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Phenotypic and genotypic identification of yeasts from cheese

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Abstract

Eighty-five yeast strains isolated from different cheeses of Austria, Denmark, France, Germany, and Italy were identified using physiological methods and genotypically using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis. Good congruence was found between the phenotypic and genotypic data for 39 of the isolates. However, 26 isolates of *Geotrichum* could only be identified to the species level using the genotypic methods and 7 isolates were correctly identified to the genus level only using phenotypic identification methods. The phenotypic identification did not agree with the genotypic data for 14 yeast isolates. Using ubiquinone analysis, yeast cell wall sugars and the diazonium blue B test 5 incorrectly identified isolates with phenotypic methods could be identified genotypically. In addition the 7 isolates identified only to the genus level by the phenotypic methods and the 26 *Geotrichum* strains were identified to the species level using the polyphasic molecular approach mentioned above. Eleven strains remained unidentified. The 76 identified yeast isolates were assigned to 39 species, the most frequent assignments were made to *Debaryomyces hansenii*, *Geotrichum candidum*, *Issatchenkia orientalis*, *Kluyveromyces lactis*, *K. marxianus*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and*Candida catenulata*. It is proposed that *Debaryomyces hansenii* (Zopf) Lodder et Kreger-van Rij and *Debaryomyces fabryi* Ota should be reinstated. The RAPD-PCR data reinforced the view that the species *Galactomyces geotrichum* is heterogeneous with all of the *Geotrichum* isolates from cheese products being assigned *G. geotrichum* group A *sensu* M.T. Smith. It is suggested that the name *Geotrichum candidum* be conserved for this rather common species.

Abbreviations: HA – 'Hefe Ascomycet'; HB – 'Hefe Basidiomycet'; RAPD-PCR – Random Amplified Polymorphic DNA Polymerase Chain Reaction; VIAM – Vienna, Institute of Applied Microbiology

Introduction

The occurrence of yeasts in dairy products is important. Yeasts have been considered to be the cause of poisoning and allergic reactions, though their public health significance in foods is considered to be negligible (Fleet 1990, 1993). In cultured milk products yeasts are a major cause of spoilage (Engel 1986; Fleet 1990, 1993; Rohm 1991; Jakobsen & Narvhus 1996) whereas in some cheese varieties, especially in semi-soft cheeses with surface films (smear-ripened cheeses), e.g. Limburger and Tilsit, and mould-ripened cheeses such as Camembert and Roquefort they are involved in maturation and form a substantial part of the microflora (Devoyod & Sponen 1970; Nakase & Komagata 1977; Nakase et al. 1977; Schmidt & Lenoir 1978, 1980; Siewert 1986; Fleet 1990; Rohm et al. 1992; Eliskases-Lechner & Ginzinger 1995; Jakobsen & Narvhus 1996).

Due to their tolerance of low pH-values and high NaCl-concentration, yeasts grow particularly well during the initial period of ripening (Eliskases-Lechner

& Ginzinger 1995). Their main contribution to ripening is the utilization of lactic acid which leads to an increase in pH that encourages bacterial growth and initiates the second stage of maturation. Bacterial growth may be stimulated by vitamins and amino acids produced by the yeasts (Siewert 1986; Jakobsen & Narvhus 1996). In addition, some proteolytic and lipolytic enzymes, and the production of aroma components, directly affect ripening (Lenoir 1984; Siewert 1986; Jakobsen & Narvhus 1996).

The predominance of *Debaryomyces hansenii* in some cheeses may reduce the risk of cheese spoilage by clostridial species through the production of antibacterial metabolites (Fatichenti et al. 1983). In certain milk products yeasts contribute to the fermentation process. Liquid milk products which derive some of their characteristic flavour from the activity of fermenting yeasts are kefir and koumiss. The yeast microflora of kefir has been described by several investigators (Marshall et al. 1984: Engel et al. 1986; Marshall 1986; Rohm & Lehner 1990).

There is little published information on the levels and species of yeasts in retail cheeses (Fleet 1990). In a survey of imported European and North American cheeses, Nakase & Komagata (1977) reported that 5 out of 12 samples had yeast counts of 10^6–10^7 cells/g. *Debaryomyces hansenii* was the species most frequently isolated from ripened cheeses while *Yarrowia lipolytica* dominated in non-ripened cheeses. Eliskases-Lechner & Ginzinger (1995) noted 12 different yeast species among 395 isolates from cheese surfaces and brine, with *Debaryomyces hansenii*, *Geotrichum candidum*, and *Yarrowia lipolytica* appearing to be the most common species. The yeasts of cheese brines were considered in detail by Seiler & Busse (1990).

A combination of physiological and morphological characteristics have traditionally been used in taxonomic studies of cheese yeasts (Wickerham 1951; van der Walt & Yarrow 1984; Barnett et al. 1990). The tests – fermentation of carbon compounds, oxidative degradation (assimilation) of carbon and nitrogen compounds, vitamin requirements, temperature tolerance – were used to characterize strains and computer assisted identifications based on them are available (e.g. Barnett et al. 1996). However, there is a lot of evidence that such tests are not satisfactory for the delineation and identification of yeast species. The genetic basis for many of these traditional tests is either unknown or known to be controlled by a single or a few genes that do not appear to be of phylogenetic significance (Scheda & Yarrow 1966, 1968; Price et al. 1978; Kurtzman et al. 1983). In addition, some tests show a high degree of variability (e.g. fermentation of glucose in *Debaryomyces hansenii*) and hence cannot be relied upon for identification purposes (Barnett et al. 1990; Rohm et al. 1992; Prillinger et al. 1996).

To overcome the problems outlined above we used genotypic approaches for correct species identification. Random amplified polymorphic DNA (RAPD)-PCR analysis was found to be a simple and highly sensitive method for differentiating species and can be used instead of nuclear DNA-nuclear DNA hybridization experiments for species identification (Welsh & McClelland 1990; Williams et al. 1990; Messner et al. 1994; Molnár et al. 1995, 1996; Cooke et al. 1996). We have checked all of the phentotypic identifications of yeasts from different cheeses by examining the appropriate type strains using RAPD-PCR. The ubiquinone and the monosaccharide patterns of purified yeast cell walls (Dörfler 1990; Prillinger et al. 1993, 1996) were used to sort out the identity of strains found to give conflicting results when the genotypic and phenotypic data were compared.

Materials and methods

Yeast strains

The test strains, which were obtained from the 'Bundesanstalt für Alpenländische Milchwirtschaft, Rotholz,' Austria, were isolated from various dairy products. The yeasts are maintained in the culture collection of the Institute of Applied Microbiology, Vienna (VIAM) (Table 1; HA = 'Hefe Ascomycet', HB = 'Hefe Basidiomycet'. Designations of the VIAM.).

Yeast isolation and identification on the basis of physiological properties

The yeasts were isolated and characterized according to Rohm et al. (1992). A compilation of the physiological data investigated is given in Table 2. The strains were identified using the computer program of Barnett et al. (1985). Additional biochemical tests and morphological criteria (i.e. budding/splitting of cells and formation of pseudomycelium, true hyphae or arthroconidia) were taken into account, when strains gave poor identification scores.Traditional physiological characterization was performed at the 'Bundesanstalt für Alpenländische Milchwirtschaft, Rotholz', Austria. The main goal of these investigations was to

Table 1. Yeast strains

Strains	Source*	Computer-assisted identification after Barnett et al. (1985)	Species identification using molecular methods
HA 754	7, Austria	*1. Candida butyrii* *(2. Candida* sp.*)*	?
HA 755	1, Austria	*Candida zeylanoides*	*Candida zeylanoides*
HA 756	1, Austria	*Candida catenulata*	*Candida catenulata*
HA 1032	3, Austria	*Candida catenulata*	*Candida catenulata*
HA 758	1, Austria	*1. Candida inconspicua* *(2. Pichia* sp.*)*	*Candida inconspicua*
HA 759	4, Austria	*1. Candida norvegica* *(2. Williopsis* sp.*)*	?
HA 760	5, Austria	*Debaryomyces hansenii*	*Debaryomyces hansenii*
HA 761	4, Austria	*Debaryomyces hansenii*	*Debaryomyces hansenii*
HA 762	4, Germany	*1. Debaryomyces hansenii* *(2. Cryptococcus laurentii)*	*Debaryomyces hansenii*
HA 763	5, France	*Debaryomyces hansenii* *(2. Cryptococcus laurentii)*	*Debaryomyces hansenii*
HA 764	5, Italy	*1. Debaryomyces hansenii* *(2. Cryptococcus laurentii)*	*Debaryomyces hansenii*
HA 765	5, Italy	*1. Debaryomyces hansenii* *(2. Cryptococcus laurentii)* *(3. Leucosporidium scottii)* *(4. Rhodotorula glutinis)*	*Debaryomyces hansenii*
HA 766	4, France	*1. Debaryomyces hansenii* *(2. Candida fennica)* *(3. Candida sake)* *(4. Sporidiobolus pararoseus)*	*Debaryomyces hansenii*
HA 767	3, Austria	*1. Debaryomyces hansenii* *(2. Debaryomyces marama)* *(3. Cryptococcus albidus)*	*Debaryomyces fabyri*
HA 768	1, Austria	*Debaryomyces hansenii*	?
HA 769	1, Austria	*1. Debaryomyces hansenii* *(2. Cryptococcus* sp.*)* *(3. Leucosporidium scottii)*	*Debaromyces hansenii*
HA 770	1, Austria	*1. Debaryomyces hansenii* *(2. Cryptococcus* sp.*)*	?
HA 771	1, Austria	*1. Debaryomyces hansenii* *(2. Sporidiobolus pararoseus)* *(3. Candida sake)* *(4. Cryptococcus laurentii)* *(5. Leucosporidium scottii)*	*Debaryomyces hansenii*
HA 772	1, Austria	*1. Debaryomyces hansenii* *(2. Cryptococcus* sp.*)*	*Debaryomyces hansenii*
HA 773	1, Austria	*1. Debaryomyces hansenii*, *(2. Cryptococcus* sp.*)*	?
HA 774	2, Austria	*1. Debaryomyces hansenii* *(2. Clavispora lusitaniae)* *(3. Candida haemulonii)*	*Clavispora lusitaniae*
HA 775	4, Germany	*Debaryomyces hansenii*	*Debaryomyces hansenii*
HA 776	WIESBY SN3 D-25893 Niebüll	*Geotrichum candidum*	*Geotrichum candidum*

Table 1. Continued.

HA 777	4, Denmark	*Geotrichum* sp.	*Geotrichum candidum*
HA 778	4, Austria	*Geotrichum* sp.	*Geotrichum candidum*
HA 779	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 780	6, Germany	*Geotrichum* sp.	*Geotrichum candidum*
HA 781	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 782	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 783	4, Austria	*Geotrichum* sp.	*Geotrichum candidum*
HA 784	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 785	1, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 786	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 787	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 788	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 789	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 790	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 791	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 792	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 793	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 794	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 795	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 796	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 797	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 798	6, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 799	2, France	*Geotrichum* sp.	*Geotrichum candidum*
HA 1011	3, Austria	*Geotrichum* sp.	*Geotrichum candidum*
HA 801	1, Austria	*Geotrichum* sp.	*Geotrichum candidum*
HA 802	7, Austria	*1. Issatchenkia orientalis* *(2. Issatchenkia occidentalis)* *(3. Issatchenkia scutulata)* *(4. Candida ethanolica)* *(5. Pichia membranifaciens)*	*Issatchenkia orientalis*
HA 803	3, Austria	*1. Issatchenkia orientalis* *(2. Issatchenkia occidentalis)* *(3. Pichia membranifaciens)* *(4. Candida ethanolica)*	*Issatchenkia orientalis*
HA 804	3, Austria	*1. Issatchenkia orientalis* *(2. Issatchenkia occidentalis)* *(3. Candida ethanolica)* *(4. Pichia membranifaciens)*	*Issatchenkia orientalis*
HA 805	3, Austria	*1. Issatchenkia orientalis* *(2. Issatchenkia occidentalis)* *(3. Candida ethanolica)* *(4. Pichia membranifaciens)*	*Issatchenkia orientalis*
HA 806	3, Austria	*1. Issatchenkia orientalis* *(2. Issatchenkia occidentalis)* *(3. Candida ethanolica)* *(4. Pichia membranifaciens)*	*Issatchenkia orientalis*
HA 807	5, France	*1. Kluyveromyces marxianus* *(2. Yarrowia lipolytica)* *(3. Candida* sp.*)* *4. Pichia* sp.*)*	*Yarrowia lipolytica*
HA 808	5, France	*1. Kluyveromyces marxianus* *(2. Cryptococcus albidus)* *(3. Debaryomyces hansenii)*	*Debaryomyces hansenii*
HA 809	5, France	*Kluyveromyces marxianus*	*Kluyveromyces lactis*

Table 1. Continued.

HA 810	7, Austria	*Kluyveromyces marxianus*	*Kluyveromyces marxianus*
HA 811	3, Austria	*Kluyveromyces marxianus*	*Kluyveromyces lactis*
HA 812	3, Austria	*Kluyveromyces marxianus*	*Kluyveromyces marxianus*
HA 813	3, Austria	*Kluyveromyces marxianus*	*Kluyveromyces lactis*
HA 814	5, Italy	*Pichia anomala*	*Pichia anomala*
HA 815	5, Italy	*1. Pichia fermentans* *(2. Pichia membranifaciens)*	*Pichia fermentans*
HA 816	3, Austria	*1. Pichia membranifaciens* *(2. Pichia kluyveri)* *(3. Issatschenkia orientalis)*	*Issatschenkia orientalis*
HA817	5, Italy	*1. Saccharomyces cerevisiae* *(2. Saccharomyces dairensis)*	*Saccharomyces unisporus*
HA 818	5, Italy	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
HA 819	5, Italy	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
HA 820	5, Italy	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
HA 821	5, Italy	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
HA 822	5, Italy	*1. Torulaspora delbrueckii* *(2. Candida sake)* *(3. Sporobolomyces roseus)*	*Torulaspora delbrueckii*
HA 823	3, Austria	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
HA 824	1, Austria	*1. Torulaspora delbrueckii* *(2. Saccharomyces* sp.*)* *(3. Candida milleri)*	*Yarrowia lipolytica*
HA 826	2, Austria	*1. Yarrowia lipolytica* *(2. Candida silvae)*	*Yarrowia lipolytica*
HA 827	4, Germany	*Yarrowia lipolytica*	*Yarrowia lipolytica*
HA 828	4, Germany	*1. Yarrowia lipolytica* *(2. Pichia pini)* *(3. Candida zeylanoides)*	*Yarrowia lipolytica*
HA 829	4, Germany	*1. Yarrowia lipolytica* *(2. Candida zeylanoides)*	*Yarrowia lipolytica*
HA 830	5, Italy	*1. Yarrowia lipolytica* *(2. Kluyveromyces marxianus)*	*Yarrowia lipolytica*
HA 831	4, France	*Yarrowia lipolytica*	*Yarrowia lipolytica*
HA 832	3, Austria	*Yarrowia lipolytica*	*Yarrowia lipolytica*
HA 833	1, Austria	*1. Yarrowia lipolytica* *(2. Candida zeylanoides)* *(3. Kluyveromyces marxianus)* *(4. Sporidiobolus salmonicolor)*	*Yarrowia lipolytica*
HA 834	1, Austria	*1. Yarrowia lipolytica* *(2. Kluyveromyces marxianus)*	*Yarrowia lipolytica*
HB 478	1, Austria	*1. Rhodotorula minuta* *(2. Rhodotorula mucilaginosa)* *(3. Rhodotorula glutinis)*	?
HB 479	7, Austria	*Trichosporon beigelli*	?
HB 480	3, Austria	*Trichosporon beigelli*	*Trichosporon sp.*
HB 481	4, Austria	*Trichosporon beigelli*	*Trichosporon sp.*
HB 704	7, Austria	*Trichosporon beigelli*	?
HB 483	7, Austria	*Trichosporon beigelli*	?

*The brine and the austrian cheese samples were obtained directly from the cheese plants. The samples from other countries were purchased locally from retail sources.
1 = smear ripened semi-hard cheeses: St. Nectaire, Tilsit.
2 = smear ripened soft cheeses: Boisange, Ejosses Bourgogne, Fra Dominique, Langres de Remparts, Limburger, Livarot Levasseur, Petit pont l'eveque Levasseur, Saint Albray, Saint Morgan, Tenoir Bridel.
3 = 'Graukäse' (a regional sour cheese variety).
4 = surface- and internal-mould ripened cheeses: Bavaria Blu, Bella Monte, Cambozola, Castello Blanc, Troubadur, Weissensteiner.
5 = blue cheeses: Bleu d'Àuvergne, Bleu de Jura, Gorgonzola, Österkron, Roquefort.
6 = surface-ripened mould cheeses: Bonifaz, Camembert, Fine des Pres, Supreme.
7 = brine.

Table 2. Compilation of the physiological data of all strains which were correctly identified phenotypically

	Yeast species											
	Cc	Ci	Cz	Dh	G	Io	Km	Pa	Pf	Sc	Td	Yl
Fermentation												
D-Glucose	–	–	–	50	17	+	+	+	+	+	–	–
D-Galactose	nd	–	nd	33	21	–	+	–	–	+	nd	–
Assimilation												
D-Galactose	+	–	+	+	+	–	+	+	–	80	+	22
L-Sorbose	–	–	+	+	84	–	–	–	–	20	–	33
D-Glucosamine	+	+	+	82	4	–	50	–	+	–	–	11
D-Ribose	–	–	–	–	–	–	+	–	–	–	–	22
D-Xylose	+	–	–	36	+	–	50	+	+	–	–	11
L-Arabinose	–	–	–	+	–	–	50	–	–	–	–	11
D-Arabinose	–	–	–	18	–	–	+	–	–	–	–	–
L-Rhamnose	–	–	–	64	–	–	–	–	–	–	–	–
Sucrose	–	–	–	91	–	–	+	+	–	80	+	–
Maltose	+	–	–	+	4	nd	–	+	–	+	–	33
Trehalose	+	–	+	+	–	–	–	+	–	20	+	11
Methyl α-D-glucoside	–	–	–	+	–	–	–	+	–	–	–	–
Cellobiose	–	–	–	+	–	–	–	+	–	–	–	–
Salicin	–	–	–	+	–	–	+	+	–	–	–	44
Arbutin	–	–	–	+	–	–	+	+	–	–	–	–
Melibiose	–	–	–	9	–	–	–	–	+	40	–	–
Lactose	–	–	–	+	–	–	+	–	–	–	–	–
Raffinose	–	–	–	91	–	–	+	–	–	80	+	–
Melezitose	–	–	–	+	–	–	–	+	–	–	–	–
Glycerol	+	+	+	+	+	60	50	+	+	–	–	+
Erythritol	–	–	–	18	–	–	–	+	–	–	–	+
Ribitol	+	–	+	+	16	–	–	+	–	–	–	44
Xylitol	–	–	–	55	–	–	+	–	–	–	–	–
L-Arabinitol	–	–	–	55	–	–	–	–	–	–	–	–
D-Mannitol	+	–	+	+	64	–	+	+	–	20	–	+
2-Keto-D-gluconate	–	–	+	+	28	–	–	–	–	–	+	33
DL-Lactate	+	+	–	+	96	+	+	+	+	+	+	+
Citrate	–	+	+	55	36	+	50	+	+	–	–	67
Nitrate	–	–	–	18	4	–	–	+	–	–	nd	–
Nitrite	–	–	–	9	20	nd	nd	+	–	–	–	50
Ethylamine	–	+	–	27	60	+	+	–	–	–	–	63
L-Lysine	+	+	–	73	92	+	+	+	+	–	–	+
Cadaverine	+	+	+	+	+	+	+	+	+	–	–	+
Creatine	50	–	–	9	4	–	–	–	–	–	–	–
Creatinine	–	–	–	27	4	–	–	–	–	–	–	–
Additional tests												
w/o Vitamins	–	–	–	27	80	+	–	+	–	–	–	–
w/o myo-Inositol	+	+	+	+	+	+	+	+	+	+	+	+
w/o Panthotenate	+	+	+	+	+	+	+	+	+	–	+	+
w/o Biotin	+	–	+	+	+	+	+	+	+	+	–	+
w/o Thiamin	–	–	+	91	+	+	+	+	–	60	+	22
w/o Biotin & Thiamin	–	–	+	82	+	+	+	+	–	80	–	44
w/o Pyridoxine	+	–	+	+	+	+	+	+	+	80	+	89
w/o Niacin	+	+	+	+	92	+	–	–	+	80	+	78
T 25 °C	+	+	+	+	+	+	+	+	+	+	+	+
T 30 °C	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. Continued.

T 35 °C	+	+	+	18	52	+	+	+	+	+	+	63
T 37 °C	+	+	–	9	8	+	+	+	+	+	–	–
T 42 °C	–	+	–	–	–	+	+	–	–	–	–	–
0.01% Cycloheximide	+	–	+	9	+	–	+	–	–	–	–	89
0.1% Cycloheximide	+	–	+	–	+	–	+	–	–	–	–	11
Urea hydrolysis	–	–	–	–	4	–	–	–	–	–	–	11
Pink colonies	–	–	–	–	–	–	–	–	–	–	–	–
Budding cells	+	+	+	+	–	+	nd	+	+	+	+	+
Fission cells	–	–	–	–	+	nd	nd	–	–	–	–	–
Filaments	+	+	–	–	+	+	+	+	+	80	+	89
Pseudohyphae	+	+	–	–	4	+	+	+	+	80	+	89
Septate hyphae	–	–	–	–	+	40	–	–	–	–	–	44

Cc: *Candida catenulata* HA 756, HA 1032; Ci: *Candida inconspicua* HA 758; Cz: *Candida zeylanoides* HA 755; Dh: *Debaryomyces hansenii* HA 760 - HA 767, HA 769, HA 771, HA 772, HA 775; *G. Geotrichum* sp. HA 777 - HA 801; Io: *Issatchenkia orientalis* HA 802 - HA 806; Km: *Kluyveromyces marxianus* HA 810, HA 812; Pa: *Pichia anomala* HA 814; Pf: *Pichia fermentans* HA 815; Sc: *Saccharomyces cerevisiae* HA 818 - HA 821, HA 823; Td: *Torulaspora delbrueckii* HA 824; Yl: *Yarrowia lipolytica* HA 826 - HA 834. The numbers show the percentage of the positive test results. +: 100%; '−0%; nd: not determined.

detect physiological variability within different species.

Diazonium blue B (DBB) test

The test was performed using modifications to the procedure described by van der Walt & Yarrow (1984). The strains were grown at least for 21 days at 25 °C on GYP agar (2%, w/v glucose, 0.5%, w/v yeast extract, 1%, w/v peptone, 2%, w/v agar). A few crystals of Fast Blue B-Salt (Serva 21270) were placed onto the yeast cultures, and ice-cold Tris-buffer (12.1 g Tris in 1 l H_2O bidest. pH 7.0) added; the appearance of a red colour within two minutes was recorded as a positive test reaction (basidiomycetous yeast). The reaction was repeated after incubating for approx. 16 hours at 55–60 °C as a control.

Analysis of the coenzyme Q system

Ubiquinone extraction was performed according to Messner et al. (1994). Purification of ubiquinones were carried out by preparative thin-layer chromatography (TLC; aluminum sheets silica gel 60 F_{254} pre-coated, 0.2 mm thickness; Merck 5554). Reverse-phase thin layer chromatography was employed to determine the prenyl side chain of the ubiquinone using Merck HPTLC $RP18F_{254}$ (Art. 13724) plates and a mixture of acetone-acetonitrile (80:20, v/v) as the developing solvent (Nakase & Suzuki 1985a).

Monosaccharide pattern of purified cell walls

The neutral sugar composition was determined after trifluoro-acetic acid hydrolysis according to the method of Dörfler (1990) with modifications as described in Messner et al. (1994).

Random Amplified Polymorphic DNA–Polymerase Chain Reaction (RAPD-PCR) analysis

The extraction of DNA from the yeast cells was performed by using the hexadecyltrimethylammoniumbromide (CTAB) method (Messner et al. 1994). PCR conditions and separation of RAPD-PCR fragments were carried out after Molnár et al. (1995). Three of the following primers were used in all cases: M13: GAGGGTGGCGGTTCT (Meyer et al. 1991); decamer 1: ACGGTCTTGG (Schäfer & Wöstemeyer 1992); decamer 2: TGCCGAGCTG (Caetano-Anolles et al. 1992); decamer 3: TGCAGCGTGG, and decamer 4: GGGTAACGCC. The primers were synthesized by Codon Genetic Systems (Vienna, Austria) using a model 392 DNA synthesizer (Applied Biosystems, Foster City, California). The levels of similarity between individual lanes were calculated as described by Nei & Li (1979) and the genetic variability values as described in Molnár et al. (1995).

Table 3. Compilation of the physiological data of all strains which were incorrectly identified phenotypically

	Yeast species												
	Cb	Cn	Dh[1]	Dh[2]	Dh[3]	Km[1]	Km[2]	Km[3]	Pm	Sc	Td	Rm	Trb
Fermentation													
D-Glucose	+	+	nd	+	+	–	–	+	+	+	+	–	–
D-Galactose	nd	–	nd	nd	nd	–	–	+	–	–	–	–	–
Assimilation													
D-Galactose	+	–	+	+	+	–	+	+	–	+	+	+	+
L-Sorbose	+	+	+	+	+	+	+	–	–	–	–	+	80
D-Glucosamjne	+	–	+	+	+	–	–	–	–	–	–	–	+
D-Ribose	+	–	+	66	–	+	–	–	–	–	–	+	+
D-Xylose	+	+	+	+	–	–	–	–	–	–	+	+	+
L-Arabinose	+	–	+	+	–	–	+	–	–	–	–	+	+
D-Arabinose	+	–	–	66	–	–	–	–	–	–	–	+	80
L-Rhamnose	–	+	–	+	+	–	–	–	–	–	–	–	20
Sucrose	+	–	+	+	+	–	+	+	–	–	+	+	+
Maltose	+	–	+	+	+	–	+	+	–	–	+	+	+
Trehalose	+	–	+	+	+	–	+	+	–	–	+	+	+
Methyl α-D-glucoside	+	–	+	+	+	–	+	+	–	–	+	–	+
Cellobiose	+	+	+	+	–	–	+	+	–	–	–	–	+
Salicin	+	+	+	+	–	–	+	+	–	–	–	+	20
Arbutin	+	+	+	+	+	–	+	+	–	–	–	+	+
Melibiose	–	+	–	33	–	–	–	–	–	+	–	–	–
Lactose	+	–	+	+	–	–	+	+	–	–	–	–	+
Raffinose	–	–	+	66	–	–	+	+	–	–	–	+	–
Melezitose	+	–	+	+	+	–	+	+	–	–	+	+	40
Glycerol	+	+	+	+	+	+	+	+	–	–	–	+	–
Erythritol	+	–	+	+	–	+	–	–	–	–	–	–	80
Ribitol	+	–	+	+	+	+	+	–	–	–	–	+	–
Xylitol	+	–	+	+	–	–	–	66	–	–	–	+	–
L-Arabinitol	+	–	+	66	+	–	+	–	–	–	–	–	–
D-Mannitol	+	+	+	+	+	–	+	+	–	–	+	+	60
2-Keto-D-gluconate	–	–	+	+	+	–	+	+	–	–	+	–	+
DL-Lactate	–	+	+	+	+	+	+	+	+	–	–	–	+
Citrate	+	+	+	–	+	+	–	–	+	–	–	+	–
Nitrate	–	+	–	–	–	–	–	–	–	–	+	–	–
Nitrite	+	+	+	66	–	–	+	–	–	–	+	–	40
Ethylamine	+	+	–	33	+	+	+	–	+	–	–	+	60
L-Lysine	+	+	+	33	–	+	+	+	+	–	+	+	50
Cadaverine	–	+	+	+	+	+	+	+	+	–	+	+	+
Creatine	–	+	+	–	–	–	–	–	+	–	–	–	–
Creatinine	–	+	+	–	–	–	–	–	–	–	–	–	–
Additional tests													
w/o Vitamins	–	–	–	–	–	–	–	–	–	–	+	–	–
w/o myo-Inositol	+	+	+	+	+	+	–	+	+	+	+	+	+
w/o Panthotenate	+	+	+	+	+	+	–	66	+	–	+	+	80
w/o Biotin –	+	–	66	–	+	–	+	+	+	+	+	60	
w/o Thiamin –	+	+	+	–	+	–	+	+	–	+	+	–	
w/o Biotin & Thiamin	–	+	–	66	–	+	–	+	+	–	+	–	–
w/o Pyridoxine	–	+	+	+	–	+	–	+	+	+	+	+	+
w/o Niacin	–	+	+	+	+	+	–	–	–	–	–	+	+
T 25 °C	+	+	+	+	+	+	+	+	+	+	–	+	+
T 30 °C	+	+	+	+	+	+	+	66	+	+	–	+	+

Table 3. Continued.

T 35 °C	+	–	–	–	+	+	–	50	+	–	–	+	+
T 37 °C	+	–	–	–	+	–	–	–	+	–	–	–	25
T 42 °C	–	–	–	–	–	–	–	–	+	–	–	–	–
0.01% Cycloheximide	–	–	–	–	–	+	–	+	–	+	–	+	+
0.1% Cycloheximide	–	–	–	–	–	+	–	+	–	+	–	–	–
Urea hydrolysis	–	–	–	–	nd	–	–	–	–	–	–	nd	+
Pink colonies	–	–	–	–	–	–	–	–	–	–	–	+	–
Budding cells	+	+	+	+	+	+	+	+	–	+	+	nd	+
Fission cells	–	–	–	–	–	–	–	–	–	–	–	–	33
Filaments	+	–	–	–	–	+	–	33	–	–	–	–	+
Pseudohyphae	+	–	–	–	–	+	–	33	–	–	–	–	25
Septate hyphae	–	–	–	–	–	–	–	–	–	–	–	–	75

Cb: *Candida butyrii* HA 754; Cn: *Candida norvegica* HA 759; Dh: *Debaryomyces hansenii*[1]: HA 767, [2]: HA 768, HA 770, HA 773, [3]: HA 774; Km: *Kluyveromyces marxianus*[1]: HA 807, [2]: HA 808, [3]: HA 809, HA 811, HA 813; Pm: *Pichia membranifaciens* HA 816; Sc: *Saccharomyces cerevisiae* HA 817; Td: *Torulaspora delbrueckii* HA 822; Rm: *Rhodotorula minuta* HB 478; Trb: *Trichosporon beigelii* HB 479 - HB 481, HB 704, HB 483.
The numbers show the percentage of the positive test results. +: 100%; – : 0%; nd: not determined.

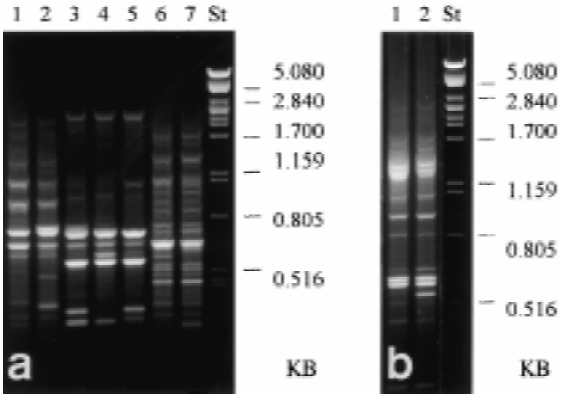

Figure 1. RAPD patterns of strains primed by M13 (GAGGGTGGCGGTTCT). (a) Lanes 1 and 2, *Clavispora lusitaniae*, HA 774 and CBS 6936^T; lanes 3–5, *Pichia anomala*, HA 1030, HA 814, and CBS 5759^T; lanes 6 and 7, *Yarrowia lipolytica*, HA 807 and CBS 6124^T. (b) Lanes 1 and 2, *Candida sake*, HA 822 and CBS 159^T. Lanes St contain lambda DNA digested with *Pst* I. Note the similarity in the patterns of the yeast strains belonging to the same species.

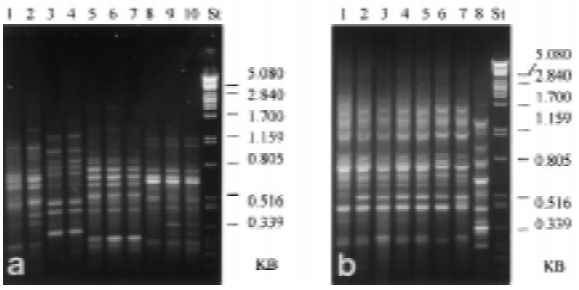

Figure 2. RAPD patterns of strains primed by decamer 2 (TGCCGAGCTG). (a) Lanes 1 and 2, *Saccharomyces bayanus* CBS 380^T and HA 1031; lanes 3 and 4, *Saccharomyces unisporus*, CBS 398^T and HA 817; lanes 5–7, *Candida catenulata* CBS 565^T, HA 800, and HA 757; lane 8, *Geotrichum javanense*, CBS 182.33^T; lanes 9 and 10, *Geotrichum candidum* HA 776, and HA 798. (b) Lanes 1–7, *Issatchenkia orientalis*, HA 802 - HA 806, CBS 5147^T, and HA 816; lane 8, *Pichia membranifaciens* CBS 107^T. Lanes St contain lambda DNA digested with *Pst* I. Note the similarity in the patterns of yeast strains belonging to the same species. HA 816 was phenotypically identified as *Pichia membranifaciens* and molecularly as *Issatchenkia orientalis*.

Results

The results of the phenotypic and genotypic identification of the test strains are summarized in Table 1. Most of the strains were correctly identified using the PC computer program of Barnett et al. (1985). A compilation of the physiological data of all of the strains which were correctly identified phenotypically is given in Table 2. Physiological data from strains which were identified genotypically but which gave a different phenotypic identification are shown in Table 3. From the 86 investigated strains, 39 species were confirmed using the molecular approach (Table 1). The strains identified using the physiological tests as *Pichia anomala* (Figure 1a, lane 4), *Pichia fermentans*, *Saccharomyces cerevisiae* (5 isolates), *Torulaspora delbrueckii* (1 isolate), *Yarrowia lipolytica*, *Candida catenulata* (Figure 2a, lanes 5–7), *C. inconspicua*, *C. zeylanoides*, and *Issatchenkia orientalis* (Figure 2b, lanes 1–6) were confirmed genotypically on the basis of the RAPD-PCR data.

Strains phenotypically identified as Saccharomyces cerevisiae. Six of the test strains were phenotypically identified as *S. cerevisiae* (Table 1). Five out of the 6 isolates (HA 818-821, and HA 823) showied identical or single differences to the data found in the description of the species. All of these strains were identified in the RAPD-PCR analysis and hence are *bona fide* members of *S. cerevisiae*. They show 84–95% similarity with the type strain of the species, and 85–100% similarity with each other in the RAPD-PCR analysis. The remaining organism, strain HA 817, showed 4 phenotypic discrepancies from the type strain of *S. cerevisiae* and was also found to be related to *S. dairensis* using the computer-assisted identification procedure. However, strain HA 817 was unambiguously identified as *S. unisporus* in the RAPD-PCR analysis showing a similarity of 96% to the type strain of this species (Figure 2a, lanes 3 and 4). Strain HA 817 differed in 8 phenotypic tests from the standard description of *S. unisporus*.

Strains phenotypically identified as Torulaspora delbrueckii. Two of the test strains were identified as *T. delbrueckii* based on the physiological data. Strain HA 824 showed a similarity of 96% with the type strain of this species in the RAPD-PCR analysis. In contrast, the second organism, strain HA 822, was not conspecific with *T. delbrueckii* according to the results obtained from the RAPD-PCR analysis. This organism, which was a Q-9 yeast, was assigned to three possible species, *T. delbrueckii* (Q-6), *Candida sake* (Q-9), and *Sporobolomyces roseus* (Q-10) using the computer-assisted identification scheme. The strain was readily identifiable as *C. sake* in the RAPD-PCR analysis showing a similarity value of 96% with the type strain (Figure 1b). It showed 3 differences from the species description of *T. delbrueckii*, and 5 from that of *C. sake* (Table 3).

Strains phenotypically identified as Issatchenkia orientalis. The 5 organisms identified as *I. orientalis* (strains HA 802-806) represented the most characteristic group amongst the yeast isolates. All the strains were identified readily using physiological characters; the RAPD-PCR analysis reinforced these results as the isolates showed 88-100% similarity with the type strain of *I. orientalis* (Figure 2b, lanes 1–6).

An additional isolate, strain HA 816, was also unambiguous identified as *I. orientalis* in the RAPD-PCR analysis showing a similarity to the type strain of 97% (Figure 2b, lanes 6, 7, and 8). However, on the basis of the phenotypic data the organism was described as *Pichia membranifaciens*, showing 2 differences in the physiological tests from the description of this species (Table 1). Strain HA 816 showed a Q-7 ubiquinone which is characteristic of *Issatchenkia orientalis.*

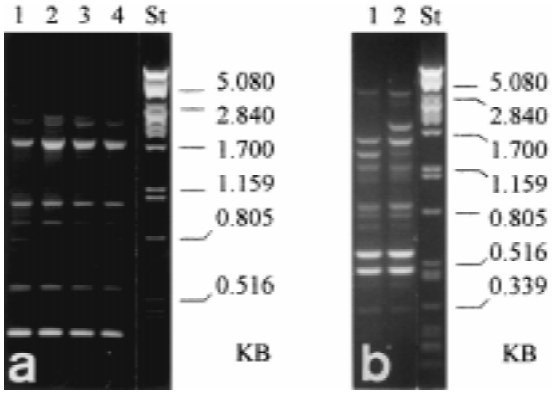

Figure 3. RAPD patterns of strains primed by decamer 3 (TGCAGCGTGG). (a) Lanes 1–4, *Kluyveromyces lactis* CBS 683^T, HA 809, HA 811, and HA 813. (b) Lanes 1 and 2, *Debaryomyces hansenii*, CBS 767^T and HA 808. Lanes St contain lambda DNA digested with *Pst* I. Note the similarity in the patterns of the yeast strains belonging to the same species.

Strains phenotypically identified as Kluyveromyces marxianus. In the case of *Kluyveromyces marxianus* and *K. lactis* (Figure 3a) the molecular approach allowed us to make accurate species identifications in the light of work by Molnár et al. (1996). We investigated 7 strains belonging to the species *K. marxianus* according to the identification program of Barnett et al. (1985). Not surprisingly *K. marxianus* (strains HA 810 and HA 812) as well as *K. lactis* (strains HA 809, HA 811, and HA 813) were represented among the isolates. These 5 strains showed up to two phenotypic differences from the species description of *K. marxianus*. The DNA similarities with the appropriate type strains were 99-100% in the case of *K. marxianus* and 92–100% with respect to *K. lactis* (Figure 3a).

The remaining 2 organisms (strains HA 807 and HA 808) showed more phenotypic differences from *K. marxianus*. Strain HA 807 showed 5 differences from *K. marxianus* in the physiological tests. The species or genera suggested by the identification program were *K. marxianus* (Q-6 yeast), *Yarrowia lipolytica* (Q-9 yeast), *Candida* sp. (Q-6 up to 9), and *Pichia sp*. (Q-7 up to 9). Strain HA 807 proved to be a Q-9 yeast and was conspecific with the type strain of *Y. lipolytica* (95% relatedness) in the RAPD-PCR analysis (Fig-

ure 1a, lanes 6 and 7). The number of the phenotypic characters not agreeing with the species description of *Y. lipolytica* was 7.

Strain HA 808 was physiologically identified as*K. marxianus* though it showed 7 differences from the species description of that taxon. The third mentioned possible species, *Debaryomyces hansenii*, differed in 9 test results from the investigated strain. The three possible species belong to three different ubiquinone type groups (Table 4). *Kluyveromyces marxianus* has a Q-6, *Cryptococcus albidus* a Q-10, and *D. hansenii* a Q-9 ubiquinone system. Our questionable strain turned out to be a Q-9 yeast. The RAPD-PCR pattern similarity with the type strain of *D. hansenii* was 92% corresponding to a genetic variability of 8% (Figure 3b).

Strain phenotypically identified as Pichia anomala. Strain HA 814 was identified as *P. anomala* on the basis of the phenotypic tests. It differed from the standard description of the species by a single test result (Table 2). This identification was underpinned in the RAPD-PCR analysis as strain HA 814 showed a similarity with the type strain of the species of 82% (Figure 1a, lanes 4 and 5).

Strain phenotypically identified as Pichia fermentans. Strain HA 815 showed 2 differences in the phenotypic tests compared with the standard description of *P. fermentans*. The RAPD-PCR analysis corroborated this result as the isolate showed a similarity of 80% with the type strain of the species.

Identification of contaminants. Five of the yeast isolates from Rotholz appeared to be contaminated by another yeast strain following morphological and physiological characterization. The contaminants were identified on the basis of the DBB-test, urease activity and on the ubiquinone and yeast cell wall sugar data (Table 4). *Candida catenulata* strain HA 1032 was contaminated with an unidentifiable *Trichosporon* species (strain HB 482), *Geotrichum candidum* strain HA 1011 by *C. catenulata* strain HA 800 (Figure 2a, lane 6), organisms phenotypically identified as *Trichosporon beigelii* (strains HB 479, HB 704, HB 483) with ascomycetous yeasts belonging to the species *Pichia anomala* (strain HA 1030; Figure 1a, lane 3), *C. catenulata* (strain HA 757, Figure 2a, lane 7), and *Saccharomyces bayanus* (strain HA 1031; Figure 2a, lane 2). The original

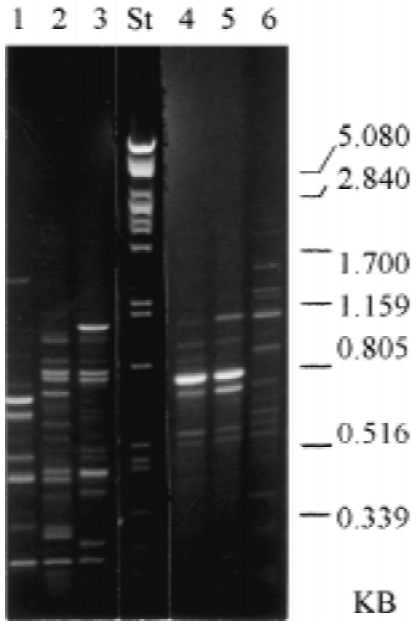

Figure 4. RAPD patterns of strains primed by decamer 3 (TGCAGCGTGG) (lanes 1–3), and decamer 1 (ACGGTCTTGG) (lanes 4–6). Lanes 1 and 6, *Debaryomyces hansenii*, CBS 767^T; lanes 2 and 5 *Debaryomyces fabryi*, CBS 789^T; lanes 3 and 4, *Debaryomyces fabryi*, HA 767. Lane St containes lambda DNA digested with *Pst* I. Note the similarity in the patterns of the yeast strains belonging to the same species. The high genetic dissimilarity value allows the separation of *Debaromyces hansenii* and *Debaryomyces fabryi* at the specific level.

Trichosporon beigelii strains HB 479 and HB 483 were lost.

Strains phenotypically identified as Debaryomyces hansenii. Based on our RAPD-PCR investigations the hitherto distinguished varieties *Debaryomyces hansenii* (Zopf) Lodder et Kreger-van Rij var. *hansenii* and *Debaryomyces hansenii* var. *fabryi* (Ota) Nakase et Suzuki were separated genotypically at the species level. The estimated similarity value between the individual patterns was 37% (Figure 4, lanes 1, 2, 5 and 6). Similarity values within the range of 30–50% are considered to be characteristic for closely related species (Messner et al., 1994; Molnàr et al., 1995; Cooke et al., 1996). We propose, therefore, to reinstate these taxa as two genotypically distinct species:

Debaryomyces hansenii (Zopf) Lodder et Kreger-van Rij

Basionym:	*Saccharomyces hansenii* Zopf Ber. Deut. Bot. Ges. 7: 94. 1889.
Typus:	CBS 767

Table 4. Cell wall monosaccharide composition and major ubiquinone system

Yeast isolate	Cell wall monosaccharide composition (mol%)					UBI
	GLC	MAN	GAL	XYL	RHA	
HA754						7
HB482	83	9		8		9
HA 759						7
HA767						9
HA 768						9
HA 770						9
HA773						9
HA774						8
HA 800						9
HA 807						9
HA 808						9
HA 816						7
HA822						9
HB 478	22	68	8		2	10
HB 479	53	47				7
HB 480	78	11		11		9
HB 481	81	10		9		9
HA 757	49	51				9
HB 483	48	52				6

GLC: Glucose, MAN: Mannose, GAL: Galactose, XYL: Xylose, RHA: Rhamnose, UBI: major ubiquinone system.

Debaryomyces fabryi Ota

Basionym: *Debaryomyces fabryi* Ota. Dermatol. Wochenschrift 78: 287. 1924.

Typus: CBS 789

We investigated 16 strains physiologically identified as *D. hansenii*. The physiological tests showed 1–6 differences from the description of this species. In two cases (strains HA 765, HA 769) the genus-specific feature, the inability to assimilate nitrate, also featured amongst the discrepancies (Table 2). However, 11 out of the 16 strains were correctly identified using the physiological methods. Strains HA 765 and HA 769, and one of the organisms showing 6 divergent tests were also identified as *D. hansenii* strains. These conclusions were underpinned by the results of the RAPD-PCR analysis. The genetic variability between the test strains and the type strain of the species *D. hansenii* was 94–100%.

The remaining 5 strains belonged to 3 distinct species according to the RAPD-PCR results. The first organism, strain HA 774 was easily identified following the ubiquinone analysis. This strain showed 6 differences from the species description of *D. hansenii*, and seven differences from the description of *Clavispora lusitaniae*. Members of these species differ in their ubiquinone systems since *D. hansenii* has a Q-9 and *C. lusitaniae* a Q-8 system, respectively. Our strain proved to be a Q-8 yeast, and – using RAPD-PCR analysis – *C. lusitaniae*. It showed a 76% similarity with the type strain of *C. lusitaniae* (Figure 1a, lanes 1 and 2). The remaining four strains including strain HA 767, and strains HA 768 = HA 770 = HA 773, belonged to two species. These strains were identified physiologically as *D. hansenii* with only 2–3 divergent tests (Table 3). In our investigations all of these strains proved to be DBB negative, Q-9 yeasts. All of the type strains of the Q-9 yeasts used in the investigation, were analysed using the RAPD-PCR procedure namely, *D. hansenii*, *D. fabryi*, *D. marama*, *Candida catenulata*, *C. fennica*, *C. intermedia*, *C. parapsilosis*, *C. sake*, *C. zeylanoides*, and*Yarrowia lipolytica*. Since none of these organisms were genotypically conspecific with strains HA 768, HA 770, and HA 773 they remained unidentifiable using our methods. However, strain HA 767 proved to be *D. fabryi* as it showed an 84% sim-

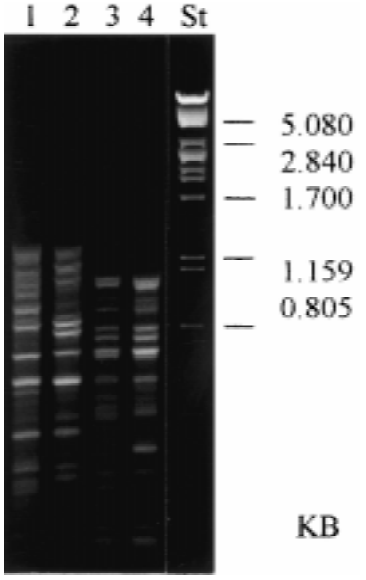

Figure 5. RAPD patterns of strains primed by decamer 2 (TGC-CGAGCTG). Lane 1, *Geotrichum candidum*, HA 776; lane 2, *Geotrichum javanense*, CBS 182.33^T; lanes 3 and 4, *Galactomyces geotrichum*, CBS 772.71^T and CBS 773.71. Lane St containes lambda DNA digested with *Pst* I. Note the similarity in the patterns of the strains belonging to *Geotrichum candidum* (lanes 1 and 2) and the strains belonging to *Galactomyces geotrichum* (lanes 3 and 4). The high genetic dissimilarity value allows the separation of the two species.

ilarity with the type strain in the RAPD-PCR analysis (Figure 4, lanes 2 through 5).

Strains phenotypically identified as Yarrowia lipolytica. Nine organisms (strains HA 826-834) were identified as *Y. lipolytica* on the basis of the phenotypic data; these strains differed by 1-6 tests from the description of this species. All of the strains were unequivocally identified as *Y. lipolytica* in the RAPD-PCR analysis showing 96–99% similarity against the type strain and 96–100% similarity amongst themselves.

Strains phenotypically identified as Geotrichum sp. We were able to distinguish the type strain of *Galactomyces geotrichum* (E.E. Butler & L.J. Petersen) Redhead & Malloch (Anamorph: *Geotrichum candidum* Link: Fries; CBS 772.71^T) from the type strain of *Geotrichum javanense* (CBS 182.33^T, obtained as *G. candidum*) in the RAPD-PCR analysis (Figure 5, lanes 2 and 3). This latter strain was isolated in 1933 from yogurt in Italy. The two strains showed only 20% DNA relatedness in the RAPD-PCR analysis. According to Messner et al. (1994) an 80% genome dissimilarity is sufficient for species distinction. We propose that the name *G. candidum* be conserved for the anamorphic yeast species commonly found in dairy and food products as shown by our results and the importance of this organism in food technology. In contrast, *Galactomyces geotrichum* was isolated from soil and seemed to be a very rare species (Smith et al. 1995). A new anamorph has to be established for *G. geotrichum*. We investigated 25 strains isolated from dairy products which were physiologically identified as *Geotrichum* sp. (strains HA 777–HA 801). All of these organisms, except for strain HA 800, were conspecific with a commercially available *Geotrichum candidum* culture (HA 776; WIESBY, SN3) in the RAPD-PCR analysis. The genetic variability among the strains were 0–10% corresponding to 90–100% RAPD-PCR pattern similarity. These strains showed 85–100% similarity with *Geotrichum javanense* (CBS 182.33^T) (Figure 2a, lanes 8–10; Figure 5, lanes 1 and 2), but only 20–25% similarity with the type strain of *Galactomyces geotrichum* (CBS 772.71^T) (Figure 5, lanes 1 and 3).

Strain phenotypically identified as Candida zeylanoides. Strain HA 755 was identified as *C. zeylanoides* on the basis of the phenotypic data. This organism completely fitted the standard description of the species given by Barnett et al. (1985). This identification was corroborated in the RAPD-PCR analysis as strain HA 755 showed a similarity with the type strain of the species of 94%.

Strains phenotypically identified as Candida catenulata. Two organisms, strains HA 756 and HA 1032, were phenotypically identified as *C. catenulata*. These strains differed from the standard description of the species in the same 2 test results. However, the fermentative ability of strain HA 1032 was not tested. Each of the strains proved to be conspecific with *C. catenulata* showing 97–98% DNA similarity with the type strain of this species.

Strain phenotypically identified as Candida inconspicua. Strain HA 758 was identified as *C. inconspicua* using the identification program of Barnett et al. (1985); no differences from the standard description of the species were observed. This result was corroborated in the RAPD-PCR analysis as the organism shared a similarity with the type strain of *C. inconspicua* of 97%.

Twenty-one strains were identified incorrectly using the classical phenotypic approach but only 10 of

these organisms were identified using the molecular methods (Table 1). Eleven strains, representing 7 distinct species remained unidentifiable. Strains HA 754 and HA 759 are Q-7 yeasts, these organisms are not conspecific with *Issatschenkia orientalis*, *Pichia anomala*, *P. fermentans*, *P. membranifaciens*, *Williopsis mrakii*, or with the originally identified *Candida butyrii* or *C. norvegica*. Strains HA 768, HA 770, and HA 773 represent a distinct species. They are Q-9 yeasts, but are not conspecific with *Debaryomyces hansenii*, *D. fabryi*, *D. marama*, *Candida catenulata*, *C. fennica*, *C. intermedia*, *C. parapsilosis*, *C. sake*, *C. zeylanoides* or *Yarrowia lipolytica*. Strains HB 480, HB 481 and HB 704 are different species of basidiomycetous yeasts, probably species of the genus *Trichosporon* with a ubiquinone Q-9 system. These strains form arthroconidia, show positive results in the diazonium blue B test, and are characterized by the presence of a glucose-mannose-xylose monosaccharide pattern in their cell walls (Table 4). They are not conspecific with the type strains of *Trichosporon aquatile*, *T. asahii*, *T. dulcitum*, *T. inkin*, *T. laibachii*, *T. ovoides* or *T. sporotrichoides*. Strain HB 478, which produces pink-coloured colonies, proved to be a Q-10 yeast that was not conspecific either with *Rhodotorula minuta* or with *R. mucilaginosa* and *R. glutinis*. It possesses the mannose-glucose-galactose-rhamnose monosaccharide pattern in its cell walls (Table 4). Two strains HB 479 and HB 483 were identified as contaminants. It was not possible to obtain the original cultures of these contaminants.

Discussion

Thirty-nine out of the 85 yeast isolates from brine and different cheeses from Austria, Denmark, France, Germany, and Italy were identified correctly using the traditional phenotypic approach. The phenotypic results were confirmed by the RAPD-PCR data. The scores for correct identification were high in the case of the ascomycetous yeasts (46%), running to 65% when the *Geotrichum* species were excluded. However, none of the basidiomycetous yeasts were identified correctly.

Smear ripened semi-hard cheeses. *Debaryomyces hansenii* and *Yarrowia lipolytica* were commonly observed in smear ripened semi-hard cheeses from Austria (e.g. Tilsit). Additional yeast species from these cheeses were *Candida catenulata*, *C. inconspicua*, *C. zeylanoides*, *Geotrichum candidum*, and *Torulaspora delbrueckii*. *Geotrichum candidum* (HA 785) was dominant in the smear ripened semi-hard cheese, St. Nectaire, from France. However, only a single sample was taken from this cheese (Table 1).

Smear ripened soft cheeses. *Clavispora lusitaniae* and *Y. lipolytica* were isolated from smear ripened soft cheese from Austria (e.g. Limburger). *Geotrichum candidum* was the dominant yeast from smear ripened soft cheese from France (e.g. Epoisses Bourgogne, Petit Pont-l'Evéque).

'Graukäse'. The common yeast species from Austrian 'Graukäse' were *Issatchenkia orientalis* and *Kluyveromyces lactis*. Additional isolates were *C. catenulata*, *D. fabryi*, *G. candidum*, *K. marxianus*, *Saccharomyces cerevisiae*, *Trichoporon* sp., and *Y. lipolytica*.

Surface- and internal-mould ripened cheeses. Common yeast species isolated from surface- and internal-mould ripened cheese were *D. hansenii*, *G. candidum*, and *Y. lipolytica*; a *Trichosporon* species from Troubadur has still to be identified.

Blue cheeses. *D. hansenii* and *Y. lioplytica* were commonly isolated from blue cheese (e.g. Bleu de Jura, Gorgonzola, Roquefort) of Austria, France, and Italy. *Saccharomyces cerevisiae* predominated in Gorgonzola from Italy. Additional yeast species from blue cheese were *C. sake* (Gorgonzola; Italy), *K. lactis* (Bleu d'Auvergne; France), *Pichia anomala* (Gorgonzola; Italy), *P. fermentans* (Roquefort; Italy), and *S. unisporus* (Roquefort; Italy). *S. unisporus* (strain HA 817) differed phenotypically from the standard description in 8 test results, but was identified unambiguosly in the RAPD-PCR analysis; the similarity with the type strain was 96%. Devoyod & Sponem (1970) isolated 81 yeast strains from Roquefort cheese and found that yeasts in the inner part of the cheese prior to salting were mainly lactose-fermenting strains such as *K. lactis* and *K. marxianus*. The yeasts isolated from the surface of the cheese after salting and during the ripening period were very salt-resistant and belonged to the genera *Debaryomyces*, *Pichia* and *Rhodotorula*.

Surface-ripened mould cheese. *G. candidum* was frequently isolated from surface-ripened mould cheese (e.g. Camembert). Schmidt & Lenoir (1978, 1980) found that *K. lactis* and *K. marxianus* were the dom-

inant yeasts of Camembert cheese beside species of *C. versatilis, D. hansenii, S. cerevisiae*, and *Zygosaccharomyces rouxii*.

Brine. *Issatchenkia orientalis, K. marxianus*, and *Trichosporon* sp. were commonly found in the brine. Seiler & Busse (1990) isolated 365 yeasts from brines of soft, semi-hard, and hard cheese and found that the predominant strains were mainly *C. versatilis* and *D. hansenii*. The specificity of the yeast flora of brines was assumed to contribute to the sensory variety of cheeses.

Brine. Nakase & Suzuki (1985a, b) studied the taxonomy of *D. hansenii* (Zopf) Lodder et Kreger-van Rij and related yeast species deriving data from the analysis of DNA base composition, DNA-DNA relatedness, ubiquinones, proton magnetic resonance spectra of mannan, and serological properties. They separated two distinct varieties within the species, *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*. The two varieties could be distinguished by the electrophoretic mobilities of their glucose-6-phosphate dehydrogenase, by different maximum temperatures for growth or by DNA-DNA hybridization. However, according to our RAPD-PCR data the two varieties of *D. hansenii* are genotypically distinct species. Our data also showed that it was not possible to separate *D. hansenii* and *D. fabryi* according to their maximum growth temperatures (Table 3).

In contrast to the species description, the 10 *Yarrowia lipolytica* strains identified in the present study were urease negative. Barnett et al. (1990) treat *Y. lipolytica* as an urease positive species on the basis of the conventional Christensen's urea agar test (CUA). Peter & Deák (1991) revised the two standard urease activity tests and found that CUA test recommended by van der Walt & Yarrow (1984) was less reliable than the Bacto Urea Rapid Broth (URB) method which was adapted for yeasts by Roberts et al. (1978). These authors also found that CUA test gave false positive reactions for *Y. lipolytica* strains; the colour change of the indicator was found to be due to a non-specific alkalization reaction, in contrast, false positive results were not recorded for the URB method. In the present study, both methods gave negative results when the *Y. lipolytica* strains were incubated at 25 °C and 35 °C. However, at 25 °C the CUA test may give doubtful results since the original orange colour of the medium turns to a light rose instead of deep pinkish red.

De Hoog et al. (1986) revised the taxonomy of the ascomycetous anamorph genus *Geotrichum* Link: Fr. and its teleomorphic genera *Dipodascus* Langerh. and *Galactomyces* Redhead et Malloch. In a comparative study of the morphology, physiology and nuclear genomes of representative strains the resultant phenotypic groups were, in general, supported by DNA-DNA reassociation data. Smith et al. (1995) found that *Galactomyces geotrichum* could be divided into four groups in DNA-DNA reassociation experiments with intergroups values of 41–59% DNA relatedness. Only a very limited number of strains were found in three of the groups, namely, *Galactomyces geotrichum* sensu stricto: (four strains), group B: (3 strains) and group C: (1 strain). The fourth taxon, group A, contained most of the strains including the type strain of *Geotrichum javanense* (CBS 182.33^T). None of the three smaller groups included a yeast strain isolated from dairy products, whereas group A contained many such isolates. De Cock et al. (1995) found the same groups using restriction fragment polymorphism of mitochondrial DNA; isolates of the same group gave identical or highly similar mitochondrial DNA patterns. In contrast, between groups patterns were very different, as was the case with RAPD patterns. The correlation of mtDNA and RAPD patterns suggests that crossing had not occurred between members of the groups. Based on the 80% genome dissimilarity between *Galactomyces geotrichum* CBS 772.71^T and *Geotrichum javanense* CBS 182.33^T in the RAPD-PCR analysis we suggested the binominal *Geotrichum candidum* be retained. This name is widely used by food technologists and medical mycologists for anamorphic yeast species showing the group A or *Geotrichum javanense* pattern (Smith et al. 1995). *G. javanense* was isolated from yogurt and *G. candidum* is a very common anamorphic yeast species in dairy products. Smith et al. (1995) have pointed out that *G. candidum* sensu stricto has been found in other habitats such as in soil, on different fruits, on *Drosophila* and on human nails and tongue, as well as in sputum. A new anamorph seems to be necessary for *G. geotrichum* sensu stricto.

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