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Identification of Fungi Storage Types by Sequencing Method

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Abstract

With the use of classical identification methods, which imply the identification of fungi by cultural and morphological features, they may not be reliable. With the development of modern molecular methods, it became possible to quickly and accurately determine the species and race of the fungus. The purpose of this work was to study bioecology and refine the species composition of fungi of the genus *Aspergillus, Penicillium* on seeds of cereal crops. The article presents materials of scientific research on morphological and molecular genetic peculiarities of storage fungi, affecting seeds of grain crops. Particular attention is paid to the fungi that develop in the stored grain. The seeds of cereals (*Triticum aestivum L., Avena sativa L., Hordeum vulgare L., Zea mays L., Oryza sativa L., Sorghum vulgare Pers., Panicum miliaceum L.*) were collected from the granaries of five districts (Talgar, Iliysky, Karasai, Zhambul, Panfilov) of the Almaty region. The pathogens of diseases of fungal etiology were found from the genera *Penicillium, Aspergillus* influencing the safety, quality and safety of the grain.

Keywords: grain cultures, seeds, fungi, identification, gene, β- тубулин, ITS, morphology.

INTRODUCTION

Fungi developing on seeds are conditionally divided into 2 groups-field and mold storage. The main representatives of storage fungi or, as they are called, the mold of storage, are the genera *Penicillium* and *Aspergillus*. Mold storage, infect the seeds after harvesting, often during transportation and actively develop storage. Specific types of *Penicillium* and less often *Aspergillus* can infect seeds before harvesting or immediately after it. The source of their inoculum can be grains themselves, infected with fungi, as well as mechanical impurities and infected plant residues found in the mass of seeds. The problem of microbiological contamination of grain continues to be global on a global scale. Phytosanitary situation in the fields, the presence of phytopathogenic microorganisms adversely affects the growth and development of plants, causing various diseases, a decrease in yield and quality of grain. Crop losses from phytopathogens are so great that they are dealt with by special state institutions, and with some by international organizations. Widespread damage to crops caused by diseases: root rot, leaf-lobe diseases (septoria, yellow spot, brown rust and others) decreases the yield on 40- 60 % [1]. Infection of seeds occurs through the damaged areas of their coverslips, formed when the grains are peeled off from the spike or cob, or through cracks that occur when threshing, drying and transporting the seeds. In the research, Zh. Zh. Kuzhantaev and A.M. Bostanova revealed the effect of temperature on the seminal microflora, which is largely determined by the various growth optima of its individual representatives. Temperature +40 had a limiting effect on the development of fungi on seeds in a grain storage facility [2].Storage fungi reduce the germination of seeds, change the color of the seeds, cause the death of the embryo, their metabolites are often toxic to plants, animals and humans [3]. About 30,000 species of various microscopic fungi were isolated from food, feed, industrial raw materials, including more than 250 toxins, and about 300 mycotoxins were identified [4]. However, the biology and taxonomy of these fungi have not been adequately studied. Until now, there are difficulties in identifying the species composition of small-fungal fungi. With the use of classical identification methods, which imply the identification of fungi by culture and morphological features, they may not be reliable. With the development of modern molecular methods, it became possible to quickly and accurately determine the species and race of the fungus. Proceeding from the above, the aim of this work was to study bioecology and refine the species composition of fungi of the genus *Aspergillus, Penicillium* on seeds of cereal crops.

MATERIALS AND METHODS

From the granaries of five districts (Talgar, Iliysky, Karasai, Zhambul, Panfilov) of the Almaty region we collected seeds of grain crops. The harvested seeds of cereal crops were left in a moist chamber. Petri dishes were kept at a temperature of 21 ° C. The growth was monitored on a daily basis. On the 7th day, spores of fungi were formed on the seeds. According to the nature of the growth of mycelium and sporulation, their species affiliation was determined. Then, in a Petri dish with Chapek's nutrient medium, these fungi were separately replaced by species to determine the nucleotide sequences (sequencing) of the beta-tubulin gene. BIf the sequencing of this area was technically impossible or the results were poorly informative, sequencing of the ITS regions of the ribosomal operon was additionally performed. Extraction of DNA from the fungal mycelium was carried out according to the standard "CTAB-chloroform" protocol [5]. The precipitation was dried in open tubes at room temperature for 30 minutes and dissolved in 25 µl TE buffer (10 мMtris-HCl, pH 7.4; 1 мM EDTA, pH 8.0) or 80% DMSO.

To amplify a fragment of the beta- tubulin gene, was used a pair of primers T1/T2 [6], for ITS regions (the region including the fragment of the 18S rRNA gene, the internal transcribed spacer 1 [ITS 1] gene 5,8S rRNA, inner transcribed spacer 2 [ITS 2] and fragment of gene 28S rRNA) –ITS1/ITS4 [7]. Amplification has been held in thermal cycler C-1000 (Bio-Rad). The reaction mixture of 25 µl contained: 1 unit of Taq polymerase (Helicon, Russia), 0.5 µM of each primer, 200 µM deoxynucleotide triphosphate, buffer (75 mM Tric-HCl (pH 8.8), 20 mM (NH4) 2SO4, 0.01% (v / v) Tween 20, 2 mM MgCl2). The amplification products were separated electrophoretically in a 1% agarose gel stained with ethidium bromide. The results of the electrophoresis were visualized on a transilluminator, and then amplitudes of the desired length were extracted using silicon oxide powder [8].

Sequencing PCR was performed according to the classical chain termination method [9] using the Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI, USA). The

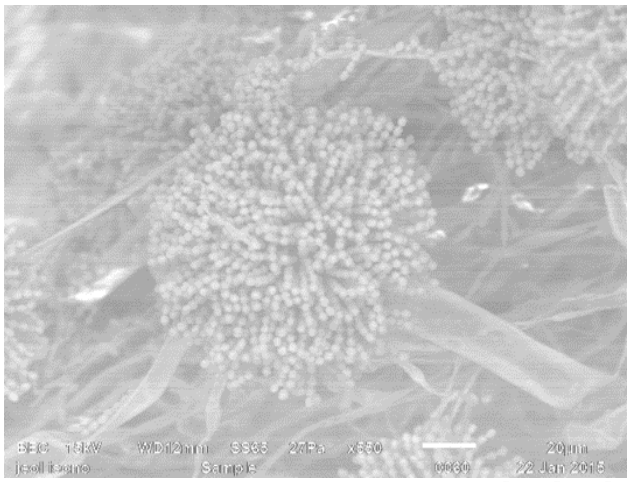
nucleotide sequences were determined by the ABI PRIZM 3500 genetic analyzer (ABI-Hitachi, Japan). The resulting nucleotide sequences were edited in the Vector NTI program.

The obtained nucleotide sequences were compared with those available in the GenBank database (http://www.ncbi.nlm.nih.gov) using the BLASTn online service. Based on the results of the comparison, a conclusion was made on the species belonging to the strains.

All received sequences are deposited in the GenBank database. The relevance of all the species names of fungi, indicated as the result of identification, and the correctness of writing the authors of these species names are verified using the nomenclatural database Mycobank.

RESULTS AND DISCUSSION

In the Ascomycota section, the Deuteromyces (Fungi imperfecti) class, belonging to the group of Hyphomycetales, belongs to the Monoiliaceae family Penicillium sp, Penicillium rugulosum Thom, Penicillium expansum Link, Penicillum glaucum Fr., Aspergillus niger Tiegh., Aspergillus tubingensis Mosseray., Aspergillus fumigatus Fres., Aspergillus ochraceum Wilhelm, Aspergillus glaucus Raper et Fennell, Aspergillus flavus Link, Aspergillus restrictus G.Sm., Aspergillus candidus Link. species were identified. *Penicillium sp., Aspergillus flavus* Link, Aspergillus ochraceum Wilhelm (Picture 1) species has been found in all cultured cereal crops.



Picture 1 - *Aspergillus ochraceus* Raper et Fennel.(550^x; 330^x; 200^x)

Genetic identification was conducted in the species shown in Table 1.

Table 1 – The resulting nucleotide sequences of the fungal gene

the name of fungi	The obtained nucleotide sequences and the accounting number in Genbank
Aspergillus flavus Link.	Beta-tubulin gene sequencing deposited in Genebank under № KJ938407): TGGTAACCAAATCGGTGCTGCTTTCTGGTATGTCTC AATGCCTTCGAGTTAGTATGCTTTGGACCAAGGAA CTCCTCAAAAGCATGATCTCGGATGTGTCCTGTTAT ATCTGCCACATGTTTGCAACAACTTTGCAGGCAAA CCATCTCTGGCGAGCACGGCCTTGACGGCTCCGGT GTGTAAGTACAGCCTGTATACACCTCGAACGAACG ACGACCATATGGCATTAGAAGTTGGAATGGATCTG ACGGCAAGGATAGTTACAATGGCTCCTCCGATCTC CAGCTGGAGCGTATGAACGTCTACTTCAACGAGGT GCGTACCTCAAAATTTCAGCATCTATGAAAACGCT TTGCAACTCCTGACCGCTTCTCCAGGCCAGCGGAA ACAAGTATGTCCCTCGTGCCGTCCTCGTTGATCTTG AGCCTGGTACCATGGACGCCGTCCGTGCCGGTCCC TTCGGTCAGCTCTTCCGTCCCGACAACTTCGTTTTC GGCCAGTCCGGTGCTGGTAACAACTGGGCCAAGGG TCACTACACTGAGGGTGC
Aspergillus tubingensis Mosseray.	Beta-tubulin gene sequencing(KJ938412): CTTGTGCTAACTGCATGTCTTCGTCGCTTCAATAGG TTCACCTCCAAACCGGCCAGTGTGTAAGTGCCAAT ATGTTCTTCGAATGATTGCCCTCCCGGGTTCTTGAT TGGTGTTCGGTGGACTAAACAACAAATGATGGTGG TTAGGGTAACCAAATTGGTGCTGCTTTCTGGTACGT ATTCACTGCCACTGGATTGGGGATGGAACATCATC TCTCAAGCTATCTCAGCTTGAGTTCAGATGTTATCC ATCGGGGATATAGCTATCGGGTTAAGAACACGTCT AACAACTCAACAGGCAGACCATCTCTGGCGAGCAC GGCCTTGACGGCTCCGGTGTGTAAGTACAACTTTTT CACACCTCTCAATTGGTCATCAATGTGGAAAGGAT TGGGTTTCCTGACGCGCAGGATAGTTACAATGGCA CCTCCGACCTCCAGCTGGAGCGCATGAACGTCTAC TTCAACGAGGTTAGATCACACCGTCCCTGAGTTTTT TCACGACAATATCATCAATGTCCTGACCACTTCAGC AGGCTAGCGGTAACAAGTATGTCCCCCGTGCCGTC CTCGTCGATCTCGAGCCCGGTACCATGGACGCCGT CCGTGCCGGTCCCTTCGGCCAGCT
Aspergillu stubingensis Mosseray.	ITS-regions sequencing (KJ938413): ACCTCCCATCCGTGTCTATTATACCCTGTTGCTTCG GCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGC CTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCC AACACGAACACTGTCTGAAAGCGTGCAGTCTGAGT TGATTGAATGCAATCAGTTAAAACTTTCAACAATG GATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC GAAATGCGATAACTAATGTGAATTGCAGAATTCAG TGAATCATCGAGTCTTTGAACGCACATTGCGCCCCC TGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCAT TGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCC GTCCCCCTCTCCGGGGGGACGGGCCCGAAAGGCAG CGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGG CTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTG CCGACGTTTTCCAACCATTTTTTCCAGGTTGACCTC GGATCAGGTAGGGATACCCGCTGAACTTAAGCATA TCAATAAGCGGAGGA
Penicillium expansum	Beta-tubulin sequencing KJ938414): AACGGCCCCTGAGCCATGACCCCACTCCCAACAGA TCTTTTGCTAACATGATCTAGGTTCACCTCCAAACC GGCCAGTGTGTAAGTTCGACATGGAACATTCTTGG AAACATTCTTGGATTCGTGGGACTAAATTGGAATT GGGTTATAGGGTAACCAAATTGGTGCCGCTTTCTG GTAAGTGCCGAGCTTTTTTTTCGCGTTGGGTATCAA TTGACAATTTACTAACTGGATTGCAGGCAAACCAT CTCTGGCGAGCACGGTCTCGATGGTGATGGACAGT AAGTTCAACGGTGATGGGTTTCTAGTAGATCACAC GTCTGATATCTTGCTAGGTACAATGGTACCTCCGAC CTCCAGCTCGAGCGTATGAACGTCTACTTCAACCAT GTGAGTACACCGACTAGTTACCGAATAATCGTGCA TCATCTGATCGGATCTTTTTCTTTGATAATCTAGGC CAGCGGTGACAAGTACGTTCCCCGTGCCGTTCTCGT CGATTTGGAGCCCGGTACCATGGACGCTGTCCGCT CCGGTCCCTTCGGCAAGCTTTTCCGCCCCGACAACT TCGTCTTCGGTCAGTCCGGTGCTGGTAACAACTGG GCCAAGGGTCACTA
Penicilliumexpansum Link.	ITS-regions sequencing (KJ938415): TGGGTCCAACCTCCCACCCGTGTTTATTTACCTCGT TGCTTCGGCGGGCCCGCCTTAACTGGCCGCCGGGG GGCTCACGCCCCCGGGCCCGCGCCCGCCGAAGACA CCCCCGAACTCTGCCTGAAGATTGTCGTCTGAGTG AAAATATAAATTATTTAAAACTTTCAACAACGGAT CTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA ATGCGATACGTAATGTGAATTGCAAATTCAGTGAA TCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGT ATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCT GCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCT CCGATTCCGGGGGACGGGCCCGAAAGGCAGCGGC GGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTT GTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCG ATCAACCCAAATTTTTATCCAGGTTGACCTCGGATC AGGTAGGGATACCCGCTGAACTTAAGCATATCAAT AAGGCGGAGGA

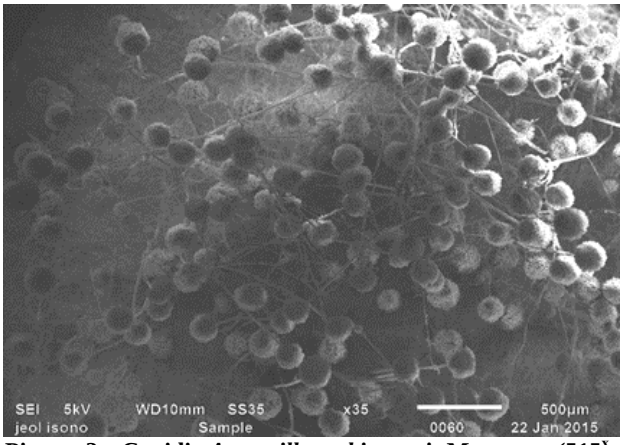
Aspergillus flavus is an opportunistic pathogen of cereals. This is important because it produces aflatoxin as a secondary metabolite in the seeds of a number of crops both before and after harvest [10]. Aflatoxin is a strong carcinogen, which is strictly regulated in most countries. Under favorable conditions, the fungus will grow, and produce aflatoxin in virtually any stored seed yield. When stored, aflatoxin can be controlled by maintaining available moisture at a level below that which will support the growth of *Aspergillus flavus [11]*. The aerial mycelium of the colony is dark green, the back side is yellow. At the edges of the aerial mycelium cobwebby. Conidiophores 300-1000x5-10 microns, colorless, rough, pitlike, depart from the hyphae of a submerged substrate mycelium, apical bubble pyriform, spherical, 20-50 microns in diameter, colorless, bearing predominantly two-row sterigms located along the entire surface. Primary sterigma -10x3-4 microns, secondary 7-10x2.5-3 microns. Conidialialides unicellular, globose, ovate less often pear-shaped 3-5 microns in diameter, almost smooth, colorless or slightly yellowish-greenish, in chains, radial, collected in the column, varying in size. Sclerotia is knobby, solid, first white, subsequently turbulent, abundant, sometimes sparse. The sequencing of the beta-tubulin gene and the ITS-5,8S ribosomal DNA of the *Aspergillus* genus showed that this nucleotide sequence with a 100% overlap (identity) and 100% similarity (homology) was assigned to the genus *Aspergillus aspergillus flavus* Link. Sequencing of the beta-tubulin gene was deposited at Genbank under No. KJ938407. 2013-2014, studies of a fungus of the species *Aspergillus tubingensis*, isolated from berries from different agroclimatic regions of Spain [12] and from soils of vineyards in various regions of Argentina, have been carried out [13]. The sclerotia formed by the *Aspergillus* section of *Nigri* was selected from the population in one field in North Carolina, USA, and identified as *A. tubingensis* on the basis of a genealogical matching analysis. It was shown that the ascospores from *A. tubingensis* differed from the species of the flavi species section in the mesh ascospores ornament and the presence of two crests forming the equatorial furrow. Sexual reproduction in *A. tubingensis* can be useful for increasing the enzyme and organic acids produced by recombination-mediated genetic engineering of industrial strains[14].

In 2013, in the Almaty region of Kazakhstan was found out the seeds of cereal crops. Colonies pinkish-gray, reddish-brown gradually change to black color. Conidiophores 200-400x7-10 microns, the final bladder 20-60 microns in diameter. Sterigmata 3-row: primary 20-30 microns, secondary 6-10 microns, conidia round, 2.5-4 microns in diameter. Sequencing of the beta-tubulin gene of fungi of the genus *Aspergillus* is deposited in Genbank.
The results of BLAST analysis: overlap of 100%, similarity of 99%. Additionally, the sequencing of the ITS regions of the ribosomal operon was carried out, since the results obtained were of little informative value. Sequencing of ITS-areas is deposited in Genbank under No. KJ938413. Results of BLAST analysis: overlap of 100%, similarity of 100%. The identification result showed that this nucleotide sequence of the gene belongs to the species *Aspergillus tubingensis* Mosseray. Among the species that cause blue mold *Penicillium expansum* are the most common and virulent species. Patulin is a mycotoxin, produced by various strains of the species *Penicillium expansum* which are the most common. This mycotoxin causes toxic effects on animals, some of which include reproductive toxicity and effects on their endocrine system. Morphologically, the species are distinguished by their brushes, as a spore-forming structure, which produce long green chains of unicellular sphyphalids. They are equally well known for biological damage of organic substances, for the production of antibiotics, such as penicillin and griseofulvin, toxic metabolites (mycotoxins) such as ochratoxin A, patulin and penitrem A in food and grain, and their main roles in camembert and blue cheeses. Sequencing of the beta-tubulin gene was deposited in Genbank. The results of BLAST analysis: overlap of 100%, similarity of 99%. Sequencing of ITS-areas is deposited in Genbank under No. KJ938415. Based on the results of the BLAST analysis, the nucleotide sequence of the ITS regions is 100% overlapped and 100% similar in nature to the genus *Penicillium* as *Penicillium expansum.*

Recently, much attention has been paid to molds that not only change the nutritional value of the grain, but also form toxic substances in the process of vital activity. Such species as *Penicillium expansum* are pathogenic for the grain, reduce its germination or are the cause of defective seedlings. They also produce the carcinogenicity of the metabolite of patulin, which is a neurotoxin that is harmful to the host. Patulin levels in food are a health issue.

In 2013, *Penicillium expansum* was isolated from wheat seeds from granaries in the Almaty region of Kazakhstan. Morphological features and genetic sequence were studied. Based on the results of gene analysis, 100% of the overlap and 100% similarity is attributed to the genus *Penicillium* as *Penicillium expansum*. As far as *Aspergillus flavus* is concerned, this species produces aflatoxins. Another characteristic of this species is the color of conidia, which is yellow. This fungus multiplies only asexually.

By morphological characteristics *Aspergillus tubingensis* is close to Aspergillus niger (Picture 2)



Picture 2 – Conidia *Aspergillus tubingensis* Mosseray. (515^x; 35^x)

It was difficult to determine the species by its morphology and in order to precisely determine the *Aspergillus tubingensis* species we sequenced it. This nucleotide sequence of gene was referred to *Aspergillus tubingensis* Mosseray species.

CONCLUSION

In overall, this research shows that molecular methods are very useful and significant tools. They can be used in addition to identification based only in morphological criteria. In the result of research of morphological As a result of studies of morphological features and genetic sequence of strains of fungi, it was possible to determine the species composition of pathogens, in comparison with the matrix of fungi found in GeneBank's database.

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