Conflicting results obtained by RAPD-PCR and large-subunit rDNA sequences in determining and comparing yeast strains isolated from flowers: a comparison of two methods

¹ Plant Ecology, Department of Biology, Philipps-University Marburg, Karl-von-Frisch-Straße, 35043 Marburg, Germany

Michael Herzberg,¹ Reinhard Fischer² and Andreas Titze¹

Author for correspondence: Michael Herzberg. Tel: +49 6421 282 2054. Fax: +49 6421 282 2093. e-mail: Herzberg@stud-mailer.uni-marburg.de

² Department of Biology, Philipps-University Marburg and Department of Biochemistry, Max-Planck-Institut, Karl-von-Frisch-Straße, 35043 Marburg, Germany

Sixty-six yeast strains isolated from the nectar of various plant species in Central Europe were characterized by randomly amplified polymorphic DNA PCR (RAPD-PCR) and by sequencing of the variable D1/D2 domain of large-subunit (26S) rDNA. The usefulness of both methods for the determination and comparison of unknown ascomycetous and basidiomycetous yeast strains was compared and evaluated. The reproducibility of RAPD-PCR was shown to be low and the information obtained by this method was clearly not as precise as that obtained from sequence analysis. Numerous imponderables make RAPD-PCR analysis unreliable, at least as a means of identifying yeasts in ecological studies. The lack of standard protocols for RAPD-PCR analysis and the absence of a general database of banding patterns made it impossible to identify unknown yeast strains or to recognize new species. In contrast to RAPD-PCR, sequence analysis of the D1/D2 domain was found to be a fast and reliable method for the rapid identification of yeast species and was also shown to be an invaluable tool for the discovery of new species.

Keywords: 26S rDNA, nectar, sequence analysis, yeasts, RAPD-PCR

INTRODUCTION

The occurrence of yeasts in floral nectaries was first reported by Boutroux (1881). Various authors, such as Boutroux (1884), Reukauf (1911/12), Schuster & Uhlela (1913), Größ (1917/18), Sydow & Sydow (1918), Schoellhorn (1919) and Hautmann (1924), have since described nectar yeast morphologically and have introduced numerous synonyms of *Metschnikowia gruessii* Giménez-Jurado and *Metschnikowia reukaufii* Pitt & Miller. Hence, it became clear that the use of morphological characteristics as an aid to species identification is limited in yeast taxonomy (Stelling-Dekker, 1931). In other investigations, physiological tests were used to characterize strains of nectar yeasts (Größ, 1927; Martin, 1954). At least for members of the genus *Metschnikowia* Kamienski, which contains several species associated with flowers, classi-

fication based on physiological properties turned out to be ineffective (Pitt & Miller, 1970).

Even in more recent studies there seems to be confusion about the identity of nectar yeast strains. Eiskowitch *et al.* (1990) reported that, in an ecophysiological study on nectar yeasts, two strains identified as *M. reukaufii* were clearly different in their physiological properties. One of the strains exhibited 'airplane' cell configurations that are characteristic of *M. gruessii*, whereas the other strain was 'more typical' of *M. reukaufii*, suggesting that the two strains did not belong to the same species. Even though *M. gruessii* had not been described at the time of the study by Eiskowitch and colleagues, the example shows that a fast, simple and reliable method for yeast identification is necessary in yeast ecology.

Price *et al.* (1978) showed genome comparison to be a valuable tool in yeast classification. Williams *et al.* (1990) and Welsh & McClelland (1990) developed the randomly amplified polymorphic DNA PCR (RAPD-PCR) and arbitrarily primed PCR (AP-PCR) methods, respectively, as simple tools to aid in classification.

Abbreviations: CBS, Centraalbureau voor Schimmelcultures; RAPD, randomly amplified polymorphic DNA.

The GenBank accession numbers for the 26S rDNA sequences reported in this paper can be found in Table 1.

Table 1. Yeasts isolated from the nectar of different flowering plant species

Site/date of sampling	Host	Yeast species	UM* no.	CBS† no.	GenBank accession no.
Marburg:					
21/04/99	<i>Arabis alpina</i>	<i>Cryptococcus heveanensis</i> sister (37s‡)	MH 40	CBS 8976	AF406890
		<i>Rhodotorula bacarum</i>	MH 46	CBS 8977	AF406891
21/04/99	<i>Arctostaphylos uva-ursi</i>	<i>Metschnikowia reukaufii</i> §	MH 120	CBS 8978	–
02/06/99	<i>Atropa bella-donna</i>	<i>Cryptococcus victoriae</i> sister (12s)	MH 39	CBS 8979	AF406892
04/06/99	<i>Ballota nigra</i>	<i>Cryptococcus oeirensis</i>	MH 38	CBS 8980	AF406902
22/08/99	<i>Epilobium angustifolium</i>	<i>Metschnikowia reukaufii</i> §	MH 105	CBS 8981	–
25/08/99	<i>Epilobium angustifolium</i>	<i>Cryptococcus albidus</i>	MH 6	CBS 8982	AF406903
25/08/99	<i>Epilobium angustifolium</i>	<i>Cryptococcus albidus</i>	MH 20	CBS 8983	AF406904
25/08/99	<i>Epilobium angustifolium</i>	<i>Cryptococcus diffluens</i>	MH 4	CBS 8984	AF406907
25/08/99	<i>Epilobium angustifolium</i>	<i>Cryptococcus diffluens</i>	MH 148	CBS 8985	AF406908
25/08/99	<i>Epilobium angustifolium</i>	<i>Rhodotorula mucilaginosa</i>	MH 146	CBS 8986	AF406910
26/08/99	<i>Epilobium angustifolium</i>	<i>Rhodotorula mucilaginosa</i>	MH 147	CBS 8987	AF406911
26/08/99	<i>Epilobium angustifolium</i>	<i>Cryptococcus diffluens</i>	MH 11	CBS 8988	AF406905
26/08/99	<i>Epilobium angustifolium</i>	<i>Cryptococcus albidus</i>	MH 103	CBS 8989	AF406906
26/08/99	<i>Epilobium angustifolium</i>	<i>Cryptococcus diffluens</i>	MH 159	CBS 8990	AF406909
27/08/99	<i>Epilobium angustifolium</i>	<i>Metschnikowia gruessii</i> §	MH 19	CBS 8991	–
16/04/99	<i>Erica carnea</i>	<i>Cystofilobasidium capitatum</i>	MH 30	CBS 8992	AF406887
15/04/99	<i>Euphorbia amygdaloides</i>	<i>Cryptococcus victoriae</i> sister (8s)	MH 23	CBS 8993	AF408633
10/04/99	<i>Helleborus foetidus</i>	<i>Metschnikowia reukaufii</i>	MH 139	CBS 8994	AF406915
10/04/99	<i>Helleborus foetidus</i>	<i>Metschnikowia reukaufii</i> §	MH 104	CBS 8995	–
10/04/99	<i>Helleborus foetidus</i>	<i>Metschnikowia reukaufii</i> §	MH 128	CBS 8996	–
15/04/99	<i>Helleborus foetidus</i>	<i>Metschnikowia reukaufii</i>	MH 101	CBS 8997	AF406916
12/02/00	<i>Helleborus foetidus</i>	<i>Saccharomyces servazzii</i>	MH 158	CBS 8998	AF406921
21/02/00	<i>Helleborus foetidus</i>	<i>Cryptococcus victoriae</i> sister (12s)	MH 166	CBS 8999	AF406893
21/02/00	<i>Helleborus foetidus</i>	Unknown§	MH 168	–	–
21/02/00	<i>Helleborus foetidus</i>	<i>Cryptococcus victoriae</i>	MH 152	CBS 9000	AF406899
16/02/00	<i>Helleborus foetidus</i>	<i>Cystofilobasidium capitatum</i> §	MH 167	CBS 9001	–
01/03/00	<i>Helleborus foetidus</i>	<i>Rhodospiridium lusitaniae</i> sister (11s)	MH 150	CBS 9002	AF406922
05/03/00	<i>Helleborus foetidus</i>	<i>Cystofilobasidium capitatum</i>	MH 153	CBS 9003	AF406887
08/03/00	<i>Helleborus foetidus</i>	<i>Cystofilobasidium capitatum</i>	MH 161	CBS 9004	AF406888
		<i>Cryptococcus cylindricus</i>	MH 160	CBS 9005	AF406933
		<i>Cryptococcus victoriae</i>	MH 155	CBS 9006	AF406900
08/03/00	<i>Helleborus foetidus</i>	<i>Cryptococcus laurentii</i> sister (10s)	MH 164	CBS 9007	AF406934
		<i>Filobasidium globisporum</i> (3s)	MH 149	CBS 9008	AF406932
		<i>Cryptococcus victoriae</i>	MH 156	CBS 9009	AF406901
08/03/00	<i>Helleborus foetidus</i>	<i>Cystofilobasidium capitatum</i>	MH 154	CBS 9010	AF406889
09/03/00	<i>Helleborus foetidus</i>	<i>Filobasidium globisporum</i> (3s)	MH 157	CBS 9011	AF406931
09/03/00	<i>Helleborus foetidus</i>	<i>Cryptococcus victoriae</i> sister (8s)	MH 162	CBS 9012	AF406894
		<i>Cryptococcus victoriae</i> sister (2s)	MH 151	CBS 9013	AF406895
		<i>Cystofilobasidium capitatum</i>	MH 163	CBS 9014	AF406885
20/04/99	<i>Lamium album</i>	<i>Sporobolomyces ruberrimus</i>	MH 134	CBS 9015	AF406930
18/04/99	<i>Lamium purpureum</i>	<i>Metschnikowia reukaufii</i>	MH 28	CBS 9016	AF406917
06/04/99	<i>Primula elatior</i>	<i>Candida bombi</i>	MH 21	CBS 9017	AF406929
15/04/99	<i>Prunus spinosa</i>	<i>Metschnikowia reukaufii</i> §	MH 31	CBS 9018	–
16/04/99	<i>Pulmonaria officinalis</i>	<i>Metschnikowia reukaufii</i> §	MH 32	CBS 9019	–
		<i>Metschnikowia reukaufii</i> §	MH 119	–	–
17/04/99	<i>Pulmonaria officinalis</i>	<i>Metschnikowia reukaufii</i>	MH 131	CBS 9020	AF406918
		<i>Metschnikowia reukaufii</i>	MH 132	CBS 9021	AF406919
17/04/99	<i>Pulmonaria rubra</i>	<i>Metschnikowia reukaufii</i>	MH 133	CBS 9022	AF406920
06/04/99	<i>Pulmonaria stiriaca</i>	<i>Cryptococcus victoriae</i> sister (8s)	MH 33	CBS 9023	AF406896
18/04/99	<i>Ranunculus bulbosus</i>	<i>Cryptococcus victoriae</i> sister (12s)	MH 27	CBS 9024	AF406897
18/04/99	<i>Ranunculus ficaria</i>	<i>Cryptococcus victoriae</i> sister (12s)	MH 13	CBS 9025	AF406898

Table 1 (cont.)

Site/date of sampling	Host	Yeast species	UM* no.	CBS† no.	GenBank accession no.
08/04/99	<i>Rhododendron maximum</i>	<i>Cystofilobasidium capitatum</i>	MH 1	CBS 9026	AF406885
08/04/99	<i>Rhododendron sulchuenense</i>	<i>Rhodotorula fujisanensis</i>	MH 2	CBS 9027	AF406928
08/04/99	<i>Rhododendron sulchuenense</i>	<i>Rhodospiridium lusitaniae</i> sister (11s)	MH 47	CBS 9028	AF406923
02/06/99	<i>Symphytum officinale</i>	<i>Metschnikowia gruessii</i> §	MH 41	CBS 9029	–
		<i>Metschnikowia gruessii</i> §	MH 129	–	–
27/05/99	<i>Trifolium pratense</i>	<i>Metschnikowia gruessii</i> §	MH 137	CBS 9030	–
		<i>Metschnikowia gruessii</i>	MH 138	CBS 9031	AF406912
Mainz:					
04/06/99	<i>Vicia villosa</i>	<i>Cryptococcus macerans</i>	MH 45	CBS 9032	AF406924
04/06/99	<i>Salvia pratensis</i>	<i>Metschnikowia gruessii</i>	MH 53	CBS 9033	AF406913
03/06/99	<i>Rhinanthus alectorolophus</i>	<i>Metschnikowia gruessii</i>	MH 127	–	AF406914
		<i>Metschnikowia gruessii</i> §	MH 102	–	–
		<i>Sporobolomyces roseus</i> sister (4s)	MH 165	CBS 9035	AF406925
03/06/99	<i>Anchusa officinalis</i>	<i>Metschnikowia hibisci</i> sister (4s)	MH 122	CBS 9036	AF406926
		<i>Metschnikowia continentalis</i> var. <i>continentalis</i>	MH 124	CBS 9037	AF406927

* UM, strains from the University of Marburg.

† CBS, strains from the Centraalbureau voor Schimmelcultures.

‡ A lower-case s with a number in parentheses indicates the number of substitutions in the D1/D2 domain compared to the type strain of the closest related species.

§ Strains that were only identified by morphological characteristics and not by sequencing.

These methods are based on the random amplification of DNA to characterize species and individuals by banding patterns. Since both methods were developed simultaneously and work on the same principle, the more common term RAPD-PCR is used for both techniques in the current study. Guadet *et al.* (1989) and O'Donnell (1993) developed a method to identify and characterize fungal species and strains by analysing the sequence of the D1/D2 divergent domain of large-subunit (26S) rDNA. Both DNA sequence analysis (e.g. Kurtzman & Robnett, 1997, 1998; Fell *et al.*, 2000; Lachance *et al.*, 2001) and RAPD-PCR (e.g. Messner *et al.*, 1994; Mollnár *et al.*, 1995; Lopandic *et al.*, 1996; Romano *et al.*, 1996; Prillinger *et al.*, 1999; Ganter & de Barros Lopes, 2000; Paramithiotis *et al.*, 2000; Naumova *et al.*, 2000) have been used frequently in the characterization and determination of yeast strains.

The initial aim of this study was to use RAPD-PCR to simplify the identification of more than 200 yeast strains isolated from the nectar of various plant species in Europe. As the ambiguity of the RAPD-PCR results became apparent, D1/D2 sequences were determined for 65 yeast strains. This allowed a critical comparison of the two approaches to be made.

METHODS

Yeast isolation and purification. The strains considered in this study are listed in Table 1. Most samples were collected in Marburg and from the surrounding area (i.e. an area of a few kilometres). Another group of samples was collected

near Mainz. The collections were made from April to August 1999 and from January to March 2000. All samples came from the nectar of the floral nectaries of various plant species. Nectar was collected from the plants by using sterile Pasteur capillary pipettes. This was then plated directly onto 40% (w/w) sugar agar (0.2%, w/w, MgSO₄; 0.3%, w/w, KH₂PO₄; 0.5%, w/w, peptone; 13.3%, w/w, sucrose; 13.3%, w/w, glucose; 13.3%, w/w, fructose; 2%, w/w, agar), as described by Hautmann (1924). The strains were further purified by cross-streaking them onto 10% (w/w) malt extract agar. Single-cell cultures were produced according to the method of Vobis (1992). Most of the strains have been deposited at the Centraalbureau voor Schimmelcultures (CBS), Oosterstraat 1, Baarn, The Netherlands.

Morphological characterization. To obtain more morphological characteristics, the strains were cultivated on GY agar (1%, w/w, glucose; 0.01%, w/w, yeast extract; 2%, w/w, agar; Lachance & Pang, 1997), dilute Gorodkova agar (1 g glucose, 5 g NaCl, 10 g peptone, 20 g agar in 1 l of tap H₂O; Lodder & Kreger-van Rij, 1952) and dilute V8 agar (Pitt & Miller, 1968). Gorodkova and V8 media were diluted 1:10, 1:15, 1:30 and 1:100 (v/v). The cultures were incubated at 14 °C. To observe the germination of cultures of *Cystofilobasidium capitatum* (Fell, Hunter & Tallman) Oberwinkler *et al.* that were rich in teliospores, slide cultures were incubated on YM at 18 °C.

Microscopy. For scanning electron microscopy, the cultures were prepared according to the methods of Vobis (1991). The samples were observed with a Hitachi S-530 microscope. Phase-contrast microscopy was performed using a Leitz Ortholux microscope. Cells were spread over a thin agar slab (Lachance, 1987). Images were recorded electronically.

DNA isolation. DNA was isolated from 24-h-old liquid cultures using the DNeasy Plant Mini Kit (Qiagen). The

DNA concentration and its purity was measured with an Ultraspec 3100 pro Photometer (Amersham Pharmacia Biotech). For each sample, the DNA concentration was adjusted to 10 ng μl^{-1} .

RAPD assay. PCR was performed in 0.5 ml thermo-tubes (Advanced Biotechnologies) using a TRIO-Thermoblock (Biometra). Three microlitres of DNA solution (10 ng μl^{-1}) and 27 μl of PCR master mix were mixed in a thermo-tube and overlaid with 35 μl of mineral oil (Sigma-Aldrich Chemie). The PCR master mix contained 30 μl sterile bi-distilled H_2O , 10 $\times$ buffer (200 mM Tris/HCl, 500 mM KCl; pH 8.4) added to a final concentration of 1 $\times$, 3 mM MgCl_2 , 0.16 mM of each dNTP, 5 U *Taq* DNA Polymerase (Gibco-BRL) and 0.5 pM of primer. The following primers were synthesized by MWG-Biotech: 1, 5'-ACGGTCTTGG-3'; 2, 5'-TGCAGCGTGG-3'; 3, 5'-GGGTAACGCC-3'. The primers were as suggested by Lopandic *et al.* (1996). The PCR protocol used was as follows. After a 10 min denaturation at 95 °C, the PCR was subjected to 35 cycles at 94 °C for 15 s, 40 °C for 30 s and 72 °C for 2 min, with a final extension at 72 °C for 5 min.

Twenty-five microlitres of the PCR product was mixed with 5 μl H_2O and 3 μl 10 $\times$ loading buffer. The DNA fragments were analysed in a 2% (w/v) agarose gel in 1 $\times$ TAE buffer; the electrophoresis unit was run at 90 V for 3 h. A 500 bp ladder (Qbiogene) and a 100 bp ladder (MBI fermentas) were included in every fifth lane in every gel. The gels were stained with ethidium bromide for 1 h and then photographed under UV light (λ 360 nm). The gel images were recorded digitally and saved as TIFF files.

The digitized images were inverted and imported into the GelCompar II software (Applied Maths) (Rademaker *et al.*, 1999). The gels were normalized using the molecular size marker. Similarity matrices of the densitometric curves of the gel lanes were calculated using the pair-wise Pearson's correlation coefficient (Häne *et al.*, 1993). 'Active zones' were defined on the images, so that the beginning and end of each lane where no bands could be observed were excluded from the analyses to minimize the background (Vinueza *et al.*, 1998). Cluster analysis of similarity matrices was performed by using the neighbour-joining algorithm.

The type strains of *Metschnikowia pulcherrima* Pitt & Miller (CBS 5833^T), *Metschnikowia lunata* Golubev (CBS 5946^T), *Metschnikowia agaves* Lachance (CBS 7745^T h⁻), *Metschnikowia continentalis* var. *continentalis* Lachance *et al.* (CBS 8429^T h⁺, CBS 8430^T h⁻), *Metschnikowia continentalis* var. *borealis* Lachance *et al.* (CBS 8431^T h⁺, CBS 8432^T h⁻), *Metschnikowia hibisci* Lachance *et al.* (CBS 8433^T h⁺, CBS 8434^T h⁻), *Metschnikowia hawaiiensis* Lachance, Starmer & Phaff (CBS 7432^T h⁺) and *Candida kofuensis* Mikita *et al.* (CBS 8058^T) were used as reference materials. As reference material for *M. gruessii* Giménez-Jurado we used the original strain of Größ (1917/18) (CBS 611), for *M. reukauffii* Pitt & Miller we used strain CBS 1903 and for *M. pulcherrima* Pitt & Miller we used CBS 610.

DNA sequence analysis. The sequence of the D1/D2 domain of the large-subunit rDNA for each sample (Table 1) was directly determined by sequencing the PCR products. Primers NL1 (5'-GCATATCAATAAGCGGAGGAAA-AG-3') and NL4 (5'-GGTCCGTGTTTCAAGAC-GG-3') (O'Donnell, 1993) were used. The primers were synthesized by MWG Biotech. PCR was performed as specified above, but with the following changes. The PCR master mix contained 30 μl sterile bi-distilled H_2O , 10 $\times$ buffer (Tris/HCl 200 mM, 500 mM KCl; pH 8.4) added to a final concentration of 1 $\times$, 1.5 mM MgCl_2 , 0.25 mM of each

dNTP, 5 U *Taq* DNA Polymerase, 0.33 pM of primer NL1 and 0.33 pM of primer NL4. After a 2 min denaturation at 95 °C, the PCR was subjected to 35 cycles at 95 °C for 15 s, 64 °C for 25 s and 72 °C for 2 min, with a final extension at 72 °C for 5 min.

Sequencing was done by MWG Biotech. The sequences for the strains were deposited in GenBank. The CLUSTAL W algorithm (Thompson *et al.*, 1994) was used to align the sequences and to construct a neighbour-joining tree. Bootstrap re-sampling was performed for the tree (1000 replications). The sequence of the type strain for each species was downloaded from GenBank and included in the analysis.

RESULTS AND DISCUSSION

All yeast strains, their CBS numbers, GenBank accession numbers, hosts of the strains and the dates of sampling are listed in Table 1. Ascomycetous yeasts belonging to the genera *Candida* Berkout, *Metschnikowia* and *Saccharomyces* Meyen *ex* Rees were found in the samples, as well as numerous basidiomycetous species belonging to the genera *Cryptococcus* Vuillemin, *Cystofilobasidium* Oberwinkler *et al.*, *Filobasidium* Olive, *Rhodospiridium* Banno, *Rhodotulula* Harrison and *Sporobolomyces* Kluyver & van Niel.

Morphological characterization

Of the ascomycetes, *M. reukauffii* ($n = 13$) and *M. gruessii* ($n = 7$) were found most often in the samples. In most cases, these two species could be distinguished on GY agar; *M. gruessii* produced its characteristic elongated 'airplane' or trident configurations when grown on this agar (Größ, 1917/18; Giménez-Jurado, 1992), whereas these were absent in *M. reukauffii*. On dilute, filtered V8 (1:15; 1:30) the two species could be distinguished by their chlamydospores, which were oval for *M. gruessii* and elongated for *M. reukauffii*. Additional morphological characteristics as described by Giménez-Jurado (1992) – e.g. the formation of thin pseudohyphae with terminal conidia or rich branched pseudomycelium formed by cylindrical cells on 10% malt extract agar – turned out not to be very helpful in distinguishing *M. gruessii* and *M. reukauffii* because these characteristics were only temporarily observed in some strains.

Of the basidiomycetous yeasts, *Cystofilobasidium capitatum* ($n = 8$) was most often found and this species could be confidently identified on the basis of morphological characteristics. On all media used in this study, cultures more than one month old exhibited pseudomycelium and true uninucleate mycelium with lateral, terminal or intercalary teliospores. Teliospores could also be observed without the formation of true mycelium. In contrast to the findings of Oberwinkler *et al.* (1983), these teliospores often germinated by the formation of septated true mycelium. Soaking the teliospores in H_2O for a prolonged period of time, as described by Kwong-Chung (1998), was not required for germination; the teliospores germinated after 8 h in slide cultures grown on YM.

Fig. 1. Analysis of the RAPD-PCR patterns for the ascomycetous yeasts. Neighbour-joining analysis was performed using the similarity matrix generated from the densitometric curves produced from the gel tracks. The similarity matrix was calculated by using the pair-wise Pearson's correlation coefficient. The results of all three primers are combined in this analysis. Primer 1, 5'-ACGGTCTTGG-3'; Primer 2, 5'-TGCAGCGTGG-3'; Primer 3, 5'-GGGTAACGCC-3'. ‡, Reference strain; s, number of substitutions in the D1/D2 domain compared to the type strain of the closest related species. The size markers at the top of each set of images are in bp.

Although *M. reukaufii*, *M. guessii* and *Cystofilobasidium capitatum* could be identified by their morphological characteristics in most cases, it must be emphasized that species determination based only on morphological criteria is inadequate for most yeast species.

Molecular characterization

Except for *M. guessii*, *M. reukaufii* and *Cystofilobasidium capitatum*, the identities of all of the species listed in Table 1 were eventually determined by large-

subunit rDNA D1/D2 domain sequence analysis. The conventional approach for species identification would have involved the determination of over 50 growth responses (Yarrow, 1998) – a very onerous task, especially for a laboratory whose primary concern is not yeast identification. Instead, the strains were subjected to RAPD-PCR, a simple and rapid method for yeast typing. Figs 1 and 2 show analyses of the results produced by this method. The banding patterns were used to construct trees from which it was hoped that species identification could be achieved, by connecting unknowns to authentic reference strains. However, the

Fig. 2. For legend see facing page.

Fig. 3. Phylogenetic tree for the ascomycetous strains, constructed using the neighbour-joining method, generated using D1/D2 rDNA sequences. Only bootstrap values above 50% are shown (based on 1000 replications). The letter s in parentheses with a number indicates the number of substitutions in the D1/D2 domain compared to the type strain of the closest related species.

cluster structure was generally weak and the banding patterns did not often fall into clearly recognizable, distinct classes. Analyses of D1/D2 sequences, based on the neighbour-joining method of tree reconstruction, for the same yeasts are shown in Fig. 3 and Fig. 4, for the ascomycetous and basidiomycetous yeasts, respectively. Following the proposal of Kurtzman & Robnett (1997), all strains that showed more than two substitutions in the D1/D2 domain when compared to their closest related type strain (found in GenBank) were suspected of being sister species (Table 1). The number of substitutions between individual isolates and their nearest neighbours are shown in Table 1. Sister species of *M. hibisci*, *Cryptococcus heveanensis* (Groeneweg) Baptist & Kurtzman, *Cryptococcus victoriae* Montes *et al.* *Cryptococcus laurentii* (Kufferath) Skinner and *Rhodospiridium lusitaniae* Fonseca & Sampaio were found, and these strains have a very high potential to represent new species. How-

ever, the isolation of more strains and their full characterization in relation to known species is required before these strains can be described as new species.

According to the analysis of RAPD-PCR patterns, the ascomycetous strains CBS 9036 and CBS 9037 would have been viewed as slightly atypical strains of *M. gruessii* (Fig. 1). However, sequence analysis (Fig. 3) showed CBS 9036 and CBS 9037 to be sister species of *M. hibisci* and *M. continentalis*, respectively. Strain CBS 9036 showed only about 30% similarity to the type strains of *M. hibisci* (CBS 8433^T h⁺, CBS 8434^T h⁻) upon the analysis of RAPD-PCR patterns, even though the two strains exhibited 99% similarity in the D1/D2 domain. Strain CBS 9016, which exhibited 100% sequence similarity to the other sequenced strains of *M. reukaufii*, appeared more closely related to the type strain of *M. hawaiiensis* (CBS 7432^T h⁺) and to the reference strain of *M. gruessii* (CBS 611) than to the reference strain of *M. reukaufii* (CBS 1903) in the analysis of the RAPD-PCR patterns (Fig. 1). In this analysis, *Candida bombi* Montrocher (CBS 9017), which belongs to the *Starmerella* clade (Rosa & Lachance, 1998), appeared to be more closely related to *M. continentalis* var. *continentalis* than the varieties of *M. continentalis* were to each other. The RAPD-PCR patterns of the reference strain used for *M. gruessii* (CBS 611) were more similar to those of the *Candida bombi* strain (CBS 9017) than to those of the other authentic strains of *M. gruessii*. Even though strain CBS 9021 showed 100% D1/D2 sequence similarity to the other strains of *M. reukaufii* (Fig. 3), including the reference strain CBS 1903, its RAPD-PCR patterns were more similar to those of the type strain of *Candida kofuensis* (CBS 8058^T; Fig. 1).

A significant discrepancy between the results of the RAPD-PCR and sequence analysis was found also for the basidiomycetous strains (Figs 2 and 4, respectively). In the RAPD-PCR analysis, strain CBS 8999 (*Cryptococcus victoriae*) was well separated from strains CBS 8979 and CBS 9025 (both *Cryptococcus victoriae*), and it clustered with strain CBS 9007 (*Cryptococcus laurentii*). However, strains CBS 8999 and CBS 9007 were quite distantly related on the basis of D1/D2 sequence data (89% similarity), whereas strains CBS 9025, CBS 8979 and CBS 8999 had identical sequences. The RAPD-PCR pattern of strain CBS 9006 (*Cryptococcus victoriae*; Fig. 2) showed less than 55% similarity with the RAPD-PCR patterns of strains CBS 9009 and CBS 9000 (both *Cryptococcus victoriae*), although all three strains were identical in their D1/D2 sequences to the type strain of *Crypto-*

Fig. 2. Analysis of the RAPD-PCR patterns for the basidiomycetous yeasts. Neighbour-joining analysis was performed using the similarity matrix generated from the densitometric curves produced from the gel tracks. The similarity matrix was calculated by using the pair-wise Pearson's correlation coefficient. Primer 1, 5'-ACGGTCTTGG-3'; Primer 2, 5'-TGCAGCGTGG-3'; Primer 3, 5'-GGGTAACGCC-3'. The results of all three primers are combined in this analysis. A lower-case s in parentheses with a number indicates the number of substitutions in the D1/D2 domain compared to the type strain of the closest related species. The size markers at the top of each set of images are in bp.

Fig. 4. Phylogenetic tree for the basidiomycetous strains, constructed using the neighbour-joining method, generated using D1/D2 rDNA sequences. Only bootstrap values above 50% are shown (based on 1000 replications). The letter s in parentheses with a number indicates the number of substitutions in the D1/D2 domain compared to the type strain of the closest related species.

coccus victoriae. In contrast to this finding, the RAPD-PCR patterns of strain CBS 9006 showed more similarity to those of *Rhodotorula fujisanensis* (Soneda Johnson & Phaff strain CBS 9027 than to any other strain (Fig. 2). In the RAPD-PCR analysis, strain CBS 8992 did not cluster with the other sequenced strains of *Cystofilobasidium capitatum*, despite the fact that it exhibits 100% sequence similarity to strains CBS 9003, CBS 9010, CBS 9014 and CBS 9004 (Fig. 4). The same discrepancy between RAPD-PCR analysis and sequence analysis was found for the *Cryptococcus albidus* (Saito) Skinner strains CBS 8982, CBS 8983 and CBS 8989 (Figs 2 and 4).

Conflicting results have been reported before when multiple isolates of *Cryptococcus neoformans* from a single patient have been characterized by RAPD-PCR and other molecular typing techniques (Casadevall &

Spitzer, 1995; Haynes *et al.*, 1995). In contrast to the findings of various authors (e.g. Messner *et al.*, 1994; Baleiras Couto *et al.*, 1994; Mollnar *et al.*, 1995; Lopandic *et al.*, 1996; Romano *et al.*, 1996; Prillinger *et al.*, 1999; Naumova *et al.*, 2000; Paramithiotis *et al.*, 2000), we conclude that RAPD-PCR is not a reliable method for the determination or comparison of unknown yeast strains. We agree with de Barros Lopes *et al.* (1998) that strains producing distinct amplification patterns cannot be assumed to be different species, given that members of a species may be represented by several banding patterns. Our results support the view of De Filippis & Magel (1998), in that the usefulness of RAPD-PCR for comparing individuals belonging to different species is low.

The background noise level in RAPD-PCR, which is inherent in this method (Messner *et al.*, 1994), seems to

be too high for the differentiation of strains and the assignment of strains to type strains. Various factors influence the RAPD-PCR patterns produced. The selection of target sites for amplification by the primers in the first cycles of the PCR does not depend only on the template sequence: the annealing of primers is also influenced by differences in the concentration of template DNA, $MgCl_2$, dNTPs and primers, the purity and quality of the template DNA, the composition of the primer and the annealing temperature (Kwok *et al.*, 1990; Rychlik *et al.*, 1990; Rychlik, 1995; Erlich *et al.*, 1991; Muralidharan & Wakeland, 1993; Meunier & Grimont, 1993; Micheli *et al.*, 1994; Davin-Regli *et al.*, 1995). Since these factors can never be completely standardized (Messner *et al.*, 1994; Mollnár *et al.*, 1995), and not all processes and mechanisms involved in RAPD-PCR are fully understood (Wolff *et al.*, 1993), they may have a great effect on the occurrence of RAPD markers as well as differences of the template sequence in different samples.

False priming and other PCR artefacts may occur (Kwok *et al.*, 1990; Sommer & Tautz, 1989; Riedy *et al.*, 1991) and, in respect to the low stringency of the RAPD-PCR method, amplification at imperfectly matching primer sites may occur frequently. At definite template concentrations, mismatches and the amplification of rare priming sites have been reported to be random events resulting in the non-reproducibility of RAPD profiles (Davin-Regli *et al.*, 1995).

Other factors may influence the RAPD-PCR patterns in later parts of the reaction. One of these is the occurrence of hairpin loops. Such secondary structures within the PCR products influence the rate at which the different fragments are amplified after the first cycles. Caetano-Anollés *et al.* (1992) showed convincingly that only a subset of all the possible target sites for amplification that are determined by the primer sequence become a RAPD marker at the end of the reaction.

Other factors, such as the annealing temperature, extension time and number of cycles, affect the specificity of every PCR (Rychlik *et al.*, 1990; Erlich *et al.*, 1991). To prevent the influence of marginal differences in the temperature program, Baleiras Couto *et al.* (1994) recommended the installation of the thermocycler in a thermoregulated room. Among other things, Halmschlager *et al.* (1994) reported that the formation of amplification products depends on the thermocycling facilities and the individuals involved. Variations in RAPD-PCR patterns can also be associated with the brand of the enzyme used in different investigations (Meunier & Grimont, 1993). One cannot guarantee the constant performance of a batch of *Taq* DNA polymerase or of a particular piece of PCR apparatus over the years (Meunier & Grimont, 1993). To prevent variations in the reaction conditions, Messner *et al.* (1994) and Mollnár *et al.* (1995) proposed that all samples for a given investigation should be processed in a single thermocycler run. This would restrict the number of samples that can be

compared in one investigation and would not allow the use of RAPD-PCR for large scale experiments, contrary to the claim made by Mollnár *et al.* (1995).

It is commonly claimed that methods such as RAPD-PCR and amplified fragment length polymorphism (AFLP) analysis sample the whole genome randomly and therefore give a representative picture of the whole genome. Consequently, consistent results should be obtained by the two methods, and with different primers. At variance with this assumption, Ganter & de Barros Lopes (2000) showed that RAPD-PCR and AFLP can result in significantly different trees and others have obtained different results with different primers (Haynes *et al.*, 1995; Baleiras Couto *et al.*, 1996; Joachim *et al.*, 1997). We conclude that the numerous imponderables of RAPD-PCR make it impossible to gain reliable results with this method, at least as a means of identifying yeasts in ecological studies. The often described high resolution of the method may be the result of 'background noise' in a lot of cases.

Beside the lack of reproducibility associated with RAPD-PCR results, another disadvantage of this method is the lack of universally established guidelines for the selection of primers (Leung *et al.*, 2000), standard methods for the interpretation of the data (Boekhout *et al.*, 1997; Leung *et al.*, 2000) and standard protocols for PCR, electrophoresis and the detection of banding patterns. Unless the method is standardized, the databases of different investigations are not comparable and it is not possible to assemble a general database of patterns for strain identification (Meunier & Grimont *et al.*, 1993). In each laboratory a new reference dataset must be generated. At least the type strains of all species considered must be included in each study. For the identification of unknown strains this leads to the paradoxical situation that one has to choose close relatives to strains whose genus is not known.

From our own data and the inconsistencies in the literature we conclude that, in agreement with de Barros Lopes (1998), the information obtained using PCR is clearly not as precise as sequencing. In contrast to RAPD-PCR, sequence analyses of the D1/D2 domain of the large-subunit rDNA turned out to be a fast, simple and completely reproducible method (100%) for yeast identification. The correlation of D1/D2 sequence divergence and species boundaries in ascomycetous and basidiomycetous yeast has been discussed sufficiently by Kurtzman & Robnett (1997) and Fell *et al.* (2000). Since a complete dataset of the D1/D2 domain for all described yeast species is available on the Internet (e.g. GenBank, EMBL) (Kurtzmann & Robnett, 1998; Fell *et al.*, 2000, and many other authors), it is usually possible to identify a single strain with only its sequence. As soon as one has a purified culture, it is possible to generate the D1/D2 sequence of a strain by amplifying the D1/D2 domain directly from whole cells, bypassing the DNA extraction procedure, within two days (Lachance *et al.*,

1999). For the discrimination of closely related strains or the analysis of intraspecific genetic diversity the sequences of the D7/D8 domain of the large-subunit rDNA, the intergenic spacers (IGSs) and 28S rDNA and the internally transcribed spacer (ITS) regions provide additional meaningful information (Fell *et al.*, 2000; Lachance *et al.*, 2000; Fonseca *et al.*, 2000; Sugita *et al.*, 2000). In view of the ever decreasing cost of sequencing and the reliability of the results it produces, contrasted with the various disadvantages of RAPD-PCR, the question arises as to why RAPD-PCR is still frequently used in laboratories.

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