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Polyphasic re-examination of *Debaryomyces hansenii* strains and reinstatement of *D. hansenii, D. fabryi* and *D. subglobosus*

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Key words

ACT1
Debaryomyces fabryi
Debaryomyces hansenii
Debaryomyces subglobosus
DNA reassociation
maximal growth temperature
PCR fingerprinting
taxonomy

Abstract The type species of the genus *Debaryomyces*, *Debaryomyces hansenii*, is a highly heterogeneous species. It has been isolated from a large diversity of natural sources including fruit, air, water, soil, but most frequently from processed food products. The species delineation of this yeast species urgently needs clarification. The heterogeneity in taxonomic characteristics has resulted in the description of varieties linked to *D. hansenii*. The aim of this study was to re-examine and classify *D. hansenii* using a polyphasic approach. A total of 65 *D. hansenii* isolates were examined, 57 representing the variety *hansenii* and nine the variety *fabryi*. The selection of strains for DNA reassociation and phylogenetic analysis was based on polymerase chain reaction mediated fingerprints using four mini- and microsatellite-specific primers. The discriminating power of growth at 35 °C and 37 °C was re-examined and ascospore production was observed. DNA reassociations and phylogenetic analyses were performed on selected isolates from each of the clusters obtained from the DNA fingerprint analyses. The data indicated the presence of three distinct species within the *D. hansenii* group, which were represented by type strains of former species and that are proposed to be reinstated: *D. hansenii* (CBS 767^T = MUCL 49680^T), *D. fabryi* (CBS 789^T = MUCL 49731^T) and *D. subglobosus* (CBS 792^T = MUCL 49732^T).

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INTRODUCTION

The ascomycetous teleomorph genus *Debaryomyces* is generally recognised by the production of persistent asci by mother cell-bud conjugation and the formation of warty ascospores, usually one or two per ascus. The most common species is the type species *D. hansenii*, described in 1952. This species has been isolated from a large diversity of natural sources, like fruit, air, water, soil, but most frequently from processed food products, in particular dairy products (Fröhlich-Wyder 2003), meat and sausages (Samelis & Sofos 2003), but also from sake-moto, wine, tobacco, coffee beans, brines, where it is important for the ripening and flavour composition of the products. Additionally, the species has been recovered from man and animals (Nishikawa et al. 1996, de Hoog et al. 2000, Pfaller et al. 2005).

Based on partial sequences of the nuclear large subunit ribosomal (LSU) DNA, Kurtzman & Robnett (1997, 1998) studied the phylogeny of ascomycetous yeasts including 15 *Debaryomyces* species. These 15 taxa were separated into four clades, exemplified by the species *D. hansenii*, *D. polymorphus*, *D. melissophilus* and *D. etchellsii*, respectively. The clade represented by *D. hansenii* included five more teleomorph species, *D. nepalensis*, *D. maramus, D. coudertii, D. robertsiae* and *D. udenii,* and is here referred to as the *D. hansenii* clade sensu Kurtzman & Robnett (1998). According to highly similar D1/D2 LSU sequences (V. Robert, unpubl. data), this clade also includes *D. prosopidis*, a species that resembles *D. hansenii* physiologically. Phaff et al. (1998) distinguished both by the inability of *D. prosopidis* to grow on cellobiose and salicin, low DNA reassociation values and opposed electrophoretic karyotypes.

Debaryomyces subglobosus, D. kloeckeri and *D. nicotianae* were combined as synonyms with *D. hansenii* by Kreger-van Rij (1970) because several strains showed intermediate reactions in lactose assimilation and formation of dry, creeping pellicles on liquid media, so far considered as distinctive characteristics of these species. Comprehensive taxonomic studies of *D. hansenii* and related species were published by Nakase & Suzuki (1985a, b). DNA reassociations of above 68 % within groups and 47–66 % between groups, the presence/absence of glucose-6-phosphate dehydrogenase activity and the maximum growth temperatures (MGTs) of 31–35 °C or 36–39 °C were used to establish two subgroups among isolates identified as *D. hansenii* for which the varieties *hansenii* and *fabryi* were introduced (Nakase & Suzuki 1985b). Twenty-two species were placed in synonymy with the variety *hansenii*, for which *Candida famata* var. *famata* was recognised as anamorph and three species were placed in synonymy with the variety *fabryi*, for which *C. famata* var. *flareri* was recognised as anamorph. Among the synonyms of *D. hansenii* var. *fabryi* was also *D. subglobosus*, although Price et al. (1978) had found only 39.7 % DNA relatedness between *D. subglobosus* CBS 792^T and *D. hansenii* CBS 767^T. Between the type strains of the anamorphs of the two varieties of *D. hansenii, C. famata* var. *famata* CBS 1795^T and *C. famata* var. *flareri* CBS 1796^T, differences of the electrophoretic patterns of ten enzymes were found (Yamazaki & Komagata 1982). *Debaryomyces subglobosus* was continued to be assigned to *D. hansenii* based on the difficulty of separating them by the commonly employed taxonomic characteristics (Kreger-van Rij 1984). Further heterogeneity was recognised

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Table 1 List of analysed strains of *Debaryomyces hansenii* and related species. The taxon assignment in the first two columns follows the catalogue of the Centraalbureau voor Schimmelcultures (2007): www.cbs.knaw.nl.

Species	Variety	New designation proposed in this study	Original species denomination of ex-type and syntype strains	CBS No.	Mol % G+C	Origin
D. coudertii			*D. coudertii*	5167$^{Ta R}$	34.5	Droppings of *Aptenodytes patagonica* (King Penguin), France
D. hansenii	var. *hansenii*			116		Cherries
			D. guilliermondii var. *nova-zeelandicus*	117$^{T R}$	34.8	Rennet, New Zealand
			Cryptococcus minor	161^{T}		Skin scale (psoriasis case), Italy
				164		Cheese, Netherlands
			D. tyrocola	766^{ST*R}	36.2	Cheese, Russia
			Saccharomyces hansenii	767^{T*R}	35.9	Unknown
				768		Atmosphere, Japan
			D. membranifaciens var. *hollandicus*	769Ta		Salted beans, Netherlands
			D. kloeckeri	770^{T}		White patch on throat of angina patient, France
			Atelosaccharomyces hudeloi	771^{Ta*}		Periostitis, France
			D. kloeckeri var. *major*	772$^{Ta R}$	35.1	Rennet, New Zealand
			D. guilliermondii	773^{T*}		Horse-meat sausage, France
				787		Unknown
			D. hildegaardi	788^{T}		Wound, Denmark
			D. gruetzii	790^{T*R}	34.4	Infected nail
			D. matruchoti var. *cesarii*	791^{T}		Beef sausage
			D. sake	795^{T}		Sake-moto, Japan
		D. fabryi	*D. mucosus*	796$^{Ta R}$	35.1	Skin lesion, France
			D. nicotianae	811^{T}		Fermenting Kentucky tobacco, Italy
			D. nicotianae var. *minor*	812^{T}		Fermenting Kentucky tobacco, Italy
			D. tyrocola var. *tyrocola*	1098$^{ST a*R}$	32.9	Cheese, Russia
				1100		Beef-and-pork sausage, France
				1101^{a}		Salt pork
				1102		Beef-and-pork sausage, France
				1103*		Spoiled pickled cucumber, Netherlands
				1119*		Refuse, Netherlands
				1120^{R}	35.4	Salt beef, France
				1122		Blastomycoses
				1123		Culture contaminant of *Sporobolomyces tenuis*, Netherlands
				1124^{*R}	36.0	Nail of corpse, Netherlands
			Monilia castellanii	1125$^{ST a}$		Persistent case of furunculosis, UK
				1126		–
				1129		Sausage, Italy
				1142*		Sake-moto, Japan
			D. marylandii	1326^{T}		Fermenting Maryland tobacco, Italy
				1780		Tinned tomato puree, Netherlands
				1781		Paper wrapping from bundle of fermenting tobacco, Netherlands
				1791		Tobacco, UK
				1792*		Chilled beef, Australia
		D. fabryi	*Saccharomyces sternoni*	1793^{Ta*}		Interdigital mycoses
			Mycotorula famata	1795^{Ta*}		Wound on hand
				1800		Cheese, Czechoslovakia
				1801		Case of paronychia, Uruguay
				1959^{*R}	35.3	Film on 15 % pickling brine, USA
				1960^{*R}	35.1	Film on 15 % pickling brine, USA
				1961^{a}		Film on 15 % pickling brine, USA
				1962		–
		D. fabryi	*D. orientalis*	2330$^{Ta R}$	32.4	Spoiled sake, Japan
			D. miso	2331^{T}		Edo-miso, Japan
				2333		Unknown
		D. subglobosus		2659$^{a R}$	35.2	Apple, Italy

			2842 *	–		–	
			2843 [a]	–		–	
			2844 [R]	35.6		–	
			4233			Salami, Netherlands	
			4890			Sausage, Belgium	
			7848 *[nd]			Takuan, salted pickle, Japan	
D. hansenii	var. *fabryi*	*D. fabryi*	789[T a * R]	35.6		Interdigital mycotic lesion, Germany	
		D. subglobosus	*Eutorulopsis subglobosa*	792[T *]	34.3		Infected nail, Austria
		D. hansenii		1099 [R]	35.3		Cheese, Russia
		D. hansenii		1121			Horse-meat pasty, France
		D. subglobosus		1128 *[R]	34.4		Nail, Netherlands
		D. subglobosus	*Blastodendrion flareri*	1796[T a * R]	33.9		Erythematous-squamatic skin lesion, Italy
				4373 *			Dry white wine, South Africa
			Paratorulopsis banhegyi	5138[T R]	36.0		Skin of man, Hungary
		D. hansenii		5139 [R]	35.4		Skin of man, Hungary
D. maramus				1794 [R]	35.5		Man
			D. maramus	1958[T R]	35.4		Atmosphere, New Zealand
				4264 [R]	34.2		Cider, UK
				6905 [R]	35.9		Soil, Iran
				7784 [R]	36.1		Surface of pear, USA
D. nepalensis		*D. cavensis*		1325[T R]	34.3		Fermenting Maryland tobacco, Italy
		D. nepalensis		5921[T * R]	35.5		Soil, Nepal
D. polymorphus	var. *polymorphus*	*Pichia polymorpha*		186[T *nd]			Soil, Denmark
D. prosopidis		*D. prosopidis*		8450[T R]	34.8		Exudates of *Prosopis juliflora* (mesquite tree), USA
				2934[T R]	39.5		Larval feed of *Xylocopa caffra* (carpenter bee), South Africa
D. robertsiae				5637 [ndR]	39.6		Pollen collected by *Xylocopa caffra*, South Africa
				6693 [ndR]	40.3		Soil, South Africa

[a] Absence of ascospores; [nd] Ascospores production not determined; [T] Ex-type strain; [ST] Ex-syntype strain; * Included in the *ACT1* phylogeny; [R] Included in the reassociations.

by Nishikawa et al. (1996), who found an intermediate DNA reassociation value of 63 % between the type strains of *D. hansenii* var. *fabryi* CBS 789[T] and its anamorph *C. famata* var. *flareri* CBS 1796[T]. Prillinger et al. (1999) examined 12 strains of *D. hansenii* isolated from different types of cheeses from four European countries phenotypically and genotypically. Using RAPD-PCR including the type strains of *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*, these authors considered the varieties *hansenii* and *fabryi* as two genetically distinct entities and reinstated the varieties at the species level. However, Barnett et al. (2000) maintained both varieties of *D. hansenii*. The number of 23 nucleotide differences between partial actin-1 (*ACT1*) gene sequences of *D. hansenii* var. *fabryi* CBS 789[T] and its anamorph *C. famata* var. *flareri* CBS 1796[T] also indicated interspecific variation (Daniel & Meyer 2003).

In view of the heterogeneity of *D. hansenii* and its inconsistent species delineation, a polyphasic approach was used in this study to provide a basis for its re-classification. A total of 65 *D. hansenii* strains, including 27 type and syntype strains, were examined using MGTs and PCR fingerprinting, an approach that has shown its value in previous studies (Cadez et al. 2002, Smith et al. 2005, Knutsen et al. 2007). In selected strains, DNA reassociations, ascospore morphology and DNA sequences were determined.

MATERIALS AND METHODS

Strains

The strains examined in this study, their origin and original designation according to the catalogue of the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl) are listed in Table 1.

PCR fingerprint analyses

Extraction of high-molecular weight DNA was performed by a combination of the Qiagen DNeasy protocol and the Invisorb® Spin Plant Mini Kit (Invitek, Germany) with modifications. From a culture grown on DYPA (2 % dextrose, 0.5 % yeast extract, 1 % peptone, 2 % agar) for 72 h at 25 °C, three 10 µL loops of cells were resuspended in 600 µL of Sorbitol Buffer (1 M sorbitol, 100 mM sodium EDTA, 14 mM β-mercaptoethanol) and 200 U of lyticase (L4025, Sigma, Belgium). The samples were shaken overnight at 30 °C, the suspension was centrifuged for 2 min at 12 000 rpm and the supernatant was discarded. Four hundred µL lysis buffer P and 20 µL proteinase K were added, the mix was homogenised with a pipette tip and incubated for 30 min at 65 °C. The solution was transferred onto a spin filter and centrifuged for 5 min at 12 000 rpm. Twelve µL of RNAse A (100 mg/mL in molecular biology grade water) were added to the filtrate, which was vortexed briefly and incubated for 30 min at room temperature. Two hundred µL of binding buffer P were added, the sample was vortexed briefly, transferred to a fresh spin filter and centrifuged for 1 min at 12 000 rpm. Five hundred and fifty µL of wash buffer I were added to the spin filter, the filter was centrifuged as before and the filtrate was discarded. A similar treatment using wash buffer II and a final treatment with 400 µL wash buffer II and centrifugation for 2 min at 12 000 rpm concluded the DNA purification. The DNA was then eluted with 100 µL molecular biology grade water (preheated to 65 °C)

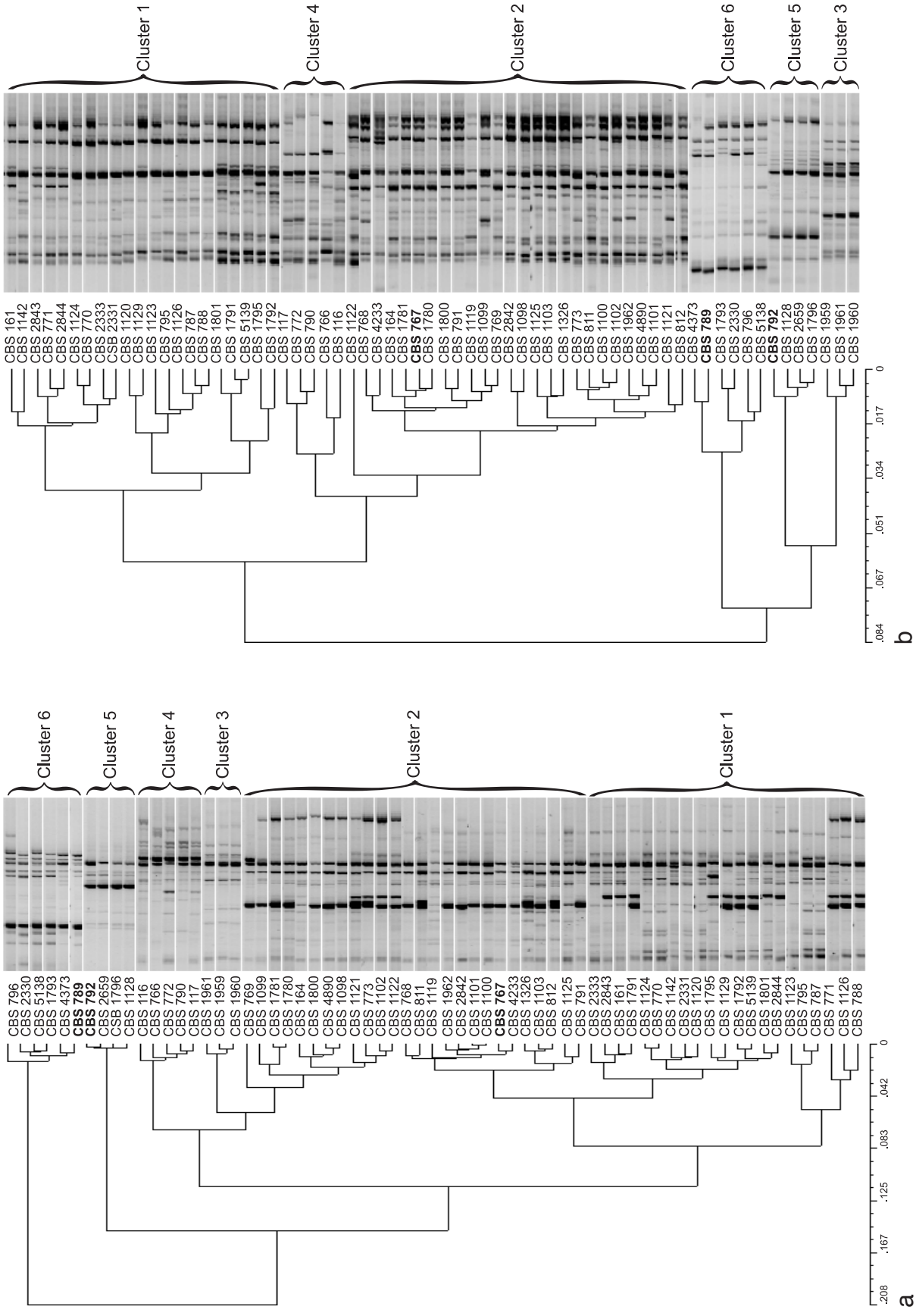

Fig. 1 UPGMA cluster analyses of a) M13 and b) $(ATG)_5$ fingerprints of 65 *Debaryomyces hansenii* strains. The type strains of the reinstated species are indicated in **bold**.

for 3 min and centrifuged for 1 min at 10 000 rpm. This DNA, stored at 4 °C for short term preservation and at -20 °C for long term preservation, was used for PCR fingerprinting and DNA sequence determinations.

PCR fingerprinting was performed using the minisatellite-specific oligonucleotide derived from the core sequence of the bacteriophage M13 (Vassart et al. 1987) with the sequence 5'-GAG GGT GGC GGT TCT-3' and the microsatellite-specific oligonucleotides $(GACA)_4$, $(GTG)_5$ and $(ATG)_5$ as single PCR primers. PCR amplifications were performed in a 25 µL reaction volume, containing 10 ng of genomic DNA, 0.13 µM primer, 0.2 mM dNTPs, 4.5 mM $MgCl_2$ and 2.5 U AmpliTaq DNA polymerase (Applied Biosystems, USA). Amplifications were carried out in an Eppendorf Mastercycler programmed for 35 cycles (20 s at 94 °C, 60 s at 50 °C, 20 s at 72 °C), followed by 6 min of final extension at 72 °C and cooling to 4 °C. Amplified DNA fragments were separated by electrophoresis in 1.4 % (w/v) agarose dissolved in 1× TBE buffer, stained with 0.6× GelRed (approximately 0.8 µg/mL, Biotium, USA) and photographed under UV light. The PCR fingerprint profiles were analysed using BioloMICS v. 7.5.71 (BioAware, Belgium). Similarities of the patterns of each primer were calculated using the CloseSym similarity coefficient. A dendrogram was generated using the UPGMA method.

DNA reassociations and DNA G+C content

Cultures were grown in 2 L yeast malt (YM) broth (Yarrow 1998) for 2 d and the DNA was extracted by hydroxyapatite column chromatography as previously applied by Smith et al. (1995a). The mol % G+C and the DNA reassociation analyses were done using the methods described by Smith et al. (1995a). The values were obtained using a UV-VIS spectrophotometer Lambda 20 (PerkinElmer, Netherlands).

Phenotypic characterisation

Growth tests at 30 °C, 35 °C, 37 °C and 40 °C were performed on GPYA medium (4 % glucose, 0.5 % peptone, 0.5 % yeast autolysate, 2 % agar) for 7 d. Ascus formation and ascospore morphology were examined on dilute V8 ascosporulation medium (Yarrow 1998) or by examining GPYA cultures aged at least 3 mo.

Ribosomal RNA and actin gene amplification, sequencing and data analysis

The FastDNA kit (BIO 101, Carlsbad, California) was used according to the manufacturer's instructions to isolate genomic DNA of cultures grown on GPYA plates for 4 d at 24 °C. Primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify the rDNA as described by Knutsen et al. (2007). A 1 kb fragment of the *ACT1* gene was amplified using the primers and conditions described by Fukuda et al. (2004). The amplification reactions were performed using a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Connecticut). The PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8 % (w/v) agarose gel containing 0.1 µg/mL ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualised under UV-light.

The amplicons were sequenced in both directions using the PCR primers for the actin gene fragment and primers NL1, NL4 (O'Donnell 1993), ITS1 and ITS4 (White et al. 1990) for the D1/D2 domain of the 26S rRNA gene and the ITS region (ITS1, ITS2 and the intervening 5.8S rRNA), respectively. The DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, The Netherlands) was used according to the manufacturer's recommendations and the products were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Foster City, California). A consensus sequence was computed from the forward and reverse sequences with SeqMan v. 7.2.1 from the Lasergene package (DNAstar, Madison, Wisconsin).

The sequences were assembled using Sequence Alignment Editor v. 2.0a11 (Se-Al; A. Rambaut, distributed by the author at http://evolve.zoo.ox.ac.uk/software/Department of Zoology, University of Oxford, Oxford, UK), and manual adjustments were made by eye where necessary. The actin sequence data were analysed by Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 (Swofford 2003). A maximum parsimony analysis was performed using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed. The robustness of the trees was evaluated by 100 bootstrap replications (Hillis & Bull 1993). Other measures calculated included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC). The resulting tree was printed with TreeView v. 1.6.6 (Page 1996). The sequences were deposited in GenBank (accession numbers ITS: EU816226–EU816290, D1/D2 LSU: EU816291–EU816355, *ACT1*: shown in Fig. 2) and the alignment in TreeBASE.

RESULTS

PCR fingerprints of D. hansenii strains

To select strains for intraspecific DNA reassociations, PCR fingerprints of 65 *D. hansenii* strains including 27 type and syntype strains were determined. The UPGMA analyses in combination with visual inspection of the banding patterns obtained with the M13 (Fig. 1a), $(ATG)_5$ (Fig. 1b), $(GTG)_5$, (data not shown) and $(GACA)_4$ (data not shown) primers separated the strains of both varieties of *D. hansenii* into six clusters. For the remainder of the manuscript these will be referred to as clusters.

Cluster 1 included 21 strains, of which all except one were catalogued as *D. hansenii* var. *hansenii.* Seven strains represented type strains of species placed in synonymy with this variety. Strain CBS 5139 was the only one catalogued as *D. hansenii* var. *fabryi*. The second cluster covered 26 strains, all, except two (CBS 1099 and CBS 1121), catalogued as *D. hansenii* var. *hansenii*, including its type strain CBS 767^T, and eight type strains of synonyms of this variety. Cluster 3 comprised three isolates from pickling brine all catalogued as *D. hansenii* var. *hansenii*. Cluster 4 covered five strains, all catalogued as *D. hansenii* var. *hansenii,* including four type strains of synonyms of this variety. Cluster 5 covered four isolates, two catalogued as

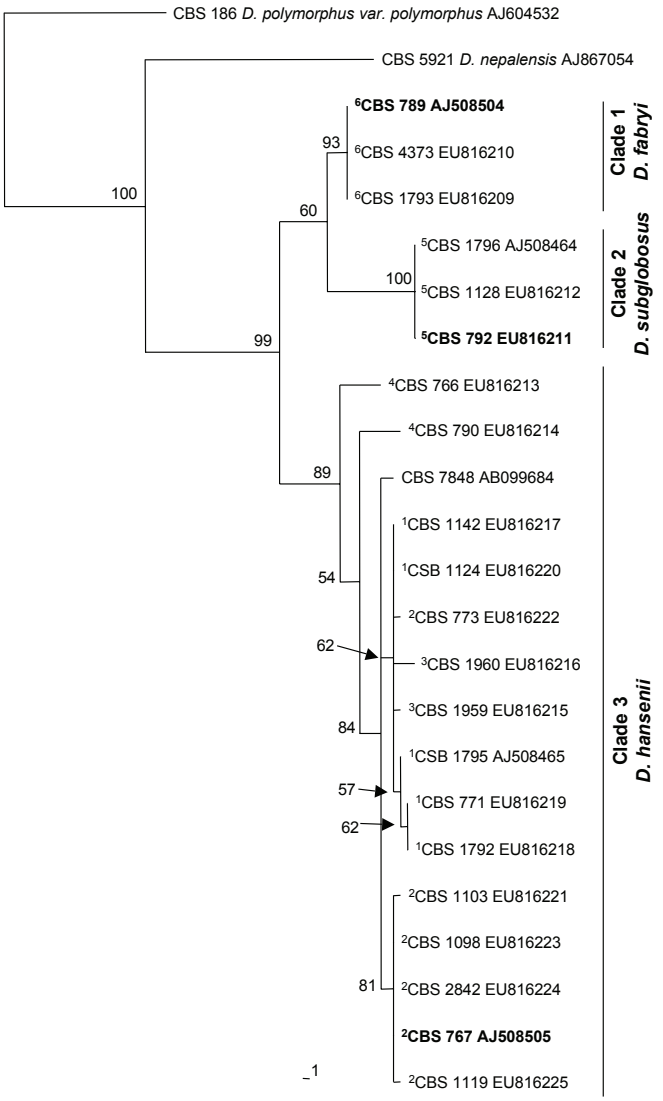

Fig. 2 One of two equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the *ACT1* sequence alignment. The scale bar shows a single change and bootstrap support values from 100 replicates are shown at the nodes. The tree was rooted to the *D. polymorphus* var. *polymorphus* strain CBS 186 and the type strains of the reinstated species are indicated in **bold**. The PCR fingerprint cluster number of each strain is indicated by the subscript number in front of the CBS number.

D. hansenii var. *hansenii*, and the remaining listed as *D. hansenii* var. *fabryi* of which two represented type strains of synonyms of *D. hansenii* var. *fabryi*. Cluster 6 consisted of six strains, including the type strain of *D. hansenii* var. *fabryi* CBS 789^{T}, and three type strains designated as synonyms of the variety *D. hansenii* var. *hansenii* (Table 1). By visual inspection of the patterns and under consideration of the identification labels, two to six strains were selected from each of the six clusters for DNA reassociation, resulting in 20 strains (Table 1) and for phylogenetic analyses of partial *ACT1* sequences, resulting in 21 strains (Fig. 2).

Intraspecific and interspecific DNA reassociations

Nuclear base compositions — The nuclear base compositions of all strains examined are presented in Table 1. The calculated G+C content ranged from 32.4–36.2 mol % except for *D. robertsiae* of which the value ranged from 39.5–40.3 mol %.

Reassociations among strains of *D. hansenii* — The DNA reassociation values among strains selected from the six clusters obtained by PCR fingerprinting are shown in Table 2. Among the selected strains of cluster 6, including the type of *D. hansenii* var. *fabryi* CBS 789^{T}, they ranged from 92–100 %, confirming conspecificity. Reassociation values ranging from 73–100 % were observed among selected strains of clusters 1–4, including the type strain CBS 767^{T} of *D. hansenii* var. *hansenii* belonging to cluster 2. Based on these values, all isolates of clusters 1–4 were considered conspecific with the latter taxon and were correctly identified except for CBS 1099, CBS 1121 and CBS 5139, catalogued as *D. hansenii* var. *fabryi,* which were here re-identified as *D. hansenii* var. *hansenii*. Among the three strains representing cluster 5, values of 98 % and 100 % were recorded between CBS 1128 and CBS 1796 and between CBS 1128 and CBS 2659 respectively, indicating their conspecificity. The reassociation values between cluster 6 strains and strains of other clusters ranged from 34–68 %, the values between cluster 1–4 strains and strains of other clusters ranged from 34–67 % and the values between cluster 5 strains and strains of other clusters ranged from 40–68 % (Table 2). These values were considered low enough to recognise the strains of these two clusters as separate species. The reassociation results correlate with the similarity tree derived from fingerprint data (Fig. 1) that shows higher similarities within the group composed of clusters 1–4 than between clusters 1–4, compared to cluster 5 and cluster 6.

Interspecific reassociations — To evaluate the genetic relatedness of the above delimited *Debaryomyces* groups to the teleomorph taxa of the *D. hansenii* clade sensu Kurtzman & Robnett (1998), interspecific DNA reassociations were performed with the inclusion of *D. prosopidis*, a species resembling *D. hansenii*, introduced by Phaff et al. (1998). *Debaryomyces udenii* could not be included in this study, due to the fact that the employed DNA extraction method did not result in suitable DNA yields. The reassociation values of the *D. hansenii* strains with five additional *Debaryomyces* species ranged from 0–47 % (Table 3). These low interspecific reassociation values confirm the separation of *Debaryomyces* clusters 1–4, cluster 5 and cluster 6 from their most closely related known species.

Sequence variation and phylogenetic analyses

No variation was found in the D1/D2 LSU and the ITS rRNA regions of the *D. hansenii* isolates and these sequences were therefore not subjected to phylogenetic analyses. At least two isolates from each of the six PCR fingerprint clusters (Table 1) and the type strains of *D. nepalensis* and *D. polymorphus* var. *polymorphus,* the latter as outgroup, were included in sequence and phylogenetic analyses of the nuclear encoded actin gene. Comparisons of partial *ACT1* gene sequences between clade 1 and clade 2 strains showed 16 nucleotide differences; between clade 1 and the type strain of *D. hansenii* CBS 767^{T}, contained in clade 3, 24 differences were found and between clade 2 and

the type strain of *D. hansenii,* 33 differences were found. Further pairwise comparisons of strains in the more variable clade 3 resulted in 11 nucleotide differences between CBS 790 and CBS 767^T and 14 differences between CBS 766 and CBS 767^T. Both basally located strains CBS 790 and CBS 766 compared to each other showed 14 nucleotide differences. For pairwise comparisons within the core of clade 3, excluding CBS 790 and CBS 766, zero to seven nucleotide differences were found.

The *ACT1* alignment, containing 24 strains including *D. hansenii* var. *hansenii* CBS 7848 (Fukuda et al. 2004) and the outgroup sequence, had a total length of 752 characters, of which 623 were constant, 73 were parsimony-uninformative, and 56 were parsimony-informative. Parsimony analysis resulted in two equally most parsimonious trees, one of which is shown in Fig. 2 (TL = 164 steps; CI = 0.841; RI = 0.876; RC = 0.737). Three distinct and well-supported clades were obtained within *D. hansenii* by phylogenetic analysis of partial *ACT1* sequences. The first clade contained the strains representing cluster 6 with 93 % bootstrap support. The second clade contained the strains representing cluster 5 with a bootstrap support of 100 % and the third clade grouped strains representing the clusters 1–4 with a bootstrap support of 89 %. Within the third clade some variation was observed among the isolates, with two strains from cluster 4 in a basal position relative to this clade and the majority of fingerprint cluster 2 representatives forming a subclade with 81 % bootstrap support.

Phenotypic characterisation

Nakase & Suzuki (1985b) proposed differences in maximum growth temperatures (MGTs) to distinguish the two varieties of *D. hansenii*. Since DNA reassociations and sequence analyses segregated the analysed strains of *D. hansenii* into three distinct groups, the ability to grow at 30 °C, 35 °C and 37 °C was re-examined in triplicate. All 55 strains in the fingerprint clusters 1–4 grew at 30 °C and most of them were unable to grow at 35 °C with the exception of CBS 771, CBS 1795 and CBS 5139. None of the strains in clusters 1–4 were able to grow at 37 °C. The four strains of cluster 5 were able to grow at 30 °C, 35 °C and 37 °C, but not at 40 °C. All six strains of cluster 6 grew at 30 °C and 35 °C, but not at 37 °C.

Table 2 DNA-DNA reassociation values among strains of the reinstated species currently classified in *Debaryomyces hansenii*. Only values of selected pairwise comparisons were determined. For better readability cells representing comparisons for which no reassociation experiments were performed were left empty. Clusters 1 through 6 refer to the grouping by PCR fingerprint analyses and thickened lines delimit comparisons that are considered as intra-species with the current reinstatements.

			Cluster 1		Cluster 2	Cluster 3	Cluster 4		Cluster 5		Cluster 6	
			CBS 2844	CBS 5139	CBS 767^T	CBS 1959	CBS 766	CBS 790	CBS 1128	CBS 2659	CBS 789^T	CBS 5138
D. hansenii	Cluster 1	CBS 1120	100		95							
		CBS 1124	100		99			83			58	
		CBS 5139	100					89	45		51	34
	Cluster 2	CBS 767^T							41		38	49
		CBS 1098	100		100	77		76		52	34	39
		CBS 1099	100									
	Cluster 3	CBS 1960	96		93	100			47	54	54	
	Cluster 4	CBS 117	100		80			76	40	45	67	
		CBS 766		74				88	61		48	52
		CBS 772						88				
		CBS 790	89		73				40			
D. subglobosus	Cluster 5	CBS 1796		42			50		98			
		CBS 2659							100			
D. fabryi	Cluster 6	CBS 789^T					48		68			
		CBS 796	62							68		100
		CBS 2330	57		40						98	92

Table 3 Interspecific DNA-DNA reassociation values among *Debaryomyces hansenii* and related species. An average value was calculated for the total number of reassociations performed among strains of two species.

Debaryomyces	*hansenii*	*subglobosus*	*fabryi*	*coudertii*	*maramus*	*nepalensis*	*prosopidis*	*robertsiae*
hansenii	90							
subglobosus	43	99						
fabryi	49	47	96					
coudertii	8	29	25	–				
maramus	26	24	16	41	89			
nepalensis	8	18	21	26	23	92		
prosopidis	42	47	45	28	10	22	–	
robertsiae	14	12	8	26	15	18	0	93

Ascus formation and ascospore morphology of strains in cluster 5 were studied to confirm their taxonomic designation. The teleomorph characteristics of mother bud conjugation and warty ascospores, typical for species assigned to the genus *Debaryomyces*, were observed in CBS 792 and CBS 1128, but were not found in the rest of the strains present in that cluster.

Taxonomy

The results obtained from PCR fingerprinting, DNA reassociations, partial *ACT1* gene sequences, and MGTs indicated that strains of the two *D. hansenii* varieties, *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*, can be separated into three distinct groups represented by the fingerprint clusters 1–4, cluster 5 and cluster 6.

The strains of clusters 1–4 have here been identified as *D. hansenii* var. *hansenii* by the inclusion of its type strain CBS 767^T in one of these clusters. Clusters 1–4 were delineated as one taxon, currently named *D. hansenii* var. *hansenii*, by DNA reassociation values of 73–100 %, highly similar PCR fingerprints with the primers M13 and $(GTG)_5$ and partial *ACT1* gene sequence differences of zero to 14 nucleotides. This taxon is proposed to be reinstated as *D. hansenii*. The detected intracluster variability was contrasted by inter-cluster differentiation by DNA reassociation values of 34–67 %, dissimilar PCR fingerprints and partial *ACT1* gene sequence differences of 24–36 nucleotides of clusters 1–4 versus clusters 5 and 6.

Cluster 5 was unified by 98–100 % DNA reassociation, identical partial *ACT1* gene sequences and highly similar PCR finger-prints with all four primers. This cluster was distinguished from the former two species by DNA reassociation values of 40–68 %, partial *ACT1* gene sequence differences of 16–36 nucleotides, differentiating PCR fingerprint patterns with all four primers and its ability to grow at 37 °C. Cluster 5 contains two type strains, CBS 1796^T *Blastodendrion flareri* (Ciferri & Redaelli 1935), later considered as *Candida famata* var. *flareri* and anamorph of *D. hansenii* var. *fabryi* and CBS 792^T *Eutorulopsis subglobosa* (Wolfram & Zach 1934), later considered under the name *D. subglobosus* as a synonym of *D. hansenii* var. *fabryi*. Based on the nomenclatural priority of *D. subglobosus*, it is proposed to reinstate this name for cluster 5 strains with CBS 792^T as type strain, which produces warty ascospores by mother cell-bud conjugation, currently named *D. hansenii* var. *fabryi*.

Cluster 6 contains *D. hansenii* var. *fabryi* CBS 789^T and CBS 2330^T as well as CBS 1793^T of species previously placed in synonymy with *D. hansenii* var. *fabryi* and CBS 796^T and CBS 5138^T of species previously placed in synonymy with *D. hansenii* var. *hansenii*, which can presently be considered as synonyms of *D. hansenii* var. *fabryi*. Based on DNA reassociation values of 92–100 %, highly similar PCR fingerprints with all four primers and identical partial *ACT1* gene sequences within cluster 6 contrasted by DNA reassociation values of 34–68 %, highly dissimilar PCR fingerprints and partial *ACT1* gene sequence differences of 16–33 nucleotides between cluster 6 and the others, it is proposed to reinstate the name *D. fabryi* for the currently named taxon *D. hansenii var. fabryi*.

The current study supports the proposal by Prillinger et al. (1999) that *D. hansenii* and *D. fabryi* are two distinct species that are part of the *D. hansenii* complex. From the data presented in the current study, a third species, *D. subglobosus*, is also hereby reinstated in this complex.

Debaryomyces fabryi M. Ota, Dermatol. Wochenschrift 78: 287. 1924

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

Debaryomyces hansenii (Zopf) Lodder & Kreger-van Rij, in: Lodder & Kreger-van Rij, The yeasts, a taxonomic study: 280. 1952.

Basionym. *Saccharomyces hansenii* Zopf, Ber. Deutsch. Bot. Ges. 7: 94. 1889.

Anamorph. *Candida famata* (F.C. Harrison) S.A. Mey. & Yarrow var. *famata,* Int. J. Syst. Bacteriol. 28: 612. 1978.

Debaryomyces subglobosus (Zach) Lodder & Kreger-van Rij, in: Lodder & Kreger-van Rij, The yeasts, a taxonomic study: 290. 1952.

Basionym. *Eutorulopsis subglobosa* Zach, Wolfram & Zach, Arch. F. Derm. & Syph. 170: 688. 1934.

Anamorph. *Candida famata* (F.C. Harrison) S.A. Mey. & Yarrow var. *flareri* (Cif. & Redaelli) Nakase & M. Suzuki, J. Gen. Appl. Microbiol. 31: 83. 1985.

DISCUSSION

The current re-classification was based on a more restrictive interpretation of intermediate DNA reassociation values in view of additional data from MGTs, PCR fingerprints and partial *ACT1* gene sequences. Nakase & Suzuki (1985a, b) delimited the varieties by a lower value of 68 % reassociation within the variety *D. hansenii* var. *fabryi* supported by the presence/absence of glucose-6-phosphate dehydrogenase activity and the MGTs of 31–35 °C or 36–39 °C. In this study, a reassociation value of 68 % was considered as indicative of different species, which was supported by PCR fingerprint profiles, partial *ACT1* gene sequences and in part also by different MGTs.

On the basis of RAPD analyses, Prillinger et al. (1999) proposed to reinstate the varieties *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi* as two genotypically distinct species, *D. hansenii* and *D. fabryi*. The estimated similarity value between the individual patterns was in the range of 30–50 % which was considered to be characteristic for closely related species (Messner et al. 1994, Cooke et al. 1996). However, Barnett et al. (2000) maintained both varieties of *D. hansenii*. The current polyphasic re-examination of the *D. hansenii* strains supports the proposal to raise the varieties *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi,* to *D. hansenii* and *D. fabryi,* respectively.

A number of strains, formerly included in one of the two *D. hansenii* varieties, were found to have phenotypic and genotypic characters that are distinct from those of *D. hansenii* and *D. fabryi.* The presence of an ascospore-producing ex-type strain of the synonymous species *D. subglobosus* among those

distinct strains allowed reinstating this group as an additional species. This reinstatement further resolves the heterogeneity of *D. hansenii* indicated already by DNA reassociations values of 39.7 % between *D. subglobosus* CBS 792^T and *D. hansenii* CBS 767^T (Price et al. 1978) and 63 % between *D. hansenii* var. *fabryi* CBS 789^T and its anamorph *Candida famata* var. *flareri* CBS 1796^T (Nishikawa et al. 1996).

Several species have previously been synonymised with the two *D. hansenii* varieties and are now proposed as synonyms of the newly proposed species (Table 1). CBS 796^T, CBS 1793^T and CBS 2330^T are ex-type strains of species previously placed in synonymy with *D. hansenii* var. *hansenii*, which should presently be considered as synonyms of *D. fabryi*. CBS 1796^T, the ex-type strain of *Blastodendrion flareri*, was placed in synonymy with *D. hansenii* var. *fabryi* by Nakase & Suzuki (1985b) and is now proposed as synonym of a different species, *D. subglobosus*.

The PCR fingerprint patterns as well as the phylogenetic analyses showed considerable variation within *D. hansenii*, which represents the most populated group in this study. This variation was interpreted as interspecies variation. Specifically the strains CBS 766 and CBS 790, located basal to the *D. hansenii* group in the phylogenetic analysis and showing a relatively high reassociation value of 88 % with each other as opposed to a lower value with another member of *D. hansenii* (74 % with CBS 5139) might give rise to different interpretations, namely to establish new varieties or more species. Although CBS 766 also showed differences in the fingerprinting profiles with primers $(GTG)_5$, $(ATG)_5$ and $(GACA)_4$ and CBS 790 showed some relatively low reassociation values with other members of *D. hansenii* (76 % with CBS 1098, 76 % with CBS 117, 73 % with CBS 767), the further splitting of *D. hansenii* was avoided as this would be based on very limited data of single strains, which bears a high potential of causing taxonomic instability. In addition, a number of high reassociation values connect CBS 790 with other members of *D. hansenii* (89 % with CBS 2844, 83 % with CBS 1124, 89 % with CBS 5139, 88 % with CBS 772). According to literature (Smith et al. 1995a, b, 2000, Knutsen et al. 2007), strains with reassociation values in this range are conspecific. Electrophoretic mobility of glucose-6-phosphate dehydrogenase showed two different relative values among strains of *D. hansenii* var. *hansenii*, also depicting a certain heterogeneity of this taxon (Nakase & Suzuki 1985b). However, CBS 766 = JCM 2098 shared the same enzyme mobility with other strains of *D. hansenii* (CBS 1795 = JCM 1521, CBS 767 = JCM1990), adding confidence to its current placement within this species.

The similarity trees calculated from the PCR fingerprints should not be regarded as reconstructions of genetic relationships because of possible co-migration of non-homologous DNA fragments and the difficulty to recognise allelic or otherwise linked variants of fingerprint markers (Weising et al. 1995). This explains the changing basal branching pattern of the similarity trees from different primers, and the distribution of clusters 1 and 2 over several branches in the M13 tree. We have judged these instabilities as an additional hint towards a closer relationship between strains of clusters 1–4 (= clade 3 = *D. hansenii*) than between cluster 5 (= clade 2 = *D. subglobosus*) and cluster 6 (= clade 1 = *D. fabryi*).

The comparison of the detected variation of partial *ACT1* gene sequences with previously determined data was difficult (Daniel & Meyer 2003) because a 227 bp shorter fragment has currently been analysed. An examination of a 979 bp fragment of the *ACT1* gene in 80 species of ascomycetous yeasts, allowing 37 intraspecific pairwise comparisons, resulted in an intraspecific variation of up to 11 nucleotides and interspecific variation starting at 17 nucleotides (Daniel & Meyer 2003). *Debaryomyces fabryi* and *D. subglobosus* were clearly differentiated from *D. hansenii* CBS 767^T by 24 and 33 nucleotide differences, respectively. The variation of 16 nucleotides between *D. fabryi* and *D. subglobosus* is in the lower range of values previously interpreted as interspecific values for this gene, likely due to the shorter alignment in the present analysis. The basal position of strains CBS 766 and CBS 790 relative to *D. hansenii* in the phylogenetic tree is a reflection of their sequence variability (11 and 14 nucleotides compared with CBS 767^T and 14 nucleotides in comparison with each other, exceeding the upper level of up to 11 nucleotide differences interpreted so far as intraspecific variation). However, taking into account the high reassociation values linking CBS 790 with other strains of *D. hansenii* and the similarity in the electrophoretic mobility of glucose-6-phosphate dehydrogenase of CBS 766 and that of other *D. hansenii* strains (Nakase & Suzuki 1985b), the placement of these two strains in *D. hansenii* is credible, despite of the variability found in the *ACT1* sequences.

MGTs have been considered a complementary characteristic in yeast identification (Yarrow 1998) and MGTs as well as growth rates at different temperatures have been used successfully in fungal identification (Groenewald et al. 2005). However, little is known about a practical and general tolerance to delimit species using this characteristic. Differences in MGTs were recognised as an important criterion in the classification of *Debaryomyces* species and introduced by Nakase & Suzuki (1985b) to distinguish the two varieties of *D. hansenii*. Among 34 strains of *D. hansenii* var. *hansenii*, Nakase & Suzuki (1985b) found MGTs of 31–35 °C and among six strains of *D. hansenii* var. *fabryi*, they found 36–39 °C, with three strains growing at 36–37 °C and the remaining three strains at 37–38 °C and 38–39 °C. Two of the strains with higher MGTs (CBS 792 = JCM 1989 and CBS 1796 = JCM 2166) were here assigned to the reinstated *D. globosus*, while the third strain was not included in the present study. The newly defined taxa delimited the MGTs of *D. fabryi* as below 37 °C and the MGTs of a subset of strains from the former variety *fabryi*, now named *D. subglobosus*, as below 40 °C, while the MGTs of *D. hansenii* was the lowest with below 35 °C. Taking into account the different methodologies of MGTs tests in both studies, these results are compatible by differentiating further the strains showing higher MGTs. This differentiation follows the trend of a more refined delimitation of potentially clinically relevant taxa. Species of potential clinical importance are often able to grow at 37 °C as is the case for *D. subglobosus,* but not for *D. hansenii* and *D. fabryi*, a characteristic of potential practical value for preliminary species assignment. Although most *D. fabryi* strains could be distinguished from *D. hansenii* strains by their ability to grow at 35 °C, this criterion is unsuitable for identification as three *D. hansenii* strains were also able to grow at 35 °C. Differences in MTGs

can be considered an adaptation to different ecological niches and therefore constitute a biologically relevant character.

Although the D1/D2 LSU and ITS rDNA are the most frequently used sequence markers for species identification of yeasts, the D1/D2 LSU has been recognised not to distinguish some closely related ascomycetous yeast species e.g. *Pichia guilliermondii* and *P. caribbica, Candida mucifera* and *Stephanoascus ciferii, Kluyveromyces marxianus* and *K. lactis* (Daniel & Meyer 2003). This problem has less frequently been encountered for the ITS region, for example in *Saccharomyces pastorianus* and *S. bayanus* (Huffman et al. 1992, Kurtzman & Robnett 2003), two species that are linked by interspecies hybridisation (Hansen & Kielland-Brandt 1995). Among several species of the genus *Debaryomyces*, including members of the *D. hansenii* clade sensu Kurtzman & Robnett (1998), highly conserved ITS and D1/D2 LSU sequences were reported (Martorell et al. 2005), while *ACT1* sequences showed more variability and were considered a suitable tool to differentiate these species. In the case of the two morphologically identical fungal species *Cercospora apii* and *C. beticola*, only one out of five gene regions sequenced, namely the calmoduline gene, was informative for species identification (Groenewald et al. 2005). The present study confirms the value of multigene approaches and the actin gene for resolving closely related species such as *D. hansenii*, *D. fabryi* and *D. subglobosus*, while other protein-coding genes e.g. RNA polymerase large subunits 1 and 2 and mitochondrial cytochrome oxidase subunit 2 (Kurtzman & Robnett 2003, Tsui et al. 2008) can also be expected to contribute to a more natural and realistic delineation of *Debaryomyces* species.

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