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Polyphasic approach to the taxonomy of the *Rhizopus stolonifer* group

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ABSTRACT

Phylogenetic analysis of *Rhizopus* strains based on the D1/D2 region of LSU rDNA sequences yielded a phylogram with four well-supported clades. The *R. microsporus* clade concurs with classification obtained by traditional methods. The *R. oryzae* group was found to include species of the genus *Amylomyces*. The traditional *R. stolonifer* group was divided into two well-supported clades in the phylogram, with one clade comprising *R. stolonifer* var. *stolonifer*, *R. sexualis* var. *sexualis*, and *R. sexualis* var. *americanus* and the other clade comprising taxa with recurved sporangiophores; *R. reflexus*, *R. stolonifer* var. *lyococcus*, and *R. circinans*, identifying recurved sporangiophores as an important taxonomic character. The molecular data supported the recognition of this clade at the species level: *R. lyococcus* (basionym: *Sporotrichum lyococcum*).

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Introduction

Members of the fungal genus *Rhizopus* are traditionally used in the preparation of fermented foods in Asia, such as tempeh, peka, and ragi. Recently, the industrial production of enzymes using *Rhizopus* has been investigated (Kumar *et al.* 2004). Some species are important post-harvest pathogens that contribute to considerable spoilage of agricultural output (Vágvölgyi *et al.* 2004) and contamination of food and animal feed through the production of mycotoxins and other biologically active compounds (Jennessen *et al.* 2005). Some *Rhizopus* species are also associated with human disease (Schipper *et al.* 1996; Voigt *et al.* 1999). *Rhizopus* was monographed by Schipper (1984) and Schipper & Stalpers (1984). Schipper (1984) divided the genus into three groups; the *R. stolonifer* group, the *R. oryzae* group, and the *R. microsporus* group, according to the characteristics of the sporangial apparatus and growth temperatures. Thereafter, the characteristics of morphology and growth temperature and the data from mating experiments became the basic criteria for the species definition of *Rhizopus*. Moreover, DNA renaturation for *R. oryzae* (Ellis 1985) and *R. microsporus* (Ellis 1986), the proteins of sporangiospores (Seviour *et al.* 1985), and antigenic studies for the *R. microsporus* group (Polonelli *et al.* 1988) were taken as additional criteria in defining taxa.

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In recent years, isozyme analysis and RAPD have been applied in taxonomic studies of *Rhizopus* species. Isozyme analysis has been used to describe the intra- and inter-specific variation (Liou *et al.* 2001). RAPD analysis revealed genetic variability in the species *R. stolonifer* (Vágvölgyi *et al.* 2004). A specific taxonomic problem was addressed by these techniques; *R. reflexus*, and *R. stolonifer* var. *lyococcus* differ from *R. stolonifer* var. *stolonifer*, suggesting they are separate species. However, the phylogenetic relationships among groups of *Rhizopus* and the criteria in defining species of the *R. stolonifer* group still remain unclear.

Combinations of phenotypic and genotypic characters, such as rDNA sequence, nuDNA base composition, and DNA–DNA reassociation values, have been successfully used in yeast taxonomy. Molecular genetic approaches are now generally used for filamentous fungi too, and are fundamental for modern taxonomy (Binder & Hibbett 2002; O'Donnell *et al.* 2001; Kurtzman *et al.* 1986; Lutzoni *et al.* 2004; Moncalvo *et al.* 2002; Tamura *et al.* 1999; Voigt *et al.* 1999). The purpose of the present study is to: (1) analyse the phylogenetic relationship of *Rhizopus* species based on D1/D2 region of LSU (28 S) rDNA sequences to evaluate the validity of the taxonomic classification by morphological characteristics; and (2) use a polyphasic approach to explore species characterisation within the *Rhizopus stolonifer* group.

Materials and methods

Fungal strains and preparation of genomic DNA

Strains used in this study were maintained at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan, Table 1 lists the names of the strains, the corresponding BCRC numbers, and the original source.

Strains were inoculated on malt extract agar (2 % malt extract DIFCO (Foster city, CA, USA), 2 % glucose, 0.1 % peptone, 2 % agar) and incubated at 25 °C for 3–7 d for activation, before being transferred to potato dextrose broth (DIFCO 254920) and then incubated at 25 °C for 3 d to collect mycelia. Additionally, DNA for PCR was extracted using the Chelex method (Chen 1998).

PCR amplification and direct DNA sequencing of rDNA

The D1/D2 region of LSU rDNA was defined using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5′-GGTCC GTGTTTCAAGACGG) (O'Donnell 1992). The reaction was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using 30 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min with an initial denaturation at 94 °C for 5 min before cycling and a final extension at 72 °C for 7 min following cycling. PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN®, Hilden, Germany). Sequencing reactions were performed using the ABI PRISM™ BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit as directed by the manufacturer. Finally, the PCR products were sequenced using the ABI PRISM™ (Applied Biosystems) 3700 DNA analyser.

Molecular phylogenetic analysis

Sequence ambiguities were identified by comparisons with their opposite strand sequences. The LSU rDNA partial sequences of each strain were aligned using CLUSTAL X 1.83 (Thompson *et al.* 1997). The alignment of all sequences was visually assessed and optimised where necessary. Phylogenetic evaluation was accomplished by applying DNADIST and NEIGHBOUR programs from the PHYLIP 3.63 package (Felsenstein 2004). Phylogenetic trees were rooted with *Mucor recurvus* var. *indicus*. Finally, a BS analysis with 1K replications was performed by applying the SEQBOOT program from the PHYLIP 3.63 package. Trees were viewed using TreeView 1.6.6 (Page 1996).

Determination of DNA base composition

The isolation and purification of DNA for DNA base composition as expressed in the G + C ratio and DNA relatedness were performed by Genomic DNA Buffer Set and Genomic-tip 500/G (QIAGEN®). The purified DNA was hydrolysed to nucleosides as described by Tamaoka and Komagata (1984), and the hydrolysate was analysed by reverse phase HPLC to determine DNA base composition (mol% G + C).

DNA relatedness

DNA–DNA reassociation values expressing the level of DNA relatedness were determined by the photobiotin-labelling microplate method (Lee *et al.* 1994a,b). This method was modified by Kaneko & Banno (Kaneko & Banno 1991) from that described by Ezaki and colleagues (Ezaki *et al.* 1988, 1989). DNA from reference strains of *Rhizopus* was labelled with photobiotin to detect DNA relatedness to other DNAs. The main modification involved immobilisation of heat-denatured *Rhizopus* DNA (400 ng) in a microplate well at 28 °C overnight, followed by hybridisation at 39 °C for 24 h. The values represent the averages of two to three determinations.

Morphological characteristics

The established taxonomic system of *Rhizopus* (Schipper 1984; Schipper & Stalpers 1984) was followed for identification of strains. The strains were cultured on Malt extract agar at 25 °C. Slide mounts were prepared and examined under a light microscope (Axiophot, Zeiss, Oberkochen, Germany). Response to temperature was determined by measuring the diameters of colonies on agar plates incubated at temperatures of 25 °C, 30 °C, 33 °C, and 35 °C.

Results

Phylogenetic analysis

The result of the NJ analysis is illustrated in Fig 1. The genus *Rhizopus* appeared monophyletic and falls into four clades with high support using NJ analysis. The species belonging to the *R. microsporus* group form clade A. Clade B comprises *R. oryzae* and *Amylomyces rouxii*. Clade C contains *R. stolonifer*

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