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EXPERIMENTAL ARTICLES

Comparative Analysis of *Pleurotus ostreatus* Natural Isolates

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Abstract—A comparative analysis was performed of the polymorphism of the oyster mushroom *Pleurotus ostreatus* (Fr.) Kumm naturally occurring strains isolated from the natural substrates from two geographically remote Russian natural preserves, the Central Forest Biosphere Tver State Preserve and the Moscow State University Zvenigorod Biological Station (Moscow oblast), and within the city of Moscow. The results of the frequency analysis for the allozyme loci alleles and for the sexual and somatic incompatibility groups are presented; the genetic structure and the interpopulation relations among 58 *Pleurotus ostreatus* dikaryotic strains are estimated. The natural samples from the Moscow and Tver oblasts are shown to have a high degree of polymorphism with a genetic differentiation of 0.743; in spite of their territorial remoteness, they are, however, actively exchanging genetic material. The natural fungal isolates form two reproductively isolated groups.

Key words: *Pleurotus ostreatus*, populations, somatic incompatibility, allozyme protein spectra.

The species *Pleurotus ostreatus* (Fr.) Kumm is widespread in various geographic zones of Russia. Although the scale of oyster mushroom industrial cultivation is second only to that of the champignon *Agaricus bisporus*, *Pleurotus* was relatively recently, only several decades ago, introduced to mass culture. A thorough genetic analysis of this species has never been performed, and wild strains from nature are therefore often introduced to industry. *P. ostreatus* has a tetrapolar system of sexual compatibility and a well-defined haplo-dikaryotic life cycle. It also has a complex taxonomic structure, including biological species (intersterile groups) which can be identified under laboratory conditions by interbreeding the natural isolates with monokaryotic tester strains [1]. The populations of higher basidial fungi are mostly formed as a result of the processes of sexual and somatic (by mycelial fragments) reproduction. It is, however, often difficult to determine the physical borders of a single mycelium; an individual mycelium can form more than a single fruiting body. Differentiation between fungal individuals in nature can be performed via an estimate of somatic incompatibility. Since the complex multiallele system of somatic incompatibility prohibits fusion and exchange of nuclei between genetically different dikaryotic mycelia, under laboratory conditions it is possible to determine whether the natural isolates are genetically different specimens or genetically identical clones originating from somatic reproduction [2]. The allozyme spectra of mycelial proteins can be considered as selectively neutral characteristics, like those often used for the population analyses of fungi and other eukaryotes [3, 4]. The estimate of genetic processes in the populations is based on analyzing allelic frequencies of the genes [5, 6].

The goal of the present work was to perform a comparative analysis of *Pleurotus ostreatus* natural isolates using a set of characteristics: somatic and sexual compatibility and allelic frequencies of allozyme loci.

MATERIALS AND METHODS

Using the route method, 58 fruiting bodies of *Pleurotus ostreatus* were collected on the grounds of the Zvenigorod Biological Station (ZBS) of Moscow State University in June–July 2000 and within the Forest Tver Preserve (Central Forest Biosphere Tver State Preserve) in June–July 2002. The collection of natural strains was supplemented by ten wild strains collected in Moscow in autumn 2002. Fallen trees, mostly of deciduous varieties, were the substrates for collection of *Pleurotus* fruiting bodies. One fruiting body was taken from every cluster. For all the fruiting bodies collected, both spore prints and dikaryotic mycelial cultures were obtained (Table 1).

Isolation of mycelial cultures and somatic compatibility and matings were performed in petri dishes with wort agar of the following composition: wort, 50 ml; water, 850 ml; agar, 20 g. Incubation was performed at 25°C. In order to suppress foreign microflora, isolation of pure dikaryotic mycelial cultures from fruiting bod-

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Table 1. Characterization of the isolated strains of *P. ostreatus*

Strains				Season	Substrate	Number of clusters per log	Intersterile group
Moscow oblast	ZBS, 2000		O.1-…	June–July	Birch	2*	I
			O.2-…	″	Aspen	2	I
			O.3-…	″	Birch	3	I
			O.4-…	″	Birch	1	I
			O.5-…	″	Willow	1	I
			O.6-…	″	Birch	6	I
			O.7-…	″	Spruce	2	I
			O.8-…	″	Birch	3	I
			O.9-…	″	Birch	4	I
			O.10-…	″	Aspen	1	I
			O.11-…	″	Aspen	2	I
	Moscow	2000	O.13-…	September	Aspen	1	II
		2002	M.2-…	September–November	Birch	1	II
			M.3-…	″	Mountain ash	4	II
			M.4-…	″	Birch	3	II
			M.5-…	″	Apple tree	2	II
Tver Forest Preserve, 2002			T.1-…	June–July	Birch	5	I
			T.2-…	″	Spruce	3	I
			T.3-…	″	Aspen	4	I
			T.4-…	″	Birch	3	I
			T.5-…	″	Birch	1	I
			T.6-…	″	Aspen	4	I

Note: Strain designation contains the information on the ordinal number of the substrate and on the number of strains isolated.
* The figures designate the number of strains collected from one log, corresponding to the number of clusters.

Table 2. Enzymes, their EC numbers, loci, and alleles with their relative mobility

Name	EC	Loci	Alleles, allele number/relative mobility	Buffer system
Acid phosphatase (ACP)	3.1.3.2	*Acp*	1/121, 2/108, 3/100, 4/86, 5/76	Tris–citrate
Alcohol dehydrogenase (ADH)	1.1.1.1	*Adh*	1/100, 2/94	Tris–EDTA–borate
Fluorescent esterase (FE)	3.1.1.2	*Fe*	1/100, 2/96, 3/88, 4/73	Tris–EDTA–borate
Leucine aminopeptidase (LAP)	3.4.11.1	*Lap1*	1/100, 2/89, 0/silent	Tris–citrate–LiOH–borate
		Lap2	1/111, 2/105, 3/100, 4/96, 5/93, 6/86, 3^s/99	
Isocitrate dehydrogenase (IDH)	1.1.4.2	*Idh*	1/106, 2/100, 3/92	Tris–citrate
Malate dehydrogenase (MDH)	1.1.1.37	*Mdh2*	1/115, 2/100	Tris–citrate
Phosphoglucomutase (PGM)	2.7.5.1	*Pgm*	1/116, 2/108, 3/100, 4/91	Tris–EDTA–borate
Phosphoglucoisomerase (PGI)	5.3.1.9	*Pgi*	1/116, 2/100, 3/83	Tris–EDTA–borate
Superoxide dismutase (SOD)	1.15.1.1	*Sod*	1/100, 2/82, 3/74	Tris–citrate
Esterase (EST)	3.1.1.1	*Est1*	1/103, 2/100, 3/95, 4/90	Tris–citrate–LiOH–borate
		Est2	1/112, 2/110, 3/100, 4/97	

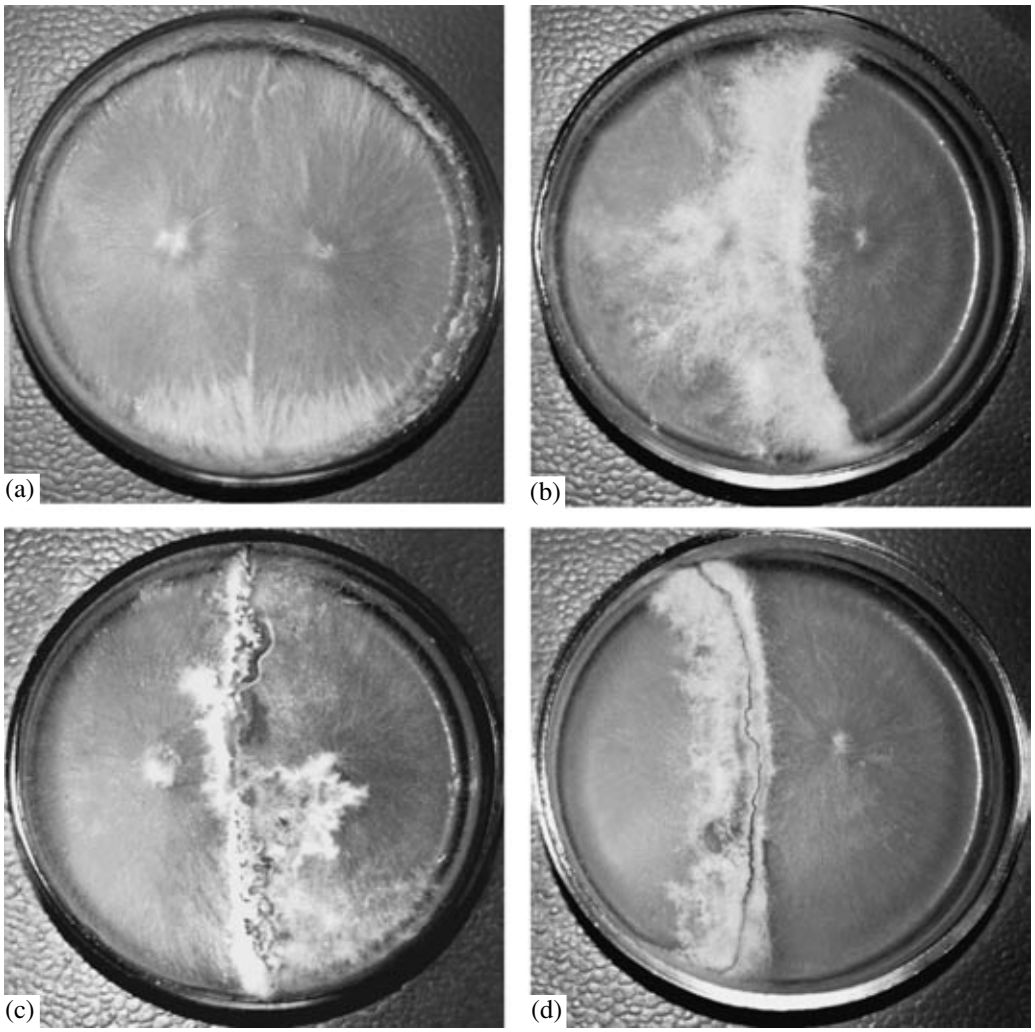

Fig. 1. Types of somatic incompatibility: (a) compatible reaction C; (b) weak reaction W; (c) strong reaction S; (d) normal reaction N.

ies was performed on the medium supplemented with fundazole (5 mg/ml) and ampicillin (10 mg/ml).

Intersterile groups were determined by hybridization of natural dikaryotic strains with the monokaryotic tester strains of groups I and II from the laboratory collection [1] (di-mon hybridization). Two to three weeks after mycelial contact, the monosporous tester colony was examined for clamps by microscopy of its outlying part on the side opposite to the dikaryon. The presence of clamps indicated sexual compatibility of the strain with a given intersterile group.

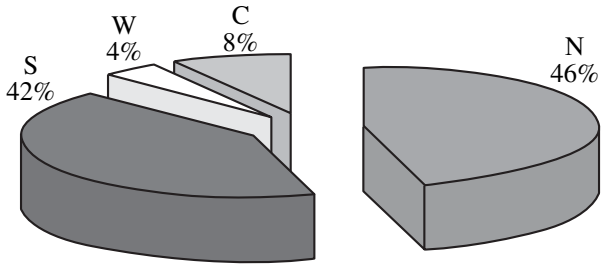

Fig. 2. The distribution of somatic incompatibility groups in natural populations of oyster mushrooms.

Somatic incompatibility was determined on petri dishes with wort agar by fusion of strains in pairs in all possible combinations. Two weeks after mycelial contact, the character of the interaction zone was determined visually by the presence of a dense mycelial ridge (barrage). The frequencies of distribution of the natural strains into somatic compatibility groups were estimated using the Shannon diversity index (I).

Extraction of water-soluble proteins was performed from a seven-day mycelium. The mycelium was removed from petri dishes with a spatula, excess moisture was removed, and proteins were extracted with 0.1 M Tris–HCl buffer, pH 6.8. Inheritance of the allozyme loci of *Pleurotus* strains was analyzed using the standard procedure developed in the Laboratory of Population Genetics, Vavilov Institute of General

Table 3. Genetic diversity and polymorphism in natural populations of *P. ostreatus*

Samplings	The number of strains	*P*, %	*A*	H_o	H_e	I	*Nm*
Moscow	38	100	3.5	0.328	0.515	0.888	0.10
Tver	20	81.8	2.64	0.268	0.348	0.612	0.16
Overall for two samplings	58	100	3.75	0.296	0.532	0.959	0.09

Note: *P*, the fraction of polymorphic loci; *A*, average number of alleles per locus; H_o, observed heterozygosity; H_e, expected heterozygosity; I, Shannon genetic diversity index; *Nm*, gene flow calculated as $Nm = 0.25\ (1 - F_{ST})/F_{ST}$.

Genetics, Russian Academy of Sciences [7, 8]. The protein fractions were separated in 13% starch gel using three buffer systems: Tris–citrate, Tris–EDTA–borate, and Tris–citrate–LiOH–borate. After electrophoresis, the starch block was sliced horizontally, and each slice was stained according to standard procedures [8]. Ten allozyme systems were studied: acid phosphatase (ACP), alcohol dehydrogenase (ADH), fluorescent esterase (FE), leucine aminopeptidase (LAP), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), superoxide dismutase (SOD), and esterase (EST) (Table 2).

The alleles most frequent among the studied sampling of strains were designated as 100; the relative mobility of other alleles was determined in relation to the 100th allele. The alleles were numbered in accordance with their decreasing electrophoretic mobility. Genetic diversity of the natural strains was estimated by the frequencies of alleles of 12 allozyme loci (Table 2). The dendrogram of genetic similarity between *Pleurotus ostreatus* strains was constructed on the basis of Nei genetic distances for 12 allozyme loci using the UPGMA algorithm [9]. The population genetic parameters were calculated using POPGENE 1.32 software [10].

RESULTS AND DISCUSSION

Distribution of the Natural Strains of the Oyster Mushroom in Groups of somatic Incompatibility and Intersterile Groups

Mutual fusion of dikaryotic strains enabled us to determine genetically isolated individuals. All dikaryotic strains were distributed in four somatic incompatibility groups according to the degree of antagonistic response: W, weak reaction, with a mildly pronounced, nonpigmented contact zone; mycelial hyphae may overlie one another without fusion; N, normal, moderate reaction with a pronounced, pigmented barrage 3–4 mm wide; S, strong reaction producing a sturdy barrage over 4–5 mm wide, often crimped and pigmented (Fig. 1). Fusion with the same strain resulted in a complete fusion of hyphae without visible antagonistic reactions; it was a complete compatibility, C. The barrage color varied from light yellow and reddish (normal reaction, N) to dark brown (strong reaction, S). The overwhelming majority of strain combinations were vegetatively incompatible; the degree of antagonism did not depend on the sampling site and on the substrate. The possibility of coexistence of several individuals on the same substrate (log, tree stump, etc.) was demonstrated for many fungal species [2]. In the *Pleurotus* populations studied, however, the strains collected from the same substrate were usually vegetatively compatible, being in fact somatic clones of the same individual. The interaction between these clones was responsible for the 8% of compatible reactions (Fig. 2). However, strong (42%) and normal (46%) reactions of somatic incompatibility prevailed in the populations. This ratio was sustained both in local populations and for the analysis of frequencies of somatic incompatibility groups in the whole collection of *P. ostreatus* strains.

According to the results of di-mon hybridization, all *Pleurotus* strains collected in summer belonged to the intersterile group I, while the ones collected in autumn belonged to the intersterile group II (Table 1).

Allozyme Loci Analysis

With ten allozyme systems, it was possible to characterize allele frequencies for 12 allozyme loci (*Acp, Adh, Fe2, Lap1, Lap2, Idh, Mdh2, Pgm, Pgi, Sod, Est1, Est2*). The loci *Mdh2* and *Pgi* of the Tver sampling were monomorphic; for the whole strain collection, however, the ratio of polymorphic loci was 100% (Table 3). Both samplings exhibited relatively high heterogeneity: the Shannon diversity index for the Moscow and Tver samplings was 0.888 and 0.612, respectively, and it was close to one (I = 0.959) for the overall samplings. Most alleles were common to the whole strain collection; some alleles were found, however, that were characteristic only to a specific sampling (Table 4). In the Moscow sampling, for example, only the alleles *Lap1-2*^{89} and *Lap1-0*^{silent} were present with respective frequencies of 0.631 and 0.369; in the Tver sampling, the *Lap1-1*^{100} allele, which was not found in the Moscow sampling (frequency 0.850), and the *Lap1-0*^{silent} allele (frequency 0.150) predominated. Five alleles of the *Acp* locus were found in the Moscow sam-

Table 4. Allele frequencies for samplings and for the whole collection

Loci	Alleles	Allele frequencies		
		Moscow oblast	Tver oblast	Total for two samplings
Acp	*Acp-1*^{121}	0.066		0.043
	Acp-2^{108}	0.460		0.302
	Acp-3^{100}	0.342	0.450	0.379
	Acp-4^{86}	0.053	0.300	0.138
	Acp-5^{76}	0.079	0.250	0.138
Adh	*Adh-1*^{100}	0.757		0.757
	Adh-2^{94}	0.242		0.243
Fe	*Fe-1*^{100}	0.317	0.950	0.570
	Fe-2^{96}	0.433	0.050	0.280
	Fe-3^{88}	0.217		0.130
	Fe-4^{73}	0.017		0.010
Lap1	*Lap1-1*^{100}		0.850	0.707
	Lap1-2^{89}	0.631		0.241
	Lap1-0^{silent}	0.369	0.150	0.052
Lap2	*Lap2-1*^{111}	0.013		0.009
	Lap2-2^{105}	0.145	0.100	0.129
	Lap2-3^{100}	0.447	0.200	0.362
	Lap2-3$^{s\ 99}$		0.150	0.035
	Lap2-4^{96}	0.158	0.175	0.164
	Lap2-5^{93}	0.210	0.275	0.189
	Lap2-6^{86}	0.026	0.100	0.112
Idh	*Idh-1*^{106}	0.329		0.216
	Idh-2^{100}	0.671	0.125	0.483
	Idh-3^{92}		0.875	0.302
Mdh2	*Mdh2-1*^{115}	0.066		0.043
	Mdh2-2^{100}	0.934	1.000	0.957
Pgm	*Pgm-1*^{116}	0.053		0.035
	Pgm-2^{108}	0.263		0.172
	Pgm-3^{100}	0.684	0.850	0.741
	Pgm-4^{91}		0.150	0.052
Pgi	*Pgi-1*^{116}	0.224		0.147
	Pgi-2^{100}	0.513	1.000	0.681
	Pgi-3^{83}	0.263		0.172
Sod	*Sod-1*^{100}	0.833	0.250	0.625
	Sod-2^{82}	0.139		0.089
	Sod-3^{74}	0.028	0.750	0.286
Est1	*Est1-1*^{103}	0.145		0.088
	Est1-2^{100}	0.468	0.700	0.559
	Est1-3^{95}	0.209	0.175	0.196
	Est1-4^{90}	0.177	0.125	0.157
Est2	*Est2-1*^{112}	0.053	0.100	0.069
	Est2-2^{110}	0.118	0.550	0.267
	Est2-3^{100}	0.513	0.250	0.422
	Est2-4^{97}	0.316	0.100	0.241

Note: Empty field, allele was not detected.

pling, and only three alleles in the Tver one. Alleles varied in frequency: from the very rare ones with frequencies below 5%, like *Fe-4*^{73}, *Lap2-1*^{111}, *Lap2-6*^{86}, and *Sod-3*^{74} in the Moscow sampling and *Fe-2*^{96} in the Tver one, to the predominant alleles with frequencies over 90%, like *Mdh2-2*^{100} and *Fe-1*^{100} in the Moscow and Tver samplings, respectively (Table 4). Rare alleles were mostly found in the Moscow oblast, where both intersterile groups were represented.

Since various selection modes, genetic drift, nonrandom hybridization, and other population dynamics factors affect heterozygosity of populations directly, heterozygosity is one of the significant parameters in the study of population genetic processes. The fraction of expected homozygotes prevailed upon the observed ones in the natural *Pleurotus* populations (in the Moscow sampling, $H_o = 0.328$, $H_e = 0.515$; in the Tver sampling, $H_o = 0.268$, $H_e = 0.348$); this finding indicates substantial heterozygote deficiency and deviation panmixia (Table 3). This may be caused by the insufficient size of the sampling and by the presence in the collection of genetically identical somatic clones from one substrate; for instance, strains O.6-1, O.6-2, O.6-3, O.6-4, O.6-5, and O.6-6, isolated on ZBS territory from a fallen birch trunk were found to be completely vegetatively compatible (Table 1).

The studied samples of *P. ostreatus* exhibited a pronounced genetic differentiation; the genetic differentiation coefficient F_{ST} was 0.718 for the Moscow oblast, 0.605 for the Tver oblast, and 0.743 between the regions (Table 4). Natural selection possibly results in adaptation of geographically remote populations to local conditions, and later on, genetic differentiation occurs, especially in the case of small population size. It is, however, difficult to determine the real sizes of the populations studied in nature.

Relatively high values of the inbreeding coefficient (the fraction of individuals homozygous in identical alleles, $F_{IS} = 0.460$) also indicated the presence of genetically identical individuals in the sampling, in this particular case, of the somatic clones collected from one log. somatic reproduction in natural populations of *P. ostreatus* is likely limited to a single substrate (log) and does not play a significant role in shaping the population structure of the species. Sexually produced basidiospores capable of migration to considerable distances are the main way of reproduction. Our data confirming the absence of geographical isolation between two regions support this notion.

An insignificant genetic drift observed between the geographically isolated samplings ($Nm = 0.09$) did not exceed migration of one individual per generation, a normal value for fungi (Table 3).

Nei genetic distances were calculated on the basis of the allozyme loci frequencies, and a dendrogram of genetic similarity between all dikaryotic *P. ostreatus* strains was constructed (Fig. 3). All the natural isolates

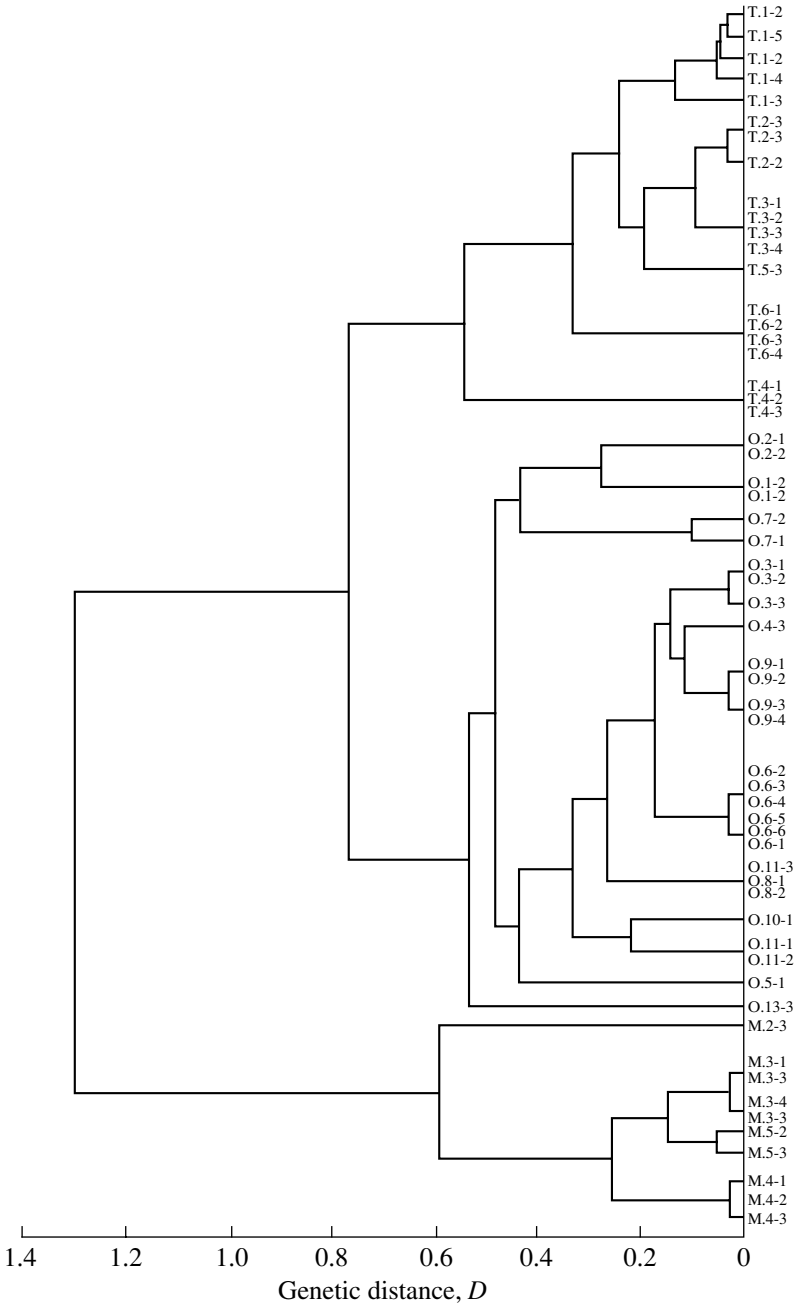

Fig. 3. The genetic similarity dendrogram for *Pleurotus ostreatus* constructed on the basis of Nei genetic distances for 12 allozyme loci using the UPGMA algorithm.

obtained from the ZBS, from Tver Forest Preserve, and from Moscow were distributed in two separate clusters corresponding to intersterile groups I and II. Vegetatively compatible strains collected from one substrate formed subgroups of genetically identical clones. No correlation was found between affiliation with a specific cluster and relation with a substrate.

In conclusion, the investigated populations of oyster mushrooms are polymorphous with a high level of heterogeneity and genetically differentiated. Sexual reproduction by basidiospores is the predominant and efficient way of reproduction, while somatic reproduction occurs within individual substrates. The functioning of two reproductively isolated intersterile groups is related to seasonal fruiting; intersterile group I dominates in summer, while intersterile group II dominates in autumn, when diurnal temperature fluctuations are substantial.

Table 5. Wright's *F*-statistics for all *P. ostreatus* populations and for the two individual regions (Moscow and Tver oblasts)

Loci	Moscow oblast		Tver oblast		Total for the whole collection	
	F_{IS}	F_{ST}	F_{IS}	F_{ST}	F_{IS}	F_{ST}
Acp	0.159	0.579	–0.163	0.418	0.144	0.572
Adh	1.000	1.000	***	***	1.000	1.000
Fe	–0.003	0.667	–0.053	0.474	0.241	0.724
Lap1	0.547	0.774	1.000	1.000	0.686	0.843
Lap2	0.257	0.628	0.075	0.538	0.222	0.611
Idh	0.583	0.791	–0.143	0.428	0.671	0.836
Mdh2	–0.070	0.465	***	0.000	–0.045	0.477
Pgm	0.771	0.885	1.000	1.000	0.834	0.917
Pgi	0.787	0.893	***	0.000	0.822	0.911
Sod	0.805	0.926	0.733	0.866	0.863	0.937
Est1	–0.272	0.550	–0.294	0.353	–0.239	0.522
Est2	0.194	0.597	0.349	0.675	0.322	0.661
Average	0.396	0.718	0.229	0.605	0.460	0.743

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