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Molecular Identification of Some Edible Mushrooms (Order: Agaricales) from Central and North-Eastern Kazakhstan

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Abstract

The aim of the work was to study the species and taxonomic composition of macromycetes of the State National Natural Parks (Kokshetau SNNP, Karkaraly SNNP, and Bayanauyl SNNP), located in Akmola, Karaganda, and Pavlodar regions of Kazakhstan, and to create a collection of strains of natural isolates of edible mushrooms. Collection and identification of mushrooms were carried out according to general procedures applied in the mycology and botany; for molecular identification of species, the method of sequencing and analysis of internal transcribed spacer (ITS) sequence of *ribosomal ribonucleic acid* (rRNA) gene cluster was used. In total, 104 species of agaricoid mushrooms were collected and identified. Morphological and cultural characteristics and growth parameters of mycelium of 57 most actively growing strains from 17 species of edible agaricoid saprotrophs and xylotrophs were isolated on solid agar medium and studied. Molecular identification was performed for two strains Psc-9 (*Psathyrella candolleana*) and Pha-4 (*Pholiota adiposa*).

Keywords

Agaricoid mushrooms; Collection of strains; Macromycetes; Mycelial culture; PCR diagnostics

Introduction

Mushroom is a valuable food product. Currently, mushroom is an ordinary commodity on the shelves of grocery stores in many countries. Previously, mainly wild (forest) mushrooms were offered which were supplied by various "silent hunters", today imported mushrooms cultivated in artificial conditions prevail in the trading network of the country. The rapid growth of production of cultivated mushrooms is evidenced by the fact that in the 1990s in the total annual consumption of mushrooms in the world, the share of the "forest" mushrooms was only about 20%, and today this figure has been steadily reducing. In a number of countries, the mushroom industry brings great income to producers and makes a significant contribution to the food security of these countries [1].

In Kazakhstan, despite its abundant natural features, cultivation of mushrooms has not been established yet on an industrial scale. In many areas, waste products of stock breeding complexes and poultry farms – the main substrate for mushroom cultivation – are not utilized and thus pollute the environment. Individual amateurs, engaged in back-yard production, work with sowing mycelium imported from other countries.

Collection of domestic high-yielding strains of edible mushrooms, required to start the mushroom production in the country, has not been created.

Traditionally, the taxonomy of mushrooms has been based on their morphological and trophic characteristics. However, features such as size and color of fruiting bodies within a species can vary greatly depending on the environmental conditions, which often leads to errors in the determination of their species. Recently, due to the development of the molecular biology and bioinformatics, a popular approach in identifying mushrooms becomes the use of phylogenetic markers of intergenic transcribed spacer (ITS) sequences of gene cluster of ribosomal RNA of a studied object.

Genes encoding the structural *rRNA* genes are conserved sequences, while the ITS region (ITS1/2) evolves more rapidly, and thereby demonstrates a high level of variability [2-4]. This fact allows to use the spacer regions for studies of affined relations between phylogenetically allied species, including agaricoid mushrooms, as well as for molecular identification of mushrooms, species identification of which by morphological character is difficult [5].

The objective of this study is to investigate the species composition of edible agaricoid mushrooms of Central and North-East Kazakhstan, select the most valuable species of mycelial cultures, and examine the cultural and morphological features of strains isolated to pure culture on different solid media. The goal of this work is also to conduct molecular identification of certain species in order to verify (confirm) them.

Materials and Methods

Collection of mushroom fruit bodies was made by the route method in three national natural parks "Kokshetau", "Karkaraly", and "Bayanauyl", located in the Central and North-Eastern parts of Kazakhstan. Morphological identification of mushrooms was performed on the basis of micro- and macro-morphometric characteristics using determinants [6-8].

Isolation of mycelial cultures of higher basidiomycetes

Isolation of mycelial cultures was performed on solid nutrient media (Wort Agar, Potato-Glucose Agar, Czapek-Dox, and Saburo agar) by the tissue method directly from the fruiting bodies. Mushroom fruiting bodies were collected during their mass appearance. Isolation of mycelial cultures was performed on the day of sample collection or from fruiting bodies, which were kept in the refrigerator not over three days. Young, strong, and uninfected

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carpophores were selected for isolation. Before isolation, the fruiting body was purified from foreign substances, and washed with running and sterile water, dried on a filter paper. Then the fruiting body was treated with a 96° – ethanol. Pieces of trams from different parts of the fruiting body (mushroom caps, stripes, junction of caps and stripes) were carried over to the nutrient medium with sterile forceps. Ampicillin (100-200 U/ml) and fundazol (50 μg/ml) were added in the nutrient medium to inhibit the growth of foreign microflora. Petri dishes with inoculum were incubated in a thermostat at a temperature of 26.5°C. Purification of cultures from foreign microorganisms was performed by repeated subculturing.

Extraction of DNA

For DNA extraction a buffer solution containing 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, proteinase K 100 mg/ml [9-11] was used. 10-12 days of mycelial cultures were taken and placed in a sterile mortar, liquid nitrogen was added and all was triturated until a homogeneous conditio. 100 mcl of the derived suspension was transferred to a sterile 1.5 ml test tube, 500 mcl of the corresponding buffer was added. Incubation lasted for 18 h. Further, the purification of DNA by phenol/chloroform method was performed. To this end, 750 mcl of chloroform/isoamyl alcohol (24:1) was added to the derived suspension, and mixed thoroughly and centrifuged at 12,000 rpm for 10 min. The aqueous phase was carried over to a new test tube, and purification with phenol/chloroform (1:1) was repeated. After centrifugation, the aqueous phase was carried over to a new clean test-tubes and DNA 600 mcl of isopropyl alcohol was precipitated. Centrifuged at 12,000 rpm for 10 min, the DNA pellet was washed once with 70% ethanol followed by centrifugation and removal of the liquid phase. The pellet was then dried in the air for 15 min. DNA samples were dissolved in 100 mcl single TE buffer and stored at −20°C. DNA concentration was measured spectrophotometrically using a NanoDrop spectrophotometer at a wavelength of 260 nm.

Amplification of the ITS sequence

The PCR was performed with primers ITS 5 5′-ggaagtaaaagtcgtaacaagg-3′ and ITS 4 5′-tcctccgcttattgatatgc-3′ in total reaction volume 30 mcl. The PCR mixture contained 40 ng of DNA, 1 unit of Taq DNA Polymerase (Fermentas), 0.2 mM of each dNTP (deoxynucleozodtriphosphate), 1-time PCR buffer (Fermentas), 2.5 mM $MgCl_2$, 10 pmole of each primer. The PCR amplification program consisted of a primary denaturation at 95°C for 4 min followed by 30 cycles: 95°C for 25 s, 52°C for 30 s, 72°C for 40 s; the final elongation for 7 min at 72°C. The PCR was performed in amplifier DNA Engine Tetrad 2 Cycler PTC-0240 (Bio-Rad).

Determination of the nucleotide sequence

Purification of PCR products from unbounded primers was performed by the fermentative method using Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas).

Sequencing reaction was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, followed by separation of fragments on the Automated Genetic Analyzer 3730xl DNA Analyzer (Applied Biosystems).

Molecular identification of mushroom strains was carried out by determining the forward nucleotide sequence of the ITS region and comparing its identity to nucleotide sequences of species deposited in the international database GenBank (www.ncbi.nlm.nih.gov).

Results and Discussion

Materials were collected in three National Natural Parks, "Kokshetau", "Karkaraly", and "Bayanauyl", located in the central and North-Eastern part of Kazakhstan. The territory of the National Parks was selected because it has been less affected by human and has rich evolutionary formed natural diversity of mushrooms.

All together 104 species of agaricoid mushrooms of the following families were collected and identified: *Russulaceae* – 26 species, *Tricholomataceae* – 20 species, *Agaricaceae* – 13 species. The rest of the families (Boletaceae, Paxillaceae, Amanitaceae, Gomphidiaceae, Cortinariaceae, Strophariaceae) included up to 10 species. Only one species of the *Hygrophoraceae* family was found (*Hygrocybe conica* (Fr.) Kumm.). The collection included edible species, mycorrhiza formers and saprotrophs. Poisonous species are rare – only three species (*Agaricus xanthodermus* Gen., *Paxillus involutus* Fr., Hygrocybe conica (Fr.) Kumm.).

Table 1 contains characteristics of the radial growth of mycelium and formation of colonies in 57 most actively growing strains out of 17 species of agaricoid edible sapro- and xylotrophs isolated on a dense agar medium (Wort Agar) in Petri dishes.

According to the A.S. Buchalo classification [12], Basidiomycetes' mycelial colonies by their growth rate can be divided into three groups: I – the fast-growing (PK $>$ 100), II – growing at a moderate speed (PK $=$ 50-100), and III – slow-growing (PK $<$ 50). All investigated strains belong to the group of slow- and moderate-growing mycelial colonies. At the initial stage of growth of colonies on solid agar medium (Wort Agar) the mycelium was arachnoid, mealy, later felted, creeping along the substrate. Through overgrowing of the nutrient medium in Petri dishes in most strains occurred after a month. Mycelium thickening began on the 12th-17th day of cultivation, but the densest cotton-felted mycelium with plaques formed after one month from the date of sowing on Petri dishes.

In a vast majority of strains (48 strains): Sg-1, Sg-8, Sg-23, Lc-1, Lc-15, Lc-18, Km-25, Km-34, Cg-3, Cg-5, Cg-7, Cg-19, Ls-2, Ls-16, Ls-18, Ls-24, Agt-6, Agt-7, Agt-12, Lacc-4, Lacc-15, Lacc-16, Lacc-22, Pi-1, Pi-6, Pi-9, Ld-5, Ld-9, Lp-2, Lp-14, Lp-23, Be-8, Be-12, Be-17, Be-20, Lv-7, Lv-13, Lv-16, Psc-4, Psc-7, Psc-9, Psc-12, Agd-3, Agd-6, Agd-8, Agd-9, Agd-13, Agd-14 – the largest radial growth of colonies was observed on the 7th-10th day of cultivation. In other strains (9 strains): Phs-3, Phs-10, Phs-15, Pp-4, Pp-6, Pp-8, Pp-12, Pha-2, Pha-4 – the highest growth rates were observed on the 17th-18th day from the beginning of cultivation. Mycelium obtained the greatest density in 4-4.5 weeks after the beginning of cultivation.

Cultural and morphological character of strains was studied on different agar media (Wort Agar, Potato-Glucose Agar, the Czapek-Dox, and Saburo Agar). The most suitable for the mycelial growth was Wort Agar. Records of the colony growth features were kept during the entire period of observation on the following parameters: texture and form of colonies, mycelium pigmentation, density and height of the aerial mycelium, daily average growth rate and growth factor. Timing of the subsequent phases of changes of mycelial colonies for five strains of two species of mushrooms, including two sequenced strains (Psc-9, Pha-4) and the other strains (Psc-7, Psc-12, Pha-2) are shown in Figure 1.

Strain Psc-9 (*Psathyrella candolleana*) by the intensity of colony growth refers to the average growing group. The shape of colonies is rounded, the color of aerial mycelium is white, the color of colonies'

Strains	Cultivation period (days)				
	Arachnoid mycelium	Dense mycelium	Dense plaques on the mycelium	Pigmented mycelium	Mycelial film
Sg 1 (*Suillus granulates*)	8	23	29	33	45
Sg 23 (*S. granulates*)	10	22	27	35	43
Sg 8 (*S. granulates*)	8	16	22	34	39
Lc 1 (*Lyophyllum connatum*)	11	20	25	43	52
Lc 15 (*L. connatum*)	11	22	24	37	50
Lc 18 (*L. connatum*)	9	16	20	33	48
Phs-3 (*Pholiota squarrosa*)	18	25	31	39	65
Phs-10 (*P. squarrosa*)	16	24	33	39	62
Phs-15 (*P. squarrosa*)	17	28	35	42	61
Km 25 (*Kuehneromyces mutabilis*)	10	23	33	35	45
Km 34 (*K. mutabilis*)	12	23	30	34	45
Pp 4 (*Pleurotus pulmonarius*)	18	28	36	42	62
Pp 6 (*P. pulmonarius*)	16	27	34	41	63
Pp 8 (*P. pulmonarius*)	19	30	37	44	64
Pp 12 (*P. pulmonarius*)	18	29	37	45	65
Cg 3 (*Clitocybe gibba*)	7	16	25	31	39
Cg 5 (*C. gibba*)	9	17	24	33	39
Cg 7 (*C. gibba*)	8	15	23	32	40
Cg 19 (*C. gibba*)	6	17	25	31	42
Lecs 2 (*Leccinum scabrum*)	7	15	26	29	39
Lecs 16 (*L. scabrum*)	8	16	23	30	41
Lecs 18 (*L. scabrum*)	10	14	21	29	43
Lecs 24 (*L. scabrum*)	9	16	21	28	46
Agt 6 (*Agaricus tabularius*)	9	16	22	29	43
Agt 7 (*A. tabularius*)	8	17	23	32	47
Agt 12 (*A. tabularius*)	10	18	24	29	45
Lacc 4 (*Lactarius controversus*)	9	16	25	32	49
Lacc 15 (*L. controversus*)	11	19	26	31	47
Lacc 16 (*L. controversus*)	8	16	24	32	48
Lacc 22 (*L. controversus*)	10	17	26	33	51
Pi 1 (*Paxilus involutus*)	9	16	22	32	47
Pi 6 (*P. involutus*)	9	16	23	32	49
Pi 9 (*P. involutus*)	11	18	24	30	41
Ld 5 (*Lactarius deliciosus*)	10	16	25	34	53
Ld 9 (*L. deliciosus*)	9	17	26	32	47
Lp 2 (*Lactarius pubescens*)	9	17	24	35	52
Lp 14 (*L. pubescens*)	11	19	25	35	50
Lp 23 (*L. pubescens*)	10	16	24	37	49
Be 8 (*Boletus edulis*)	9	17	26	34	47
Be 12 (*B. edulis*)	10	18	27	33	49
Be 17 (*B. edulis*)	10	18	25	31	53
Be 20 (*B. edulis*)	11	17	25	32	54
Lv 7 (*Lactarius vellereus*)	10	16	26	31	51
Lv 13 (*L. vellereus*)	9	15	24	32	49
Lv 16 (*L. vellereus*)	11	17	26	32	51
Psc-4 (*Psathyrella candolleana*)	9	14	23	30	39
Psc-7 (*P. candolleana*)	8	15	24	32	39
Psc-9 (*P. candolleana*)	7	15	23	31	38
Psc-12 (*P. candolleana*)	9	13	24	30	39
Agd 3 (*Agrocybe dura*)	9	14	25	32	40
Agd 6 (*A. dura*)	8	14	24	30	37
Agd 8 (*A. dura*)	7	13	25	31	39
Agd 9 (*A. dura*)	7	14	26	30	37
Agd 13 (*A. dura*)	8	14	24	31	36
Agd 14 (*A. dura*)	8	13	24	31	37
Pha-2 (*Pholiota adiposa*)	13	19	27	37	47
Pha-4 (*P. adiposa*)	12	19	28	37	47

Table 1: Characteristics of growth of mycelial colonies of different strains of mushrooms on solid agar medium (Wort Agar)

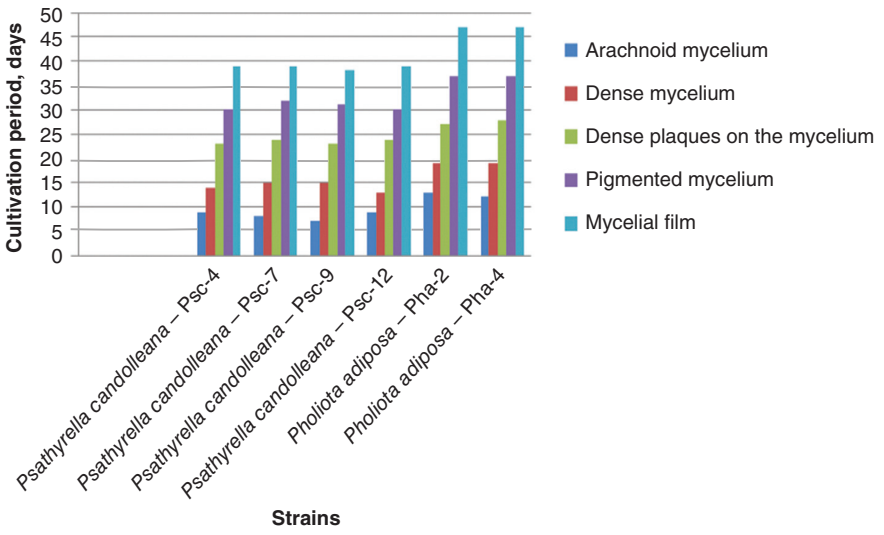

Figure 1: Characteristics of growth of mycelia colonies of certain strains of mushrooms

reverse is light yellowish tinge, the nature of colonies' surface is velvety, edges of colonies are smooth, colonies' growth is radial. Timing of the subsequent phases of morphological changes of the colonies is outlined in Figure 1. Formation of the arachnoid mycelium was noted on the 7th day from the beginning of incubation, arachnoid mycelium thickening was observed on the 15th day, dense plaques appeared on the mycelium on the 23rd day, mycelium pigmentation started on the 31st day, and a dense mycelial film was formed on the 38th day of cultivation.

Strain Pha-4 (*Pholiota adiposa*) – by the intensity of colony growth refers to the slow-growing group. The shape of colonies is rounded, the color of the aerial mycelium is yellowish, the color of colonies' reverse is yellowish-gray, the nature of colonies' surface is velvety, the edges of colonies are smooth, colonies' growth is radial. Timing of the above mentioned phases of colonies' morphological changes was observed, respectively, on the 12th, 19th, 28th, 37th, and 47th days of incubation.

Nucleotide Sequence Analysis

For more accurate species identification of two strains Psc-9 (P. *candolleana*) and Pha-4 (Ph. *Adiposa*), the molecular identification was performed, using the sequencing method and analysis of the ITS sequences. A phylogenetic tree of kinship was built between the species based on sequenced ITS sequences of these strains, and sequences deposited in the international Gene Bank database. To build a phylogenetic tree, the method of maximum likelihood (ML) from the MEGA 5 software package was used [13]. The counting was performed on 705 sites of sequenced ITS sequences.

The nucleotide sequences were analyzed and combined into a common sequence in the SeqMan (DNAStar) program. Then terminal fragments (nucleotide sequences of primers and fragments with low quality levels) were removed. The derived sequences were identified using the GenBank database by the BLAST algorithm.

The molecular identification of the nucleotide sequences of two strains (Psc-9, Pha-4), examined is belonging to two species of agaricoid mushrooms (Psc-9 *Psathyrella candolleana* and Pha-4 *Pholiota adiposa*), and comparative analysis with homologous ITS sequences from the GenBank (HQ436117.1 *Psathyrella candolleana*; HQ436122.1 *Pholiota adiposa*) showed a 99% similarity for the both strains. For a more reliable confirmation of the Psc-9 and Pha-4 strains' species, a phylogenetic analysis of kinship was conducted based on the ITS sequence between these strains and samples of ITS sequences of strains deposited in the Genbank (Figure 2). On the dendrogram, the Psc-9 strain fell into the group that united all strains *P. candolleana*, the sequences of which were derived from the Genbank. Therefore, we believe that the Psc-9 strain belongs to *Psathyrella candolleana* species. The Pha-4 strain got to a clade that combined the two closely related species Ph. *adiposa* and Ph. *aurivella*, and this Pha-4 strain fell into the subclade with strains Ph. *aurivella* from the Genebank. According to the Index Fungorum, the species names – Ph. *adiposa* and Ph. *aurivella* – are synonymous, so we assign the Pha-4 strain from our collection to the *Pholiota adipose* species, as determined on the basis of macro-morphological characteristics using the determinant "Kazakhstan's Spore Plants Flora, Volume 13, Part 1.2".

Thus, as a result of the research, the collection of mycelial cultures of agaricoid Basidiomycetes has been created and defined; this collection comprises 57 strains belonging to 17 species. These species are the most common in Kazakhstan's national parks explored by us. Optimal environment and culture conditions were provided for cultivation in the laboratory of basidiomycetes' natural isolates, isolated to culture. The species of two strains from the collection has been confirmed by the molecular method.

Conclusion

In three national parks located in Central and North-East Kazakhstan 104 species of agaricoid mushrooms belonging to ten families were detected. The studies on solid agar medium of features of the mycelium radial growth and the nature of colony formation in 57 strains of 17 the most valuable edible species of sapro-xylotrophs showed the following results. All investigated strains belonged to the group of slow- and moderate-growing mycelial colonies. The vast majority of strains (48 strains) showed a high growth rate on the 7th-10th day of cultivation, and the remaining strains (9 strains) showed the highest

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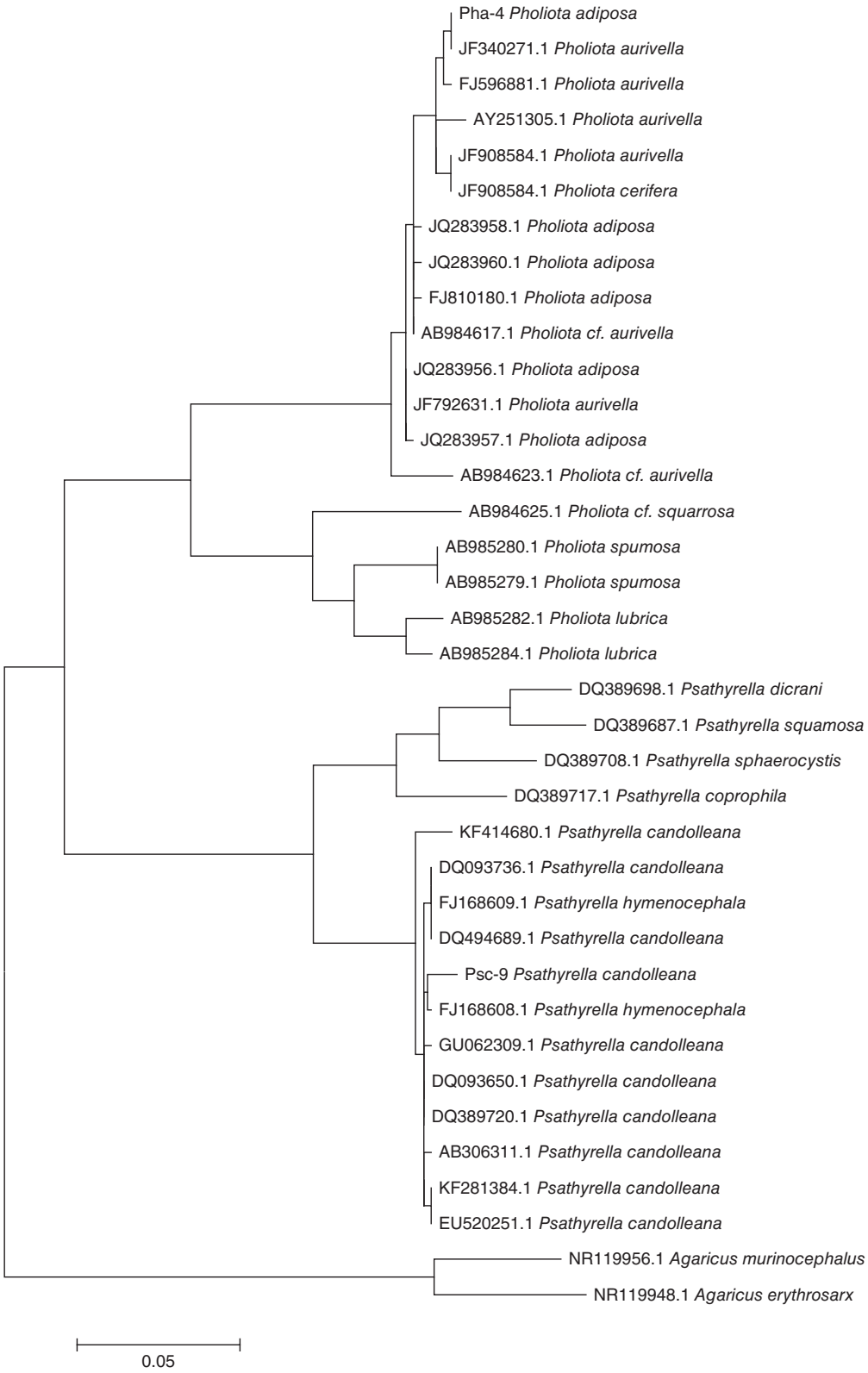

Figure 2: The phylogenetic tree of kinship based on the ITS sequences between the Psc-9 *Psathyrella candollean* and Pha-4 *Pholiota adiposa* strains and sequences from the Genbank. The tree was built using the ML (Maximum Likelihood) algorithm in the MEGA5 program

growth rates on the 17th-18th day from the beginning of cultivation. Mycelium obtains the biggest density in 4-4.5 weeks after the beginning of cultivation. Pigmentation of colonies was observed on the 33rd-42nd day, a mycelial film was formed on the 39th-62nd day of cultivation.

Molecular identification of the nucleotide sequence of the two strains (Psc-9, Pha-4) of two species of agaricoid mushrooms (*Psathyrella candolleana* and *Pholiota adiposa*) and its comparative analysis with the Genebank data (HQ436117.1 *Psathyrella candolleana*; HQ436122.1 *Pholiota adiposa*) for both strains equally showed a 99% coincidence.

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