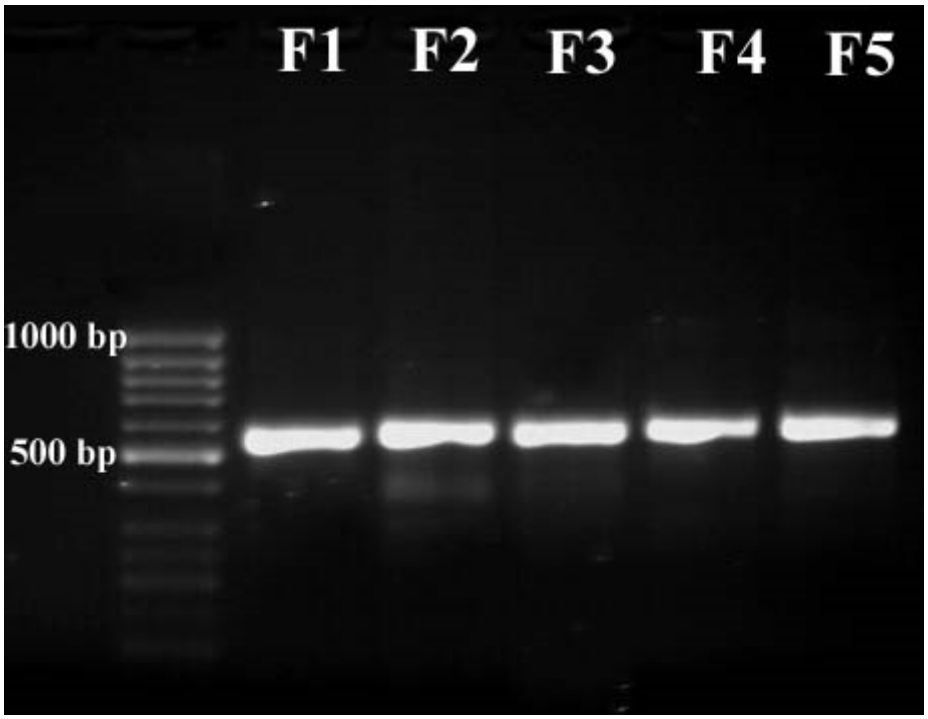

Figure 1. Full ITS PCR products amplified from all of the strains with ITS1/ITS4 primers. M, molecular-weight markers (50 bp GeneRuler DNA Lader, Fermentas)

Phylogeny based on the ITS region in this study showed a successfully topology for these genera and indicated main phylogenetic position of them as closely related but distinctly different members (Figure 2).

Table 1. Newly generated ITS sequences with their closest GenBank sequences (according to Blast searches)

Species	Collection	GenBank accession number	Closest Blast hit (% identity/%coverage)
Fusarium solani (Mart.) Sacc.	F1	KX958415	*Fusarium solani* KP992939 (99/100)
Gibberella tricincta El-Gholl, McRitchie, Schoult. & Ridings 1978	F2	KX958416	*Fusarium tricinctum* KU556038 (98/97)
Gibberella avenacea R.J. Cook 1967	F3	KX958417	*Fusarium avenaceum* KX839156 (99/99)
Bionectria ochroleuca (Schwein.) Schroers & Samuels	F4	KX958418	*Bionectria ochroleuca* AF358237 (99/100)
Nectria inventa Pethybr.	F5	KX958419	*Nectria inventa* KR709185 (100/100)

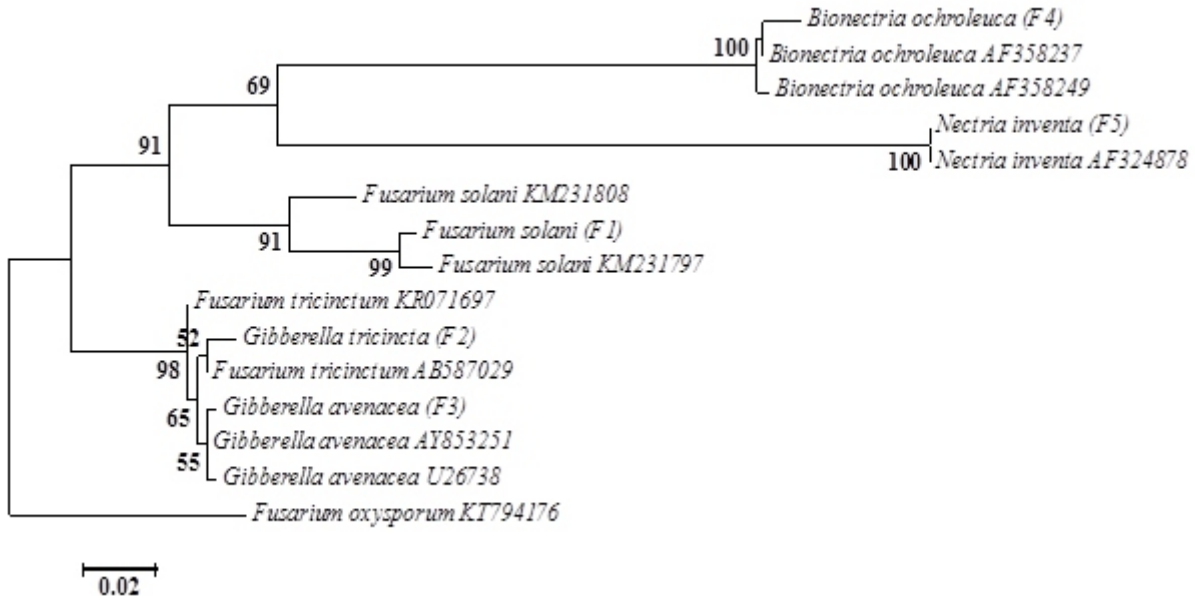

Figure 2. Best-scoring maximum likelihood tree based on the Tamura–Nei model calculated using MEGA 6.0 based on ITS sequences showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The scale bar denotes 0.02 substitutions per position. The tree with the highest log likelihood (-1499.8212) is shown. Initial tree for the heuristic search were obtained by applying the neighborjoining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. All positions with less than 50% site coverage, containing gaps, or missing data were eliminated. There were a total of 374 positions in the final dataset. The tree is rooted with *Fusarium oxysporum* (KT794176) (bootstrap 1000).

Conclusions

The results of this study demonstrated the efficiency of rDNA region and phylogenetic analysis in taxonomic studies of closely related members of *Hypocreanlean* fungi. In particular, ITS region was found to be success because of its high performance with regard to easy application, topology, identification and clearly discrimination. In addition, high quality sequences of the ITS locus obtained in the present study have been deposed in the NCBI database for bridge over to other taxonomic studies of *Hypocreanlean* fungi.

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