An integrated approach to taxonomical identification of the novel filamentous fungus strain producing extracellular lipases: morphological, physiological and DNA fingerprinting techniques

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A new filamentous fungal strain was isolated into pure culture and initially named as strain L-1. The strain was found to secrete a high level of extracellular lipase at high temperatures. The identification of the isolate was performed by the combination of conventional morphological-physiological methods, scanning electron microscopy and RAPD. Phenotypic and genotypic characteristics of the L-1 strain were compared to reference strains. The morphological characteristics, radial growth rate at different temperatures and surface ornamentation of sporangiospores of the isolate almost completely match the reference strain *Rhizopus* [= *microsporus* var. *rhizopodiformis* VKM F-3693. The strain L-1 was characterized by high growth rate and the spore maturation abilities at 50°C. These characteristics are unique among all other strains of *Rhizopus*. The results of RAPD-diagnosis indicate the high degree of genetic similarity between strains L-1 and F-3693. We therefore identified strain L-1 as *Rhizopus microsporus* var. *rhizopodiformis*. The strain has been submitted and included in the All-Russian Collection of Microorganisms as VKM F-3688.

Key words: fungal taxonomy, identification, lipase, RAPD, *Rhizopus microsporus*-group, SEM.

Introduction

The extent of biological diversity has generated the need to isolate and culture the large numbers of microorganisms that remain to be studied. The number of fungal species presently described is only a small proportion of those that actually exist (Hawksworth, 1991, 1997, 2001). Accordingly the

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isolation and correct identification of fungi is of great practical importance. The fungi of *Rhizopus* genus are widespread in nature, and many species of this genus have significant industrial applications, particularly the production of lipases (Iwai and Tsujisaka, 1984). For the efficient lipase production and functioning, it is strongly desirable to have thermophilic producers and thermostable lipolytic systems. Previously we reported the isolation of a new thermophilic lipase-producing strain (Lusta *et al*., 1999). Here we discuss the identification of this isolate.

Different concepts have been used to define the fungal species. The phenotypic concept is the classic approach based on the morphological characteristics (Inui *et al*., 1965). The polythetic concept is based on a combination of characters (Guarro *et al*., 1999). Identifications based on colour and physical appearance of growing colonies and microscopic detail of morphological structures in some fungi may be equivocal because the shapes and sizes of different fungal organs are variable (Schipper and Stalpers, 1984). Current methods to identify Zygomycetes are incorporate sporangiospore topography. SEM is capable of allowing the surface ornamentation of spores to be defined and these characteristics appeared to be distinctive for individual species (Ellis, 1981).

A variety of biochemical and physiological methods for fungal identification have been also devised (Kohn, 1992; Carlile and Watkinson, 1994; Guarro *et al*., 1999). These alternative approaches to identifying fungi are labourious, time-consuming and provide insufficient taxonomic resolution. The major disadvantages are that all the assays based on phenotypes are too sensitive to growth conditions and depend on gene expression (De Bernardis *et al*., 1998).

These problems may be obviated by adopting DNA-based methods. The basic DNA sequences of an organism are insensitive to short-term environmental change and thus should provide a more stable alternative for strain identification (Kohn, 1992; Weising *et al*., 1995; Liew *et al*., 1998; Soll, 2000). Methods that directly detect DNA differences among species and strains are referred to as DNA fingerprinting methods (Soll, 2000).

One approach uses a random amplified polymorphic DNA (RAPD) technique to generate markers for any specific genome (Williams *et al*., 1990). This RAPD assay is genotypic method based on PCR technique in combination with single short arbitrary primers and requires no post-amplification sample manipulation (Poonwan *et al*., 1998). A single random primer hybridizes to homologous sequences in the genome, and DNA region between the two hybridization sites will be amplified using *Taq* polymerase. Each random primer gives a different pattern of PCR products (Brandt *et al*., 1998). The data

generated by RAPD can be used to determine the degree of genetic relatedness between different strains on the basis of similarity coefficients (Nei and Li, 1987; Soll, 2000).

The present paper describes the identification and species assignment of a new fungal isolate that makes use of several different methods: traditional morphological-physiological techniques, SEM and the PCR-based RAPD. In this study, twenty random primers were tested to identify individual fingerprints by PCR. The results of the analysis performed on the new isolate, strain L-1, by conventional and RAPD methods are compared with those of several reference strains from *Aspergillus* and *Rhizopus* and particularly the *Rhizopus microsporus*-group.

Materials and methods

Microorganisms

The following reference strains have been used for comparison by morphological-physiological techniques or RAPD: *Rhizopus microsporus* var. *rhizopodiformis* VKM F-3692 (= CBS 343.29); *R. microsporus* var. *rhizopodiformis* VKM F-3693 (= CBS 607.73); *R. microsporus* var. *chinensis* VKM F-1218, MT- (= ATCC 1227b); *R. microsporus* var. *chinensis* VKM F-1360, MT- (= CBS 262.28; ATCC 52812); *R. microsporus* var. *chinensis* VKM F-1361, MT+ (= CBS 261.28; ATCC 52811; DSM 2193); *R. microsporus* var. *microsporus* VKM F-773, MT+ (= CBS 699.68; ATCC 52813; CCRC 31140); *R. microsporus* var. *microsporus* VKM F-774, MT- (= CBS 700.68; ATCC 52814; CCRC 31141; CCF 1570); *R. microsporus* var. *microsporus* VKM F-1063; *R. stolonifer* var. *stolonifer* VKM F-601; *R. oryzae* VKM F-605 and *Aspergillus terreus* VKM F-3687. Strains were obtained from the All-Russian Collection of Microorganisms at the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. Strain L-1 was isolated from soil.

Strain isolation

The combined technique of dilution plating and direct isolation were used for fungal strain isolation into pure culture on selective media. A mold growing in the soil around a hot spring in Ashkhabad City (Turkmenistan) was maintained in a moist chamber with the soil until sporulation. Spores were dispersed in sterile water and after following serial dilutions were inoculated into selective liquid medium: 0.3% (w/v) $(NH_4)_2SO_4$ and 0.1% (w/v) $CaCl_2$ which contained 1% (v/v) of olive oil as the sole carbon and energy source (Lusta *et al*., 1999). Cultures were grown on a shaking incubator at 40°C, over

two days, and then transferred evenly onto the surface of malt agar medium (MEA). After maturation of the spores (~ four days at 40°C), spores from a particular fungus were transferred to sterile culture medium using a stereomicroscope and an inoculating sterile needle. Isolated strains were maintained on slants of MEA medium and stored at 4°C.

Strain cultivation and description

The MEA used as the basal medium for morphological and growth studies was contained in 90 mm Petri dishes (20 ml in each plate). Growth/temperature relationships were established as the average radial growth rate (cm/h) of the fungal strain. Petri dishes with nutrient medium were inoculated with equal amounts of spore suspension centrally and were cultivated at different temperatures. Measurements of colony diam. were made after 4, 24, 48 and 120 hours (Kochkina *et al*., 1978). The length and colour of the sporangiospores and rhizoids, and sizes of sporangia, columellae, and sporangiospores were determined using light microscopy (magnification ×160, ×400 and ×640).

Electron microscopy techniques

For SEM (Goldstein *et al*., 1992) the specimens were fixed in glutaraldehyde-paraformaldehide and then in OsO_4 solutions, dehydrated in ethanol and dried using a Critical Point Dryer BAL-TEC CPD 030 (Switzerland). Gold sputtering was performed using BAL-TEC SCD 050 Sputter Coater (Switzerland). Specimens were examined and photographed with Zeiss Scanning Electron Microscope DSM 962 (Germany).

DNA preparation

Two gram (wet weight) of fresh mycelium of each strain was frozen with liquid nitrogen and then ground with a -20°C cooled mortar and pestle. The powder (0.4 ml) was dispersed in 0.6 ml of extraction buffer (Tris HCl 100 mM, Na_2 EDTA 50 mM, NaCl 700 mM, $Na_2(SO_3)$ 100 mM, 1% (w/v) SDS, pH 7.5) and heated to 65°C for 20 minutes. Then 0.6 ml of chloroform/isoamyl alcohol was added and incubated on ice for 30 minutes. After centrifugation at 12000 × g for 20 minutes the aqueous phase was mixed with an equal volume of isopropanol. The mixture was incubated at room temperature for 10 minutes and then centrifuged at 2000 × g for 5 minutes. The pellet was air dried, resuspended in 200 µl of double distilled water, incubated at 37°C for 15 minutes, mixed with 100 µl of ammonium acetate and incubated on ice for 1 hour. Following centrifugation at 12000 × g for 20 minutes the supernatant was mixed with 0.54 volumes of isopropanol, incubated at room temperature for 10

minutes and recovered by centrifugation at 12000 × g for 5 minutes. The pellets were rinsed in 500 µl of 70% ethanol prior to air drying. The DNA pellets were then resuspended in 100 µl of TE buffer (Tris-HCl 10 mM, Na_2EDTA 1 mM), pH 7.5 containing of 100 µg ml^{-1} of RNase A, incubated at 37°C for 60 minutes, examined for DNA concentration using 0.8% agarose gel electrophoresis and then stored at -20°C until use.

RAPD assay

Amplification reactions were carried out in 25 µl volume containing KCl 50 mM, Tris-HCl 10 mM (pH 9.0), $MgCl_2$ 15 mM and 0.1% (v/v) TritonX-100, 200 µM each of dNTPs, 25 pmole of each random primer (Table 1), 1.5 U of Taq DNA polymerase and 50 ng of template DNA. The reaction mixture was overlaid with approximately 40 µl of mineral oil. Amplifications were performed by Minicycler (MJ Research). The temperature cylces were 95°C for 5 minutes, followed by 40 cycles of 40 seconds at 94°C, 40 seconds at 36°C and 1 minute at 74°C with a final extension of 5 minutes at 74°C. The amplified products were separated by electrophoresis on the 0.8% agarose gel in 1 × TBE buffer at 10 V cm^{-1} for 90 minutes and visualized by staining with ethidium bromide on an uv transilluminator.

Analysis of RAPD data

Amplified DNA fragments, reproducible in three reactions, were scored by the binary values on two possible character states 0 and 1. Estimation of genetic relationships between all pairs of strains were performed using Jaccard's coefficients calculated from the following formula (Nei and Li, 1987):

$$S_j = 2C_{xy}/(U_x + U_y + 2C_{xy})$$

in which C_{xy} is the number of bands common in lanes x and y, and U_x and U_y represent the number of unique bands in each sample. The total number of mismatches is $U_x + U_y$. S_j values range from 0 to 1. A measure of 0 reflects no common bands and lowest degree of genetic similarity indicated, while a measure of 1 reflects all common bands and highest degree of genetic similarity. Measures of 0.01 to 0.99 represent increasing degrees of commonness (Soll, 2000).

Results

Morphological identification

Strain L-1 formed very fast growing dark-grayish colonies, which became slightly brown at higher temperatures. Hypha were not septate. The

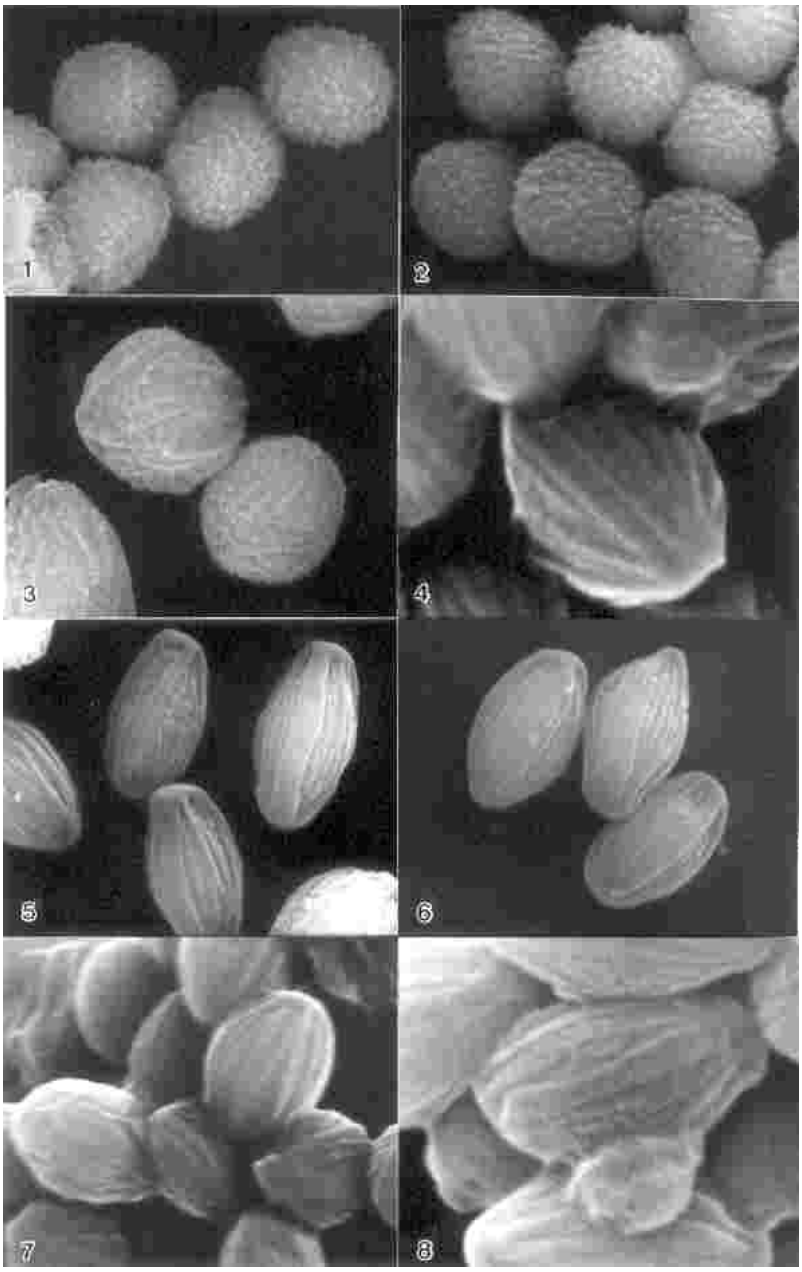
Table 1. RAPD primer sequences used for the experiments (OPERON company, kit F).

Primer code	Seqeunces	Primer code	Seqeunces
F01	ACGGATCCTG	F11	TTGGTACCCC
F02	GAGGATCCCT	F12	ACGGTACCAG
F03	CCTGATCACC	F13	GGCTGCAGAA
F04	GGTGATCAGG	F14	TGCTGCAGGT
F05	CCGAATTCCC	F15	CCAGTACTCC
F06	GGGAATTCGG	F16	GGAGTACTGG
F07	CCGATATCCC	F17	AACCCGGGAA
F08	GGGATATCGG	F18	TTCCCGGGTT
F09	CCAAGCTTCC	F19	CCTCTAGACC
F10	GGAAGCTTGG	F20	GGTCTAGAGG

Table 2. Comparative average data on the dimensions of different organs (diagnostic characters) in various *Rhizopus* isolates.

Strains	Diagnostic characters		
	Sporangia, µm	Columellae, µm	Sporangiospores, µm
VKM F-1218	(63.8-)80.2(-89.9)	Pyriform, (52.2-)60.2(-72.5) × (46.4-)52.3(-60.9)	Globose,-ellipsoidal, (4.6-)4.8(-5.4) × (3.6-)4.1(-4.6)
VKM F-1063	(72.5-)90.3(-107.3)	Subglobose, (52.2-)77.9(-95.7) × (49.3-)68.1(-78.3)	Broadly- ellipsoidal, (5.1-)5.5(-6.0) × (3.5-)3.9(-4.4)
VKM F-773	(72.5-)87.9(-104.4)	Subglobose, (46.4-)55.2(-69.6) × (37.7-)45.0(-49.3)	Broadly-ellipsoidal, (4.2-)4.9(-5.6) × (2.4-)2.8(-3.0)
VKM F-3693	(55.5-)76.4(-92.5)	Ellipsoidal, (51.8-)66.2(-81.4) × (44.4-)57.6(-66.6)	Globose, subglobose, (3.8-)4.1(-4.7) × (3.0-)3.3(-3.6)
L-1	(55.0-)78.2(-100.5)	Subglobose, ellipsoidal, (49.3-)70.2(-81.2) × (46.4-)57.9(-72.5)	Globose, subglobose, (3.9-)4.2(-4.8) × (3.0-)3.3(-3.6)

strain was characterized as bearing stolons and rhizoids, forming globular sporangia with columellae and apophyses. Unbranched sporangiophores were borne on stolons singularly or in clusters. They were straight or slightly curved, with smooth walls, and ranged from about 400 to 700 µm in height and 5-8 µm diam. Grayish-black sporangia were globular. Cylindrical sporangiophores were slightly and gradually swollen near the columellae to form funnel-shaped apophysis of about 10-25 µm diam. Comparative data on different organs in strain L-1 and reference *Rhizopus microsporus*-group strains are presented in Table 2.



Figs. 1-8. Scanning electron micrographs of sporangiospores of different *Rhizopus microsporus* strains: **1.** *Rhizopus* sp. L-1. **2.** *R. microsporus* var. *rhizopodiformis* VKM F-3693. **3.** *R. microsporus* var. *chinensis* VKM F-1218. **4.** *R. microsporus* var. *microsporus* VKM F-1063. **5.** *R. microsporus* var. *microsporus* VKM F-773. **6.** *R. microsporus* var. *microsporus* VKM F-774. **7.** *R. microsporus* var. *chinensis* VKM F-1360. **8.** *R. microsporus* var. *chinensis* VKM F-1361. Spores of both strains (VKM F-1360 and 1361) are ellipsoidal and striated ranging from 3.5 to 6 µm. Bars = 1 µm.

Table 3. Radial growth rate (cm/h) of *Rhizopus microsporus* strains at different temperatures.

Temperature (°C)	Strains				
	VKM F-1218	**VKM F-1063**	**VKM F-773**	**VKM F-3693**	**L-1**
8	–	–	–	–	–
16	0.025±0.005	0.033±0.003	0.010±0.003	0.016±0.004	0.028±0.003
20	0.032±0.007	0.062±0.009	0.056±0.008	0.057±0.007	0.045±0.005
26	0.063±0.009	0.062±0.009	0.083±0.008	0.087±0.011	0.054±0.005
30	0.120±0.014	0.125±0.019	0.100±0.010	0.125±0.013	0.131±0.0015
37	0.138±0.022	0.162±0.024	0.137±0.014	0.138±0.018	0.136±0.008
45	0.089±0.011	0.098±0.009	0.130±0.010	0.156±0.018	0.150±0.010
50	–	0.001±0.001	–	0.034±0.009	0.067±0.007
52	–	–	–	–	–

–: No growth.

Morphological parameters of strain L-1 were dependent on growth temperature. As temperatures increased the mycelium becomes more closely adhered to the agar surface and all asexual reproductive structures were smaller. For instance, the average size of sporangium reduced by 26% at a temperature of 45°C and by 39% at 50°C, as compared to those grown at 20-26°C. At higher temperatures the size and shape of the columellae changed; these became smaller (30-40%) and more globose without collars.

It is important to emphasize that strain *Rhizopus* sp. L-1 did not to produce zygospores in the contact with any strains from the *Rhizopus microsporus*-group tested.

The morphological characteristics of *Rhizopus microsporus* var. *rhizopodiformis* VKM F-3693 reference strain, were mostly close to strain L-1, and are as follows: colonies were dark-grayish; rhizoids were simple and unbranched; sporangiophores varied from 200-600 μm high, 7-11 μm wide. Sporangia were grayish-black.

Characteristics of sporangiospores

Strain L-1 has one-celled black, globose to slightly elliptical spores, which have distinctive surface warts or spines (Fig. 1). Comparative average data on shape and size of strain L-1 spores and some reference strain spores are presented at Table 2. Ultrastructural characteristics of these spores obtained by SEM are shown in Figs. 1-8. Spores of strain L-1 are very similar to that of the VKM F-3693 reference strain (Fig. 2), but are distinctly different from spores of the other strains examined (Figs. 3-8). These features provide convincing evidence that strain L-1 is *Rhizopus microsporus* var. *rhizopodiformis*.

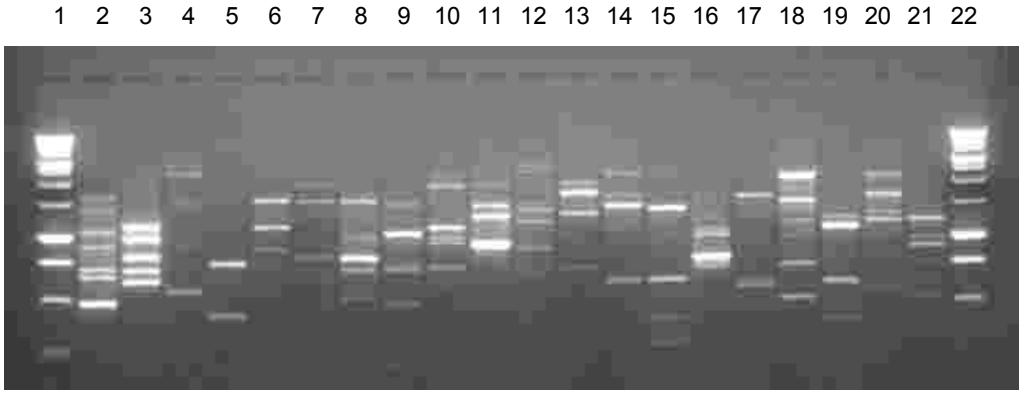


Fig. 9. Comparative fingerprinting of L-1 strain and *Aspergillus terreus* VKM F-3687 DNAs obtained by RAPD with ten different primers (F01- F10). Lanes 1 and 22: marker (Promega's 1kb DNA Ladder); Lane 2: VKM F-3687 with primer F01; Lane 3: L-1 (primer F01); Lane 4: VKM F-3687 (primer F02); Lane 5: L-1 (primer F02); Lane 6: VKM F-3687 (primer F03); Lane 7: L-1 (primer F03); Lane 8: VKM F-3687 (primer F04); Lane 9: L-1 (primer F04); Lane 10: VKM F-3687 (primer F05); Lane 11: L-1 (primer F05); Lane 12: VKM F-3687 (primer F06); Lane 13: L-1 (primer F06); Lane 14: VKM F-3687 (primer F07); Lane 15: L-1 (primer F07); Lane 16: VKM F-3687 (primer F08); Lane 17: L-1 (primer F08); Lane 18: VKM F-3687 (primer F09); Lane 19: L-1 (primer F09); Lane 20: VKM F-3687 (primer F10); Lane 21: L-1 (primer F10).

Growth at various temperatures

The growth rate/temperature relationships of various fungal strains at different temperatures are presented in the Table 3. Strain L-1 only displayed rapid growth at 50°C, and its growth optimum was 45°C. Reference strain VKM F-3693 is mostly similar to strain L-1 in this respect, providing evidence that it belongs to the *Rhizopus microsporus* var. *rhizopodiformis* -group.

Differentiation of fungal isolate and reference strains using random primers

Initially RAPD patterns were assessed in genomic DNA extracted from the L-1 fungal isolate and a reference strain, *A. terreus* VKM F-3687. On the basis of amplified DNA patterns the two fungal species were distinctive. Both strains had characteristic DNA fingerprinting patterns for each particular primer as shown in Fig. 9. The numbers of bands detected using 10 different primers (F01-F10) ranged from two to ten. The greatest number of PCR products was found in *A. terreus* VKM F-3687 genomic DNA (10 bands with F01 primer, followed by 9 bands with F06 and F09 primers). In general, the numbers of PCR fragments showed in the reactions with strain L-1 genomic DNA were fewer than in the respective reactions with *Aspergillus* DNA. Product sizes typically ranged between approximately 0.3 up to 6 kbp. The

Table 4. Jaccard's coefficients of genetic relatedness between *Rhizopus* sp. L-1 strain and reference strains from *Rhizopus* genus.

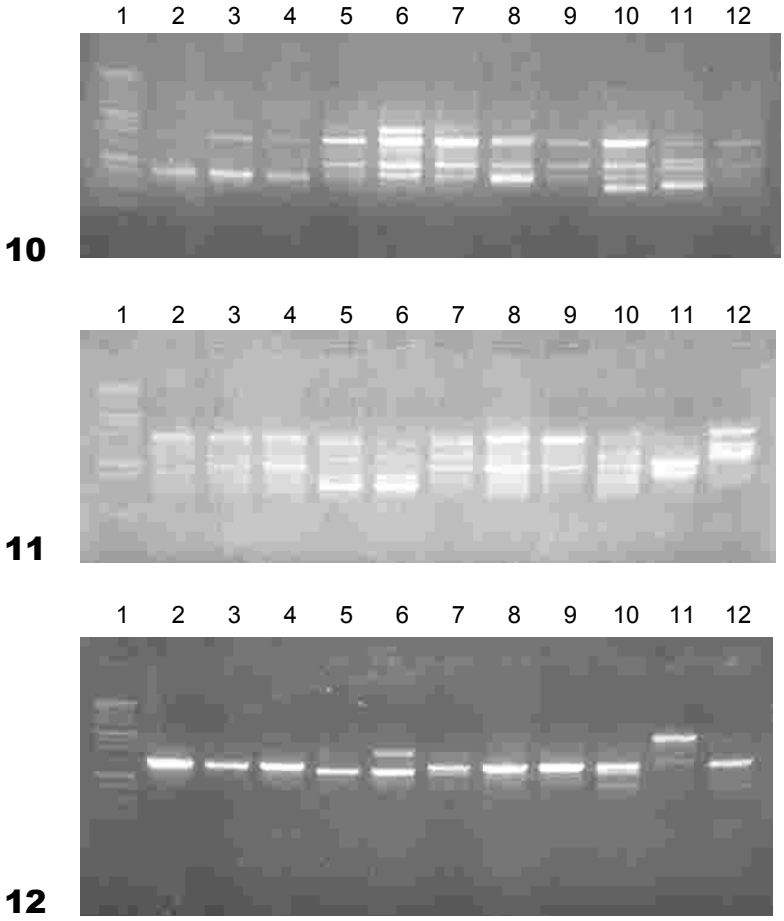
Reference strains	**S_j**
R. microsporus var. *rhizopodiformis* VKM F-3693	0.89
R. microsporus var. *rhizopodiformis* VKM F-3692	0.89
R. microsporus var. *chinensis* VKM F-1218	0.67
R. microsporus var. *microsporus* VKM F-1063	0.60
R. microsporus var. *microsporus* VKM F-773	0.58
R. microsporus var. *microsporus* VKM F-774	0.53
R. microsporus var. *chinensis* VKM F-1361	0.51
R. microsporus var. *chinensis* VKM F-1360	0.43
R. stolonifer var. *stolonifer* VKM F-601	0.36
R. oryzae VKM F-605	0.33

banding patterns for strain L-1 DNA were markedly different from the patterns for the *A. terreus* VKM F-3687 DNA (Fig. 9).

DNA fingerprinting patterns of strain L-1 were also compared to ten reference strains of the *R. microsporus*-group and other species of *Rhizopus* using different random primers. Using 20 decamer primers more than 200 different reproducible RAPD markers were generated from 11 *Rhizopus* strains. Amplification carried out with all the primers used in this study showed very similar banding pattern in strain L-1 and those of VKM F-3692 and VKM F-3693 strains (Figs. 10-12) suggesting they are genetically very similar. On the other hand the RAPD results presented in Figs. 10-12 indicate that banding patterns from strain L-1 and those of other eight strains (*R. microsporus* var. *microsporus* VKM F-773; *R. microsporus* var. *microsporus* VKM F-774; *R. microsporus* var. *microsporus* VKM F-1063; *R. microsporus* var. *chinensis* VKM F-1218; *R. microsporus* var. *chinensis* VKM F-1360; *R. microsporus* var. *chinensis* VKM F-1361; *R. stolonifer* var. *stolonifer* VKM F-601; *R. oryzae* VKM F-605) differ in various extent.

Differences can be also recognised between RAPD electrophoretic patterns of two sexual forms of one strain. Figures 11, 12 shows that the banding patterns of *R. microsporus* var. *microsporus* VKM F-773, MT+ and *R. microsporus* var. *microsporus* VKM F-774, MT- are different with primers F 13 and F 14. *Rhizopus microsporus* var. *chinensis* VKM F-1361, MT+ and *R. microsporus* var. *chinensis* VKM F-1360, MT- have slightly different fingerprints as well when primers F 12, F 13 and F 14 (Figs. 10-12) are used.

The degree of genetic variation within the strains of *Rhizopus* studied here were assessed using the average genetic distance values calculated as Jaccard's similarity coefficients (S_j). The object of the pattern comparison is to obtain a measure of commonness or difference between the gel banding patterns of the two isolates. S_j values have been computed between all pairs of



Figs. 10-12. Comparative fingerprinting of 11 different *Rhizopus* strain DNA's obtained by RAPD with primer F12 **(10)**, or with primer F13 **(11)**, or with primer F14 **(12)**. Lane 1: marker (Promega's 1kb DNA Ladder); Lane 2: L-1; Lane 3: *R. microsporus* var. *rhizopodiformis* VKM F-3693; Lane 4: *R. microsporus* var. *rhizopodiformis* VKM F-3692; Lane 5: *R. microsporus* var. *microsporus* VKM F-773; Lane 6: *R. microsporus* var. *microsporus* VKM F-774; Lane 7: *R. microsporus* var. *microsporus* VKM F-1063; Lane 8: *R. microsporus* var. *chinensis* VKM F-1218; Lane 9: *R. microsporus* var. *chinensis* VKM F-1360; Lane 10: *R. microsporus* var. *chinensis* VKM F-1361; Lane 11: *R. stolonifer* var. *stolonifer* VKM F-601; Lane 12: *R. oryzae* VKM F-605.

a collection of isolates for each of the 20 decamer primer used. Jaccard's coefficients for every particular pair of strains are presented as an average value for 20 primers in Table 4. The average genetic distance value between strain L-1 and VKM F-3692 and VKM F-3693 strains was 0.89 and indicates the highest degree of genetic similarity between these taxa. S_j values presented in Table 4 reflect the decreasing degrees of commonness between strain L-1

and the *Rhizopus* reference strains of a collection. Data from the RAPD experiments using 20 primer sets suggested that strain L-1 is genetically very similar to *R. microsporus* var. *rhizopodiformis*-group strains.

Discussion

Classical work on the circumscription and identification of *Rhizopus* species has primarily been based on morphological criteria (Inui *et al*., 1965). The identification by traditional methods, however, is not clear-cut as there are few character suites to use to classify taxa. This has resulted in an excessive number of species, with few character differences, having been described. There is a need for an alternative approach in the precise naming of fungal strains.

Schipper and Stalpers (1984) suggested that a useful approach to *Rhizopus* taxonomy was to classify species based on the combination of maximum growth temperature rates and sporangiospore characteristics. This approach is very effective for initial species identification. It enables the division of the genus *Rhizopus* into several large groups. The *Rhizopus microsporus*-group is characterized by: sporangiophores up to 1 mm in length and 9 μm in width; sporangia up to 80 μm diam.; rhizoids are small, and slightly pigmented; temperature maximum for growth is 45°C or higher. The evidence from the data here indicates that strain L-1 belongs to this group.

Species and variety delimitation in the *Rhizopus microsporus*-group relies on the shape, size and, especially, the surface ornamentation of the sporangiospores (Ellis, 1981). There are four types of spore ornamentation in this group (Kochkina and Mirchink, 1989). The representatives of *R. microsporus* var. *microsporus* have distinct, smooth ridges on the spore surface running from end to end. *Rhizopus microsporus* var. *chinensis* spores lack striations, but possess distinctive warts on the surface (Ellis, 1981). *Rhizopus microsporus* var. *oligosporus* spores are almost smooth or slightly wrinkled. *Rhizopus microsporus* var. *rhizopodiformis* spores are covered with randomly spaced and distinct warts or spines (Schipper and Stalpers, 1984). The globular form of spores and lack of striations on their surfaces differentiate strain L-1 from *R. microsporus* var. *microsporus* representatives. Strain L-1 could be included in *R. microsporus* var. *chinensis*, based on spore size and surface ornamentation according to the description of the group made by Ellis (1981). However, the SEM micrographs presented here show that the spores of strains *R. microsporus* var. *chinensis* VKM F-1360 (Fig. 7) and F-1361 (Fig. 8) are both ellipsoidal and striate. On the basis of sporangiospore characteristics strain L-1 must be grouped with the *R. microsporus* var. *rhizopodiformis*

group. The above discussion exemplifies how difficult it is to classify species and varieties of *Rhizopus* using only morphological characteristics.

The growth characteristics at various temperatures have been also used as a valuable complementary tool in the identification of fungi (Kochkina *et al*., 1978; McGinnis and Salkin, 1986; Kochkina and Mirchink, 1989). Growth rate/temperature relationships are of key importance for species delimitation in in *Rhizopus*. This study found this method to be effective in species delimitation.

Identification based on the genotype of an isolate, including RAPD assays, have been applied in the last decade for many different groups of fungi (Kubelik and Szabo, 1995; Brandt *et al*., 1998; Poonwan *et al*., 1998). Nevertheless, there has been no work on the delimitation of *Rhizopus* species using RAPDs. Species and subspecies concept in *Rhizopus* is unsatisfactory (Kochkina and Mirchink, 1989). Classification is based mainly on morphological criteria, and fungi are usually recognized and identified by their phenotypes (Carlile and Watkinson, 1994). The use of DNA fingerprinting together with conventional morphological-physiological and SEM methods for identification of the *Rhizopus* species has not previously been reported. In this study 20 oligonucleotide were tested for their abilities to generate RAPD markers from genomic DNAs of 11 different isolates of *Rhizopus* and *Aspergillus terreus* VKM F-3687. Strain L-1 shared no DNA fingerprinting patterns with the reference strain *Aspergillus terreus*. All primers generated RAPDs that indicated strain L-1 was *R. microsporus* var. *rhizopodiformis*. The results demonstrate the usefulness of RAPD techniques for distinguishing between *Rhizopus microsporus* isolates.

We also identified by RAPD techniques the differences in banding patterns between + and - mating tipes of several strains of *Rhizopus*. Similar results have been obtained by DNA fingerprinting methods for + and - strains of *Mucor circinelloides* (Diaz-Minguez *et al*., 1999) and *Mucor piriformis* (Papp *et al*., 1997).

It is interesting to note that *R. microsporus* var. *chinensis* 1218 was similar strain L-1 besides *R. microsporus* var. *rhizopodiformis*. Polonelli *et al*. (1988) have suggested that this subspecies is a natural hybrid between '*rhizopodiformis*' and '*microsporus*'.

Nevertheless, it is not possible to assign *Rhizopus* species based on the RAPDs because sequence coding for some physiological and morphological characters might not have been amplified by the RAPD technique (Soll, 2000). The complexity of RAPD fingerprinting makes the taxonomical analyses of isolates by this method difficult. Combining RAPD methodology with conventional morphological-physiological and SEM methods for the identification of

Rhizopus species would simplify the interpretation of the results and can improve the accuracy of taxonomical classificaton.

Among the several characteristics examined in this study, those suitable as keys for classification may be listed in order of reliability as follows: (i) RAPD-diagnosis; (ii) radial growth rate at different temperatures; (iii) colour, shape and size of sporangiophores and sporangia; (iv) colour and branching of rhizoids; (v) colour, shape, size and surface ornamentation of sporangiospores.

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