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EXPERIMENTAL ARTICLES

A Comparative Analysis of *Stachybotrys chartarum* Strains Isolated in Russia

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Abstract—This work deals with a comparative analysis of *Stachybotrys chartarum* strains isolated from various artificial cellulose-containing materials and natural substrates in geographically distant regions of Russia. The analysis included determination of the spore size; the strain toxicity to *Paramecium caudatum*; the strain resistance to the fungicides Benomil, Olilen, and Tilt; and the PCR study of the genome structure with the aid of a primer that was complementary to the core sequence of the SINE retrotransposon. It was found that some of the strains that were isolated from different areas and from different substrates differ in their toxicity, fungicide resistance, and genome structure. PCR analysis showed the absence of any correlation between the genome structure, the strain properties, the geographic area, and the substrates from which the strains were isolated. The pheno- and genotypic diversity of the strains and their different vegetative compatibility suggest the existence of an intraspecies diversity of the *S. chartarum* strains that were isolated in different geographic areas. The absence of any correlation between the pheno- and genotypic properties of the strains and the substrates from which they were isolated implies that the colonization of artificial substrates by *S. chartarum* occurred occasionally from natural habitats. The *S. chartarum* populations that live on artificial substrates are unlikely to have their own evolutionary history.

Key words: *Stachybotrys chartarum*, biodamage, mycotoxins, fungicide resistance, analysis of fungal populations.

Stachybotrys chartarum (Ehrenberg) S. Hughes is a saprotrophic micromycete fungus, which naturally lives in soil and on plant debris, with the dead stalks, leaves, and grains of cereals being preferred [1]. The fungus is found to cause soybean root rot [2], to affect the fibers and unopen balls of cotton, and to induce lesions on sugarcane stalks and fruit trees [3]. In the anthropogenic environment, the fungus infects various cellulose-containing materials, such as paper, paperboard, wallpaper, wood, and plywood.

S. chartarum synthesizes a number of compounds that are toxic to humans and animals, such as roridin E; satratoxins F, G, and H; trichoverrols; trichoverrins; verrucarol; and verrucarin J. The toxins were found in the culture liquid and all the morphological structures of the fungus. In addition to mycotoxins, the fungus synthesizes immunosuppressors (such as cyclosporin) [4, 5] and volatile organic compounds, including 1-butanol, 3-methyl-2-butanol, 3-methyl-1-butanol, and thujopsene [6].

An intense development of the fungus on cellulose-containing materials (such as wallpaper, plasterboard, and fillers) inside water-damaged buildings threatens human health. The extensive development of *S. chartarum* on the water-damaged lower floors of buildings after the high flood in Cleveland, the United States, in the mid-1990s afflicted many people and even killed some of them [4]. The *S. chartarum* spores and volatile toxic compounds, when present in the air-conditioning systems of many-storied buildings, can also affect people. Some researchers observed a correlation between human illness related to indoor air quality and the prevalence of *S. chartarum* [7, 8].

S. chartarum strains differ in their hemolytic activity, toxin production, and genome structure [9] and show different growth rates on the same nutrient media or cellulose-containing building materials [1, 10].

The aim of this work was to investigate the possible relationship between the substrates and the geographic areas from which particular *S. chartarum* strains were isolated and the morphometric characteristics, toxicity, fungicide resistance, and the genome structure of these strains.

MATERIALS AND METHODS

The 54 strains of *Stachybotrys chartarum* used in this study were isolated from soil samples, plant debris, and various cellulose-containing building materials in the Moscow region (MR), Tomsk region (TR), Tver

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Table 1. The number of the *S. chartarum* strains that were isolated from different substrates and from different geographic areas

Area	Substrate	Literal strain designation	Number of isolates	Total number of isolates from a given area
Moscow and Moscow region	Filler	MSh	6	22
	Paperboard	MB	10	
	Plant debris	MRO	2	
	Textile	MT	2	
	Air	MV	1	
	Unknown	MN	1	
Tomsk and environs	Paperboard	TB	12	23
	Plant debris	TRO	5	
	Air	TB	2	
	Cockroach feces	TT	3	
	Filler	TSh	1	
Karachay-Cherkessia	Soil	KP	4	4
Buryatia	Soil	BP	4	4
Tver region	Plant debris	TRO	1	1
Total				54

region, Buryatia, and Karachay-Cherkessia (Table 1). The strains were isolated by standard microbiological techniques with the use of humid chambers and selective media [11, 12]. All the strains were tested for resistance to three fungicidal biopreparations (Benomil, Olilen, and Tilt) and for toxicity to *Paramecium caudatum* Ehrb. Thirty-two *S. chartarum* strains, isolated from different habitats, had the structure of their genomes investigated.

To determine the size of spores, strains were cultivated for 10 days on malt extract agar, and 100 spores of each strain were analyzed for their length and width. The confidence level of comparisons was evaluated in terms of Student's *t*-test for $P < 0.05$ by using the SPSS 11.5 software package.

To determine the toxicity of the *S. chartarum* strains, they were grown in wort broth for 10 days and the culture liquid was tested for toxicity to *P. caudatum.* Paramecia were grown in an infusion of 10 oat grains in 200 ml of water. Two drops (50 μl) of the culture liquid of a *S. chartarum* strain and one drop (50 μl) of the paramecium culture were placed onto a microscope slide and incubated in a humid chamber. After 5, 20, 40, and 60 min of incubation, the cell integrity and activity of paramecia were estimated. A paramecium was considered dead if it became nonmotile and morphologically degraded [11]. The tested *S. chartarum* strain was considered to be severely toxic, toxic, and relatively toxic if no less than 70% of the paramecia died after 5, 20, and 40 min of incubation, respectively. If most of the paramecia remained morphologically unaltered after 60 min of incubation, the tested strain was considered nontoxic.

The fungicide resistance of the *S. chartarum* strains was tested by using three fungicidal biopreparations (Benomil, Olilen, and Tilt) [10], which showed high activity against the *S. chartarum* strains. Olilen is a systemic fungicide with a broad specificity, which is active against cereal diseases. Tilt is a systemic protective and curative fungicide, which is efficient against ascomycetes, basidiomycetes, and imperfect fungi [13]. Benomil is a systemic protective and curative fungicide, which is efficient against powdery mildew, *Fusarium* wilt, and *Cercospora* leaf spot [14]. The sensitivity of an *S. chartarum* strain to a fungicide was evaluated by estimating the radial growth rate of the colonies of this strain during its cultivation on nutrient media with and without the fungicide [15]. The fungicide was taken at a concentration of 10 μg/ml, although in some experiments, at a concentration of 1 μg/ml. The tested *S. chartarum* strain was considered to be sensitive, relatively sensitive, and resistant to the fungicide if the ratio of the growth rates in the presence and absence of the fungicide (10 μg/ml) was lower than 0.1, fell within the interval 0.1–0.4, or was higher than 0.4, respectively. The fungicide tolerance test was carried out in triplicate at 25°C (the optimal growth temperature of *S. chartarum*) [1].

To obtain DNA, the fungal mycelium was grown in wort broth at 25°C for 14 days, separated from the medium by filtration, washed with sterile distilled water, and laid onto several layers of filter paper for drying. An aliquot (about 0.5 cm^3) of the dry mycelium was placed in a frozen mortar, flooded with liquid nitrogen, and ground with a pestle. The ground mycelium (about 0.25 cm^3) was transferred into a microtube and flooded with 0.8 ml of CTAB buffer (100 mM Tris–HCl

(pH 8.0) with 1.4 M NaCl, 20 mM EDTA, and 2 wt % cetyltrimethylammonium bromide). The microtube was vortexed and incubated at 65°C for 1 h with vortexing after each 20 min. The contents of the microtube was then suspended in 0.5 ml of chloroform and centrifuged for 10 min. The supernatant (0.7 ml) was transferred into a new microtube, mixed with 0.4 ml isopropanol and 1/10 volume of CH_3COOK (5 M, pH 4.6), and centrifuged for 10 min. The supernatant was mixed with 0.2 ml of cold 70% ethanol, and the mixture was centrifuged for 5 min. The supernatant was decanted, and the liquid that remained on the DNA pellet was removed with filter paper. The DNA was resuspended in 50 µl of TE buffer.

The reaction mixture (20 µl) for PCR contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.01% Tween 20, 100 µM each dATP, dGTP, dCTP, and dTTP (Silex, Russia), 2 µM RevSine primer, 5 mM $MgCl_2$, 20 ng DNA (2 µl of the DNA preparation), and 0.1 unit of *Taq* polymerase (Silex). PCR was performed with the initial DNA denaturation step at 94°C for 3 min, followed by 30 cycles of DNA denaturation at 94°C for 40 s, primer annealing at 49°C for 40 s, and primer extension at 72°C for 90 s, with the final extension step at 72°C for 5 min. The amplified DNA fragments (about 20 µl of the reaction mixture) were purified by electrophoresis in 2% agarose gel, which was prepared by using a solution containing 0.1 M Tris–HCl (pH 8.3), 0.1 M borate, 0.025 M EDTA, and 1 µg/ml ethidium bromide. The electrophoresis was carried out at an electric field strength of 7 V/cm with a 1 kb DNA ladder (Sibenzyme, Russia) as the molecular marker. The RevSine primer 5'-GGCATCAGTGAAGACCAAGCTAGGG-3' was complementary to a part of the A–B box of the SINE retrotransposon [16, 17]. The cluster analysis of the strains was performed with respect to 14 distinct DNA fragments from 200 to 750 bp in size with the aid of the Treecon software package.

RESULTS

The cultural and morphological characteristics of the *S. chartarum* strains were found to be similar to those reported in the literature for the species *S. chartarum* (atra) [3, 18, 19]. The mean length of the *S. chartarum* spores according to our estimates (7.36 µm) was slightly shorter than the minimal value reported by Ellis (8–11 µm) [19], although the mean width of the spores estimated by us (5.56 µm) fell within the interval reported by this researcher (5–10 µm) (Table 2). The mean length of the spores produced by the strains isolated in the Moscow region varied from 5.4 to 8.6 µm, and the mean width of the spores of these strains varied from 3.9 to 6.7 µm. For the strains isolated in the Tomsk region, the mean length of spores varied from 5.6 to 9.2 µm, and the mean width varied from 4.3 to 7.6 µm. The mean length of the spores produced by the strains isolated from paperboard substrates in the Moscow region varied from 6.1 to 7.5 µm, and the mean width of the spores of these strains varied from 4.6 to 5.9 µm. For the strains that were isolated from paperboard substrates in the Tomsk region, the mean length of spores varied from 6.1 to 9.2 µm, and the mean width varied from 4.6 to 7.6 µm. Statistical analysis failed to reveal any difference between the Moscow and Tomsk isolates either in the mean spore length ($t = 0.052$) and the mean spore width ($t = 0.15$) or in the length-to-width ratio ($t = 0.39$). At the same time, this analysis revealed a statistically significant difference in the mean spore length ($t = 0.01$) between the *S. chartarum* strains that were isolated from paperboard substrates in the Moscow and Tomsk regions although the mean widths of the spores of these strains differed insignificantly ($t = 0.08$). The other groups of the *S. chartarum* strains were too small to perform reliable statistical analysis.

The *S. chartarum* strains under study differ greatly in the growth rate of their colonies on malt extract agar (Table 2). The mean growth rates of the strains that were isolated in the Moscow and Tomsk regions differed insignificantly. A relatively large percent (from 17 to 60%) of the strains of different groups produced sectors during their growth on malt extract agar.

The toxicity of the *S. chartarum* strains greatly differed (Table 2). The groups of the strains that were isolated in the Moscow and Tomsk regions were both dominated by toxic strains. At the same time, the second-most-abundant subgroup of the Moscow isolates comprised the relatively nontoxic strains, whereas the second-most-abundant subgroup of the Tomsk isolates comprised the severely toxic strains. Most of the strains isolated in Buryatia were relatively nontoxic. The strains isolated in Karachay-Cherkessia were mainly toxic, whereas nontoxic and severely toxic isolates were absent. The strains isolated from paperboard substrates in the Moscow and Tomsk regions were mainly toxic and severely toxic, although some of the Tomsk isolates were nontoxic. Among the strains isolated from fillers in the Moscow region and from plant debris in the Tomsk region, nontoxic isolates were absent.

A comparative analysis of the *S. chartarum* strains with respect to fungicide tolerance showed that they were most sensitive to the fungicide biopreparation Benomil, with only one of the strains isolated in the Moscow region being resistant to this fungicide. At the same time, the strains under study differed greatly in their tolerance to the fungicides Olilen and Tilt (Table 2).

A comparative analysis of the *S. chartarum* strains in terms of the Rogers distances showed that the strains that were isolated in the Moscow and Tomsk regions differed slightly in this statistical parameter (Table 3). More distinct differences were observed between the strains that were isolated in these regions from paperboard substrates and between the strains that were isolated in the same region but from different substrates (paperboard, filler, and plant debris). However, the possibility cannot be excluded that the observed

Table 2. A comparative analysis of the *S. chartarum* strains that were isolated from geographically distant areas

Area, substrate	Number of isolates	Percentage of isolates forming sectors	Growth rate, mm/day	Spore size, μm		Percentage of strains with a given toxicity				Percentage of strains tolerant to					
				length	width	NT	RT	T	ST	Olilen			Tilt		
										S	*SR*	*R*	*S*	*SR*	*R*
Moscow region	22	33	1.94	6.9	5.4	14	24	48	14	30	60	10	32	42	26
Tomsk region	23	27	1.96	7.5	5.7	10	10	50	30	48	43	9	14	62	24
Buryatia	4	50	2.06	8.4	6.1	0	75	25	0	67	0	33	67	0	33
Karachay-Cherkessia	4	25	1.17	7.1	5.2	0	25	75	0	50	25	25	0	100	0
Moscow region, paperboard	10	22	1.96	6.6	5.3	0	0	78	22	43	57	0	37	50	13
Moscow region, filler	6	33	2.17	6.9	5.1	0	49	34	17	0	83	17	20	20	60
Tomsk region, plant debris	5	60	2.22	7.9	5.8	0	20	60	20	60	40	0	0	60	40
Tomsk region, paperboard	12	17	2.03	7.6	6	22	0	33	45	27	64	9	9	73	18
Mean value		33.38	1.94	7.36	5.58	5.75	25.38	50.37	18.5	40.63	46.5	12.87	22.38	50.87	26.75

Note: NT, RT, T, and ST stand for nontoxic, relatively toxic, toxic, and severely toxic, respectively. *S*, *SR*, and *R* stand for sensitive, relatively sensitive, and resistant, respectively.

Table 3. Rogers distances between the various groups of the *S. chartarum* strains

Groups compared	R_{tox}	Fungicide tolerance		R_{mean}
		R_{oli}	R_{tilt}	
Moscow region vs. Tomsk region	0.2	0.25	0.27	0.24
Moscow region vs. Tomsk region (paperboard)	0.55	0.2	0.36	0.37
Paperboard vs. filler (Moscow region)	0.66	0.53	0.58	0.59
Paperboard vs. plant debris (Tomsk region)	0.47	0.42	0.27	0.39

Note: $R = [\Sigma(p_i - q_i)^2]^{1/2}$, where p_i and q_i are the frequencies of the ith parameter in the populations compared. R_{tox}, R_{oli}, and R_{tilt} are the Rogers distances between the strain groups in toxicity, tolerance to Olilen, and tolerance to Tilt, respectively. R_{mean} is the arithmetic mean of R_{tox}, R_{oli}, and R_{tilt}.

differences between the different groups of the strains is due to an insufficient number of the strains being taken for analysis.

The genome of the *S. chartarum* strains was analyzed using the PCR with the RevSine primer. The resulting PCR products gave rise to a large number of electrophoretic bands, the most distinct of which (14 in number, from 200 to 750 bp in size) were taken for a statistical analysis of the *S. chartarum* genome. The analysis allowed the *S. chartarum* strains to be divided in several groups (see figure). Three strains (9KP2/1, 1TB1, and 9MSh1) differed considerably from all the other *S. chartarum* strains (it should be noted that 9MSh1 is the only strain that is resistant to the fungicide Benomil). The other strains fell into three groups, two of which included strains that were isolated from different substrates and from different areas, and one group comprised three strains that were isolated from paperboard (two strains) and plant debris (one strain) in the Tomsk region. In general, PCR analysis failed to reveal any correlation between the genome structure, toxicity, fungicide tolerance, substrates, and the geographic areas from which the analyzed strains were isolated.

DISCUSSION

The study showed that the *S. chartarum* strains that were isolated from different substrates and from different areas differ in the toxicity, fungicide tolerance, and genome structure. Of particular interest is the different

The cluster diagram of the *S. chartarum* strains constructed on the basis of PCR analysis with the RevSine primer.

fungicide tolerance of the *S. chartarum* isolates, which could be due to their contact with the fungicides that are used in agriculture for pest control. As was reported by Frolova *et al.* [20], the species *S. chartarum* is a common micromycete that resides in soil below cereal crops. The different toxicity of the *S. chartarum* strains is likely to be their inherent property. The existence of differences between some characteristics of the *S. chartarum* strains isolated from distant habitats, as well as the different vegetative compatibility of these strains [10], suggests their intraspecies diversity. The absence of correlation between the pheno- and genotypic properties of the groups of *S. chartarum* strains that were isolated from different artificial substrates implies that these substrates were occasionally colonized by *S. chartarum* from natural habitats. The *S. chartarum* populations on artificial substrates may be considered as population waves, which are unlikely to have their own evolutionary history.

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