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Molecular identification and diversity of Aspergillus storage fungi in chickpea seeds from the Akmola region, Kazakhstan

R.S. Uakhit[1], A.M. Smaggulova[2], A.S. Abash[3], V.S. Kiyan[4], K.A. Dyussembayev*[5]

[1,2,4,5]Laboratory of Biodiversity and Genetic Resources, National Center of Biotechnology, Astana, Kazakhstan
[3]Higher School of Natural Sciences, Astana International University, Astana, Kazakhstan
[5]Department of Biotechnology and Microbiology, L.N. Gumilyov Eurasian National University, Astana, Kazakhstan

*E-mail: [1]erken.uakhitrabiga@gmail.com, [2]smagulova0114@gmail.com, [3]altın_jan82@mail.ru, [4]vskiyan@gmail.com, *[5]dyussembayev@biocenter.kz*

Abstract. Chickpea (*Cicer arietinum* L.) is a vital legume whose post-harvest quality is frequently compromised by opportunistic storage fungi, posing both economic losses and potential health risks due to mycotoxin contamination. Despite its agricultural importance, information regarding storage fungi affecting chickpea seeds in Kazakhstan remains limited. This study aimed to isolate and identify *Aspergillus* species contaminating chickpea seeds cultivated in the Akmola region. Over 200 seed samples representing local agricultural production were evaluated. Initial microbiological analysis and microscopic examination were followed by accurate molecular identification through the amplification sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA. Phylogenetic analysis of the obtained sequences confirmed the presence of two distinct species: *Aspergillus niger* and *Aspergillus fumigatus*. The isolation of these ubiquitous saprophytes indicates significant post-harvest fungal proliferation, likely exacerbated by local climatic factors and storage microclimates. These findings highlight a critical vulnerability in regional post-harvest infrastructure and underscore the necessity for rigorous phytosanitary monitoring. Implementing improved, sustainable storage management strategies is essential to mitigate fungal contamination and ensure the safety of local agricultural commodities.

Keywords: *Cicer arietinum*, storage fungi, *Aspergillus niger*, *Aspergillus fumigatus*, ITS rDNA, phytosanitary monitoring, post-harvest quality

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*Corresponding author

Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes cultivated worldwide and represents a major source of plant protein for human nutrition [1]. The crop is widely grown in semi-arid and temperate regions of Asia, the Middle East, and Africa, and is increasingly grown in Europe and Central Asia [2]. By fixing atmospheric nitrogen and offering high nutritional value, chickpea is integral to sustainable agricultural systems and soil fertility management. [3]. However, the productivity of chickpea is strongly constrained by numerous fungal diseases affecting plants at different developmental stages [4-6].

Aspergillus spp. are ubiquitous filamentous fungi widely distributed in soil, air, and plant debris [5-7]. Numerous *Aspergillus* species opportunistically colonize agricultural commodities, particularly when elevated temperature and humidity during storage promote fungal proliferation. In chickpea seeds, *Aspergillus* species can develop under inadequate storage conditions, such as high moisture content, poor ventilation, or prolonged storage periods [8]. These fungi can rapidly colonize seed surfaces and internal tissues, leading to deterioration of seed quality, reduced germination capacity, and potential loss of market value [9].

In addition to causing economic losses, contamination of food products by *Aspergillus* spp. represents a potential risk to human health [10-14]. Some species in this genus produce mycotoxins, including aflatoxins and ochratoxins, which can accumulate in contaminated food products. Furthermore, Aspergillus spores and fungal metabolites may trigger allergic reactions in susceptible individuals. Exposure to fungal spores through inhalation or consumption of contaminated food may lead to hypersensitivity reactions, respiratory symptoms, or other allergic manifestations. These effects are associated with the presence of allergenic proteins and secondary metabolites produced by certain *Aspergillus* species [15].

Because chickpea seeds are widely consumed and often stored for extended periods before processing or consumption, monitoring for fungal contamination is essential to ensure food safety and quality [16]. The identification of *Aspergillus* species associated with stored chickpea seeds is therefore important for understanding the sources of contamination and assessing potential risks for consumers [17]. Traditional morphological identification of fungi may be limited by similarities among species; therefore, molecular methods, such as PCR amplification and sequencing of ribosomal DNA regions, provide a reliable approach to accurate species identification [18].

Despite the importance of chickpea production in many regions, information regarding the occurrence and molecular identification of storage fungi associated with chickpea seeds remains limited in several countries, including Kazakhstan. Therefore, studying the diversity of *Aspergillus* species contaminating chickpea seeds is necessary to improve phytosanitary monitoring and to support the development of effective storage management strategies aimed at reducing fungal contamination and associated health risks [19, 20].

Materials and methods

Sample collection

Chickpea (*Cicer arietinum* L.) seed samples were collected from agricultural fields located in the Akmola region of Kazakhstan. The samples were provided by the A.I. Baraev Scientific Production Center for Grain Farming (Shortandy, Kazakhstan). In total, more than 200 chickpea seed samples were examined during the study period. Seeds were collected from different batches representing local agricultural production. All samples were placed in sterile containers, labeled, and transported to the laboratory for further mycological analysis.

Prior to microbiological analysis, seeds underwent visual inspection to detect overt fungal contamination and morphological anomalies. Individual seeds from each sample were selected randomly for further microscopic and molecular investigations.

Microbiological analysis and microscopic examination

Chickpea seeds were subjected to microbiological analysis to isolate fungal contaminants. Individual seeds were placed on Petri dishes containing potato dextrose agar (PDA) medium and incubated at 25 ± 2 °C for 5–7 days. Emerging fungal colonies were monitored daily, and colonies with morphological characteristics were selected for further examination.

For microscopic analysis, small fragments of fungal colonies were transferred onto microscope slides in a drop of sterile distilled water and covered with a coverslip. Microscopic observations were performed using a Zeiss Axio Scope light microscope (Carl Zeiss, Germany).

DNA extraction and PCR amplification

Genomic DNA was extracted from fungal mycelium of *Aspergillus* spp. grown on potato dextrose agar (PDA). A small fragment of fungal mycelium (approximately 0.5 × 0.5 cm) was transferred from the culture plate into a sterile mortar. Then, 300 µL of extraction buffer containing 2 M Tris–HCl (pH 8.0), 0.5 M EDTA, NaCl (3.27 g), and CTAB (0.8 g) was added together with Proteinase K. The mixture was thoroughly homogenized and incubated at 65 °C for 14–16 h. Genomic DNA was subsequently extracted using the standard phenol–chloroform purification method [21]. The quality and concentration of the isolated DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

For molecular identification of *Aspergillus* spp., the internal transcribed spacer (*ITS*) region of ribosomal DNA was amplified using the universal fungal primers (*ITS1* F: TCCGTAGGTGAACCTGCGG, *ITS4* R: TCCTCCGCTTATTGATATGC) [22]. PCR reactions were performed in a total volume of 25 µL containing template DNA (50 ng/µL), PCR Mastermix 2x (Biolabmix, Russia) 12,5 µL, and nuclease-free water 8µL. PCR amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; with a final extension step at 72 °C for 7 min. Amplified PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination.

DNA sequencing and sequence analysis

Successful PCR products were purified and subjected to Sanger sequencing. The obtained nucleotide sequences were edited and assembled using BioEdit software version 7.0.5. Sequence quality was manually inspected and trimmed where necessary. Species identification was performed by comparing the obtained sequences with reference sequences available in the NCBI GenBank database using the BLASTn algorithm. Identification of fungal isolates was based on the highest sequence similarity with previously deposited sequences of *Aspergillus* spp.

Phylogenetic analysis

To confirm the taxonomic placement of the obtained isolates, phylogenetic analysis was conducted. Multiple sequence alignment of *ITS* sequences was performed using the ClustalW algorithm implemented in MEGA version 11 software. Reference sequences of related Aspergillus species retrieved from GenBank were included in the analysis. A phylogenetic tree was constructed using the Maximum Likelihood method to infer evolutionary relationships among the studied isolates and reference sequences [23]. The reliability of the phylogenetic tree topology was assessed by bootstrap analysis with 1000 replicates.

Results

Microbiological analysis of chickpea seeds resulted in the isolation of fungal colonies belonging to the genus *Aspergillus*. Fungal growth was observed on potato dextrose agar (PDA) after 5–7 days of incubation. Initial morphological and microscopic characterization tentatively placed the isolates within the genus *Aspergillus*. Subsequent molecular identification using ITS rDNA sequencing confirmed the presence of two species, *Aspergillus niger* and *Aspergillus fumigatus*, among the isolates obtained from chickpea seeds. The morphological characteristics of the colonies and microscopic structures of the identified species are presented in Figure 1, while the phylogenetic relationships inferred from ITS sequences are shown in Figure 2.

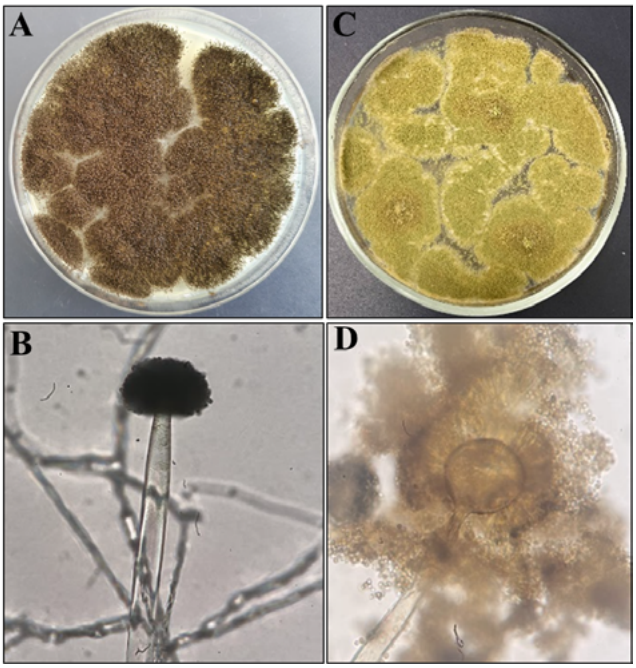

Figure 1. Cultural morphological characteristics of *Aspergillus niger* 1A and 1B; *Aspergillus fumigatus* 1C and 1D

Aspergillus niger colonies (Figure 1A and 1B) on PDA are characterized by rapid growth and intense sporulation. Initially, the mycelium is whitish, but as conidia form, the colony surface becomes dark brown or black. The colonies are dense, velvety-powdery, with a distinct radial structure and jagged edges. The colony diameter rapidly increases and can occupy a significant portion of the Petri dish. The colony reverse is usually light, yellowish-beige, or almost colorless.

Microscopic examination reveals a well-developed, septate, hyaline mycelium. The conidiophores are long, smooth, and colorless, ending in a spherical vesicle. The vesicle is completely covered with metulae and phialides, forming a radial structure. The phialides produce long chains of darkly pigmented conidia, giving the conidial head its characteristic black color. The conidia are spherical, rough-surfaced, and collected in dense radial heads.

Aspergillus fumigatus (Figure 1C and 1D) colonies on PDA medium are characterized by rapid growth and a velvety surface. Young colonies are initially whitish, later acquiring a charac-

teristic gray-green or olive-green color as conidia form. The colony surface is dense, sometimes slightly folded, with jagged or slightly serrated edges. Colonies may form isolated areas of intense sporulation. The colony reverse is usually pale yellow or cream.

Microscopic examination reveals thin, septate mycelium and smooth conidiophores extending from the substrate mycelium. A club-shaped or slightly rounded vesicle form at the apex of the conidiophores. The phialides are arranged in a single row (uniserial) and cover only the upper portion of the vesicle. The conidial chains form a compact columnar head. The conidia are small, round, smooth or slightly rough, form long chains, and give the colonies their characteristic green color.

The phylogenetic tree included the *ITS* sequences obtained in this study together with reference sequences of *Aspergillus* species retrieved from the GenBank database.

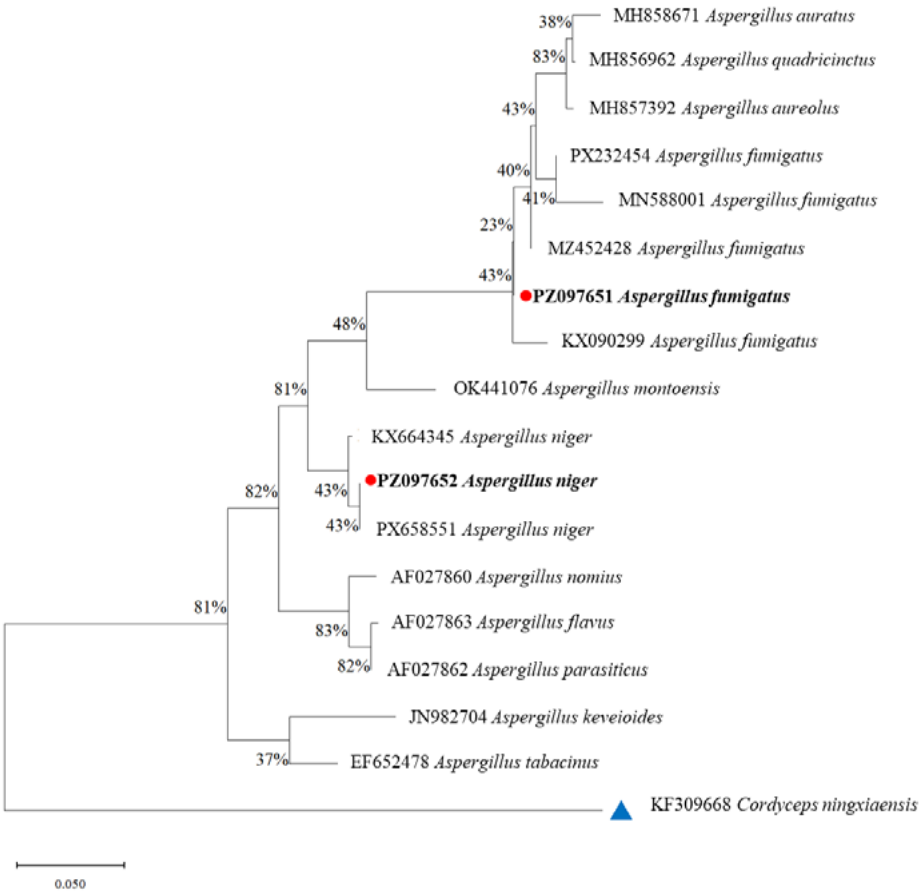

Figure 2. Maximum likelihood phylogenetic analysis of *Aspergillus* species based on *ITS* gene among the studied isolates and reference sequences

In the phylogenetic tree, isolates obtained in the present study are indicated by red circles and bold text. One isolate (PZ097651) clustered with reference sequences of *A. fumigatus* (PX232454, MN588001, MZ452428, and KX090299), forming a clade corresponding to this species. Another isolate (PZ097652) grouped with reference sequences of *A. niger* (KX664345 and PX658551), confirming its placement within the *A. niger* lineage.

Bootstrap values indicated moderate to strong support for several nodes within the tree. To root the phylogenetic tree, *Cordyceps ningxiaensis* (KF309668) was used as an outgroup and

is indicated by a blue triangle in the figure. The resulting topology clearly separated the analyzed isolates into distinct clades corresponding to recognized *Aspergillus* species.

Discussion

The phylogenetic analysis based on the *ITS1* region confirmed that the fungal isolates obtained from chickpea seeds belong to the species *Aspergillus fumigatus* and *Aspergillus niger*. Both species are widely distributed saprophytic fungi commonly associated with stored agricultural products and are frequently reported as storage contaminants of grains and legumes [24, 25].

Species of the genus *Aspergillus* are well known for their ability to colonize seeds during post-harvest storage, particularly under conditions of elevated humidity and temperature [26]. Consequently, the isolation of *A. niger* and *A. fumigatus* from chickpea seeds indicates post-harvest fungal proliferation rather than active field infection. Similar findings have been reported in previous studies investigating fungal contamination of stored legumes and cereal grains [27, 28].

The detection of these species in chickpea seeds is also important from a food safety perspective. Several *Aspergillus* species are known to produce allergenic spores and secondary metabolites that may affect human health [29]. Consumption or inhalation of fungal spores may trigger allergic reactions or respiratory symptoms in susceptible individuals [30]. Beyond direct health implications, the contamination of chickpea batches by potential mycotoxin-producing *Aspergillus* strains represents a significant economic barrier.

As chickpea cultivation expands in Central Asia to meet growing global demand for plant-based proteins, adherence to strict international phytosanitary frameworks – such as the stringent mycotoxin limits enforced by the European Union – becomes paramount. Consequently, the identification of these specific storage pathogens is not merely a localized quality control issue, but a fundamental step in ensuring the export viability of regional agricultural commodities. Therefore, rigorous molecular monitoring of *Aspergillus* species in stored legumes is essential for maintaining food safety and mitigating health risks. Moving forward, addressing this contamination requires a shift toward integrated, agroecological storage management. Rather than relying on chemical fungicidal treatments, which may compromise organic certification and consumer safety, strategies should prioritize holistic environmental control. Implementing advanced moisture monitoring, optimizing natural aeration systems to disrupt condensation cycles, and exploring the application of bio-based fungal inhibitors or antagonistic microbial agents offer promising, sustainable pathways to enhance the resilience and safety of stored chickpea crops.

Conclusion

This study successfully utilized ITS rDNA sequencing to identify *Aspergillus niger* and *Aspergillus fumigatus* as prominent storage contaminants in chickpea seeds cultivated in the Akmola region of Kazakhstan. The isolation of these opportunistic saprophytes underscores a critical vulnerability in current post-harvest storage conditions, where fungal proliferation threatens the economic and export viability of the crop while posing significant health risks due to potential allergenic and mycotoxigenic contamination. To ensure the safety and quality of these vital agricultural commodities, it is imperative to implement rigorous molecular phytosanitary monitoring. Ultimately, mitigating these post-harvest losses requires a paradigm shift away from conventional chemical treatments and toward integrated, agroecological storage management. Future strategies must emphasize the adoption of sustainable agricultural technologies, such as

utilizing bio-based fungal inhibitors and optimizing natural environmental controls, to sustainably enhance crop resilience and ensure food safety.

Author Contributions

K.D., R.U., V.K., A.S. - Conception and design; K.D., R.U., A.S. - Collection and assembly of data; K.D., R.U., A.S. - Data analysis and interpretation; K.D., R.U. - Manuscript writing; K.D., V.K. - Final approval of manuscript.

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Conflicts of Interest

All authors have no conflict of interest to declare.

Compliance with ethical standards

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Қазақстанның Ақмола облысындағы ноқат тұқымдарындағы Aspergillus сақтау саңырауқұлақтарының молекулалық идентификациясы және әртүрлілігі

Р.С. Уахит[1], А.М. Смагулова[2], А.С. Абаш[3], В.С. Киян[4], К.А. Дюсембаев[*5]

[1,2,4,5]Биоәртүрлілік және генетикалық ресурстар зертханасы,
Ұлттық биотехнология орталығы, Астана, Қазақстан
[3]Жаратылыстану ғылымдарының жоғары мектебі,
Астана халықаралық университеті, Астана, Қазақстан
[5]Биотехнология және микробиология кафедрасы,
Л.Н. Гумилев атындағы Еуразия ұлттық университеті, Астана, Қазақстан

Аңдатпа. Ноқат (*Cicer arietinum* L.) – егін жинаудан кейінгі сапасы жиі оппортунистік сақтау саңырауқұлақтарымен төмендейтін, микотоксиндермен ластануына байланысты экономикалық шығындарға және денсаулыққа әлеуетті қауіп төндіретін маңызды бұршақ дақылы. Оның ауылшаруашылық маңызына қарамастан, Қазақстанда ноқат тұқымдарын зақымдайтын сақтау саңырауқұлақтары туралы ақпарат шектеулі күйде қалып отыр. Бұл зерттеудің мақсаты Ақмола облысында өсірілген ноқат тұқымдарын ластайтын *Aspergillus* түрлерін бөліп алу және анықтау болды. Жергілікті ауылшаруашылық өндірісінен алынған 200-ден астам тұқым үлгілері бағаланды. Бастапқы микробиологиялық талдау мен микроскопиялық зерттеуден кейін рибосомалық ДНҚ-ның ішкі транскрипцияланатын спейсері (ITS) аймағын амплификациялау және секвенирлеу арқылы нақты молекулалық идентификация жасалды. Алынған тізбектердің филогенетикалық талдауы екі түрлі түрдің: *Aspergillus niger* және *Aspergillus fumigatus* бар екенін растады. Осы барлық жерде таралған сапрофиттердің бөлініп алынуы жергілікті климаттық факторлар мен қойма микроклиматы әсерінен ушығатын, егін жинаудан кейінгі саңырауқұлақтардың айтарлықтай көбеюін көрсетеді. Бұл нәтижелер аймақтық егін жинаудан кейінгі инфрақұрылымдағы маңызды осалдықты көрсетеді және қатаң фитосанитариялық мониторинг қажеттілігін баса айтады. Жақсартылған, тұрақты сақтауды басқару стратегияларын енгізу саңырауқұлақтармен ластануды азайту және жергілікті ауылшаруашылық өнімдерінің қауіпсіздігін қамтамасыз ету үшін өте маңызды.

Түйін сөздер: *Cicer arietinum,* сақтау саңырауқұлақтары, *Aspergillus niger*, *Aspergillus fumigatus*, ITS рДНҚ, фитосанитариялық мониторинг, егін жинаудан кейінгі сапа

Молекулярная идентификация и разнообразие грибов хранения *Aspergillus* в семенах нута в Акмолинской области Казахстана

Р.С. Уахит[1], А.М. Смагулова[2], А.С. Абаш[3], В.С. Киян[4], К.А. Дюсембаев[*5]

[1,2,4,5]Лаборатория биоразнообразия и генетических ресурсов, Национальный центр биотехнологии, Астана, Казахстан;
[3]Высшая школа естественных наук, Международный университет Астана, Астана, Казахстан;
[5]Кафедра биотехнологии и микробиологии, Евразийский национальный университет имени Л.Н. Гумилева, Астана, Казахстан

Аннотация. Нут (*Cicer arietinum L.*) – важная зернобобовая культура, послеуборочное качество которой часто снижается из-за оппортунистических грибов хранения, что приводит к экономическим потерям и потенциальным рискам для здоровья в связи с контаминацией микотоксинами. Несмотря на его сельскохозяйственное значение, информация о грибах хранения, поражающих семена нута в Казахстане, остается ограниченной. Целью данного исследования было выделение и идентификация видов *Aspergillus*, контаминирующих семена нута, выращенного в Акмолинской области. Было оценено более 200 образцов семян, полученных из местного сельскохозяйственного производства. После первичного микробиологического анализа и микроскопического исследования была проведена точная молекулярная идентификация путем амплификации и секвенирования региона внутреннего транскрибируемого спейсера (ITS) рибосомной ДНК. Филогенетический анализ полученных последовательностей подтвердил наличие двух различных видов: *Aspergillus niger* и *Aspergillus fumigatus*. Выделение этих повсеместно распространенных сапрофитов указывает на значительное размножение послеуборочных грибов, усугубляемое местными климатическими факторами и микроклиматом хранилищ. Эти результаты выявляют значительную уязвимость в региональной послеуборочной инфраструктуре и подчеркивают необходимость строгого фитосанитарного мониторинга. Внедрение улучшенных, устойчивых стратегий управления хранением имеет решающее значение для снижения грибковой контаминации и обеспечения безопасности местной сельскохозяйственной продукции.

Ключевые слова: *Cicer arietinum*, грибы хранения, *Aspergillus niger*, *Aspergillus fumigatus*, ITS рДНК, фитосанитарный мониторинг, послеуборочное качество

Авторлар туралы мәлімет:

Уахит Рабиға Сейтбатталқызы – PhD, Ұлттық биотехнология орталығы Биоәртүрлілік және генетикалық ресурстар зертханасының аға ғылыми қызметкері, 010000, Астана, Қазақстан.

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Абаш Алтынгүл Сембайқызы – Астана халықаралық университеті Жаратылыстану ғылымдары жоғары мектебінің докторанты, 010000, Астана, Қазақстан.

Киян Владимир Сергеевич – PhD, профессор, Ұлттық биотехнология орталығы Биоәртүрлілік және генетикалық ресурстар зертханасының меңгерушісі, 010000, Астана, Қазақстан.

Дюсембаев Казбек Амзеұлы – хат-хабар авторы, PhD, Ұлттық биотехнология орталығы Биоәртүрлілік және генетикалық ресурстар зертханасының ғылыми қызметкері, 010000, Астана, Қазақстан; Л.Н. Гумилев атындағы Еуразия ұлттық университеті Биотехнология және микробиология кафедрасының аға оқытушысы, 010000, Астана, Қазақстан.

Сведения об авторах:

Уахит Рабиға Сейтбатталқызы – PhD, старший научный сотрудник Лаборатории биоразнообразия и генетических ресурсов Национального центра биотехнологии, 010000, Астана, Казахстан.

Смагулова Айнура Муратовна – кандидат биологических наук, старший научный сотрудник Лаборатории биоразнообразия и генетических ресурсов Национального центра биотехнологии, 010000, Астана, Казахстан.

Абаш Алтынгүл Сембайқызы – докторант Высшей школы естественных наук Международного университета Астана, 010000, Астана, Казахстан.

Киян Владимир Сергеевич – PhD, профессор, заведующий Лабораторией биоразнообразия и генетических ресурсов Национального центра биотехнологии, 010000, Астана, Казахстан.

Дюсембаев Казбек Амзеұлы – автор корреспондент, PhD, научный сотрудник Лаборатории биоразнообразия и генетических ресурсов Национального центра биотехнологии, 010000, Астана, Казахстан; старший преподаватель кафедры биотехнологии и микробиологии Евразийского национального университета имени Л.Н. Гумилева, 010000, Астана, Казахстан.

Authors' information:

Rabiga Uakhit – PhD, senior research scientist, Laboratory of Biodiversity and Genetic Resources, National Center for Biotechnology, 010000, Astana, Kazakhstan.

Ainura Smagulova – Candidate of biological sciences, senior research scientist, Laboratory of biodiversity and genetic resources, National Center for Biotechnology, 010000, Astana, Kazakhstan.

Altyngul Abash – PhD student, Higher school of natural sciences, Astana international university, 010000, Astana, Kazakhstan.

Vladimir Kiyan – PhD, professor, head of the Laboratory of Biodiversity and genetic resources, National Center for Biotechnology, 010000, Astana, Kazakhstan.

Kazbek Dyussembayev – corresponding author, research scientist, Laboratory of biodiversity and genetic resources, National Center for Biotechnology, 010000, Astana, Kazakhstan; senior lecturer, Department of biotechnology and microbiology, L.N. Gumilyov Eurasian National University, 010000, Astana, Kazakhstan.