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EXPERIMENTAL ARTICLES

Geographical Races of Certain Species of Ascomycetous Yeasts in the Moscow and Novosibirsk Regions

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Abstract—Strains of three species of the ascomycetous yeasts *Hanseniaspora guilliermondii, Torulaspora delbrueckii*, and *Debaryomyces hansenii*, isolated from the above-ground parts of plants in similar biocenoses of distant geographic regions (Moscow and Novosibirsk regions), have been investigated. The strains in each species were indistinguishable with respect to phenotypic features and general DNA characteristics as determined by restriction analysis. However, comparison of the strains using PCR analysis with nonspecific primers revealed considerable intraspecific variability. From their electrophoretic patterns, the strains of the three species studied were found to cluster in accordance with the region of isolation. This phenomenon is interpreted as an example of the existence of geographical races in the major eurytopic species of yeasts.

Key words: yeasts, forest, microbial communities, geographical populations, restriction analysis, microsatellite primers.

Modern yeast taxonomy is rapidly developing due to the introduction of molecular and biological methods. The number of known yeast species has substantially increased over recent decades not so much owing to the discovery of new forms in nature but rather as a result of splitting the already known large phenotypic species that exhibit considerable genotypic heterogeneity. The tendency toward multiplication of "elementary" genotypic species contradicts the established concept of a biological species as a sufficiently complex and heterogeneous system.

Substantial intraspecific variability is an indispensable attribute of a species in this context. One of its manifestations is differentiation of species into geographical races.

Despite the established notion of microorganisms as cosmopolitans that do not encounter geographical barriers to their propagation, the phenomenon of geographical divergence seems to be sufficiently pronounced in yeast fungi. It has been shown that the taxonomic composition of the yeast population of close plant species and of the insects pollinating them largely differs in different geographical regions, e.g., on various islands and archipelagoes in Oceania [1]. Different geographical populations are known in the complex of the twin species of the genus *Saccharomyces* [2–8] and in the species of the genera *Williopsis* [9] and *Arthroascus* [10]. The existence of geographical populations has been noted in certain phylogenetically compact species, e.g., *Saccharomyces paradoxus* Bachinskaya [2, 6–8] and *Cryptococcus neoformans* (Sanfelice) Vuillemin [11].

As a rule, strains from different populations are virtually indistinguishable using the set of standard morphological and physiological features but can be distinctly differentiated by the structure of their conservative genes, as revealed by means of molecular-genetic methods such as restriction fragment length polymorphism (RFLP) analysis [12–15] and analysis with nonspecific microsatellite primers (RAPD) [6, 11, 12, 15, 16]. At the same time, most of the recent molecular-taxonomic studies of yeasts have been conducted using irregular sampling of collection strains, often with very limited information on the sources of their isolation. In order to reveal microbial geographical races, i.e., varieties that appeared as a result of geographical isolation, it is necessary to compare populations influenced by similar ecological factors. Molecular-biological comparison of phenotypically similar strains isolated within one natural climatic zone from the same substrate but in different geographical regions is the most productive approach. In this communication, we report the results of just such an analysis performed for three species of ascomycetous yeast fungi.

In order to assess the role of geographical isolation in the formation of yeast communities, we carried out a comparative analysis of the yeast population structure in two biogeocenoses similar with respect to vegetation and soil type but geographically isolated [17]. Birch forests, which are widespread in the territory of Russia, including its Asian part, were chosen as an example of such biogeocenoses. It has been shown, as a result of many years of research, that biogeocenoses so similar in their flora composition and soil type and occurring within the same geographical zone (although remote one from another in space) are very similar in relation

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Table 1. The strains studied and the regions of their isolation

Species	Novosibirsk oblast	Moscow oblast
Hanseniaspora guilliermondii	nss-1-4, nss-8-1, nss-8-2	nss-31-1, nss-32-5, nss-32-7
Torulaspora delbrueckii	nsd-3-1, nsd-3-3, nsd-3-7	Tor-1, Tor-2, Tor-3
Debaryomyces hansenii	nso-17-2, nso-18-4, nso-19-2, nso-21-5	nss-15-6, ned-10-1, ned-86-1

to the structure of their yeast populations. The ratio of the abundances of dominant large (*sensu lato*) agamic species of basidiomycetous affinity and the pattern of their microbiotopic distribution are approximately the same in both cases [17]. In the course of this study, we also isolated, from similar substrates in both regions, cultures of several species of ascomycetous yeasts and conducted a more thorough investigation using the methods of RFLP analysis and PCR analysis with nonspecific primers (RAPD).

MATERIALS AND METHODS

The studies were conducted in two regions: (1) western Siberia, in the vicinity of Novosibirsk and on the territory of the Altai State Reservation, and (2) localities near Moscow, specifically, near the village of Burtsevo; Shakhovskoi raion; and in the vicinity of the Malino biological station, Institute of Ecological and Evolutionary Problems, Russian Academy of Sciences. The natural conditions in the regions of study are very similar: the average temperature in July in these regions is 18 and 19°C, and that in January is –12 and –19°C; the annual rainfall is 400–500 and 400 mm, respectively. The soils and vegetation of the biogeocenoses are also very similar: mixed-grass birch forests with the dominance of birch, mountain ash, *Aegopodium*, wild strawberry, ferns, and sylvan horsetail.

Samples of these plant species were collected from August to September 2002–2003. The yeasts were isolated by inoculation of a wort agar with the pH adjusted to 4–4.5 with 40% lactic acid to inhibit bacterial growth. In order to inhibit the growth of mycelial fungi, the cultures were incubated at 5°C for two to four weeks. The grown yeast colonies were examined under a binocular magnifying glass and subdivided into different types based on their macromorphological characteristics. Several colonies of each type were used to obtain pure cultures, which were then identified on the basis of the morphological and physiological characteristics using a determinative manual [18] and the keys described in [12, 13].

Among the 32 yeast fungi isolated in the birch forests of the localities near Moscow and in western Siberia [17], three species were selected for further study based on the following criteria: (a) constant occurrence on similar substrates in both regions; (b) complete absence of significant phenotypic differences from the corresponding standard descriptions; (c) a minimal level of phenotypic intraspecific variability; and (d) availability of published data on the genetic heterogeneity of the species. The following three species of ascomycetous yeasts met these criteria to the greatest extent: *Hanseniaspora guilliermondii* Pijper, *Torulaspora delbrueckii* (Lindner) Lindner, and *Debaryomyces hansenii* (Zopf) Lodder et Kreger-van Rij. Three to four strains of these species from each geographical region were selected for further studies (Table 1). All the strains of these species were isolated from the above-ground parts of plants.

DNA isolation and amplification were carried out according to the procedure described in [19]. Amplification of 5.8S rDNA and the internal transcribed spacers ITS1 and ITS2 (the 5.8S-ITS fragment) was carried

Table 2. Results of the restriction analysis of amplified 5.8S-ITS fragments with different endonucleases

Strains	Size of PCR product, bp	Sizes of fragments yielded by endonucleases:	
		*Hae*III	*Hin*fI
Hanseniaspora guilliermondii			
nss-1-4, nss-8-1, nss-8-2, nss-31-1, nss-32-5, nss-32-7	775	775	385 + 200 + 160 + 80
CECT 11029^T (CBS 465) [12, 14]	775	775	385 + 200 + 160 + 80
Torulaspora delbrueckii			
nsd-3-1, nsd-3-3, nsd-3-7, Tor-1, Tor-2, Tor-3	800	800	410 + 380
CECT 1880, 10558, 10589, 10651, 10676, 10683, 10693, 10694, 11146, 11199 [14]	800	800	410 + 380
Debaryomyces hansenii			
nso-17-2, nso-18-4, nso-19-2, nso-21-5, nss-15-6, ned-10-1, ned-86-1	650	420 + 150 + 90	325 + 325
CECT 10386 [14]	650	420 + 150 + 90	325 + 325

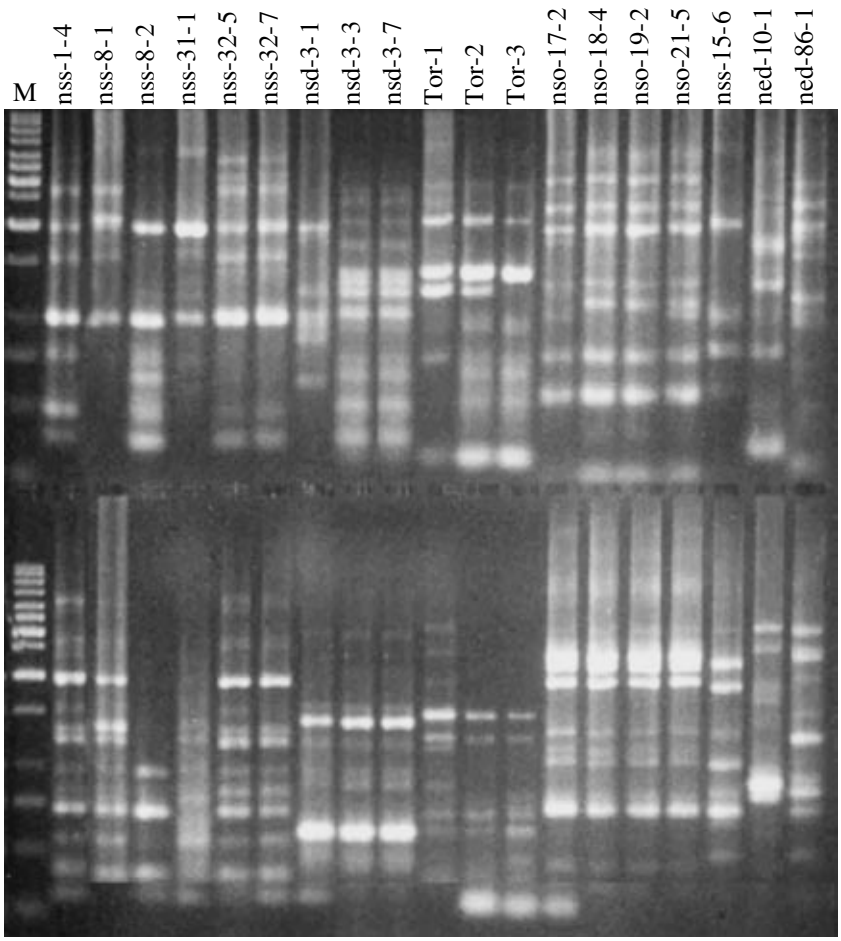

Fig. 1. Results of the PCR with microsatellite primers: upper row, $(GTG)_5$; lower row, $(ATG)_5$. M indicates the 1 kb DNA Ladder marker (Fermentas, Lithuania).

out with the primers pITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and pITS4 (5'-CCTCCGCTTATTGATATGC-3'). The PCR was performed in 30 μl of a reaction mixture containing PCR buffer, 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.25 mM dNTP, 0.30 μM of each primer, 1.25 units of *Taq* polymerase (Sintol, Russia), and 20 ng of the genomic DNA to be analyzed. A Tertsik DNA amplifier (DNK-tekhnologiya, Russia) was used in the following mode. The initial denaturation, 3 min at 94°C, was followed by 30 cycles of DNA denaturation for 2 min at 94°C; primer annealing for 1 min at 60°C; and DNA synthesis for 2.5 min at 72°C, with final elongation for 10 min at 72°C. The amplification products were subjected to electrophoresis in 1% agarose gel at 60–65 V in 0.5× TBE (45 mM Tris, 10 mM EDTA, and 45 mM boric acid) for 2 h and stained with ethidium bromide.

The polymorphism of the restriction fragment lengths was assayed after treatment with the endonucleases *Hae*III and *Hin*fI (Fermentas, Lithuania). Separation of the restriction fragments was performed in 1.6% agarose gel at 60–65 V in 0.5× TBE for 3 h. The gel was stained with ethidium bromide and photographed in UV light using a Vilber Lourmat transilluminator (France).

The intraspecific polymorphism was studied using the nonspecific microsatellite primers M13, $(GTG)_5$, and $(ATG)_5$. This method has previously been successfully used to differentiate genetic populations of cultured saccharomycetes [16, 20]. The PCR was performed in 30 μl of a reaction mixture containing PCR buffer, 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.25 mM dNTP, 030 μM of each primer, 1.25 units of *Taq* polymerase (Sintol), and 20 ng of the genomic DNA to be analyzed.

The Tertsik DNA amplifier was used in the following mode. The initial denaturation for 5 min at 94°C was followed by 40 cycles of DNA denaturation for 1 min at 94°C, primer annealing for 2 min at 52°C, and DNA synthesis for 3 min at 74°C, with final elongation for 10 min at 74°C.

The amplification products were subjected to electrophoresis in 1.2% agarose gel at 60–65 V in 0.5× TBE (45 mM Tris, 10 mM EDTA, and 45 mM boric acid) for 3 h and stained with ethidium bromide. Photography was performed in ultraviolet light using a Vilber Lourmat transilluminator.

The image was analyzed using the Scion Image 4.0.2 program (Scion Corp., 2000). Pairwise genetic distances were calculated taking into account the color

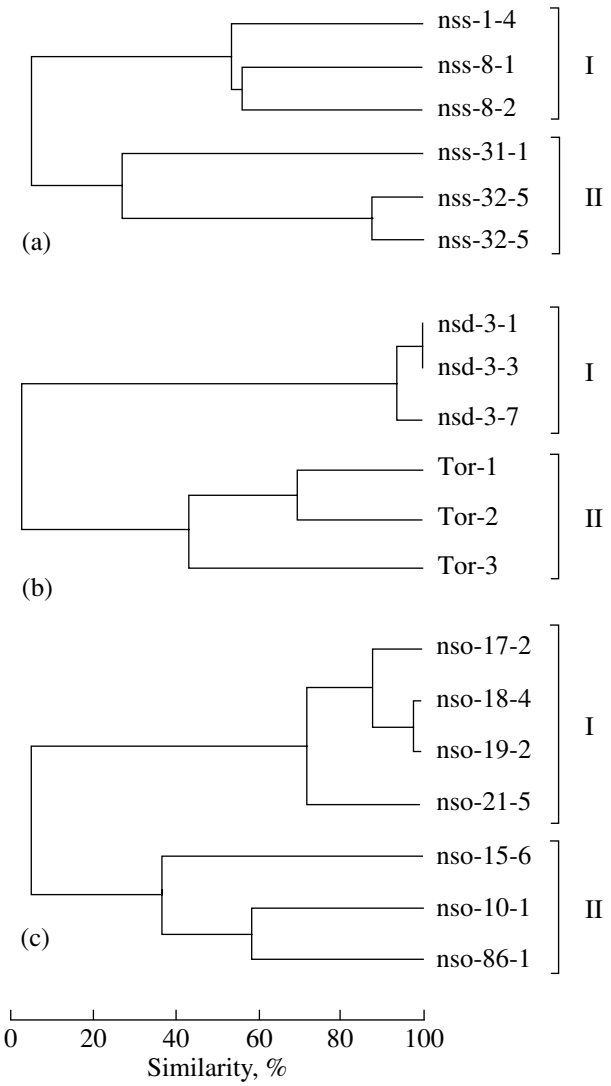

Fig. 2. Cluster analysis of the strains of ascomycetous yeasts based on the similarity between their microsatellite profiles. Species: (a) *Hanseniaspora guilliermondii*, (b) *Torulaspora delbrueckii*, and (c) *Debaryomyces hansenii.* Isolation regions: (I) western Siberia and (II) localities near Moscow.

intensity of bands of the same size. Dendrograms were constructed using the Statistica program (StatSoft Inc., 1984–2001); pairwise calculation of distances with the arithmetic mean (UPGMA) was used as the clusterization algorithm.

RESULTS AND DISCUSSION

A complete description was compiled for each of the strains selected based on study of the morphological and physiological characteristics used in yeast taxonomy. By the aggregate of these characteristics, strains belonging to the same species did not differ from each other or from the corresponding standard descriptions in the manual [18]. In order to confirm their species identity and conspecificity, an RFLP analysis was also employed. All the strains studied demonstrated a high intraspecific similarity between their restriction profile patterns, as well as a high similarity to the profiles of the corresponding type strains and the strains with known species identity [12–15] (Table 2).

Thus, according to both phenotypic characteristics and the general DNA characteristic obtained by restriction analysis, the strains isolated in different regions should be considered as conspecific. However, when comparing the strains by means of the PCR analysis with nonspecific primers, we revealed significant intraspecific variability of these species.

Earlier, the use of this method allowed the polymorphism within the extensive European geographical population of the yeast *Saccharomyces paradoxus* to be demonstrated. This species has an extensive geographic range of distribution and is represented by a number of diverged populations. Among the latter, European [2, 6], Far East [7], Hawaiian [5], and North American [8] populations have been recognized. The primers $(GTG)_5$, OPA-04, and OPA-09 were used in a study on characterization of the European population [6].

A preliminary experiment showed that the use of primer M13 resulted in a poor PCR resolving capacity; therefore, it was excluded from the rest of the experiment. Using the microsatellite primers $(GTG)_5$ and $(ATG)_5$, electrophoretic patterns demonstrating noticeable distinctions between the strains of one species were obtained (Fig. 1). The constructed dendrograms, which were identical for both of the primers used, showed that the strains of all three species studied clustered in accordance with their isolation region (Fig. 2). The closest similarity was observed between the strains isolated from samples of the same plant species within each region.

The intraspecific heterogeneity of the *Hanseniaspora* yeasts was shown not once [12, 13]. However, the researchers did not succeed in linking this variability to any factor, e.g., to the substrate used for isolation or to the region of isolation. Earlier, the intraspecific variability of cultured yeasts of the species *Debaryomyces hansenii* isolated from substrates of the same type in several localities within one geographical region was noted [15]. However, attempts to reliably link this intraspecific variability to the isolation sites were unsuccessful. Maximum standardization of the substrates used for the isolation enabled us to level the influence of the ecological factors and to reveal the influence of the isolation site on the genotypic characteristics of strains belonging to one species. Thus, the differences in the PCR profiles revealed in the yeast species that we studied seem to provide evidence for the existence of geographical differentiation that yields geographical races determined by geographical isolation rather than by the action of environmental factors. Strains of one species isolated from similar habitats in remote regions with the same climatic conditions exhibited no distinctions with respect to morphological or physiological characteristics but differed rather

clearly in their more conservative features, namely, in their rDNA sequences.

Our results show that descriptions of the yeast species based on phenotypic and genotypic characteristics may have different meanings. The species described on the basis of morphological and physiological characteristics occupy the same ecological niche, and their distribution is determined by ecological factors. However, comparative analysis of the representatives of these species shows that they consist of different populations consistent with the concept of geographical race. The recognition of such races in yeasts is possible due to the use of conservative nucleotide sequences. However, the use of rDNA sequences for characterization of the ecological structure of microbial communities at the species level may be inappropriate, since they reflect the degree of relatedness and the natural history of the development of an organism but not its function in an ecosystem.

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