Multilocus microsatellite typing for *Rhizopus oryzae*

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Rhizopus oryzae is the most frequent causative agent of zygomycosis. Although zygomycosis causes considerable morbidity and mortality in immunocompromised patients, the epidemiology of the disease is not well studied and no standard molecular typing method has been described for any of the causative agents. Here we describe a multilocus microsatellite typing (MLMT) method for *R. oryzae*. *R. oryzae* genome sequences were downloaded from the Fungal Genome Initiative database (Broad Institute). The intergenic regions and ORFs of approximately 5.7 Mb were screened for repeat regions with the help of the online repeat search tool Repeat Masker. Of the 30 microsatellite loci identified, 3 microsatellites [RO3, (CCT)_n; RO4, (TA)_n; and RO8, (GAA)(GGA)_n] were selected after validation of the ability to amplify them and their size variation in 8 randomly selected clinical isolates of *R. oryzae*. Nucleotide sequence analysis of these loci demonstrated polymorphism in the microsatellite repeat number. The capabilities of these microsatellite loci were assessed for strain differentiation on 30 clinical isolates, based on fragment size determination in an automated capillary electrophoresis using fluorescent labelled primers. These three polymorphic microsatellite loci were found to have good discriminatory power (*D*) (RO3, *D*=0.846; RO4, *D*=0.747; RO8, *D*=0.742; with a combined *D*=0.986) and stability for seven subcultures. It was also confirmed that the MLMT method may be applied to both *R. oryzae* and *Rhizopus deleamar* (a proposed new species), although MLMT analysis could not differentiate them into two clusters. The MLMT system, described here for what is believed to be the first time for a zygomycotic fungus, holds promise as a powerful tool for the strain typing of *R. oryzae*.

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INTRODUCTION

Classically, the class Zygomycetes has been subdivided into two orders, Mucorales and Entomophthorales, although recently a modified classification of these fungi has been proposed, wherein the classical Zygomycota has been classified into the phylum Glomeromycota and four subphyla comprising Mucormycotina, Kickxellomycotina, Zoopagomycotina and Entomophthoromycotina (Hibbett *et al.*, 2007). The fungi in the order Entomophthorales produce indolent subcutaneous and mucocutaneous mycoses in healthy hosts, whereas those in the Mucorales produce rapidly fatal infections in immunocompromised hosts (Ribes *et al.*, 2000). An alarming rise in the incidence of invasive zygomycosis in recent years is a matter of

concern worldwide (Greenberg *et al.*, 2004; Kauffman, 2004; Roden *et al.*, 2005), with special interest in India due to the large case series reported (Chakrabarti *et al.*, 2006; 2009; Nithyanandam *et al.*, 2003; Sundaram *et al.*, 2005). Occasionally, outbreaks of zygomycosis have been reported that were linked with excavation, construction or contaminated air-conditioning filters. In hospitals, nosocomially acquired zygomycosis due to *Rhizopus oryzae* had also been reported (Keys *et al.*, 1978; Mead *et al.*, 1979). Nosocomial zygomycosis has been associated with immunosuppression, antifungal prophylaxis and a variety of procedures, and has been related to the use of devices including bandages or medication patches, intravenous catheters and tongue depressors (Keys *et al.*, 1978; Mead *et al.*, 1979; Ribes *et al.*, 2000; Spellberg *et al.*, 2005).

R. oryzae is the most common Mucorales to cause zygomycosis (Chakrabarti *et al.*, 2006; Ribes *et al.*, 2000; Roden *et al.*, 2005). The fungus is identified by its microscopic morphology and growth temperature. Recently, *R. oryzae* strains have been divided into two species based on

Abbreviations: 6-FAM, 6-carboxyfluorescein; AFLP, amplified fragment length polymorphism; MLMT, multilocus microsatellite typing; MLST, multilocus sequence typing; NCCPF, National Culture Collection of Pathogenic Fungi.

The GenBank/EMBL/DBJ accession numbers for the microsatellite sequences of RO3, RO4 and RO8 are GU132512–GU132525.

organic acid production: *R. oryzae* (lactic acid producers) and *Rhizopus delemar* (fumaric/malic acid producers) (Abe *et al.*, 2007). To understand the molecular epidemiology of *R. oryzae* and *R. delemar*, molecular strain typing is essential. For strain differentiation in fungi, various pattern-based typing techniques, such as random amplified polymorphic DNA and RFLP analysis have been used, but a major problem with such techniques is the poor inter-laboratory reproducibility and difficulty in exchanging the results obtained by these techniques (Gil-Lamaignere *et al.*, 2003). Multilocus sequence typing (MLST) has been used as a reproducible typing method for a few fungal agents (Bougnoux *et al.*, 2003; Dodgson *et al.*, 2003), but its strain discriminatory power is poorer than the multilocus microsatellite typing (MLMT) method (Klaassen, 2009). Moreover, MLST is a laborious and expensive technique. The MLMT method has been used successfully for strain typing in *Aspergillus fumigatus*, *Penicillium marneffei*, *Candida albicans* and *Candida glabrata* (de Valk *et al.*, 2007a; Dodgson *et al.*, 2003; Fisher *et al.*, 2004; Sampaio *et al.*, 2005). The method targets multiple loci that contain di-, tri- or tetranucleotide repeats, and has the potential to be a highly discriminatory and reproducible typing method. Here, we describe what is believed to be the first report of development of the MLMT method for strain typing of *R. oryzae* and *R. delemar*.

METHODS

Isolates. Thirty clinical isolates of *R. oryzae* (identified on the basis of microscopic morphology and growth temperature) stored at -80°C in National Culture Collection of Pathogenic Fungi (NCCPF) (previously the Mycology Culture Collection Laboratory) at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, were used in this study (Table 1). The isolates were isolated from patients with invasive zygomycosis who attended the Nehru Hospital, PGIMER, Chandigarh, India. The isolates were considered unrelated because they were isolated from multiple wards at different time intervals. After growth of the isolates, their identity was confirmed by morphological features (broad, aseptate, ribbon-like hyphae having sporangioophores and rhizoids; sporangioophores and rhizoids borne from creeping aerial hyphae known as stolons; sporangioophores, single or in tufts, brown, mostly unbranched, sometimes with brownish swelling; brownish rhizoids sparingly branched; spherical sporangia with ellipsoidal columellae; apophysis short or absent; greyish-green sporangiospores, longitudinally striated and rhomboid to lemon-shaped) and by the sequencing of the internal transcribed spacer region of the rRNA gene of the isolates.

Identification of lactic acid- and fumaric/malic acid-producing strains of *Rhizopus* species. The organic acids produced by the strains were determined by the method described by Abe *et al.* (2007) with certain modifications. Fermentation of the isolates was carried out in 10 ml organic acid production medium containing 50 g glucose l^{-1} , 6.7 g yeast nitrogen base without amino acid (Difco) l^{-1} and 5 g casamino acids l^{-1} . Just before inoculation, calcium carbonate (sterilized in an oven at 180°C for 5 h) was added to a final concentration of 2.5% (w/v). The medium was inoculated with a small amount of mycelia, pre-grown on Sabouraud dextrose agar (SDA; Hi Media), and incubated at 27°C on an incubator shaker (90 r.p.m.) for 3 days. The organic acids (lactic acid and fumaric/

malic acid) in the culture supernatant were analysed by HPLC (Shimadzu). After 3 days of incubation, the fermentation medium was centrifuged, and the culture supernatant filtered through a $0.22\text{ }\mu\text{m}$ membrane filter and injected into the HPLC column. The HPLC used a C-18 column with 0.1% phosphoric acid solvent, at a flow rate of 1 ml min^{-1} and a column temperature of 40°C , with a UV detector.

Isolation of genomic DNA. Whole-cell DNA from the mycelia of each isolate was extracted following a slightly modified protocol of the small-scale fungal DNA extraction method developed by Lee & Taylor (1990). Briefly, spores harvested from SDA were allowed to grow in Sabouraud dextrose broth (HiMedia) at 37°C on a rotary shaker (HT Infors) at 120 r.p.m. for 3–5 days. The mycelial mat was recovered by filtration and washed with sterile saline. Approximately 0.2–0.3 g mycelial mat was ground in the presence of liquid nitrogen, and the resultant powder was transferred to a 1.5 ml microcentrifuge tube containing 600 μl lysis buffer [100 mM Tris/HCl (pH 8.0), 50 mM EDTA, 3% SDS]. The tubes were vortexed briefly and proteinase K (Sigma) was added to a final concentration of $20\text{ }\mu\text{g ml}^{-1}$. The tubes were incubated at 56°C for 1 h. Finally, the DNA was extracted using a phenol:chloroform extraction procedure. The DNA was precipitated with an equal volume of 2-propanol in the presence of 3 M sodium acetate. The pellet was washed with 70% alcohol, dissolved in 100 μl TE [10 mM Tris/HCl (pH 7.5), 1 mM EDTA] and stored at -20°C for further use.

Identification of microsatellites and PCR primer design. To identify microsatellite repeats, the available *R. oryzae* genome sequences were downloaded from the website of the Fungal Genome Initiative (Broad Institute) (www.broad.mit.edu/annotation). The intergenic regions and ORFs of approximately 5.7 Mb were screened for microsatellites with the help of an online bioinformatics tool, Repeat Masker (www.repeatmasker.org). Di-, tri- and tetranucleotide repeats were selected on the basis of loci with the highest repeat numbers and counterselected on loci containing two or more repeat sequences within the boundaries of potential PCR primer regions. Interrupted repeats, which might have a lower chance of displaying inter-strain repeat number variation, were not taken into consideration.

Screening of microsatellites by PCR and sequencing. Each individual microsatellite locus was amplified by PCR in the presence of 2 mM MgCl_2 , 200 μM each dNTP (Bangalore Genei), 0.25 μM each primer (Integrated DNA Technologies), 0.25 U *Taq* polymerase (Bangalore Genei) and 5–10 ng fungal genomic DNA in a total volume of 10 μl . The amplification reactions were performed in a thermocycler (Eppendorf Mastercycler). PCR cycling conditions consisted of an initial denaturation step for 5 min at 94°C , followed by 35 cycles of 94°C for 1 min, $55\text{--}59^{\circ}\text{C}$ for 30 s (varied according to microsatellite loci) and 72°C for 1 min, with a final extension step at 72°C for 5 min. The annealing temperature for the three selected microsatellite loci was 58°C for 30 s. The amplified products were separated in an 8% polyacrylamide gel to ascertain the amplicon band sizes. The presence of all microsatellite loci was screened in eight randomly selected *R. oryzae* isolates by apparent size variation on PAGE. The microsatellite loci that were available in all isolates (confirmed by amplification), and having better discrimination power, were subjected to DNA sequencing to confirm whether the apparent size variation seen on PAGE was due to changes in the repeat numbers of the microsatellites. Sequencing reactions were performed with a BigDye terminator cycle sequencing kit, version 3.1/1.1 (Applied Biosystems). All the sequencing reactions were purified and analysed on an ABI 3130 genetic analyser (Applied Biosystems).

Automated capillary electrophoresis. The chosen microsatellites of *R. oryzae* were analysed further by fragment size analysis in an

Table 1. *R. oryzae* and *R. delemar* isolates included in the study, fragment sizes of the three microsatellite loci and acid production patterns

<i>R. oryzae</i> NCCPF no.	Age (years)/ sex of patient	Month and year of isolation	Source of isolate	Fragment size (bp)			Acid production
				RO3	RO4	RO8	
710016	35/M	August 2001	Nasal tissue	285	195	303	LA
710024	60/M	October 2002	Nasal tissue	285	189	313	LA
710031	11.5/F	March 2003	Nasal tissue	279	191	303	FA
710034	60/F	May 2003	Nasal tissue	288	189	313	LA
710050	36/F	August 2004	Nasal tissue	285	189	333	FA
710056	50/M	December 2004	Maxillary tissue	289	189	306	FA
710058	35/M	February 2005	Nasal tissues	297	196	333	FA
710059	19/F	April 2005	Nasal crust	278	189	303	LA
710060	45/M	June 2005	Nasal tissue	278	191	303	FA
710068	36/F	December 2005	Nasal tissue	288	191	303	FA
710070	12/F	December 2005	Nasal tissue	283	195	303	FA
710073	2/F	May 2006	Nasal crust	288	189	309	LA
710078	25/M	August 2006	Nasal tissue	298	186	309	LA
710079	28/M	August 2006	Nasal tissue	285	189	303	FA
710083	30/F	October 2006	Palate ulcer	298	186	309	FA
710084	64/M	October 2006	Nasal swab	285	194	335	FA
710085	NA	November 2006	Nasal tissue	286	186	303	FA
710086	30/M	April 2007	Nasal tissue	293	189	313	FA
710088	50/M	August 2008	Nasal tissue	297	196	333	LA
710089	35/M	January 2008	Nasal crust	278	191	303	FA
710093	6/M	January 2008	Nasal tissue	288	189	313	LA
710094	25/M	August 2008	Nasal wash	283	191	303	LA
710097	28/M	June 2008	Nasal tissue	285	195	313	FA
710099	50/M	September 2008	Nasal tissue	283	189	303	FA
710100	2/F	November 2008	Palatal ulcer	278	186	306	LA
710101	45/F	January 2009	Nasal crust	285	195	303	FA
710103	52/F	February 2009	Nasal tissue	288	189	309	FA
710116	45/F	February 2009	Nasal crust	285	186	335	FA
710117	50/F	January 2009	Nasal tissue	278	197	303	ND
710118	60/M	July 2009	Tissue from cutaneous lesion	293	189	303	LA

F, Female; FA, fumaric acid; LA, lactic acid; M, male; ND, not detected.

automated capillary electrophoresis system for all 30 isolates. Individual microsatellite loci were amplified by PCR as described above using a fluorescently labelled primer. The primer for RO8 was labelled with the fluorescent dye 6-FAM, whilst RO3 and RO4 were labelled with VIC and NED, respectively. After PCR, a 2 µl aliquot of each sample was diluted 200-fold and added to 10 µl Hi-Di formamide containing 0.5 µl GeneScan 500 6-carboxytetramethyl-rhodamine size standards (Applied Biosystems). Amplicons were denatured at 95 °C for 5 min and rapidly chilled on ice. Denatured samples were resolved by capillary electrophoresis with a 35 cm capillary filled with POP-7 polymer (Applied Biosystems) in an ABI Prism 3130 genetic analyser (Applied Biosystems). The molecular sizes of the amplified alleles were automatically analysed and calculated using GENESCAN analysis software (version 2.1; Applied Biosystems). As a result of the length polymorphisms, isolates were assigned to different microsatellite genotypes based on one or more differences in band size.

Discriminatory power and stability of the markers. The discriminatory power of the microsatellite markers was calculated

using Simpson's index of diversity (*D*). This index is a statistical measure that any two randomly chosen isolates are of the same genotype for a given marker(s). A *D* value of 1 indicates that all isolates are unique, whereas a *D* value of 0 indicates that all isolates are identical. The stability of the typing method was evaluated by analysing the DNA preparations after each passage for up to seven passages. Briefly, in this experiment, a *Rhizopus* species spore suspension (3×10^2 spores ml⁻¹) was prepared and approximately 100 µl of this suspension was plated on SDA and incubated at 37 °C. A single non-sporulating colony was then picked and placed on a new SDA plate and incubated until sporulation. The spores were collected and a portion was used to inoculate Sabouraud dextrose broth for DNA extraction, whilst the rest was plated on SDA to obtain growth of the fungus with spores. This procedure was repeated seven times, and the extracted DNA in each passage was used for microsatellite analysis.

Data analysis. Typing data were imported into BioNumerics version 5.0 software (Applied Maths). Microsatellite data were analysed using the multistate categorical similarity coefficient.

RESULTS

After analysing 30 *R. oryzae* isolates (identified by morphology and growth temperature) for organic acid production, the isolates were classified into 17 *R. delemar* isolates (fumaric/malic acid producers), 12 *R. oryzae* isolates (lactic acid producers) and one isolate that could not be classified as either of the above as there was no organic acid production by this isolate (Table 1).

Computational analysis of *R. oryzae* (the strain identified later as *R. delemar* on the basis of organic acid production) genome sequences yielded 30 microsatellite loci with the help of the online tool Repeat Masker. A total of 10 of these 30 microsatellites loci were intergenic, whilst the other 20 were within the putative coding region. The nomenclature used for the microsatellite loci was RO for *R. oryzae*, followed by a number. The intergenic microsatellites could not be amplified in all isolates tested. Of the 20 putative coding-region microsatellite loci, 3 that could be amplified with good discrimination from 8 randomly selected isolates were selected for analysis of 30 clinical isolates of *R. oryzae*. These microsatellite loci were: RO3 [(CCT)_n] from ORF RO3G 04330 (*R. oryzae* predicted protein); RO4 [(TA)_n] from ORF RO3G 04103 (*R. oryzae* hypothetical protein); and RO8 [(GAA)(GGA)_n] from ORF RO3G 13155 (*R. oryzae* predicted protein). The PCR products of RO3, RO4 and RO8 showed apparent size variation by PAGE (data not shown).

Sequence analysis confirmed the presence of the microsatellites and demonstrated polymorphism in the microsatellite repeat number. The sequence information for the various alleles of these three microsatellite loci was submitted to GenBank (accession numbers given in Table 2). Accurate sizes of the selected loci were determined by an automated capillary electrophoresis system for all the clinical isolates of *R. oryzae* (Fig. 1). The PCR products of RO3, RO4 and RO8 varied in the range of 278–298, 186–197 and 303–335 bp, respectively (Table 1). These 3 microsatellite loci identified 21 genotypes among the 30 *R. oryzae* isolates.

The discriminatory power (*D*) was highest for locus RO3 (0.846), whereas for RO4 and RO8 the *D* value was 0.747 and 0.742, respectively. The combined *D* value was 0.986 (Table 2). All three microsatellite loci were found to be reasonably stable, as they produced the same genotypes even after seven passages. A dendrogram of the 30 *R. oryzae* isolates included in the MLMT analysis using these three

microsatellite loci showed clustering of the isolates into four groups. (Fig. 2)

DISCUSSION

This study describes what is believed to be the first report of the development of a stable and discriminatory MLMT system for *R. oryzae*. Microsatellite markers have been used successfully for strain typing and population genetics studies of several fungal species because of their hypervariable nature, ease of PCR amplification and interpretation, co-dominance and potential use in automated assays. The ideal MLMT scheme should amplify the same loci from all studied isolates and should demonstrate sufficient repeat number diversity to develop a discriminatory typing scheme (Klaassen, 2009). Recently, sequencing and assembly of the entire *R. oryzae* genome has been completed (Ma *et al.*, 2009). This has provided the opportunity to search for more potential microsatellite loci *in silico* by using software available in the public domain. The *Rhizopus* genome is approximately 46 Mb. As the genome sequence was quite large, we started screening sequences with a National Center for Biotechnology Information tool, which yielded ten intergenic (non-coding) microsatellites. When these intergenic microsatellites failed to qualify as suitable markers for strain typing, we screened approximately 5.7 Mb of ORFs. Although we found 20 microsatellite loci in the coding region, only three – RO4 [(TA)_n], RO3 [(CCT)_n] and RO8 [(GAA)(GGA)_n] – fulfilled the criteria for an ideal microsatellite marker. In general, it is believed that non-coding region microsatellites are far more discriminatory than those in coding regions, and these have been utilized for the typing of various fungi (Fisher *et al.*, 2004; de Valk *et al.*, 2007a; Sampaio *et al.*, 2005). However, in the present study, the non-coding region microsatellites could not be amplified in most of the isolates tested. This may have been due to high levels of sequence variation in the primer-binding sites. In seven out of ten cases, attempts to amplify the non-coding region microsatellites generated a number of non-specific bands (results not shown). Thus, we suggest that the non-coding region microsatellites are not good candidates for an MLMT system for *R. oryzae*. The three coding-region microsatellites described here were found to have reasonably good discriminatory power and stability. The discriminatory power of the assay was greater when all three loci were used for analysis, compared with the

Table 2. Salient characteristics of selected microsatellite loci

Microsatellite	No. of genotypes	Size (bp)	Repetitive motif	<i>D</i> value	Dye label	GenBank accession no.
RO3	7	278–298	(CCT) _n	0.846	VIC	GU132512–GU132516
RO4	5	186–196	(TA) _n	0.747	NED	GU132517–GU132519
RO8	6	303–335	(GAA)(GGA) _n	0.742	6-FAM	GU132520–GU132525

Fig. 1. GENESCAN profile showing the results of automated fragment sizing for microsatellite analysis. Each microsatellite locus is represented by a different colour: RO4 (black), RO3 (green) and RO8 (blue). Size standards (GeneScan LIZ 500) are shown in orange.

discriminatory powers of individual loci. A combined discriminatory power of 0.986 was achieved for *R. oryzae*, which is in good agreement with reports for typing of other

fungi by microsatellite analysis (de Valk *et al.*, 2007a; Sampaio *et al.*, 2005). For the pathogenic yeast *C. albicans*, the discriminatory power based on microsatellite analysis

Fig. 2. Dendrogram based on the three microsatellite loci RO4, RO3 and RO8. The results denote the NCCPF strain numbers, sex of the patients, age of the patients (years), the year of isolation and body site source of isolate. F, Female; M, male; RhOrySTR, *Rhizopus oryzae* short tandem repeats MLMT.

has been reported to range from 0.87 to 0.97 for the typing of 114 isolates (Sampaio *et al.*, 2005). Likewise, a high degree of discrimination ($D=0.989$) was achieved using polymorphic microsatellite markers for typing 100 *A. fumigatus* isolates (de Valk *et al.*, 2007a). In the present study, we evaluated only 30 isolates, so it is possible that there would be an improvement in discriminatory power when a larger numbers of isolates are included and more microsatellite loci are analysed. As we screened only about 5.7 Mb of the genome, there remains scope for identifying many more ideal microsatellite markers by screening the rest of the genome and validating any potential markers.

The ability to assign an identical genotype to the same isolate after multiple passages defines *in vitro* stability. This is another important criterion for evaluating a typing system (Gil-Lamaignere *et al.*, 2003). The reproducibility as well as the *in vitro* stability of the present MLMT method was found to be 100 % for up to seven passages. Recently, Sabino *et al.* (2010) described the stability of microsatellite markers for *Candida parapsilosis* for 300 generations (Sabino *et al.*, 2010). As the genotypes were the same for up to 300 generations, they predicted a mutation rate of less than 3.33×10^{-3} for these microsatellites. However, for *Aspergillus* species, the stability of microsatellites has only been determined for 14–30 passages (Balajee *et al.*, 2007; de Valk *et al.*, 2005; Lasker & Ran, 2004). For organisms with a short generation time, it may be necessary to assess the stability of the markers for up to several hundred generations, whereas for filamentous fungi, which have a longer generation time, it remains to be seen whether they have to be followed for a similarly high number of generations. The level of stability of the markers obtained in the current study may be sufficient to use them to answer epidemiological questions concerning outbreaks in a hospital or in a given geographical location. However, future studies are required to assess the stability of microsatellite markers in filamentous fungi. In addition to good reproducibility and discrimination power, microsatellite analysis has other advantages. Because the procedure is PCR based, MLMT analysis requires relatively small amounts (~30–40 ng per reaction) of template DNA compared with other methods. Large numbers of isolates may be typed easily, as preparation is simple and does not require the tedious and labour-intensive procedures needed for highly purified DNA. Using a standard panel of microsatellite loci and test isolates, inter-laboratory comparisons and creation of databases are feasible (de Valk *et al.*, 2009).

At present, for molecular strain typing of fungi, three methods are popular: MLST, MLMT and amplified fragment length polymorphism (AFLP) analysis (Bougnoux *et al.*, 2003; de Valk *et al.*, 2005, 2007b; Dodgson *et al.*, 2003). Each has its advantages and disadvantages. Klaassen (2009) compared MLST and MLMT techniques and pointed out that the different microsatellite markers may display much heterogeneity with regard to stability. Within one locus, alleles with a low number of repeat units may be more stable

than the alleles with a high number of repeats. In contrast, the stability of single-nucleotide polymorphisms in housekeeping genes makes MLST a more reliable typing method for population genetic studies, although the discriminatory power of MLST is expected to be lower than MLMT. However, MLST is laborious and time-consuming. MLMT is versatile for strain discrimination because of its inherent instability compared with the single-nucleotide polymorphisms in housekeeping genes. The degree of stability offered by the presence of microsatellite markers in the coding regions should be sufficient to balance the stability versus its variability that gives rise to many genotypes. This balancing phenomenon is only a feature of MLMT. In comparison with the AFLP method, the MLMT approach is found to be highly biased towards arbitrarily selected loci, whereas AFLP provides an overview of the entire genome variability. Furthermore, the AFLP technique is applicable to any organism without the need for prior genome sequence information. In contrast, MLMT is usually species specific and requires species sequence information for the identification of microsatellite loci (de Valk *et al.*, 2007b). MLMT also requires specialized reagents, software and expensive equipment, which may not be available in many laboratories. This problem may partially be circumvented by running the PCR products in a high-resolution agarose gel system or using denaturing PAGE.

Abe *et al.* (2007) have divided *R. oryzae* isolates into two species based on organic acid production, *R. oryzae* (lactic acid producers) and *R. delemar* (fumaric/malic acid producers). They proposed *R. delemar* as a new species. The *R. oryzae* genome sequence information available from the database of the Fungal Genome Initiative is actually from a *R. delemar* strain (later confirmed by organic acid production). We wanted to see whether the protocol worked with both species and thus isolates of both species were included in this study. The microsatellite markers worked for members of both species but could not distinguish them into two clusters. Inclusion of additional microsatellites in the future may help in species differentiation.

In conclusion, the present study describes a MLMT system for *R. oryzae* and *R. delemar* that seems to have a good degree of discriminatory power, stability, ease of use and interpretation, typeability and high-throughput potential. The method may be improved further with the use of additional microsatellites, and it could be used to address important epidemiological issues such as tracking the transmission of infection and strain specificity for a specific disease manifestation.

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