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EXPERIMENTAL ARTICLES

Comparative Morphological, Ecological, and Molecular Studies of *Aspergillus versicolor* (Vuill.) Tiraboschi Strains Isolated from Different Ecotopes

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Abstract—Cultural, morphological, ecological, and trophic properties (growth at different temperatures and on various organic substrates), as well as molecular and genetic peculiarities of *Aspergillus versicolor* (Vuill.) Tiraboschi strains of different origins, were determined. The strains were isolated from different ecotopes (upper horizons of modern soils of several geographic regions, ancient soils and peat, and permafrost). No essential distinctions in cultural and morphological properties were revealed between the strains. Strains obtained from peat of the Aleutian Islands were characterized by the highest radial rates of colony growth. Some variations in the ITS loci of rDNA were observed in strains isolated from different ecotopes; the distinctions were most pronounced (1.7%) in the strain isolated from 100000-year-old permafrost.

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Key words: microscopic fungi, *Aspergillus versicolor*, populations, growth rates, sequencing, rDNA.

Fungi of the genus *Aspergillus* are a dominant group of micromycetes in soil ecosystems. They are especially abundant in soils of southern latitudes [1]; their spores frequently occur in air [2]. Aspergilli are typical inhabitants of anthropogenic urban ecosystems [3]. At present, fungi of the genus *Aspergillus* attract increased interest due to their toxic, allergenic, and, particularly, pathogenic properties for humans. Representatives of the genus *Aspergillus* do not belong to obligate human or animal parasites; however, they exhibit properties of opportunists: they can grow as saprophytes and, at the same time, they are able to cause secondary mycoses in humans [4].

Information on the extent of variability in the properties of fungal species isolated from different ecotopes is of great interest. Saprophytic strains of microscopic fungi isolated from various ecotopes were shown to have different temperature optima for mycelium growth. Comparative studies of fungi isolated from different geographic zones revealed the existence of ecotypes among such species as *Penicillium nigricans* (synonym, *P. janczewskii*), *Cladosporium cladosporioides, Aspergillus alliaceus, A. flavus*, and *A. niger* [5].

Thus, strains of one species isolated from different populations may differ in a number of properties, such as morphology, kinetics of colony growth under various conditions, biochemical properties (enzyme activity, production of secondary metabolites, etc.), and genetic characteristics.

At present, new molecular methods are usually applied to reveal intraspecific variability of morphologically similar organisms [6]. However, information on variations in the genetic properties of microscopic fungi from different populations is scarce.

It is also of interest to compare the properties of modern and ancient microorganisms. Most species of microscopic fungi isolated from ancient soils were shown to be entire analogues of modern inhabitants of terrestrial ecosystems [7]. This raises the question of whether the properties of age-differentiated representatives of these species are identical.

This work was carried out with strains of the species *Aspergillus versicolor*, which is widespread in terrestrial ecosystems from polar to southern latitudes [1]. It can survive for a long time in natural environments under conditions unfavorable for growth (more than tens of thousands of years) [7]. At present, this species is considered to be an important causative agent of secondary aspergilloses in humans, much like such well known potential pathogens as *A. fumigatus, A. flavus*, and *A. terreus* [4].

The aim of this work was to compare cultural, morphological, ecological, and trophic properties and molecular characteristics of *A. versicolor* strains isolated from modern soils of different geographic regions and from soils of different ages.

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Table 1. Strains of *Aspergillus versicolor* used in the study

Strain designation	Short name	Source of isolation	Geographic location	GenBank accession number
NHRC-FE043 (DSB MSU 20.1)	E43	Leached chernozem	Voronezh oblast	AJ937749
NHRC-FE060 (DSB MSU E60)	E60	Urban soil	Rostov-on-Don	AJ937750
NHRC-FE061 (DSB MSU E61)	E61	Buried soil from an ancient town of the third century B.C.	Rostov-on-Don	AJ937751
NHRC-FE079 (DSB MSU E79)	E79	Brown semidesert solonetzic soil	Astrakhan oblast	
VKM FW–681 (NHRC-FE075)	E75	Permafrost (100000 years old)	Kolyma Lowland	AJ937752
NHRC-FE076	E76	Peat (7000 years old)	Shemia Island, Aleutian Islands, United States	AJ937753
NHRC-FE077	E77	Peat (7000 years old)	Shemia Island, Aleutian Islands, United States	AJ937754
NHRC-FE078	E78	Peat (7000 years old)	Shemia Island, Aleutian Islands, United States	AJ937755

MATERIALS AND METHODS

Strains. This study was carried out with eight strains of *A. versicolor* isolated from soils of different ages in various geographic regions (Table 1).

Investigation of cultural and morphological properties. Description of cultural and morphological properties of the strains studied was performed according to modern recommendations [8]. Reproductive structures were measured under a Carl Zeiss AxioSkop microscope at a magnification of 800×. Identification of strains was performed according to [9, 10].

Investigation of ecological and trophic properties. To study ecological and trophic properties of *A. versicolor* strains isolated from various ecotopes, the fungi were cultivated on media with different organic substrates, such as Czapek agar with sucrose and protein-containing media; Mycosel agar; and blood agar. The growth was studied in a wide temperature range, at 5, 15, 20, 25, 27, 29, 31, 33, 35, and 37°C. Since representatives of *A. versicolor* are frequently encountered in soils of high salinity and can be isolated from permafrost of marine origin [11], the fungi were cultivated on Czapek media with different NaCl concentrations: 0.05, 0.9, and 3.5%, which corresponded to the concentrations of NaCl in standard Czapek medium, saline solution, and seawater, respectively. Strain growth was evaluated by calculating the radial rate of colony growth: $K_r = \Delta D/\Delta t$. The results were statistically processed using Microsoft Excel and Statistika.

Isolation of genomic DNA. A 5-day culture grown in liquid Czapek medium was treated with an alkaline solution of sodium dodecyl sulfate (SDS) (0.5% SDS in 25 mM NaOH) in a 1.5-ml Eppendorf tube and heated at 94°C for 5 min. Then, cryodestruction of cells was carried out by repeated freezing in liquid nitrogen and thawing (20 cycles). Finally, the suspension was heated at 94°C for 5 min and centrifuged; the supernatant was transferred into a clean tube, whereas the sediment was repeatedly extracted with a solution of guanidine thiocyanate (7 M, pH 5.5). Supernatants were combined, acidified with 1 M acetic acid to pH 5.5–6.5, and supplemented with isopropyl alcohol (20 vol%) and 50% (v/v) "glass milk" (no less than 16 μl/ml mixture) prepared from Silica Gel (Merck, Germany). Typically, the mixture contained 100 μl of extract with SDS, 170 μl of a guanidine solution, 1 μl of acetic acid, 67.5 μl of isopropyl alcohol, and 5 μl of a glass milk suspension. The Eppendorf tube with the mixture was shaken in a vortex and incubated at 55–60°C in a thermostat for 5 min with periodic shaking. The glass was precipitated by centrifugation, resuspended in 300–400 μl of a guanidine thiocyanate solution (7 M, pH 5.5), and then washed thrice with a solution containing Tris–HCl (10 mM, pH 7.0), EDTA (1 mM), NaCl (100 mM), and ethanol (about 75 vol %). DNA was eluted with Tris–HCl (10 mM, pH 8.5). The concentration of DNA was measured spectrophotometrically.

PCR analysis and DNA sequencing. Polymerase chain reaction was carried out with universal fungal primers, ITS1F [12] and NL4 [13]. DNA sequencing was performed with the use of an ABI PRISM® BigDye™ Terminator v. 3.1 kit; the reaction products were analyzed on an automatic ABI PRISM 3100-Avant sequencer using the following primers: ITS1F, ITS3, ITS4 [14], and NL4.

Analysis of the sequences obtained and assembly of common contigs were performed with the use of the Chromas 2.3 program. Cladistic analysis was carried out by the neighbor-joining method with the corrections for the possible multiple substitutions according to Kimura. The bootstrap analysis was performed with the use of 1000 replications. All these analyses, including the alignment of nucleotide sequences, were performed with the aid of the ClustalX 1.83 software package. The program TreeView 1.6.6. was applied to visualize cladograms, and the program BLAST-3 was used for searching for and ranking homologous nucleotide sequences in the GenBank/EMBL/DDJB databases.

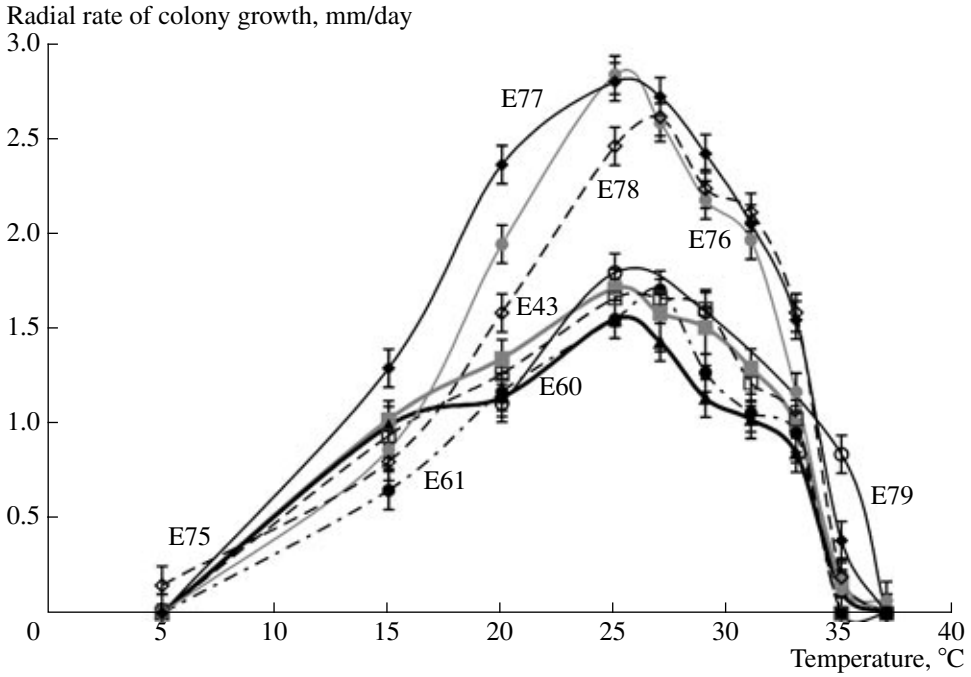

Fig. 1. Radial rates of colony growth of *A. versicolor* strains on Czapek medium at different temperatures. The strains are described in Table 1.

RESULTS

Under standard conditions of micromycete cultivation, cultural and morphological properties of *A. versicolor*, such as pigment excretion and the size and color of colonies, corresponded to the description of this species and were similar in different strains. Conidia were 2.5 to 3.5 μm in size and had a rough surface; vesicles were 10 to 15 μm in diameter, with the shape varying from clavate to ellipsoid; sterigmas were two-layered [9, 10].

Although the strains studied were isolated from regions with different temperature regimes, they had the same temperature optima and similar temperature ranges for mycelium growth (Fig. 1).

All the strains grown on Czapek medium exhibited the highest rate of colony growth at 25–27°C. Minimal temperature for the growth of the strains (including those isolated from permafrost) was 5°C; maximal temperatures varied from 35 to 37°C.

However, strains of *A. versicolor* isolated from various ecotopes differed in the rates of colony growth in the temperature range from 20 to 30°C. The strains isolated from peat of the Aleutian Islands (E76, E77, and E78) were characterized by the highest growth rates; the time courses of their growth were similar.

It is known that the effect of adverse ecological factors on the growth of micromycetes often decreases on rich organic media. To study whether the medium composition can affect the temperature range of mycelium growth, we cultivated the strains on protein-containing Mycosel agar at temperatures above 20°C. However, the optimum temperature (25–27°C) and the maximal temperature for strain growth proved similar to those observed on Czapek medium.

Blood agar, containing a wide range of animal proteins, is an even richer organic medium. All the strains studied exhibited higher growth rates on blood agar than on other media (Fig. 2). At temperatures close to optimal (25–30°C), the growth rates on blood agar were 1.5–2 times higher than on standard Czapek medium. At a higher temperature (35°C), the rates of strain growth on blood agar were similar to those observed on Czapek medium. The increase in the rate of strain growth on blood agar is in agreement with the known ability of *A. versicolor* to exhibit pathogenic properties [4].

Since *A. versicolor* is often encountered in environments with increased salinity, we studied the effect of the NaCl concentration on the growth of the isolates. It was found that increased concentrations of NaCl were favorable for the growth of all the strains studied. The strains isolated from ecotopes with increased salinity, such as permafrost (E75) and seaside peat (E76, E77, and E78), showed the highest growth rates in media with maximal concentrations of NaCl, in contrast to the strains isolated from continental soils (modern urban soil and brown semidesert solonetzic soil) (Fig. 3).

To study strain distinctions at the genetic level, we analyzed sequences of the ITS1, 5.8S, and ITS2 loci (500 bp) and an adjoining 600-bp fragment of 28S rDNA, which are standard objects of molecular and phylogenetic analyses at the species level.

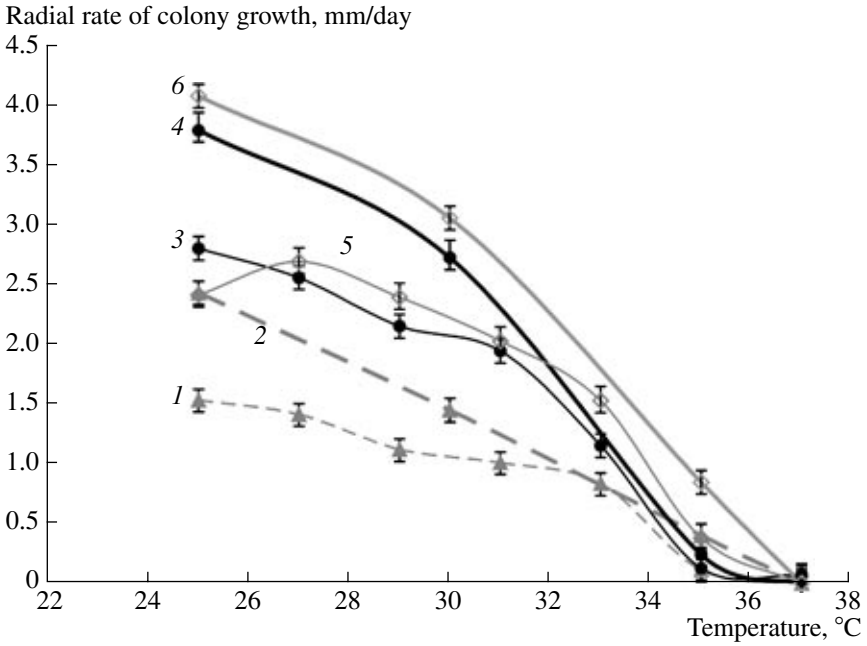

Fig. 2. Comparison of radial rates of colony growth of *A. versicolor* strains at different temperatures on (*1*, *3*, and *5*) Czapek medium and (*2*, *4*, and *6*) blood agar. Strains: (*1* and *2*) E60; (*3* and *4*) E76; (*5* and *6*) E77.

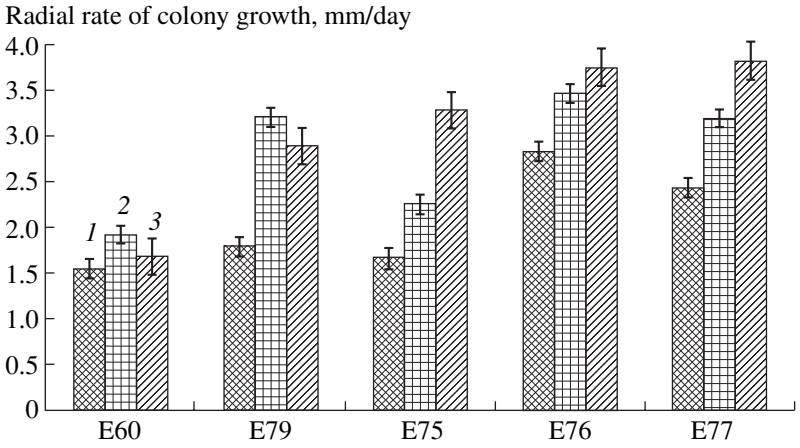

Fig. 3. Radial rates of colony growth of *A. versicolor* strains at 25°C on Czapek media with different NaCl concentrations (%): (*1*) 0.05; (*2*) 0.9; (*3*) 3.5. The strains are described in Table 1.

Sequences of the ITS1, 5.8S, ITS2, and 28S rDNA loci of the strains isolated from southern soils of European Russia, such as leached chernozem (E43), soil from an ancient town (E61), and modern urban soil (E60), were completely identical throughout the sequenced fragment of rDNA (Fig. 4).

Similarly, nearly identical sequences were revealed in strains isolated from peat of the Aleutian Islands (a distinction in one nucleotide was observed between strain E78 and strains E76 and E77). On the whole, the group of strains isolated from peat differed from the group of soil strains in two nucleotides in the ITS1–5.8S–ITS2 locus (the similarity was 99.6%) (Table 2).

Strain E75 isolated from permafrost of Kolyma Lowland differed in six nucleotides (one in the ITS1

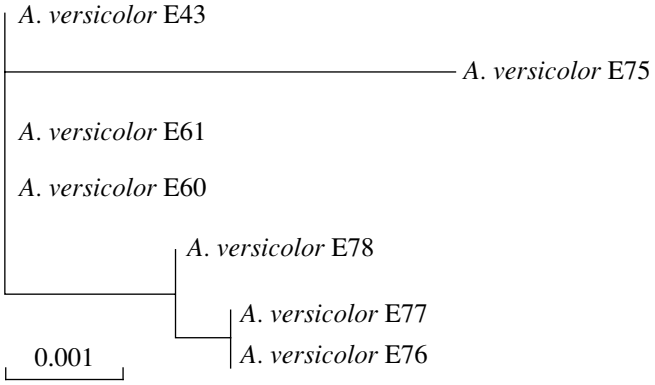

Fig. 4. Cladogram based on the sequences of ITS1, 5.8S, and ITS2 loci and partial sequence of 28S rDNA of strains.

Table 2. Similarity (%) of *A. versicolor* strains in the ITS1–5.8S–ITS2 fragments

Strains	From soils of European region of Russia (E43, E60, and E61)	From 7000-year-old peat of Aleutian Islands(E76, E77, and E78)	From 100000-year-old permafrost of the Kolyma Lowland (E75)
E43, E60, E61	100		
E76, E77, E78	99.6	100	
E75	98.7	98.5	100

locus and five in the ITS2 locus) from the group of soil strains obtained from European Russia, and in seven nucleotides (in the ITS loci) from the strains isolated from peat of the Aleutian Islands (Table 2).

It should be emphasized that the distinctions between the strains were revealed only in the ITS1 and ITS2 loci, whereas the sequences of the more conservative 5'-terminus of the 28S rRNA gene were identical.

Thus, the most pronounced distinctive features were revealed in the strain obtained from 100000-year-old ancient permafrost, whereas the differences were less pronounced between the other strains isolated from less ancient soils, modern soils, and peat.

DISCUSSION

For a number of micromycete species, it was shown that strains isolated from different geographic regions differ in their growth characteristics [1].

In our study, the ecological and trophic characteristics of the strains investigated correlated with the ecological characteristics of the ecotopes from which they had been isolated. Thus, strains obtained from peat were characterized by the highest growth rates, especially on rich protein-containing media. The growth rates of the strains isolated from ecotopes with a high salinity increased with increasing NaCl concentration in the medium.

The strains studied by us can be divided into three groups with respect to the geographic characteristics of their ecotopes: strains isolated from southern soils of the European part of Russia; strains isolated from peat of the Aleutian Islands (United States); and strains obtained from permafrost of Kolyma Lowland. However, no correlation between climatic characteristics of the habitat and the temperatures favorable for strain growth was revealed. All of the strains studied were characterized by the same temperature optima and similar temperature ranges for mycelium growth.

It was of interest to compare the properties of strains belonging to one species but isolated from ecotopes of different age. The study was carried out with four groups of isolates: strains E43, E60, and E79 isolated from modern soils; strain E61 obtained from buried cultural layer of the third century B.C.; strains E76, E77, and E78 isolated from ancient (7000-year-old) seaside peat; and strain E75 isolated from 100000-year-old permafrost. No essential morphological, ecological, or trophic distinctions were revealed between the strains of these four groups. However, the strain isolated from ancient permafrost differed considerably from the other strains in its genetic properties.

At present, an important problem of phylogenetic studies is the investigation of intraspecies and interspecies variations in genetic properties of microorganisms. In recent years, sequencing of rDNA loci has usually been used for the determination of genetic characteristics of different micromycete species. It was shown that distinctions in genetic properties of strains belonging to one species can be associated with different loci of rDNA. For instance, it was revealed that the difference in the ITS1 and ITS2 loci of different species of the genus *Aspergillus* can reach 20.7%, whereas intraspecies variations do not exceed 1% [15]. A fundamental investigation performed with ascomycetous yeasts [16] revealed that the variable D1/D2 regions of 28S rDNA can be successfully applied for determining phylogenetic relationships at the species or higher levels. Variations of these regions in strains of one yeast species usually do not exceed 0.5%.

The phylogenetic structure of the multispecies and complicated genus *Aspergillus* was also verified by the sequence analysis of this site of rDNA [17]. Recently, it was assumed that biologically different species can have identical ITS1 and ITS2 loci or identical 5' termini of 28S rDNA, or even both sites may be identical in them [18].

The results of this study allow us to conclude that strains of the species *A. versicolor* exhibit certain distinctions in genetic properties. Thus, in the strains studied, the differences in the sequences of ITS fragments reached 1.7%. Genetic distinctions were observed between both strains isolated from geographically distant biotopes and from substrates of different age. However, maximal differences were revealed between the ancient strain obtained from permafrost and the strains isolated from younger soils rather than between the strains of geographically distant populations, such as strains isolated from soils of the European Russia and strains isolated from peat of the Aleutian Islands.

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