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VARIABILITY OF ISOZYME AND RAPD MARKERS AMONG ISOLATES OF *MUCOR GENEVENSIS**

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Mucor genevensis is a dimorphic and homothallic fungal species (Zygomycetes). Ten *M. genevensis* strains, each strain of the recently described new homothallic species (*M. meguroense* and *M. hachijyoensis*) and strains of *M. hiemalis* and *M. piriformis* (as outgroups for numerical analysis) were investigated. Five different enzyme systems (CAT, GDH, G6D, MDH and SOD) and five 10-bp random primers were used in isoenzyme and random amplified polymorphic DNA analyses, respectively. The data from these studies were subjected to numerical analyses. Substantial intraspecific variability was detected in *M. genevensis* with both of the methods applied. Though both the *M. meguroense* strain and the *M. hachijyoensis* strain revealed characteristic differences, they grouped closer to the homothallic *M. genevensis* than to the heterothallic *M. piriformis* and *M. hiemalis* strains.

Keywords: Genetic variability – homothallism – isoenzyme analysis – *Mucor genevensis* – RAPD analysis

INTRODUCTION

The species *Mucor genevensis* is of interest as concerns both its dimorphism and its homothallism. The latter feature is rather rare among fungi belonging in the genus *Mucor*, where most of the known species are heterothallic. The comprehensive taxonomic work of Schipper [13] listed only 4 homothallic species (*M. azygosporus, M. bacilliformis*, *M. bainieri* and *M. genevensis*). There are a few other descriptions of homothallic *Mucor* species, but with a somewhat uncertain taxonomic status, e.g. *M. philippovi* [9] and *M. parvisporus* [6]. These doubtful taxa are generally represented by a single isolate. This list of homothallic *Mucor* species was recently extended with 2 new species (*M. meguroense* and *M. hachijyoensis* [19]), on the basis of the morphological and physiological traits observed for 2 *Mucor* isolates collected in Japan.

The investigation of micro- and macromorphological characteristics comprises the classical basis for strain identification and species delimitation in fungal taxonomy. However, with the significance of conventional strain typing left partially untouched,

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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different molecular methods have gained widespread application and special importance both alone and as part of integrated approaches in fungal systematics. The main advantage of different molecular markers is that they allow the detection of practically unlimited numbers of independent polymorphic genetic loci. As well-established methods, isoenzyme analysis and random amplification of polymorphic DNA (RAPD) analysis are useful for providing genetic markers for investigations of genetic polymorphism, and have also proved to be valuable tools in the handling of certain taxonomic questions in the genus *Mucor* and related genera [10, 16–18].

In the present study, the applicability of these approaches for elucidation of the intraspecific variability of *M. genevensis* (the most ubiquituous homothallic *Mucor* species) and its taxonomic relationship to the recently described homothallic *Mucor* species (*M. meguroense* and *M. hachijyoensis*) was investigated.

MATERIALS AND METHODS

Fungal strains and cultivation

The names and origins of the fungal strains used in this study are given in Table 1. Mycelia were grown in 150 ml of yeast extract-glucose (1% glucose, 0.5% yeast extract and 1% KH_2PO_4) or potato-dextrose liquid medium for DNA or protein extraction, respectively. After culturing at 37 °C on a rotary shaker at 200 rpm for 3 days, mycelia were harvested by filtration and stored at –70 °C until use.

Isoenzyme analysis

The preparation of protein extract and gel electrophoresis were performed essentially as described earlier [16]. The enzyme systems used were catalase (CAT, EC 1.11.1.6 [21]), glutamate dehydrogenase (GDH, EC 1.4.1.4 [1]), glucose-6-phosphate dehydrogenase (G6D, EC 1.1.1.49 [8]), malate dehydrogenase (MDH, EC 1.1.1.38 [3]) and superoxide dismutase (SOD, EC 1.15.1.1 [2]). After incubation at 21 °C (in the dark), the gels were rinsed, first in distilled water, and next in 7% acetic acid, and then read.

RAPD analysis

DNA was extracted by using a variation of the rapid lithium chloride procedure [7]. Pieces of frozen mycelia (0.5 g) were powdered with liquid nitrogen, transferred immediately into a sterile Eppendorf tube and mixed with 0.5 ml of ice-cold LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris/HCl, pH 8.0, 0.5% SDS). The mixture was vortexed until a fine mycelial slurry was obtained, which was quickly frozen by immersion in liquid nitrogen. The tube was transferred into a water bath at 65 °C

Table 1
Fungal strains investigated in the present study.

Species name	Original code	Source
M. genevensis	NRRL 1411	u.s.
M. genevensis	NRRL 1821	u.s., Wisconsin, USA
M. genevensis	NRRL 1410	u.s.
M. genevensis	NRRL 1412	u.s.
M. genevensis	MUFS 038	leaf litter, Knysna forest, South Africa
M. genevensis	NRRL 1407	u.s.
M. genevensis	NRRL 1755	u.s.
M. genevensis	NRRL 1758	u.s., Wisconsin, USA
M. genevensis	NRRL 1756	u.s.
M. genevensis	NRRL 1408	u.s.
M. hachijyoensis	FFPRI TW70-1179	soil, Hachijo-jima, Japan
M. meguroense	FFPRI TW92-268	leaves of *Phellodendron amurense,* Meguro, Tokyo, Japan
M. hiemalis f. hiemalis	WRL CN(M) 328	soil, Halfield College, UK
M. hiemalis f. luteus	NRRL 3632	u.s., Germany
M. piriformis	17-C-1	Persimmon, California, USA

u.s. = unknown source
*Abbreviations: NRRL, Agricultural Research Service C. C., Peoria, Illinois, USA; WRL, Wellcome Bacterial Collection, Beckenham, UK; FFPRI, Herbarium, Forestry and Forest Product Research Institute, Tsukuba, Japan; MUFS, Department of Microbiology and Biochemistry, Orange Free State, South Africa.

for few minutes, 0.2 ml of 10% SDS was added and the mixture was vortexed. The slurry was extracted once with phenol : chloroform (25 : 24), then with phenol, and next again with the phenol : chloroform (25 : 24) mixture. Finally, the sample was extracted with an equal volume of a chloroform : isoamyl alcohol (24 : 1) mixture. DNA was precipitated from the aqeuous phase with two volumes of ethanol at –20 °C. After centrifugation (15 min at 10 000 *g*), the resulting pellet was dried under vacuum, and resuspended in 30 µl of TE buffer.

Amplification of template DNA was carried out as described by Williams et al. [20] with slight modifications. Each 25 µl of reaction mixture contained 2.5 µl of 10×PCR buffer, 200 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 2.5 mM $MgCl_2$, 0.2 µM primer (Random primer kit C, Operon Technologies), 0.5 U of Taq DNA polymerase (Zenon), 2.5 µl of genomic DNA extract (50 ng/µl) and sterile distilled water. Sterile mineral oil (40 µl, Sigma) was used as overlay. Control reactions contained all reagents except genomic DNA extract.

Amplifications were performed in a PTC-100-60 DNA thermocycler (MJ Research) programmed for a denaturation step at 94 °C for 2 min, followed by 1 cycle at 38 °C for 1 min and 72 °C for 2 min, and then 36 cycles at 93 °C for 1 min, 38 °C for 1 min and 72 °C for 2 min. The final cycle concluded with an extension step at 72 °C for 10 min. DNA from each isolate was amplified with five different 10-base primers in three separate experiments. The arbitrary primers used were OPC-01

(TTCGAGCCAG), OPC-02 (GTGAGGCGTC), OPC-04 (CCGCATGTAC), OPC-07 (GTCCCGACGA) and OPC-13 (AAGCCTCGTC).

Ten μl of each amplification product was separated by electrophoresis on 1.0% agarose/TBE (90 mM Tris/borate, pH 8.0, 2 mM EDTA) gels and visualized by UV fluorescence after ethidium bromide (0.5 μg/ml) staining, using *Hind*III- and *Hind*III-*Eco*RI-digested λ DNA as size standards.

Numerical analysis

From the isoenzyme and RAPD data, matrices based on the presence or absence of a given activity band or an amplification product were created. Jaccard coefficients (S_J) were calculated and a dendrogram was produced with a UPGMA (unweighted pair-group method using arithmetic averages [15]) linkage. Computations were performed with the SYNTAX 5.0 software package [11].

RESULTS

All the enzyme systems investigated revealed some polymorphism among the fungal strains studied (results not shown). The highest variability was found for CAT; 6 different electrophoretic patterns (electromorphs) were detected, while for G6D (Fig. 1) and MDH 4 electromorphs were found. The least variation was observed when the GDH and SOD (Fig. 1) activity stains were used: each indicated 3 different electromorphs.

When all the isoenzymes were taken into account, a definite intraspecific variability was found in *M. genevensis*; the 10 *M. genevensis* strains revealed 6 different electrophoretic phenotypes (EPs). The differences between these EPs were in some cases substantial; some of them exhibited different activity patterns in 3 of the 5 enzyme systems investigated. When a dendogram was created from the isoenzyme data (Fig. 2), all the homothallic *Mucor* strains clustered together (cluster A) and differed substantially ($D \geq 0.84$) from 2 heterothallic *Mucor* strains (17-C-1 and WRL CN(M) 328). However, strain NRRL 3632 (which is a heterothallic *M. hiemalis f. luteus*) surprisingly grouped together with the homothallic isolates (cluster A/2). While the isolate *M. hachijyoensis* remained unclustered, the M. *meguroense* isolate grouped together with a South African *M. genevensis* strain (cluster B).

To provide further characters of distinctive value, RAPD analysis of the isolates was carried out. Reproducible amplifications were obtained with all 5 primers tested (results not shown); the number of amplified bands varied between 1 and 17.

All the primers tested revealed high DNA polymorphism among the *Mucor* isolates. As a result of this, the amplification patterns from the 5 primers together provide a strain-specific characterization (Fig. 3).

A total of 190 RAPD markers were generated from the DNA of the 15 fungal strains investigated. A matrix was calculated from the binary scores of these charac-

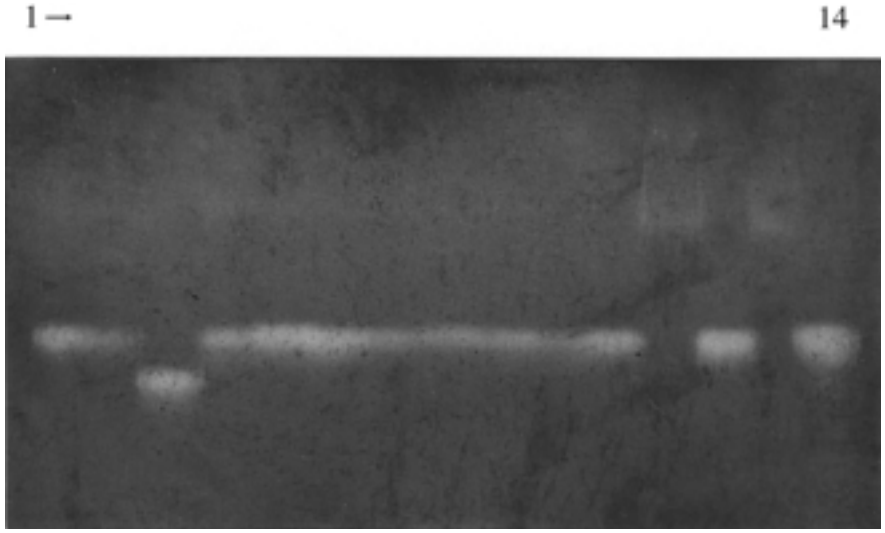

Fig. 1. SOD activity patterns of the *Mucor* strains. Lanes 1–14: strains NRRL 1407, NRRL 1408, TW70-1179, NRRL 1410, NRRL 1411, NRRL 1412, NRRL 1756, NRRL 1758, NRRL 1821, MUFS 038, WRL CN(M)328, TW92-268, NRRL 3632 and NRRL 1755, respectively

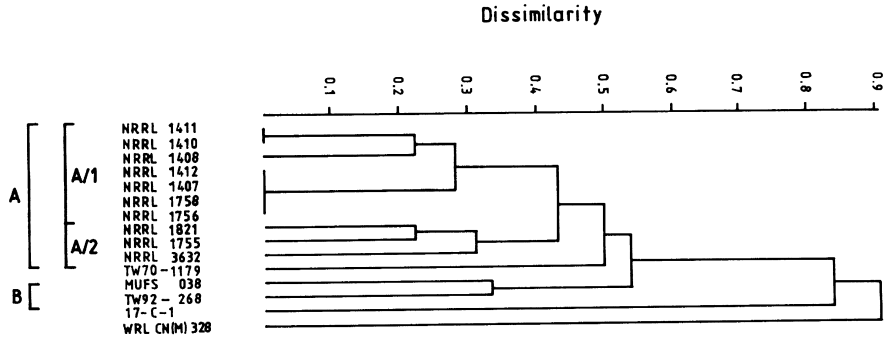

Fig. 2. UPGMA dendrogram produced from isoenzyme analyses of the *Mucor* strains. The scale represents dissimilarity (squared distance)

ters and a dendrogram was generated by means of the UPGMA clustering (Fig. 4). Fifteen composite amplification types were identified for *Mucor* isolates, indicating a higher degree of variability than found in isoenzyme studies. Although this dendogram is more complex than that created from the isoenzyme data, it also reveals the above-mentioned clustering of the homothallic strains.

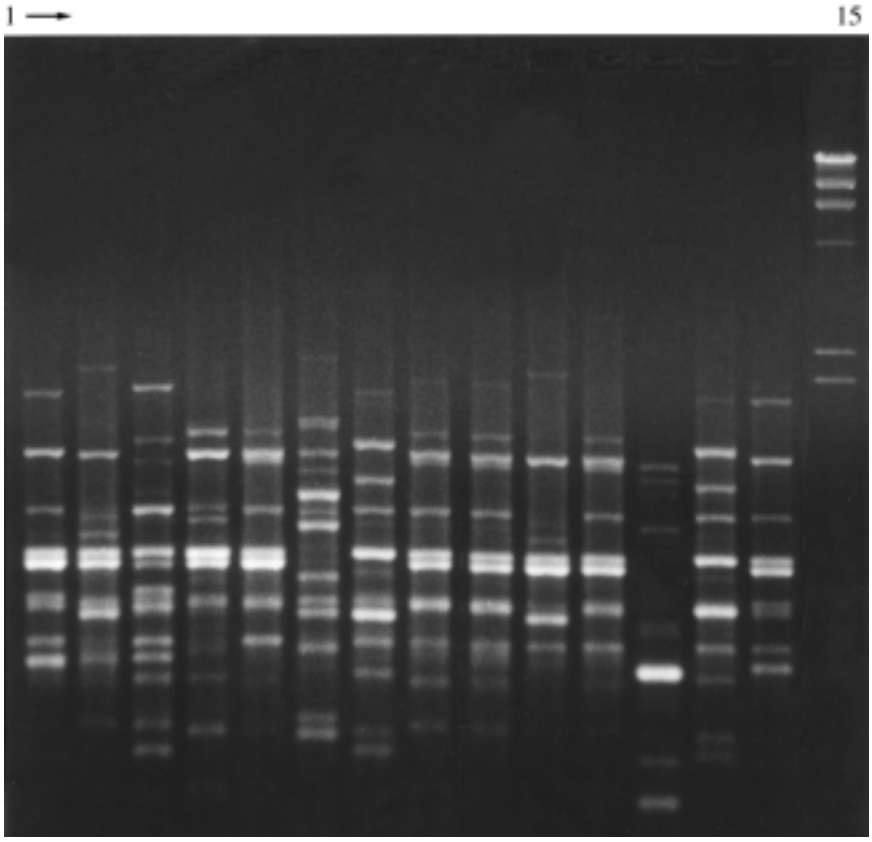

Fig. 3. Amplification products of *Mucor* DNA with primer OPC-13. Lanes 1–14: strains NRRL 1411, NRRL 1821, TW70-1179, NRRL 1410, NRRL 1412, NRRL 3632, MUFS 038, NRRL 1407, NRRL 1755, NRRL 1758, NRRL 1756, WRL CN(M)328, TW92-268 and NRRL 1408, respectively. Lane 15: *Hind*III-digested λ DNA as size standard

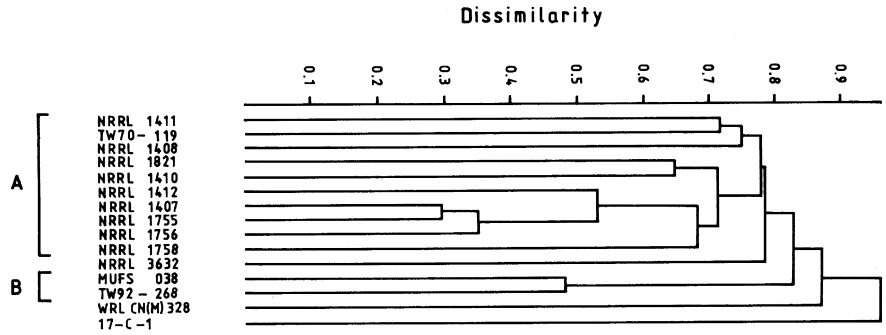

Fig. 4. UPGMA dendrogram produced from RAPD analyses of the *Mucor* strains. The scale represents dissimilarity (squared distance)

DISCUSSION

The members of the genus *Mucor* have few and even variable morphological characters; strains of a single species may vary in their cultural characteristics (macromorphology), and to a lesser degree in their micromorphologies. This has a serious impact on the precision of species identification on a morphological basis, and leads in several cases to ambiguity when new isolates have to be identified.

Inclusion of a mating study evidently does not simplify this scheme. The mating behaviour of the heterothallic *Mucor* species could be rather complex [14]: some heterothallic strains react either as (+) or as (–) strains; some have lost their mating potencies (neutral strains); zygospore production in various matings under the same conditions with one common partner may differ greatly due to unknown physiological factors; and azygospores could be formed or in intraspecific and interspecific crosses, and even apparently normal zygospores could be produced between strains of different species (e.g. *M. indicus* and *M. variosporus* [13]).

Mating studies are evidently of no use when homothallic *Mucor* isolates have to be identified. Though abundantly produced zygospores could be good sources of additional morphological characters, their proper evaluation is no less problematic as than that of the vegetative structures. Homothallic strains are rather rare in the genus *Mucor* and, partly in consequence of the above-mentioned reasons, both the number of accepted homothallic species and their taxonomic status have long remained subjects of debate.

Hesseltine [5] included *M. genevensis* in the section *Genevensis*, together with 3 other homothallic species. Schipper [13] also accepted 4 homothallic species, but she emphasized their connections to different heterothallic species: *M. genevensis* was handled as a counterpart of the heterothallic *M. hiemalis*.

The results of our experiments revealed a rather high genetic polymorphism in the species *M. genevensis*. In the case of *Rhizomucor miehei*, another homothallic zygomycetes, the intraspecific genetic polymorphism was found to be low [18]. For example, there was no polymorphism in the 26 isoenzyme markers determined. As the heterothallic *Rhizomucor pusillus* revealed much higher genetic variability in the same study, the different sexual strategies were presumed to be responsible for the very different levels of the intraspecific polymorphisms of these two closely related species. Interestingly, the high genetic variability of *M. genevensis* does not fit into this scheme. Unfortunately, homothallic *Mucor* isolates are rather rare (several species are represented by only a single or very few isolates), and it is therefore difficult to decide whether this is a general phenomenon of mesophilic homothallic *Mucor* species.

Among the taxonomic deductions that could be drawn from our results, the most important is that the single isolates of *M. meguroense* and *M. hachijyoensis* seem to represent separate species: on the basis of the molecular markers investigated, the latter clearly displays a greater similarity to *M. genevensis*. Surprisingly, the 2 *M. hiemalis* strains involved in this study as outgroups were found to be very different: the strain representing *M. hiemalis f. luteus* (NRRL 3632) displayed a rather close

connection to the homothallic *Mucor* strains, while the strain representing *M. hiemalis f. hiemalis* (as the other heterothallic *Mucor* strain) was clearly separated from homothallic isolates. This problem could not be solved from the present study, as it requires a more detailed investigation of the intraspecific genetic polymorphism of *M. hiemalis*. From this point of view, it is worth while to mention the investigation of Havens [4] carried out in the section *Hiemalis* of the genus *Mucor*: mating tests were coupled with isoenzyme analysis to reduce the possibility of misidentifications. The investigated "species" exhibited a relatively high polymorphism; the isozyme similarity and sexual compatibility displayed a correlation coefficient of only 0.61.

As both dendograms, based on independently created data matrices from different molecular methods, show the high similarity of the *M. meguroense* isolate with a South African homothallic *Mucor* isolate (obtained as *M. genevensis* MUFS 038), they might be handled as representatives of the same homothallic *Mucor* species.

This study again demonstrates the merits of the use of different molecular markers in resolving taxonomic problems of fungal taxa when the investigation of morphological traits does not supply sufficient solid information.

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