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GENERAL GENETICS

Differentiation between Commercial Strains of Oyster and Button Mushrooms Using Molecular Markers

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Abstract—Analysis of commercial strains of two edible mushrooms, *Pleurotus ostreatus* and *Agaricus bisporus*, using PCR and isozyme electrophoresis techniques allowed us to differentiate groups of genetically similar and distant strains. Among the commercial strains of *P. ostreatus*, the level of genetic variation was higher suggesting a broader genetic basis employed in breeding of this mushroom. The cultivars and hybrids of *A. bisporus* showed a higher level of homology. The isozyme markers (nonspecific esterase, leucinaminopeptidase, and phosphoglucoisomerase) are recommended for identification of the commercial strains of edible mushrooms.

INTRODUCTION

Edible oyster mushroom *Pleurotus ostreatus* Jacq.: (Fr.) P.Kumm., and button mushroom *Agaricus bisporus* L.E.(Lange) Imbach belong to a class of basidial fungi extensively commercial in Russia. While *Agaricus* cultivars have been subjected to long-term European breeding, the oyster mushroom was introduced to agriculture only several decades ago and has not yet been extensively bred in Russia. In our country, most oyster mushroom cultivars are either European by origin or somewhat improved wild strains. Unlike *Agaricus* grown predominantly in large enterprises, successful production of oyster mushroom is possible in small farms due to a relatively simple and flexible growing process. However, the optimal choice among different cultivars is difficult for several reasons: production of fungal spawn mycelium by numerous small laboratories, uncoordinated commercial collections, and, primarily, the absence of unified specification for oyster mushroom strains. Therefore, the necessity of comparative analysis of the fungal strains commercial in Russia is evident. Biochemical and molecular markers were already repeatedly used to characterize European commercial *A. bisporus* cultivars and identify new genotypes in natural populations of species of the genus *Agaricus* [1–5]. The most frequent markers used for mushroom genotyping are isozymes and RAPD (random amplified polymorphic DNA fragments obtained in polymerase chain reaction with random primers). The amplified DNA homology testifies to genomic similarity of the strains and, hence, to their relatedness and common genetic origin; PCR patterns may be strain- and species-specific [6, 7].

In this study, we aimed to determine the degree of genetic similarity/relatedness and variation of the of oyster and button mushroom commercial strains as well as to define molecular markers most appropriate for diagnosing these strains.

MATERIALS AND METHODS

A collection of oyster and button mushroom commercial strains used in this study was from the Kushnarev joint-stock company Zarech'e (Moscow oblast). We have examined 11 oyster mushroom and 18 button mushroom strains (Table 1).

The oyster mushroom strains were grown on wort-agar in Petri dishes for ten days at 25°C. Mycelium was removed from the dish surface with a spatula and used for protein and DNA extraction. The button mushroom strains were grown for 25 days at 25°C in the dark and without agitation in liquid medium containing 20 g of barley extract, 8 g of yeast extract, and 20 g of glucose per 1 l of water. Mycelium collected by filtration was washed with distilled water and used for protein and DNA extraction.

Cell lysate preparation and protein separation in horizontal 13% starch gel in three buffer systems (Tris–citrate, Tris–EDTA–borate, and Tris–citrate–LiOH-borate) were conducted by conventional techniques used in Vavilov Institute of General Genetics, Russian Academy of Sciences, and described previously [8]. After electrophoresis, the starch slab was cut into several horizontal slices each stained by a standard technique [9]. The following 11 enzymes were studied: alcohol dehydrogenase (ADH, EC 1.1.1.1.), isocitrate dehydrogenase (IDH, EC 1.1.4.2.), malate dehydrogenase (MDH, EC 1.1.1.37.), phosphoglucomutase (PGM, EC 2.7.5.1.), phosphoglucoisomerase (PGI, EC 5.3.1.9.), glutamate dehydrogenase (GDH, EC 1.4.1.2), leucinaminopeptidase (LAP, EC 3.4.11.1), acid phosphatase

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Table 1. Commercial strains of oyster and button mushrooms from collection of Joint-Stock Company Zarech'e

Cultivar, strain	Source of origin/obtaining	Strain description
	Pleurotus ostreatus	
27/6*	Duna company, Hungary	–
P77/6	Institute of Agricultural Biotechnology, Moscow	–
L/4 (Lvov)	Laboratory for Mycelium Production, Lvov	–
Som/5 (Sommerauster)	Dieskau co-operative, GDR	Hybrid *P. ostreatus* × *P. florida*
F-3725/4	Laboratory of Spawn Mycelium, Zarech'e	Hybrid of European breeding
BP/8	Donetsk State University, Ukraine	Spruce form
D103/10	The same	
Don112/10	″	
Fl/16	Duna company, Hungary	*florida* variety
Mich/6 (Michigan)	Oknitsa Laboratory, Moldova	–
P.m./3 (Michigan)	The same	–
	Agaricus bisporus	
Y217/7	Horst experimental station, Netherlands	White cultivar
2008/5	The same	The same
19/2	? European breeding	″
2001/1	? Ireland	White cultivar
F-3718/2	Horst experimental station, Netherlands	The same
F-3714/2	The same	″
F-3715/2	″	″
F-3709/2	″	″
F-3717/2	″	″
F-3710/2	″	″
F-3712/3	″	″
F-3711/4	″	″
F-3713/2	″	″
12/7	″	″
1/1	″	″
273/7	Department of Mycology, Moscow State University, L.V. Garibova	Brown variety, multisporous reproduction
MKSh-5/2	Horst experimental station, Netherlands	Brown variety
GDR-2/7	Dieskau co-operative, GDR	The same

* Figures after slash indicate succession of passage; ** origin unknown.

(ACP, EC 3.1.3.2.), superoxide dismutase (SOD, EC 1.15.1.1.), esterase (EST, EC 3.1.1.1.), and peptidase (PEP, EC 3.4.1.1).

DNA was extracted from mycelium according to a standard procedure [10]. PCR was conducted in a LIYaF thermocycler (St. Petersburg) in 25 μl of a reaction mixture containing DNA, 40–100 ng; Tris–HCl, 10 mM (pH 8.3); KCl, 50 mM; $MgCl_2$, 1.5 mM; deoxytriphosphates (dATP, dCTP, dGTP, and dTTP), 50 μM of each one; 15 pmol of a primer, and 1–2 units of *Taq* polymerase (Sileks M). PCR was conducted according to the following scheme: denaturing at 95°C for 5 min followed by 35 cycles: DNA denaturing at 94°C for 60 s; primer annealing at 39°C for 60 s; DNA synthesis at 72°C for 120 s; a cycle at 72°C for 10 min. A sample loaded on the gel contained 10–15 μl of a reaction mixture. The amplified DNA fragments were analyzed by electrophoresis in 1.2% agarose prepared in Tris–acetate buffer, which was followed by staining with ethidium bromide (standard techniques [11]). The Lambda DNA treated by *Eco*RI +*Hind*III as well as GeneRuler 1kb DNA Ladder (MBI Fermentas) served as markers for DNA fragments. In control reaction mixtures, no

mushroom DNA was added. After staining, the gels were photographed in UV light. All amplification reactions were run two to three times and yielded reproducible results. The primers and PCR conditions were developed in the Department of Mycology and Algology, Moscow State University. Eight out of 12 primers used yielded polymorphic DNA fragments from 360 bp to 2.8 kb:

A-2, 5'-ggaagcttgg-3'; A-3, 5'-caaagcgctc-3'; A-4, 5'-agtcagccag-3'; S-13, 5'-gtcgttcctg-3'; V-08, 5'-ggacggcgtt-3'; N-14, 5'-tcgtgcgggt-3'; U-18, 5'-gaggtccaca-3'; AP-09, 5'-gtggtccaga-3'.

The PCR patterns and isozyme spectra were separately compared to reveal genetic similarity between the strains examined. The genetic diversity was assessed from the frequencies of isozyme loci to calculate the pairwise coefficients and Nei's genetic distances [12]. On the basis of these parameters, similarity/relatedness dendrograms were constructed for the studied cultivars of oyster and button mushrooms. The results obtained were mathematically processed using the POPGENE software package version 1.32 (MBBCU of Alberta and CIF) [13].

The PCR patterns were compared in a different manner. The presence or absence in the gel of the bands corresponding to DNA fragments of a certain length was regarded as an individual character, on the basis of which the matrices of character alternative states were constructed. The combined matrices for all PCR patterns were calculated using the TREECON software package for Windows, version 1.1 [14]. Like in isozyme spectrum analysis, unrooted dendrograms that reflect proximity and similarity between the strains were constructed using the UPGMA algorithm based on paired-group comparison of unweighted characters with the arithmetic mean.

RESULTS

Analysis of Isozyme Spectra

In this study, staining for 11 specific isozymes was conducted. Under conditions used for electrophoresis, the genotypes of commercial oyster mushroom strains were reliably determined for 13 active loci (*Acp, Lap-1, Lap-2, Mdh-1, Mdh-2, Idh, Adh, Pgm, Pgi, Sod, Gdh, Est-1*, and *Est-2*), whereas genotypes of button mushroom strains were determined for six active loci (*Acp, Lap, Sod, Est-1, Est-2, Pep*). The combined isozyme spectra are shown in Fig. 1. In strains of button mushroom *A. bisporus*, only four out of six active isozyme loci proved to be variable (*Est-1, Est-2, Pep*, and *Acp*). At a clear-cut separation of the *P. ostreatus* fractions, the esterase (*Est-1* and *Est-2*) and phosphoglucoisomerase (*Pgi*) loci displayed the highest polymorphism (Table 2). Multiple alleles were characteristic of nonspecific esterases, phosphoglucoisomerases, and peptidases, which was informative for the quantitative estimation of the strain genetic diversity.

In similarity dendrograms constructed on the basis of genetic diversity, commercial oyster mushroom strains formed three clear-cut groups (clusters) (Fig. 2). Note that clusters are formed by genetically related (similar) cultivars. The more distant are the clusters on a dendrogram, the greater is the genetic distance between these clusters (and the strains included).

The strains Mich/6 and P.m/3 proved to be completely genetically identical. Along with Fl/16 cultivar they contributed to a cluster remote from all remaining strains examined, which suggests that the members of this cluster differ in their origin from the other oyster mushroom strains. The strains Mich/6, P.m/3, and Fl/16 probably belong to the same genetic selection line.

The strains combined in the second cluster, 27/6, P77/4, F-3725/4, Som/5, and L/4 represent another genetic line (Fig. 2). On the dendrogram, strain P77/4 is genetically identical to cultivar 27/6 and both of them have identical isozyme loci. European hybrid strain F-3725/4 is somewhat genetically similar to the former two strains as well as to the hybrid cultivar Som/5 and strain L/4. The domestically bred strains D103/10 and Don112/10 form a detached cluster; they are also genetically related, which testify to their common origin. Note that the first and second clusters are not far apart (Fig. 2). They might be bred from a common genetic line. As for the third cluster including cultivars Florida (Fl/16) and Michigan (Mich/6 and P.m/3), it is distant from the first two clusters. Hence, these strains seem to differ in their genetic origin.

Clustering of strains of the button mushroom *A. bisporus* is less pronounced. On the dendrogram, only two isolated groups of white and brown cultivars can be seen (Fig. 3). Isozyme analysis revealed relative genetic homogeneity of white cultivars and button mushroom hybrids. The most abundant first cluster includes both white cultivars and white hybrids of the European breeding between which only insignificant genetic differences are observed. White Dutch cultivars Y217/7 and 19/2 are completely identical for multiple alleles of six isozyme loci. The white hybrid of Irish origin, 2001/1, is some distance apart from the Dutch cultivars. Cultivar 2008/5 is somewhat different from the three above cultivars but is genetically similar to hybrids F-3709/2 and F-3717/2, which testifies to their common origin.

Brown button mushroom cultivars 273/7, GDR-2/7, and MKSh-5/2 constitute a cluster isolated from white cultivars and hybrids, which is not surprising because their genetic origin differ from that of white cultivars.

RAPD–PCR Analysis

All eight RAPD primers produced PCR patterns containing a great number of distinct bands (from nine to fourteen) in all 30 oyster and button mushroom strains examined. Irrespective of the primer used, the white cultivars and hybrids of the button mushroom

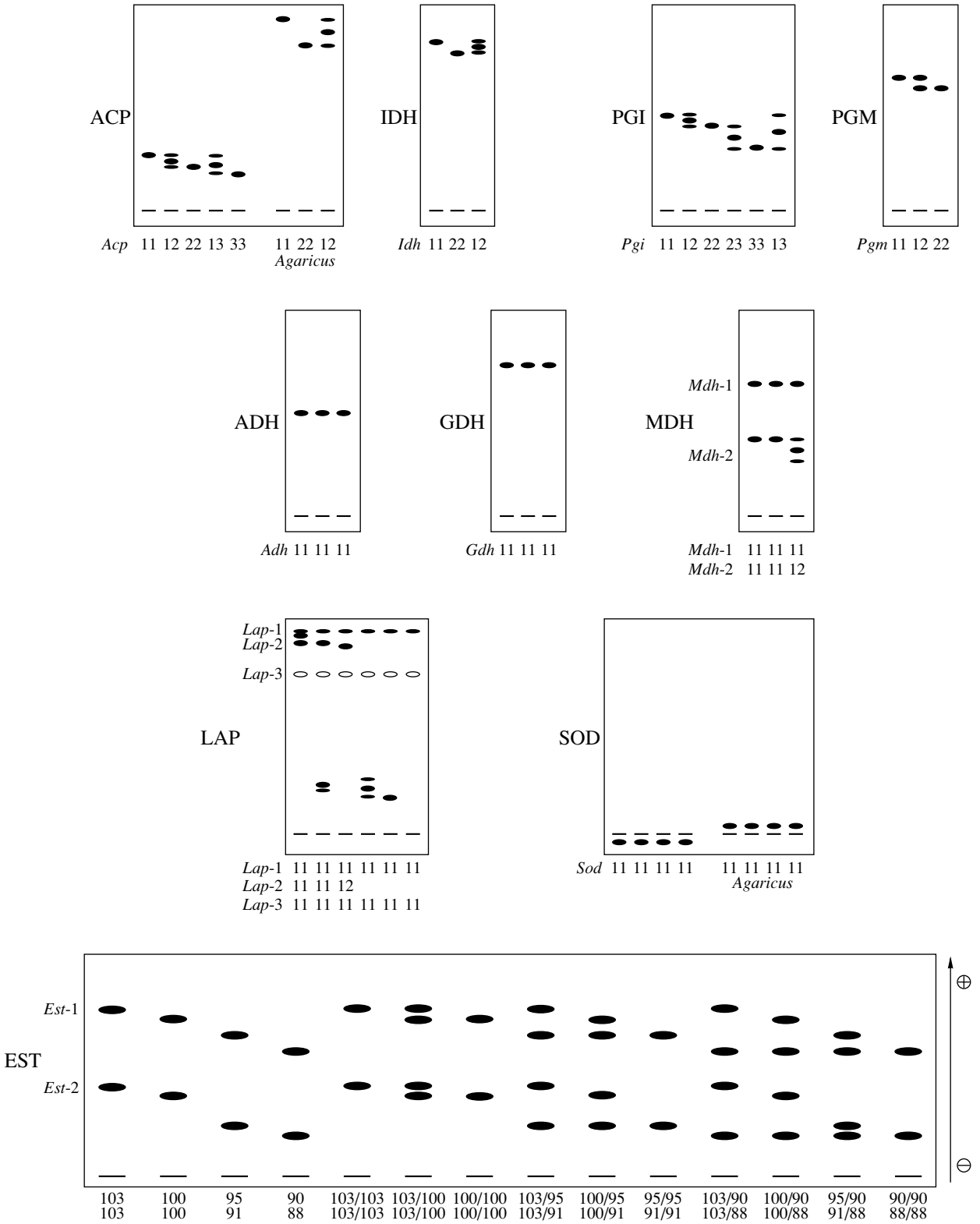

Fig. 1. Electrophoretic isozyme patterns of loci studied in button mushroom (*Agaricus*) and oyster mushroom ACP, acid phosphatase; IDH, isocitrate dehydrogenase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; ADH, alcohol dehydrogenase; GDH, glutamate dehydrogenase; MDH, malate dehydrogenase; LAP, leucine aminopeptidase; SOD, superoxide dismutase; EST, esterase. Numerals indicate alleles and their combinations; dotted line indicates sample loading.

Table 2. Genetic diversity of the commercial strains of oyster and button mushrooms estimated from isozyme spectra of mycelial proteins

Locus	Number of alleles	Heterozygosity[1]	Genetic diversity[2]
Pleurotus ostreatus			
Idh	2	0.2975	0.4741
Pgi	3	0.6281	1.0362
Mdh-1	m*	0.0000	0.0000
Mdh-2	3	0.1694	0.3676
Pgm	2	0.3967	0.5860
Adh	m	0.0000	0.0000
Gdh	m	0.0000	0.0000
Lap-1	3	0.3967	0.5860
Lap-2	2	0.4339	0.6255
Acp	3	0.5248	0.8961
Sod	m	0.0000	0.0000
Est-1	4	0.6074	1.0729
Est-2	4	0.6550	1.1674
Averaged over all loci		0.3161	0.5240
Polymorphism for all loci, 69.23%			
Agaricus bisporus			
Lap	m	0.0000	0.0000
Acp	3	0.5000	0.6931
Sod	m	0.0000	0.0000
Est-1	3	0.5900	0.9659
Est-2	3	0.6073	1.0139
Pep	4	0.6869	1.2508
Averaged over all loci		0.3973	0.6540
Polymorphism for all loci, 66.67%			

[1] Expected heterozygosity according to Nei; [2] Shannon's index of genetic diversity: $H = 1 - \sum p_i^2$, where p_i is the frequency of i allele for the given locus.

* Monomorphic loci.

displayed no differences between strains except for variation of individual minor bands (fragments) (Fig. 4). Even white hybrids of *A. bisporus* exhibited relative homogeneity which testifies to the lack of genetic diversity and confirms the opinion of Khush *et al.* [15] that genetic basis of commercial button mushroom strains is rather limited.

The dendrogram in Fig. 5 shows similarity between some oyster mushroom strains. For example, strains 27/6 and P77/6 are almost identical, and strain P77/6 is in fact a Hungarian cultivar 27/6. Analysis of the protein spectrum confirmed their genetic identity (Fig. 2). The strains Mich/6 and P.m./3 are also completely genetically identical. Along with the Fl/16 cultivar they constitute a cluster and, presumably, all of them are cultivars of Hungarian breeding stemming from a common ancestor. European hybrid strain F-3725/4 and Lvov cultivar L/4 seem to be related to Hungerian cultivar 27/6 (genetic distances between them do not exceed 0.3).

In general, all 11 strains of the oyster mushroom *P. ostreatus* form two clusters with a single separated strain BP/8, which is a natural isolate collected from a spruce and introduced into cultivation in Donetsk University.

Two commercial Donetsk strains D103/10 and Don112/10 differ insignificantly and have presumably a common genetic ancestor. They might be also generated by breeding from the German cultivar Som/5.

Eighteen commercial cultivars and hybrids of the button mushroom *A. bisporus* form two clusters including white and brown cultivars (Fig. 6). Note that all white hybrid strains of European breeding (marked as F-…) and the white hybrid strains of Dutch breeding, 12/7 and 1/1 (Horst), show low genetic polymorphism and little differrence from each other (genetic distances between them are lower than 0.2). They have presumably a common genetic basis and represent the same inbred line. In fact, the commercial hybrids F-3712/3, F-3711/4, F-3709/2, and F-3713/2 (genetic distances lower than 0.07) and hybrids F-3717/2, F-3710/2, F-3718/2, F-3714/2, and F-3715/2 (genetic distances lower than 0.1) are reisolates of the same hybrid commercial strain, which is in a good agreement with data of Spanish researchers [5]. The strains F-3709/2 and F-3713/2 as well as the strains F-3717/2 and F-3710/2 are in fact twin hybrids (completely genetically identical). White hybrids of Dutch breeding 12/7 and 1/1 also contribute to a cluster of closely related hybrid strains of European breeding. The hybrid strains of European breeding marked as F-…, the strains 12/7 and 1/1 of Dutch selection, and the Dutch cultivar Y217 are presumably genetically related. The European cultivar 19/2 might be also similar in origin to the Y217 cultivar (genetic distance between them is at most 0.3). The white hybrid 2001/1 of Irish breeding is little different from the European cultivar 19/2 and genetically similar to a Dutch cultivar Y217.

Three brown cultivars of the button mushroom, 273/7, GDR-2/7, and MKSh-5/2 form a cluster of brown cultivars (Fig. 6). Despite different origin of these cultivars (Ukraine, Germany, and Netherlands), genetic distances between them do not exceed 0.32. These cultivars might also have a common origin. All brown cultivars have a specific pattern of DNA fragments in gel. The PCR patterns obtained with primers N-14 and S-13 were most illustrative of cultivar specificity in commercial strains of button mushrooms with brown fruit bodies (Fig. 4). These primers were the best to differentiate between the white and brown cultivars of *A. bisporus.*

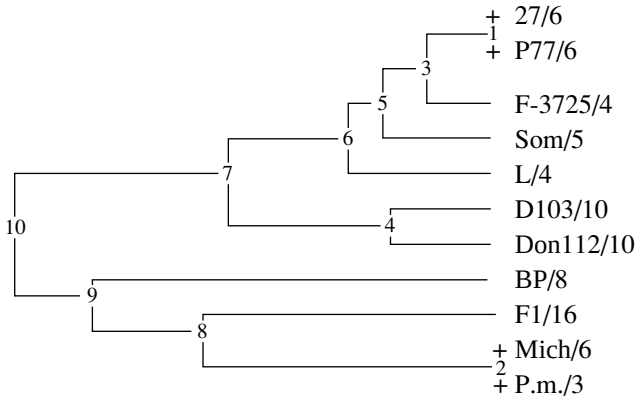

Fig. 2. Dendrogram of genetic similarity between the commercial strains of *P. ostreatus* constructed from Nei's genetic distances using UPGMA (based on 13 isozyme loci).

DISCUSSION

In this study, two approaches were used to determine molecular genotypes of commercial strains of oyster and button mushrooms: analysis of genetic similarity inferred from DNA polymorphisms and analysis of frequencies of isozyme loci. Both approaches yielded comparable dendrograms of genetic similarity/relatedness between the strains, which allowed us to make some conclusions on origin of some cultivars and strains of edible mushrooms commercial in Russia. Both species- and cultivar-specific molecular markers were isolated to reliably identify genetic lines and to determine the degree of similarity between strains originating from different breeding sources.

The higher polymorphism and genetic diversity among the *P. ostreatus* cultivars suggest that breeding of this mushroom is based on a broader genetic basis. Conversely, the button mushroom commercial cultivars are mostly reisolates originating from a common ancestor, i.e., they represent in fact the same genetic breeding line. Our results obtained by analyses of isozyme loci and RAPD markers confirm the opinion of foreign authors that new hybrid cultivars of button mushrooms exhibit low genetic diversity and close relatedness and originate from the hybrid strains that appeared on the market [2, 4, 15]. To improve the desired characteristics of the button mushroom cultivars, the genetic basis of breeding should be broadened, i.e., the wild strains isolated from natural sources should be involved into breeding and hybridization.

Among the commercial strains of oyster mushroom commercial in Russia, three breeding lines can be differentiated. The first seems to be connected with Hungarian cultivar 27 and German hybrid Sommer; the second line is related to strain hybridization with *florida* variety; the third independent line represented by BP/8 cultivar is connected with the introduction of the natural strains into commercial cultivation.

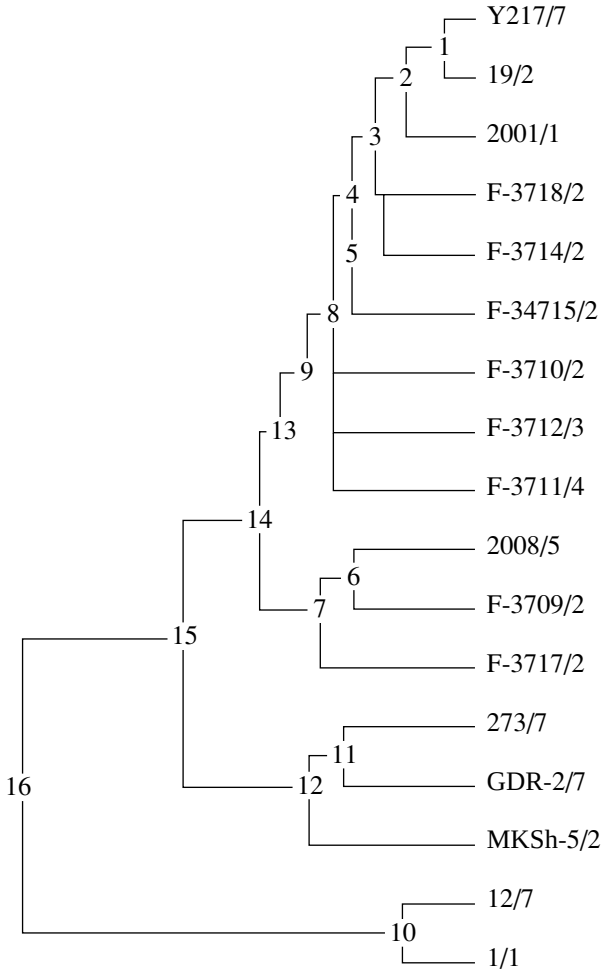

Fig. 3. Dendrogram of genetic similarity between the commercial strains of *A. bisporus* constructed from Nei's genetic distances using UPGMA (based on 6 isozyme loci).

The isozyme marker analysis is proposed as the most rapid method of genotype discriminating among the natural strains of edible mushrooms introduced into cultivation and the inbred cultivars. Esterase loci (*Est-1* and *Est-2*) are the best for marking the cultivars and hybrid strains of both oyster and button mushrooms. PCR pattern is also proposed to be used as "certificate" for commercial strains of these mushrooms.

We have been the first to perform a comparative analysis of two most widely cultivated species of edible mushrooms in Russia. This analysis is of importance for both small mushroom farming and large-scale producers of edible mushrooms, which often maintain in their laboratories abundant collections of genetically identical cultivars under different names. The proposed analysis is also important for submerged cultivation of edible mushroom strains (as control for purity of the grown biomass).

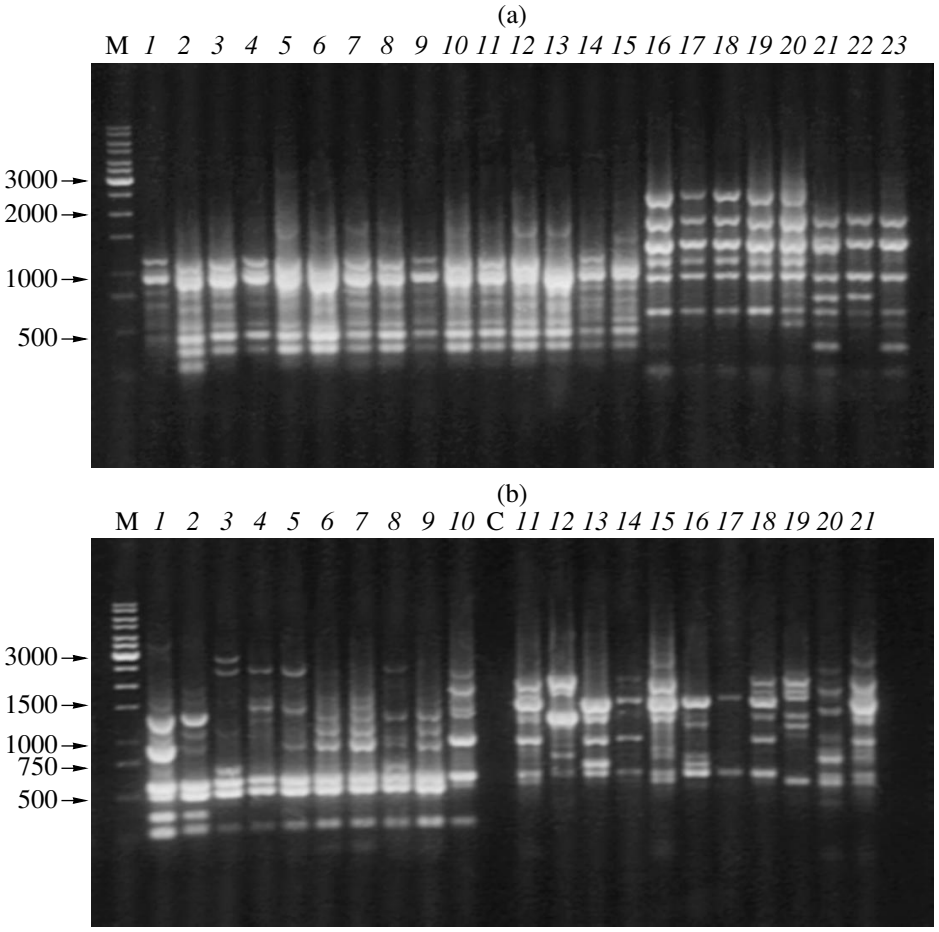

Fig. 4. PCR patterns of the button mushroom (a) and of oyster mushroom (b) strains, which were obtained using oligonucleotide primers. (a) Lanes *1–15*, N14 primer was used; lanes *16–23*, V08 primer was used; *1*, F-3718; *2*, F-3714; *3*, F-3713; *4*, F-3717; *5*, F-3710; *6*, 3712; *7*, Y217/7; *8*, 2008/5; *9*, 19/2; *10*, 2001/1; *11*, 12/7; *12*, 1/1; *13*, 273/7; *14*, GDR-2/7; *15*, MKSh-5/2; *16*, F-3714; *17*, F-3717; *18*, F-3710; *19*, F-3711; *20*, 1/1; *21*, 273/7; *22*, GDR-2/7; *23*, MKSh5/2; M, markers of fragment length (bp). (b) Lanes *1–10*, S-13 primer was used; lanes *11–21*, A-2 primer was used; *1*, D103/10; *2*, Don112/10; *3*, Fl/16; *4*, Mich/6; *5*, P.m./3; *6*, 27/6; *7*, P77/4; *8*, Som/5; *9*, F-3725; *10*, BP/8; *11*, 27/6; *12*, BP/8; *13*, Fl/16; *14*, P77/4; *15*, Som/5; *16*, P.m./3; *17*, Mich/6; *18*, F-3725; *19*, D103/10; *20*, Don112/10, *21*, L/4; C, control without DNA.

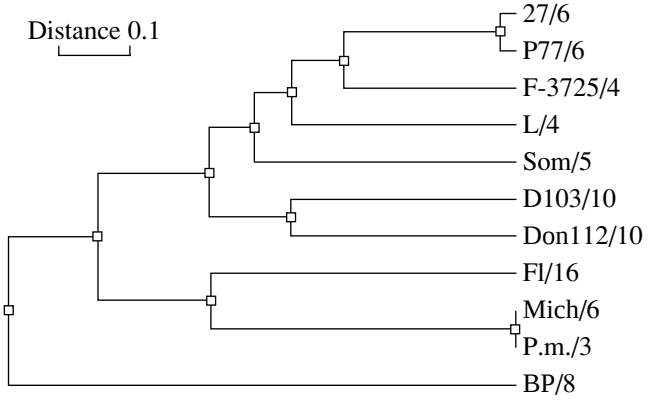

Fig. 5. Dendrogram of similarity between the commercial strains of oyster mushroom based on RAPD–PCR analysis.

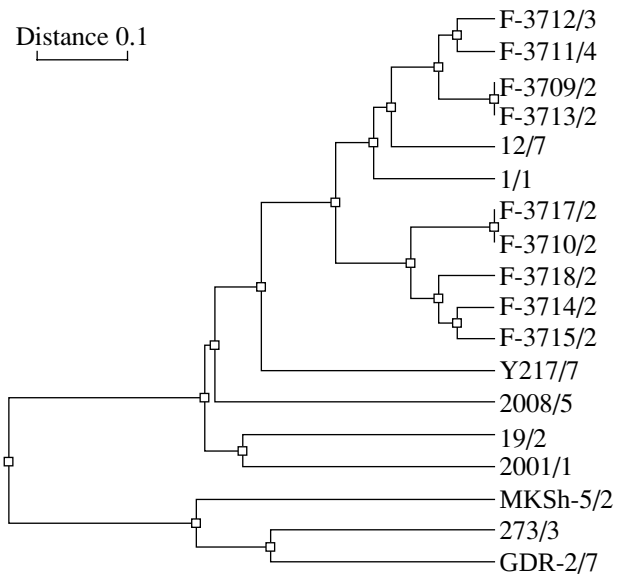

Fig. 6. Dendrogram of similarity between the commercial strains of button mushroom based on RAPD–PCR analysis.

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