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Research Article

Phylogenetic analysis of some fungi species in West Kazakhstan based on nuclear ribosomal DNA ITS sequences

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Abstract

In this study, phylogenetic analysis of some fungal species distributed in West Kazakhstan was performed based on ITS sequences. All of the mushroom samples were collected from different regions of West Kazakhstan and brought to the laboratory. Total genomic DNA was extracted using a GeneMark commercial kit. ITS1 and ITS4 primers were used for the amplification of the ITS region in PCR analyses. The resulting DNA sequences were then edited using BioEdit and FinchTV. For phylogenetic analysis used MEGA 6.0 program. As a result of the study, ITS sequences ranged from 532 to 715 nucleotides, while the divergence values of the sequences differed between 0.000 and 0.468. The maximum likelihood tree constructed using ITS sequences consists of two clades. According to the phylogenetic analysis results obtained using other fungal species, ITS results were found to be a good indicator for the differentiation of fungal genera.

Keywords: fungi; phylogenetic analysis; ITS; West Kazakhstan

Introduction

The territory of Kazakhstan is about 2,725,000 square kilometers from the temperate and humid forest-steppes of the Siberian Depression to the hot deserts of Central Asia, and the Tien Shan Mountain ranges stretching for more than 1.6 thousand kilometers from north to south and almost three thousand kilometers from west to east (Spanbayev *et al*., 2009). Fungi have been widely used as a valuable source of food and medicine since ancient times (Panda *et al*., 2021), and are an important part of all ecosystems as saprotrophs, are decomposers of organic matter and are diverse groups playing a regulatory role in terrestrial and aquatic ecosystems by making nutrients available for plant uptake (Newbound *et al*., 2010; Zagryadskaya, 2017; Gómez-Hernández *et al*., 2019; Kautmanová *et al*., 2021). Fungi are rich in biodiversity (about 5.1 million

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species) and include both micromycetes and macromycetes. They are also considered among the most important organisms for an ecosystem due to their essential role in the nutrient cycle and their function as pathogens and mutualists (Angelini *et al*., 2016; Caiafa *et al*., 2017; Gómez-Hernández *et al*., 2021). Basidial macromycetes, one of the largest groups of fungi incorporating important ecological groups such as agaricoid, aphyllophoroid and gasteroid fungi, have been recognized worldwide as sources of medicine and food (Shiryaev *et al*., 2018; Martínez-Escobedo *et al*., 2021). In many countries around the world, edible macromycetes species are dietary staples for humans mainly due to their organoleptic properties such as aroma, taste and texture, in addition to their culinary and market value (Stachowiak and Reguła, 2012). Currently, molecular identification of PCR-based methods is believed to be a reliable alternative tool for accurate authentication (Sun *et al*., 2014). rDNA genes exist as parts of repeat units arranged in tandem sequences located at chromosomal sites known as NORs (Dizkirici *et al*., 2016). The ITS (internal transcribed spacer) regions of rDNA genes are considered the primary barcode sequence and DNA marker for phylogenetic studies on fungi due to their respective properties such as ease of amplification in a very reproducible manner (Kalmer *et al*., 2018; Feitosa *et al*., 2019). In this study, non-coding ITS regions consisting of two variable regions were used. This region is located between the SSU and the 5.8 S subunit (ITS1) as well as the region between the LSU rRNA genes and the 5.8 S subunit (ITS2) (White *et al*., 1990; Kılıçoğlu and Özkoç, 2008). In this study, phylogenetic analyses of some macromycetes species distributed in West Kazakhstan were performed using ITS sequences to elucidate phylogenetic relationships among the taxa.

Materials and Methods

Study area, genomic DNA isolation and polymerase chain reaction

The research area is located in the north-west of the Republic of Kazakhstan. Due to its geographical location, the study area in the depths of the temperate climatic belt is extensive and quite homogeneous in climatic terms of a continuous latitudinal strip. This zone of air domination of moderate latitudes is located between the summer position of the polar and the winter position of the Arctic main atmospheric front. The study materials were authors' own mycological collections in the amount of more than 570 specimens and observations in nature collected during field research on forest communities of the Ural River Valley within the West Kazakhstan region. The field investigations were performed by the classical route method. The field studies were carried out in 2019-2022 (Figure 1 and Table 1).

Table 1. Location of fungi species in West Kazakhstan region

No	Species	Ecology	Location/Date
14	*Russula purpureovirescens*	West Kazakhstan region, floodplain forest, left bank of the Ural River, near the village of Pogromnoye, on the ground, this species has been noted in these forest communities: *Populus alba+Rubus caesius; P. alba+Galium boreale; P. alba, Carex acutiformis, P. alba+ Convolvulus arvensis.*	N50° 9683' E51° 3135'/ h-25 m / 11.07.2020
137	*Leucoagaricus barssii*	West Kazakhstan region, floodplain forest (*Populus alba+Populus nigra+Calamagrostis epigejos*), right bank of the Ural River, forest near the village of Atameken, on the ground, this species has been noted in these forest communities: *Populus nigra+Glycyrrhiza aspera*.	N49° 23.130' E51° 47.464', h-(-4) m/ 06.09.2020
272	*Battarrea phalloides*	West Kazakhstan region, floodplain forest, left bank of the Ural River, near the relict lake Bobrovoye, near the destroyed deadwood, this species has been noted in these forest communities: *Populus nigra+Agropyron desertorum,*	N50° 26.010' E51° 08.510', h -15 m/ 07.11.2020

		Populus nigra+ *Carex acutiformis* in combination *Ulmus laevis*+ *Rubus caesius*	
302	*Agaricus pampeanus*	West Kazakhstan region, Ural river valley, right bank of the Ural River, floodplain forest in the vicinity of the village of Budarin, western slope of the dell in the floodplain forest, this species has been noted in these forest communities: clumps of *Elaeagnus commutate* +*Urtica dioica, Ulmus laevis*+*Glechoma hederacea,* littered with such species *Artemisia absinthium, Atriplex oblongifolia, Euphorbia virgata, shoots Ribes rubrum, Arabidopsis thaliana, Capsella bursa-pastoris.*	N50°30.823'E50°58.919',h-21 m./16.05.2021
320	*Amanita vittadinii*	West Kazakhstan region, the central floodplain of the Ural River in the vicinity of the village of Kogalytobek, this species has been noted in these forest communities: in depressions and at the edge of black poplar and white poplar forests *Glechoma hederacea*+*Poa pratensis*, *Dracocephalum thymiflorum*+*Bromopsis inermis,* has also been found in *Populus alba*+ *Elytrigia repens*+ *Carex praecox, Agropyron pectinatum*+*Bromopsis inermis, Erytrigia repens* +*Bromopsis inermis, Potentilla bifurca*+*Carex praecox* communities.	N50° 25.999' E51° 08.408', h - 16 m./ 22.05.2021
374	*Russula exalbicans*	West Kazakhstan region, ravine forest, birch forest *Betula pendula, B. pubescens*, left bank of the Ural River, near the village of Kabyltobe, lower slope, on the ground, this species has been noted in these forest communities: *Betula pendula, B. pubescens*+ multigrass community+ *Cerasus fruticose, Betula pendula, B. pubescens*+ *Vicia cracca*	N51° 19.336' E51° 55.085', h-71 / 20.06.2021
385	*Amanita* sp.	West Kazakhstan region, ravine forest, right bank of the Ural River, near the village of Kabyltobe, birch forest (*Betula pendula, B. pubescens*+*P. tremula),* on the ground, near a fallen birch tree, this species has been noted in these forest communities: *Betula pendula*+ *Vicia cracca*, *B. pubescens*+*Senecio campestris, Betula pendula, B. pubescens*+*Stachys palustris, Betula pendula*+*Galium boreale*	N51° 19.349' E51° 55.066', h - 71m./20.06.2021
386	*Russula anthracina*	West Kazakhstan region, ravine forest, right bank of the Ural River, birch forest (*Betula pendula, B. pubescens*+*P.tremula),* on the ground, near a fallen birch tree (*Betula pendula*+ *Vicia cracca*), this species has been noted in these forest communities: *Betula pendula, B. pubescens*+*Poa pratensis, Betula pendula, B. pubescens*+*Silaum silaus*+*Achillea millefolium*	N51° 19.346' E51° 55.067', h - 70 m./ 20.06.2021
396	*Russula pectinata*	West Kazakhstan region, floodplain forest, Ural River valley, right bank of the Ural River, near the village of Krugloozernyi, on the ground, this species has been noted in these forest communities*: P. alba*+*Aristolochia clematites*+ *Carex acutiformis*	N51° 04.670' E51° 20.447', h - 28 m**./**26.06.2021
418	*Russula exalbicans*	West Kazakhstan region, ravine forest, on the ground, this species has been noted in these forest communities: *Betula*	N51° 19.200' E51° 53.491', h - 60 m**./**05.07.2021

		pubescens+ *Equisetum arvense, E. palustre*. Wheatgrass birch, also found in horsetail birch, lily of the valley birch and sedge birch. Wheatgrass birch, also found in horsetail birch, lily of the valley birch and sedge birch *E. arvense, E. palustre.*	
424	*Russula exalbicans*	West Kazakhstan region, ravine forest (*Quercus robur+Populus tremula+Betula pendula, B. pubescens*), left bank of the Ural River, near the village of Kabyltobe, on the ground, this species has been noted in these forest communities*: Betula pendula, B. pubescens+Galium boreale+Carex acutiformis, Betula pendula, B. pubescens+Rubus caesius.*	N51° 19.213' E51° 53.488', h - 50 m**.**/05.07.2021
443	*Russula purpureovirescens*	West Kazakhstan region, floodplain forest, right bank of the Ural River, white poplar forest in the vicinity of the village of Yanaikino, on the ground, this species has been noted in these forest communities*: Populus alba+Carex melanostachya*	N50° 40.311' E51° 08.617', h - 14 m./06.07.2021
444	*Amanita vittadinii*	West Kazakhstan region, floodplain forest, right bank of the Ural River, white poplar forest in the vicinity of the village of Yanaikino, on the ground, this species has been noted in these forest communities*: Populus alba+Carex praecox*, has also been found in the central floodplain *Populus alba+Glycyrrhiza glabra+Carex praecox, Carex praecox+Festuca ovina* communities.	N50° 40.289' E51° 08.639', h - 14 m./06.07.2021
500	*Calvatia fragilis*	West Kazakhstan region, right bank of the Ural River, near the village of Kirsanov, on the ground, this species has been noted in these forest communities*:* ruderal *Cannabis ruderalis+Artimisia abrotanum.*	N51° 25.846' E52° 24.572', h - 46 m./21.08.2021
537	*Trichaptum biforme*	West Kazakhstan region, ravine forest, left bank of the Ural River, near the village of Kabyltobe, on a fallen birch tree, this species has been noted in these forest communities: *Betula pendula, B. pubescens+Equisetum arvense, E. palustre, Betula pendula, B. pubescens+Rubus caesius* in combination *Convallaria majalis*, *Petasites hybridus.*	N51° 19.361' E51° 54.711', h - 51 m./26.09.2021
548	*Tulostoma fimbriatum*	West Kazakhstan region, Ural River valley, left bank of the Ural River, in the vicinity of the village of Chapaevo, steppe meadow, at the edge of white poplar and black poplar forests *Populus alba+P.nigra,* on the ground, this species has been noted in these forest communities: *Artemisia absinthium+ Agropyron pectinatum, Limonium gmelinii+Agropyron desertorum*.	N50° 12.334' E51° 12.167', h - 11 m./03.10.2021
549	*Geastrum schmidelii*	West Kazakhstan region, Ural River valley, left bank of the Ural River, in the vicinity of the village of Chapaevo, steppe meadow, at the edge of white poplar and black poplar forests *Populus alba+P.nigra,* on the ground, this species has been noted in these forest communities: *Artemisia absinthium+ Agropyron pectinatum*	N50° 12.332' E51° 12.168', h - 11 m**.**/03.10.2021

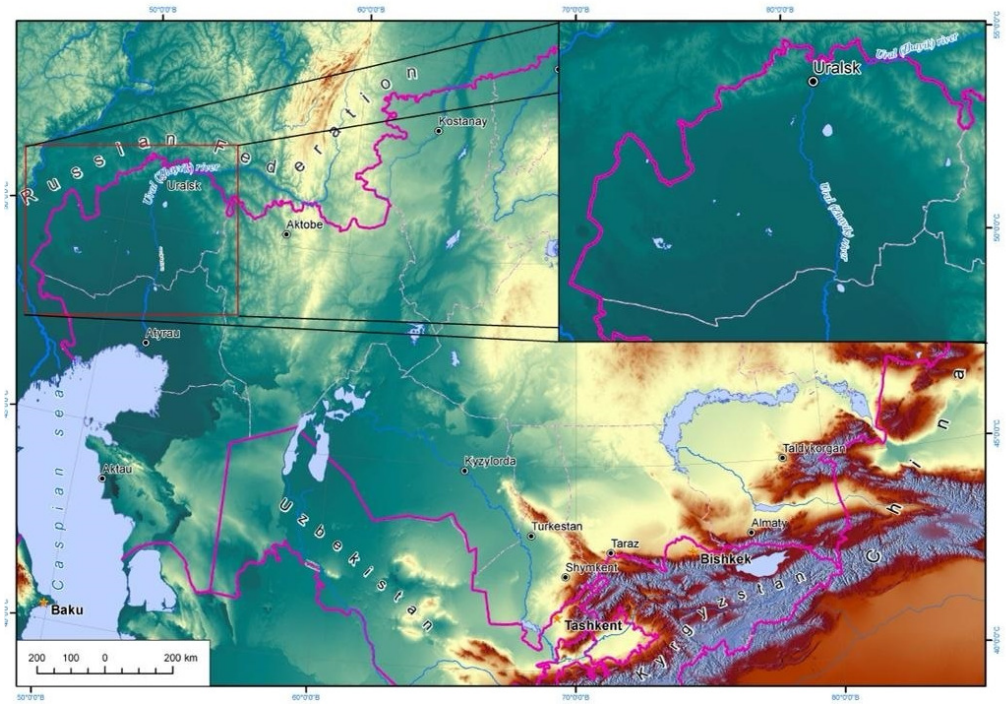

Figure 1. Location of the West Kazakhstan region

Genomic DNA isolation was performed using the Commercial kit (GeneMark) as a genomic DNA isolation method from fungi. The obtained gDNA samples were stored at −20 °C. PCR amplifications were performed with primers from the selected ITS region. The base sequence of the primers, references and PCR protocol are given in Table 2.

Table 2. ITS primers used in this study with PCR compotents and PCR amplification

Primer name	5' to 3' Primer sequence	References	PCR components	PCR amplification
Forward ITS1	TCCGTAGGTGAACCTGCGG	White *et al.* (1990)	1 µL genomic DNA 1 µL primer (forward), 1 µL primer (reverse), 5 µL master mix (PCR buffer, 2Mm $MgCl_2$, dNTP, 0.75U Taq DNA polymerase) and 17 µL dH_2O	94°C/4 min 94°C/1 min 50°C/1 min 72°C/1 min 72°C/10min 35 cycles
Reverse ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990)		

PCR applications were performed with Thermocycler Gradient device. Ready mix was used as an alternative way in the reactions. PCR products were analyzed by electrophoresis on a 1% agarose gel and the amplified products were detected after staining with ethidium bromide.

ITS analysis

For the sequencing and purification of the PCR products, services were obtained from the TRIOGEN (Istanbul/Turkey). For these analysis results to be healthy, it was necessary to visually check the accuracy of DNA sequences one by one, and therefore professional software called BioEdit (Hall, 1999) and Finch TV,

which are frequently used internationally in molecular systematic studies, were used, and contig sequences were obtained. The BioEdit program was used to align the sequences. Appropriate parameters of MEGA 6.0 (Tamura *et al*., 2013) phylogenetic analysis software, which is widely used all over the world, were used to reveal the phylogenetic relationships of macromycetes species whose sequences were obtained. Maximum likelihood was selected from these parameters. To evaluate the degree of support for given clades, a bootstrap analysis (1,000 replicates) was applied (Felsenstein, 1985). In addition, data such as genetic distance and nucleotide content between fungi species were obtained with this program. ITS sequences were uploaded into NCBI and GenBank numbers are given in Table 3.

Table 3. Length, adenine (A), thymine (T), guanine (G), cytosine (C) contents and Genbank numbers of ITS sequences of fungi species

Species	T(U)	C	A	G	Total bp	GenBank numbers
Russula purpureovirescens (14)	21,0	24,2	28,9	25,9	595,0	OP268621
Leucoagaricus barssii (137)	22,1	24,6	29,1	24,2	691,0	OP268629
Battarrea phalloides (272)	22,9	22,9	31,0	23,1	715,0	OP271578
Agaricus pampeanus (302)	23,0	23,4	33,6	20,0	684,0	OP269540
Amanita vittadinii (320)	24,0	21,0	33,6	21,3	675,0	OP271577
Russula exalbicans (374)	22,1	23,7	26,8	27,3	615,0	OP269541
Amanita sp. (385)	25,5	20,7	32,4	21,4	537,0	OP270696
Russula anthracina (386)	21,5	23,4	29,5	25,6	633,0	OP269546
Russula pectinata (396)	22,3	22,6	29,9	25,1	645,0	OP269547
Russula exalbicans (418)	22,4	23,5	27,0	27,1	630,0	OP269555
Russula exalbicans (424)	22,3	23,6	26,5	27,6	615,0	OP269597
Russula purpureovirescens (443)	20,8	24,2	28,7	26,2	595,0	OP269598
Amanita vittadinii (444)	24,1	21,3	33,2	21,3	684,0	OP271579
Calvatia fragilis (500)	24,6	23,0	31,1	21,3	695,0	OP270221
Trichaptum biforme (537)	23,0	23,4	31,8	21,9	636,0	OP288032
Tulostoma fimbriatum (548)	24,4	23,6	29,6	22,4	675,0	OP271295
Geastrum schmidelii (549)	28,6	20,7	30,5	20,3	532,0	OP270695
Average	23,2	23,0	30,2	23,6	638,4	

Results and Discussion

The ITS region in the nucleus evolves more rapidly and is useful for comparing strains within a species or fungal species within a genus (Abacı and Haliki, 2005; Kılıçoğlu and Özkoç, 2008). There are many studies using ITS regions on fungal species (Ko *et al*., 1997; Mitchell and Bresinsky, 1999; Zhang *et al*., 2004; Zamora *et al*., 2014; Guo *et al*., 2014; Loizides *et al*., 2018; Caboň *et al*., 2019; Rusevska *et al*., 2019; Chen *et al*., 2021). This study presents the first attempt to apply molecular-based identification in Kazakhstan macromycetes classifications and an insight into their phylogeny. The ITS sequence lengths of the fungal species ranged from 532 (*Geastrum schmidelii*) to 715 (*Battarrea phalloides*). The mean nucleotide ratio was determined as 23.2% for Thymine, 23.0% for Cytosine, 30.2% for Adenine, and 23.6% for Guanine (Table 3).

Tajma's Neutrality Test (Tajima, 1989) was calculated based on ITS sequences of Fungus species. Numbers of sequences (m) yielded one segregation site (S) revealing very low nucleotide diversity (π) of 0.001422 (Table 4).

Table 4. Tajima's Neutrality Test Values based on ITS of date Fungi species

No. of sequences "m"	No. of segregating sites "S"	$Ps=S/n$	$\Theta = p_s/a_1$	nucleotide diversity " π "	Tajima test statistic "D"
17	179	0.539157	0.159479	0.237708	2.104760

Figure 2 shows the maximum likelihood phylogenetic tree using ITS sequences. This phylogenetic tree consists of 2 large clades.

Clade 1 is divided into two sub-clades. Subclade A, consists of *Russula* spp. (*Russula exalbicans* (418), *Russula exalbicans* (424), *Russula exalbicans* (374), *Russula purpureovirescens* (14), *Russula purpureovirescens* (443), *Russula anthracina* (386), *Russula pectinate* (396)) and this branch is supported with a bootstrap value of 100%. *Russula* It is a cosmopolitan group and is found in a variety of habitats. Some species are both edible and traded worldwide (Caboň *et al*., 2019; Chen *et al*., 2022). Buyck *et al*. (2018) using five loci (nucLSU, mitSSU, *RPB*1, *RPB*2, *TEF*1) in phylogenetic analysis detected *Russula exalbicans* and *Russula gracillima* species in a group. Panda *et al*. (2021) in the ITS analysis of *Russula* species, *Russula anthracina, Russula nigricans* and *Russula densifolia* detected in same group. Subclade B consists of *Amanita (Amanita* sp. (385), *Amanita vittadinii (*320), *Amanita vittadinii (*444)) species and has a bootstrap value of 96%. Drehmel *et al*. (1999) genus *Amanita* is rich in morphological, anatomical and developmental characters that have been used to support subgroups. In addition, phylogenetic evidence using large-subunit ribosomal DNA sequences provides a context for reexamining key characters that have been used to classify *Amanita* species. Zhang *et al.* (2004) reported that molecular analyses results from polyphyletic groups of many *Amanita* specimens of the same name, that are widely distributed in the Northern Hemisphere.

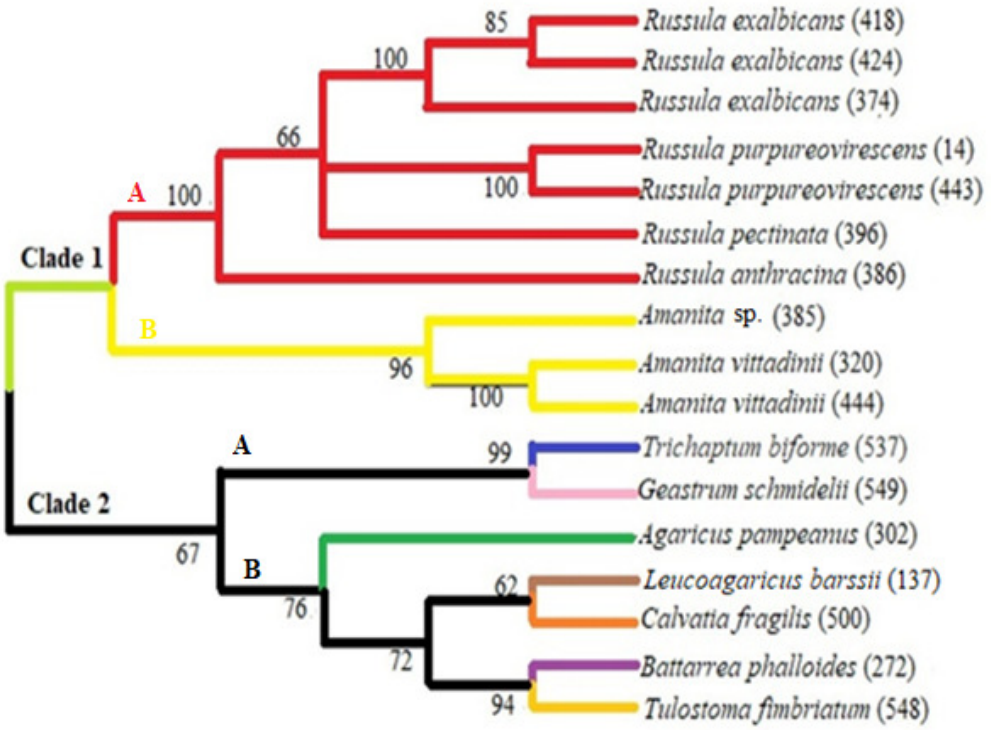

Figure 2. The maximum likelihood tree generated using ITS sequences some fungi species in West Kazakhstan

Clade 2 is divided into two subclades, and subclade A consists of *Trichaptum biforme* (537) and *Geastrum schmidelii* (549) species with a 99% bootstrap support value. For Subclade B, *Calvatia fragilis* (500) and *Leucoagaricus barssii* (137) sister group (bootstrap 62%), *Battarrea phalloides* (272) and *Tulostoma fimbriatum* (548) sister group (bootstrap 94%) and *Agaricus pampeanus* (302) species were found to be related to these two groups. Vellinga *et al.* (2011) determined that in the phylogeny of the *rpb*2 gene region, *Agaricus* species are related to *Chlorophyllum, Clarkeinda trachodes, Heinemannomyces, Coniolepiota, Eriocybe* taxa, *Tulostoma* sp., *Coprinus comatus*, *Lepiota*, and *Leucoagaricus* species are related to *Leucocoprinus* and *Micropsalliota* species. Based on the ITS1 phylogenetic tree, Lagiotis *et al*. (2021) reported that various macromycetic species were clustered in vastly different branches for the majority of taxonomic groups especially characteristic for the groups *Agaricus, Cantharellus*, and *Morchella* forming monophyletic clades, while the topology for a few individual species and clades revealed that it is not so clearly defined. In the ITS1 results, they determined that *Amanita vittadinii* was in a group with *Russula virescens* and *Russula palidospora*. In the nrLSU phylogenetic tree, some *Russula* species were determined to be in a separate group from some *Amanita* species. The ITS sequence results revealed that fungal genera were effective both in differentiation from each other and within species. In addition, the genetic distance between species obtained using ITS sequences is given in Table 5.

Table 5. Pairwise genetic distance matrix obtained from ITS sequences

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Russula purpureovirescens (14)	--																
Leucoagaricus barssii (137)	0.376																
Battarrea phalloides (272)	0.401	0.140															
Agaricus pampeanus (302)	0.387	0.148	0.141														
Amanita vittadinii (320)	0.388	0.273	0.258	0.297													
Russula exalbicans (374)	0.151	0.367	0.392	0.374	0.344												
Amanita sp. (385)	0.409	0.332	0.309	0.297	0.262	0.337											
Russula anthracina (386)	0.165	0.371	0.355	0.351	0.360	0.123	0.351										
Russula pectinata (396)	0.123	0.375	0.367	0.345	0.333	0.105	0.343	0.105									
Russula exalbicans (418)	0.158	0.367	0.403	0.385	0.354	0.012	0.343	0.133	0.116								
Russula exalbicans (424)	0.147	0.362	0.397	0.379	0.349	0.003	0.343	0.126	0.109	0.009							
Russula purpureovirescens (443)	**0. 000**	0.376	0.401	0.387	0.388	0.151	0.409	0.165	0.123	0.158	0.147						
Amanita vittadinii (444)	0.388	0.273	0.258	0.297	**0.000**	0.344	0.262	0.360	0.333	0.354	0.349	0.388					
Calvatia fragilis (500)	0.408	0.129	0.126	0.141	0.259	0.366	0.293	0.353	0.359	0.377	0.371	0.408	0.259				
Trichaptum biforme (537)	0.374	0.263	0.293	0.274	0.362	0.357	0.396	0.363	0.373	0.368	0.362	0.374	0.362	0.272			
Tulostoma fimbriatum (548)	0.401	0.132	0.102	0.148	0.266	0.382	0.301	0.350	0.362	0.398	0.388	0.401	0.266	0.118	0.279		
Geastrum schmidelii (549)	0.464	0.378	0.360	0.298	0.430	0.428	0.441	0.427	**0.468**	0.423	0.422	0.464	0.430	0.352	0.341	0.369	--

The distances between the populations ranged from 0.000 (*Amanita vittadinii* (320-444) and *Russula purpureovirescens* (14-443)) to 0.468 (*Russula pectinata* and *Geastrum schmidelii*) (Table 5). The ITS region can often be widely used to distinguish between closely related species and acts as an efficient primary DNA barcode and for phylogenetic analysis in fungi when other DNA markers are less effective (Avin *et al*., 2012; Badotti *et al*., 2018; Akata *et al*., 2021).

Conclusions

In conclusion, in this study, the phylogenetic relationship of some macromycetes (*Leucoagaricus, Battarrea, Agaricus, Russula, Amanita, Calvatia, Trichaptum, Tulostoma*, and *Geastrum*) species distributed in

Western Kazakhstan was determined and ITS sequence variation could be used effectively for similar studies shown. The ITS phylogenetic tree revealed 2 clades that clearly demonstrate the relationship between macromycetes species. 100% monophyletic *Russula* species and 96% monophyletic *Amanita* species appeared related to each other. As a result of this study, the phylogenetic relationship of some macromycetes species in Kazakhstan was revealed for the first time and a significant contribution was made to the relevant existing phylogenetic data.

Authors' Contributions

S.A.N. collected mushroom samples. A.S.A., D.T.E. identified plant species. S.A.N., E.S, E.S, C.E. Molecular experiments carried out. S.A.N., E.S, E.S. analyzed the data. S.A.N., E.S, C.E. wrote the draft manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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