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Evaluation of ribosomal RNA and actin gene sequences for the identification of ascomycetous yeasts

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Abstract

Highly similar gene sequences of the 5' region of the large subunit (LSU) are commonly interpreted to predict the organism's identity. However, it was recognised that closely related taxa do not always show sufficiently diverged D1/D2 LSU sequences to differentiate between them. The effectiveness of species separation using D1/D2 LSU sequences, small subunit (SSU) sequences and actin gene sequences was determined by pair-wise comparisons. The LSU data showed coinciding similarities among and within species. The actin data resolved all investigated species. Examples strengthened the value of almost complete SSU sequences for species separation. The larger number of differences in the highly conserved actin gene, compared to the overall more variable LSU gene, is due to the tolerance of protein coding genes to synonymous nucleotide changes. In contrast, the pairing in secondary structures of the rRNA, ensuring the functionality of the molecule, relies on longer and uninterrupted sequence sections. In conclusion, D1/D2 LSU sequences are not specific enough to identify closely related taxa. The actin gene is a better marker in these cases. However, because of the availability of a large database of fungal D1/D2 LSU sequences, this gene region is currently still the preferred target for sequence-based identification.

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Keywords: Ribosomal RNA; Actin gene; Species identification of yeasts

1. Introduction

The identification of yeasts based on phenotypic characters is difficult because of the paucity of such

characters and the recognition that some characters are subject to intra-group variability, convergent or parallel evolution (Fuson et al., 1980; Kurtzman, 1984; Price et al., 1978). The preferred but laborious basis to establish pairs of sibling species, in contrast to con-specific strains, are the percent of nuclear DNA relatedness and genetic crosses. DNA sequence data provide a more readily accessible source of evidence. However, their interpretation is difficult for closely related strains. The establishment of cut-off values to distinguish conspecific strains from sibling species

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Table 1
Strains of the studied ascomycetous yeasts and reference species

Species	Strain designation		Actin gene accession no.	LSU gene accession no.	SSU gene accession no.
	Lab no.	Collection no.			
<i>Arxiozyma telluris</i>	WM 784	CBS 2685 T	AJ508456	U72158	Y15806
<i>Candida albicans</i> ST A	WM 2	CBS 562 NT	AJ389057	U45776	M60302, X53497
<i>Candida albicans</i> ST B	WM 231	CBS 5983	AJ508457	AJ508555	n.d.
<i>Candida bovina</i>	WM 786	CBS 2760 T	AJ508458	AJ508556	Y15808
<i>Candida cacaoi</i>	WM 802	CBS 2020 T	AJ508459	U45744	AB013513
<i>Candida castellii</i>	WM 790	CBS 4332 T	AJ508460	U69876	n.d.
<i>Candida catenulata</i>	WM 6	CBS 565 T	AJ508461	U45714	AB013539
<i>Candida ciferrii</i>	WM 814	CBS 4856 T	AJ508462	AJ508557	AB018141
<i>Candida ciferrii</i>	n.a.	CBS 5295	n.d.	U40138	n.d.
<i>Candida colliculosa</i>	WM 820	CBS 133 T	AJ508463	AJ508558	X98119
<i>Candida dubliniensis</i>	WM 602	CBS 7987 T	AJ389058	U57685	X99399
<i>Candida famata</i> var. <i>famata</i>	WM 279, 280	CBS 1795 T	AJ508465	AJ508559	AB013568
<i>Candida famata</i> var. <i>flareri</i>	WM 60	CBS 1796 T	AJ508464	AJ508560	AB013567
<i>Candida fermentati</i>	WM 1092	CBS 2022 T	AJ508466	CPK	AJ508269
<i>Candida fukuyamaensis</i>	WM 1091	CBS 7921 T	AJ508467	U62311	AB013566
<i>Candida glabrata</i>	WM 54	DUMC 19-1112	AJ389072	n.d.	n.d.
<i>Candida glabrata</i>	WM 53	CBS 138 T	AJ389073	U44808	X51831
<i>Candida globosa</i>	WM 1094	CBS 162 T	AJ508468	AJ508561	AB018164
<i>Candida guilliermondii</i> var. <i>carpophila</i>	WM 829	CBS 5256 T	AJ508469	CPK	AJ508270
<i>Candida guilliermondii</i> var. <i>guilliermondii</i>	WM 826	CBS 566 T	AJ389064	AJ508562	AB013587
<i>Candida guilliermon.</i> var. <i>membranifaciens</i>	WM 806	CBS 1950 T	AJ508470	AJ508563	n.d.
<i>Candida haemulonii</i>	WM 822	CBS 5149 T	AJ508471	U44812	AB013572
<i>Candida haemulonii</i> Type II	WM 823	CBS 7798	AJ508472	AJ508564	n.d.
<i>Candida haemulonii</i> Type II	n.a.	CBS 6915	n.d.	U44819	n.d.
<i>Candida holmii</i>	WM 788	CBS 135 NT	AJ508473	AJ508565	X97808
<i>Candida humilis</i>	WM 800	CBS 5658 T	AJ508474	U69878	n.d.
<i>Candida inconspicua</i>	n.a.	DSM 70631	n.d.	n.d.	AF201301
<i>Candida inconspicua</i>	WM 11	CBS 180 T	AJ508475	U71062	n.d.
<i>Candida inositophila</i>	WM 818	CBS 6736 T	AJ508476	AJ508566	CR3358seq1 ^a
<i>Candida intermedia</i>	WM 811	CBS 572 T	AJ508477	U44809	AB013571
<i>Candida kefir</i>	WM 13	CBS 834 T	AJ389079	AJ508567	n.d.
<i>Candida krusei</i>	WM 14	CBS 573 T	AJ389087	AJ508568	M55528
<i>Candida lambica</i>	WM 16	CBS 1876 T	AJ389083	AJ508569	n.d.
<i>Candida lipolytica</i>	WM 290	CBS 599 T	AJ508478	AJ508570	n.d.
<i>Candida lodderae</i>	WM 1.182	CBS 1924 T	AJ508479	U45755	AB013533
<i>Candida lusitanae</i>	WM 18	CBS 4413 T	AJ389066	AJ508571	M55526
<i>Candida maltosa</i>	WM 603	CBS 5611 T	AJ389060	U45745	D14593
<i>Candida milleri</i>	WM 801	CBS 6897 T	AJ508480	U94923	n.d.
<i>Candida molischiana</i>	WM 795	CBS 136 T	AJ508481	U70178	AB018169
<i>Candida mucifera</i>	WM 815	CBS 7409 T	AJ508482	AJ508572	AB019362
<i>Candida nitrativorans</i>	WM 76 A	CBS 6152 T	AJ508483	AJ508573	AJ508271
<i>Candida norvegensis</i>	WM 235	CBS 1922 T	AJ389085	AJ508574	n.d.
<i>Candida norvegica</i>	WM 19	CBS 4239 T	AJ508484	U62299	n.d.

Table 1 (continued)

Species	Strain designation		Actin gene accession no.	LSU gene accession no.	SSU gene accession no.
	Lab no.	Collection no.			
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T	AJ508485	U45754	AB013588
<i>Candida parapsilosis</i> Type 1	WM 23	DUMC 31-1070	AJ389062	n.d.	n.d.
<i>Candida parapsilosis</i> Type 2	WM 1089	CBS 8825	AJ508486	AJ508575	n.d.
<i>Candida parapsilosis</i> Type 2	WM 1.57	PL 452	AJ508487	AJ508576	n.d.
<i>Candida parapsilosis</i> Type 3	WM 1.56	PL 448	AJ508488	AJ508577	n.d.
<i>Candida pelliculosa</i>	WM 825	CBS 605 T	AJ508489	AJ508578	n.d.
<i>Candida pintolopesii</i>	WM 785	CBS 1787 T	AJ508490	AJ508579	Y15810
<i>Candida pulcherrima</i>	WM 25	CBS 610 NT	AJ389071	AF017402	AB013578
<i>Candida rancoensis</i>	WM 799	CBS 8174 T	AJ508491	AJ508580	AJ508272
<i>Candida robusta</i>	WM 604	CBS 1907 T	AJ389076	AJ508581	n.d.
<i>Candida rugosa</i>	WM 234	CBS 613 T	AJ508492	U45727	AB013502
<i>Candida saitoana</i>	WM 52	CBS 940 T	AJ508493	U45762	AB013523
<i>Candida sake</i>	WM 27 A	CBS 159 T	AJ508494	U45728	AB013529
<i>Candida shehatae</i>	WM 809	CBS 5813 T	AJ508495	U45761	AB013582
<i>Candida slooffiae</i>	WM 787, 1083	CBS 2419 T	AJ508496	AJ508582	Y15809
<i>Candida</i> spec.	WM 1169	contamination on CBS 6857 T	AJ508497	n.d.	n.d.
<i>Candida sphaerica</i>	WM 28	CBS 141 T	AJ389081	AJ508583	n.d.
<i>Candida steatolytica</i>	WM 816	CBS 5839 T	AJ508498	AJ508584	AB108152
<i>Candida tropicalis</i>	WM 1087	CBS 94 T	AJ508499	U45749	M60308
<i>Candida tropicalis</i>	WM 30	DUMC 29-3711	AJ389059	n.d.	n.d.
<i>Candida utilis</i>	WM 31	CBS 621 T	AJ389091	AJ508585	n.d.
<i>Candida valida</i>	WM 32	CBS 638 T	AJ389089	AJ508586	n.d.
<i>Candida veronae</i>	WM 804	CBS 5815 T	AJ508500	U45783	AB013505
<i>Candida viswanathii</i>	WM 33	CBS 4024 T	AJ389061	U45752	AB013589
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	AJ508501	U45707	AB013517
<i>Candida zeylanoides</i>	WM 64	CBS 619 NT	AJ389069	U45832	AB013509
<i>Citeromyces matritensis</i>	n.a.	CBS 2764 T	n.d.	U75959	AB018176
<i>Citeromyces matritensis</i>	WM 1095	CBS 864	AJ508502	AJ508587	n.d.
<i>Clavispora lusitaniae</i>	WM 35	CBS 6936 T	AJ389065	U44817	n.d.
<i>Clavispora opuntiae</i>	WM 58	CBS 7068 T	AJ389067	U44818	n.d.
<i>Debaryomyces carsonii</i>	WM 41	CBS 2285 T	AJ508503	U45743	n.d.
<i>Debaryomyces etchellsii</i>	WM 69	CBS 2011 T	AJ389068	U45809	n.d.
<i>Debaryomyces hansenii</i> var. <i>fabryii</i>	WM 66	CBS 789 T	AJ508504	U94927	AJ508273
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	WM 36	CBS 767 T	AJ508505	U45808	X62649
<i>Filobasidiella neoformans</i>	n.a.	ATCC 208821T	U10867	n.d.	n.d.
<i>Filobasidiella neoformans</i>	n.a.	ATCC 24067	n.d.	CPC5825	n.d.
<i>Filobasidiella neoformans</i>	n.a.	CBS 6886	n.d.	n.d.	D12804
<i>Issatchenkia orientalis</i>	WM 37	CBS 5147 T	AJ389086	U76347	n.d.
<i>Kluyveromyces cellobiovorus</i>	WM 812	CBS 7153 T	AJ508506	AJ508588	X89528
<i>Kluyveromyces delphensis</i>	WM 791	CBS 2170 T	AJ508507	U69576	X83823
<i>Kluyveromyces lactis</i>	WM 67	CBS 683 NT	AJ389080	U94922	n.d.
<i>Kluyveromyces lactis</i>	n.a.	CBS 2359	n.d.	n.d.	X51830
<i>Kluyveromyces marxianus</i>	WM 39	CBS 712 T	AJ389078	U94924	X89523

(continued on next page)

Table 1 (continued)

Species	Strain designation		Actin gene accession no.	LSU gene accession no.	SSU gene accession no.
	Lab no.	Collection no.			
<i>Kluyveromyces polysporus</i>	WM 40	CBS 2163 T	AJ389074	U68548	X69845
<i>Kodamae ohmeri</i>	WM 807	CBS 5367 T	AJ508508	U45702	n.d.
<i>Lodderomyces elongisporus</i>	WM 68	CBS 2605 T	AJ508509	U45763	X78600
<i>Metschnikowia pulcherrima</i>	n.a.	CBS 5833 T	n.d.	U45736	AB023473
<i>Metschnikowia pulcherrima</i>	WM 80	CBS 2255	AJ389070	n.d.	n.d.
<i>Metschnikowia reukaufii</i>	WM 798	CBS 5834 T	AJ508510	U44825	AB023469
<i>Neurospora crassa</i>	n.a.	FGSC 987	U78026	n.d.	n.d.
<i>Neurospora crassa</i>	n.a.	NRRL 13141	n.d.	U40124	n.d.
<i>Neurospora crassa</i>	n.a.	n.a.	n.d.	n.d.	X04971
<i>Pichia anomala</i>	WM 824	CBS 5759 NT	AJ508511	U74592	X58054
<i>Pichia burtonii</i>	WM 794	CBS 2352 T	AJ508512	U45712	AB018177
<i>Pichia capsulata</i>	WM 796	CBS 1993 T	AJ508513	U75516	AB018178
<i>Pichia farinosa</i>	WM 803	CBS 185 T	AJ508514	U45739	n.d.
<i>Pichia fermentans</i>	WM 43	CBS 187 T	AJ389082	U75726	n.d.
<i>Pichia guilliermondii</i>	WM 827	CBS 2030 T	AJ389063	U45709	AJ508274
<i>Pichia guilliermondii</i>	WM 828	CBS 2031	AJ508515	AJ508589	n.d.
<i>Pichia holstii</i>	WM 797	CBS 4140 T	AJ508516	U75722	AB018180
<i>Pichia jadinii</i>	n.a.	CBS 5609	n.d.	n.d.	AF239662
<i>Pichia jadinii</i>	WM 45	CBS 1600 T	AJ389090	U73570	n.d.
<i>Pichia membranifaciens</i>	WM 46	CBS 107 T	AJ389088	U75725	X58055
<i>Pichia mexicana</i>	n.a.	CBS 6306	n.d.	n.d.	AB013570
<i>Pichia mexicana</i>	WM 805	CBS 7066 T	AJ508517	U45797	n.d.
<i>Pichia norvegensis</i>	n.a.	DSM 70760	n.d.	n.d.	AF201302
<i>Pichia norvegensis</i>	WM 236	CBS 6564 T	AJ389084	U75730	n.d.
<i>Pichia norvegensis</i>	WM 808	CBS 8403	AJ508518	n.d.	n.d.
<i>Pichia segobiensis</i>	NRRL Y-11571	CBS 6857 T	AJ508519	U45742	n.d.
<i>Pichia stipitis</i>	WM 810	CBS 5773 T	AJ508520	U45741	n.d.
<i>Pichia sydowiorum</i>	WM 81	CBS 5995 T	AJ508521	U74594	AJ508275
<i>Saccharomyces bayanus</i>	n.a.	CBS 380 T	n.d.	U94931	n.d.
<i>Saccharomyces bayanus</i>	n.a.	CBS 395	n.d.	AJ279065	n.d.
<i>Saccharomyces barnettii</i>	n.a.	CBS 6946	n.d.	U84231	n.d.
<i>Saccharomyces barnettii</i>	WM 813	CBS 5648 T	AJ508522	AJ50590	X97778
<i>Saccharomyces bayanus/pastorianus</i>	WM 1167	CBS 2165	AJ508523	AJ50591	n.d.
<i>Saccharomyces bayanus/pastorianus</i>	WM 1168	CBS 2440	AJ508524	AJ50592	n.d.
<i>Saccharomyces carlsbergensis</i>	WM 1080	RH 6136	AJ508525	AJ50593	n.d.
<i>Saccharomyces cerevisiae</i>	WM 48	CBS 1171 NT	AJ389075	U44806	Z75578
<i>Saccharomyces exiguus</i>	WM 789	CBS 379 T	AJ508526	U68553	X98868
<i>Saccharomyces kluyveri</i>	WM 50	CBS 3082 T	AJ389077	U68552	Z75580
<i>Saccharomyces paradoxus</i>	n.a.	CBS 432 NT	n.d.	U68555	n.d.
<i>Saccharomyces pastorianus</i>	n.a.	CBS 1538 NT	n.d.	U68547	n.d.
<i>Saccharomycopsis capsularis</i>	WM 51	CBS 2519 NT	AJ389092	U40082	X69847
<i>Schizosaccharomyces pombe</i>	n.a.	CBS 356 T	n.d.	U40085	X58056
<i>Schizosaccharomyces pombe</i>	n.a.	L 975	Y00447	n.d.	n.d.
<i>Torulaspora delbrueckii</i>	WM 821	CBS 1146 NT	AJ508527	U72156	X53496
<i>Trichosporon appendiculare</i>	WM 1.26	CBS 5265 T	AJ508528	CPK	AJ508276
<i>Trichosporon fennicum</i>	WM 792	CBS 5928 T	AJ508529	U45715	AB018162, AJ508277
<i>Trichosporon hellenicum</i>	WM 817	CBS 4099 T	AJ508530	U40125	AJ508268
<i>Trichosporon melibiosaceum</i>	WM 793	CBS 6087 T	AJ508531	AJ50594	AJ508279
<i>Yarrowia lipolytica</i>	WM 17	CBS 6124 T	AJ508532	U40080	AB018158

Table 2
Primers used for the amplification and sequencing of the actin gene fragment

Primer	Sequence (5' → 3')	bp	GC%	Position ^a	Reference
CA1	GCCGGTGACGACGCTCCAAGAGCTG	25	68	720–744	Kan, 1993
CA2R	CCGTGTTCAATTGGGTATCTCAAGGTC	27	48	851–877	Kan, 1993
CA3	GACATCAAGGTATCATGGTTGGTATGGGTGC	31	50	772–802	Kan, 1993
CA4	CCATCATGAAGTGTGACATGGATGTTAG	28	43	1498–1525	Kan, 1993
CA5R	GTGAACAATGGATGGACCAGATTCTGTCG	28	50	1742–1769	Kan, 1993
CA7R	CCATCACCAGAATCCAAAACAATACCGG	28	46	1102–1129	Daniel et al., 2001
CA8	TGTACTCTTCTGGTAGAACTACCGG	25	48	1081–1105	Daniel et al., 2001
CA9R	GGTCAATACCAGCAGCTTCCAAAACCT	26	50	1457–1482	Daniel et al., 2001
CA11	AACAATGGACGGTGGTATGT	20	45	–3–16	Daniel et al., 2001
CA13	CCAGATGGTCAAGTTATCAC	20	45	1383–1402	Daniel et al., 2001
CA14	AACTGGGATGACATGGAGAAGATCTGGC	28	50	888–915	Daniel et al., 2001
CA15R	TCGGTCAAATCTCTACCAGC	20	50	1197–1216	Daniel et al., 2001
CA16	TTTACGCTGGTTTCTCCATGCCTCACGG	28	54	1150–1177	Daniel et al., 2001
CA17R	TTGTGGTGAACAATGGATGGACC	23	47	1752–1774	Daniel et al., 2001
CA18	TTTCCCCTCCATCGTCGGTCG	21	62	746–766	Daniel, 2003
CA19R	ACTTTCGTCGTATTCTGGCTTGG	23	48	1729–1751	Daniel, 2003
CA20	TATACTCCACTGGTAGAACCACCGG	25	52	1081–1105	Daniel, 2003
CA21	ATTGATAACGGTTCGGTATGTG	23	43	684–706	Daniel, 2003
CA22R	TCGTCGTATTCTTGCTTGGATCCAC	27	44	1721–1747	Daniel, 2003
CA23R	GTRATRACTTGDCRC	17	35–47	1386–1402	Daniel, 2003

R, reverse.

^a Position relative to the *Candida albicans* sequence X16377; first base of the start codon set 1.

might not be possible as species are continuous entities rather than distinct units. The genes of the ribosomal gene complex (rRNA), especially the 5' region of the large subunit gene (LSU), have been sequenced for the majority of ascomycetous yeasts (Kurtzman and Robnett, 1998). Therefore, they have become prime targets for DNA sequence based identification of these organisms (Yang et al., 2001; Kano et al., 2002; Herzberg et al., 2002).

The 5' end of the LSU contains the variable regions D1 and D2 of about 600 bp. The more variable D2 region showed 0–1% divergence in conspecific strains and most sibling species could be distinguished based on higher sequence divergence in the genera *Issatchenkia*, *Pichia* and *Saccharomyces* (Peterson

and Kurtzman, 1991). However, exceptions were noted for *Saccharomyces bayanus*/*Saccharomyces pastorianus* and *Williopsis saturnus* and its variety *sargentensis* (Kurtzman and Blanz, 1998). Comparisons of two and more strains from 103 ascomycetous yeast species by Kurtzman and Robnett (1998) showed intra-species variation of up to two nucleotides in the D1/D2 region of most strains, three nucleotide differences in three species and up to six nucleotide differences in one species in which the higher numbers of differences were explained by contiguous deletions rather than single substitutions. However, also 13 comparisons between sibling species with low nDNA relatedness of 10–59% showed D1/D2 divergences of up to two nucleotides. These

Note to Table 1:

ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DMS, Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany; DUMC, Duke University Medical Center, Durham, NC, USA; FGSC, Fungal Genetics Stock Center, Kansas City, USA; NRRL, Northern Regional Research Center of the United States Department of Agriculture (ARC), Peoria, IL, USA; WM, Culture collection of Dr. Wieland Meyer, Westmead Hospital, Sydney, NSW, Australia; strain RH 6136 kindly provided by Prof. Stahl, TU Berlin, Institut für Biotechnologie, Berlin, Germany; strains PL 448 and PL 452 kindly provided by Prof. S.A. Meyer/E. O'Neill, Georgia State University, Atlanta, GA, USA; strain L 975 was used by Mertins and Gallwitz (1987) as provided by Dr. Gutz, Braunschweig, Germany; ST, serotype; T, ex-type culture; NT, ex-neotype culture; CPK, unpublished sequences kindly provided by Dr. C.P. Kurtzman, ARC, Peoria, IL, USA. n.a., not available; n.d., not determined; accession numbers printed in bold represent the sequences determined in the current study.

^a Sequence from the CBS database. (<http://www.cbs.knaw.nl/>)

Table 3

Primers used for the amplification and sequencing of the D1/D2 region of the large subunit rRNA (LSU) gene (LR) and the small subunit (SSU) gene (S)

Primer	Sequence (5' → 3')	bp	GC%	Position ^a
SR1R	TACCTGGTTGATTCTGCCAGT	21	48	1–21 SSU
5.8S	CGCTGCGTTCTTCATCG	17	59	51–35 5.8S
LR0R	ACCCGCTGAACTTAAGC	17	53	26–42 LSU
LR16	TTCCACCCAAACACTCG	17	53	675–691 LSU

These primer sequences were identified by the Vilgalys laboratory, Duke University, Durham, NC, USA (Moncalvo et al., 2000). A list of useful rRNA primers can be found at <http://www.biology.duke.edu/fungi/mycolab/primers.htm>.

R, reverse.

^a *Saccharomyces cerevisiae* numbering.

were regarded as exceptions or were previously established as variety pairs. It was predicted that strain pairs with more than three nucleotide differences can be recognised as different species and strain pairs with up to three nucleotide differences as conspecific or as sibling species (Kurtzman and Robnett, 1998). It was further implicated that this prediction must be interpreted in the context of the whole organism and its life history. It is often generalised that strains with less than three nucleotide differences are to be regarded as conspecific. This might be a misleading conclusion in view of the overlap between intra- and interspecies sequence variation.

The small subunit (SSU) rRNA gene has been used extensively for the inference of phylogeny of ascomycetous yeasts (e.g. Barns et al., 1991; Hendriks et al., 1991, 1992; Cai et al., 1996; Suzuki et al., 1999; James

et al., 2001). However, not many data exist for multiple strains of single species making the assessment of the variability of closely related strains difficult. In general, the SSU gene is more conserved than the LSU gene and its utility for identification purposes appears rather limited as the determination of almost complete gene sequences of about 1800 nucleotides is often required to detect sufficient variation.

The *act1* gene encodes actin, a protein that is abundant in all eukaryotic cells, where it is the major component of cytoplasmic microfilaments. Due to structural constraints, the amino acid sequence of actin proteins is highly conserved (e.g. Hightower and Meagher, 1996). However, the DNA sequences, in particular the third codon positions, show substantial variation among closely related taxa (Daniel et al., 2001).

Here, we present a comparative analysis of partial LSU and actin gene sequences in regard to their ability to resolve closely related yeast species reliably. In addition, selected comparisons of almost complete small subunit RNA gene sequences were evaluated.

2. Material and methods

The strains considered in this study are listed in Table 1. Genomic DNA was extracted as described by Meyer et al. (1997). PCR amplification of specific fragments was conducted using a touch-down PCR program. It consisted of 20 cycles of denaturation at 94 °C for 5 min in cycle 1 and 30 s in cycles 2 to 20, annealing at 60 °C for 30 s reduced by 0.5 °C per cycle and an extension at 72 °C for 1 min; followed by 15 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, an extension at 72 °C

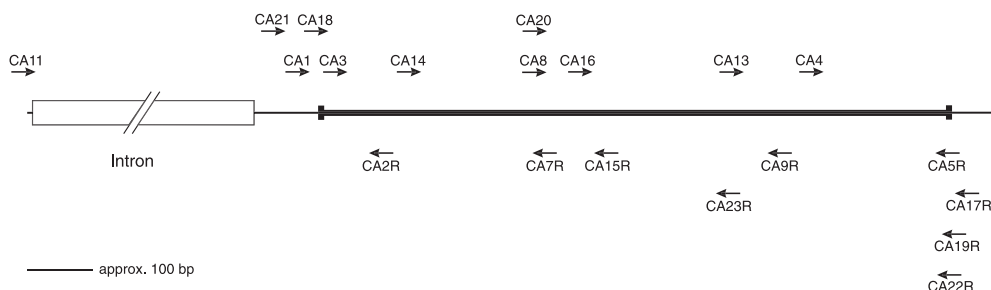


Fig. 1. Actin primer binding sites in a schematic drawing of the actin gene. The Intron was shortened by 50% based on its length in the *C. albicans* sequence X16377. The bold line delimits the analysed sequence. For the primers names CA1–CA22R see Table 2.

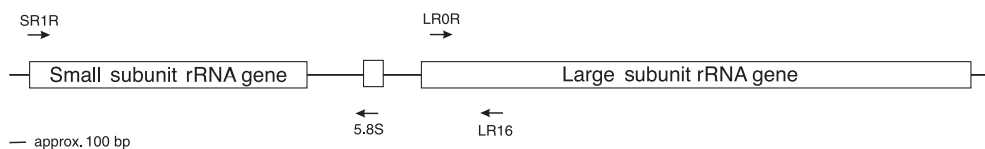


Fig. 2. Ribosomal RNA primer binding sites in a schematic drawing of the rRNA of *Saccharomyces cerevisiae* Z73326, Y13138. For the primer names SR1R, 5.8S, LR0R and LR16 see Table 3.

for 1 min and a final extension at 72 °C for 10 min. The amplification reactions were performed in a PTC-100™ Programmable Thermal (MJ Research, Waltham, MA, USA) Controller in 50 µl containing: 50 ng genomic DNA; 0.2 mM each of dATP, dCTP, dGTP, dTTP (Roche, Basel, Switzerland); 0.5 mM

magnesium acetate (Sigma, St. Louis, MO, USA); 0.3 µM each of the two primers, 2.5 U AmpliTaq Polymerase® (Perkin Elmer, Boston, MA, USA) and the buffer provided by the manufacturer with 10 mM Tris-HCl pH 8.3; 50 mM KCl; 1.5 mM MgCl₂. After the reaction amplification products were assayed by

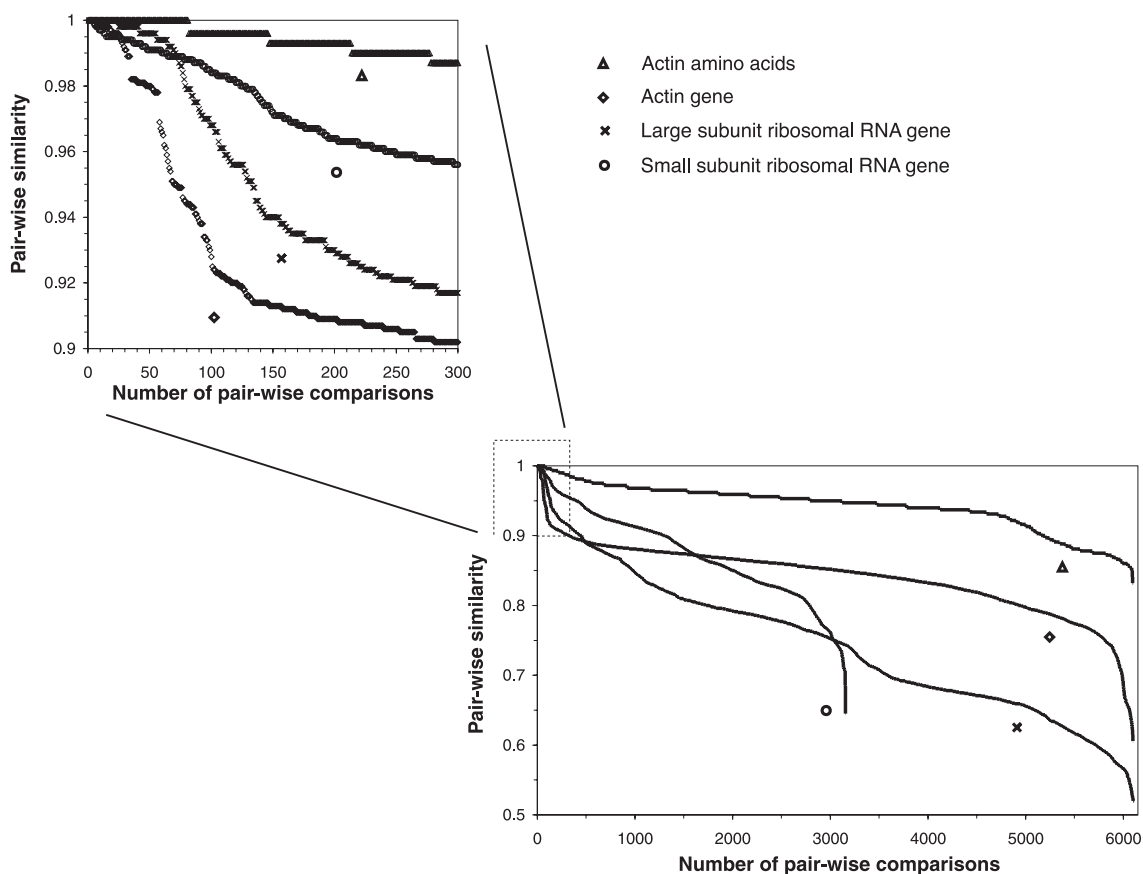


Fig. 3. Scatter plots of pair-wise similarity between pairs of taxa in order of decreasing relatedness. The enlarged upper part of the figure shows the most informative region for the separation at the strain and species level. The actin gene shows the steepest decline of similarities in this region.

Table 4

Intraspecies sequence similarity in the actin gene and DNA relatedness data for all multiple strains that were considered to belong to the same species

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Candida glabrata</i>	WM 53	CBS 138 T			
<i>Candida glabrata</i>	WM 54	DUMC 19-1112	0	100	n.a.
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T			
<i>Candida parapsilosis</i> Type 1	WM 23	DUMC 31-1070	0	100	n.a.
<i>Issatchenkia orientalis</i>	WM 37	CBS 5147 T			
<i>Candida krusei</i>	WM 14	CBS 573 T	0	100	99 (1)
<i>Kluyveromyces lactis</i>	WM 67	CBS 683 NT			
<i>Candida sphaerica</i>	WM 28	CBS 141 T	0	100	n.a.
<i>Pichia guilliermondii</i>	WM 828	CBS 2031			
<i>Pichia guilliermondii</i>	WM 827	CBS 2030 T	0	100	n.a.
<i>Candida guilliermondii</i> var. <i>guilliermondii</i>	WM 826	CBS 566 T	0	100	100 (2)
<i>Candida fukuyamaensis</i>	WM 1091	CBS 7921 T			
<i>Candida fermentati</i>	WM 1092	CBS 2022 T	0	100	68 (2)
<i>Trichosporon appendiculare</i>	WM 1.26	CBS 5265 T	1	99.8	n.a.
<i>Saccharomyces cerevisiae</i>	WM 48	CBS 1171 NT			
<i>Saccharomyces carlsbergensis</i>	WM 1080	RH 6136	0	100	n.a.
<i>Saccharomyces bayanus/pastorianus</i>	WM 1167	CBS 2165	1	99.8	n.a.
<i>Candida robusta</i>	WM 604	CBS 1907 T	2	99.7	86 (3)
<i>Candida albicans</i> ST A	WM 2	CBS 562 NT			
<i>Candida albicans</i> ST B	WM 231	CBS 5983	1	99.8	n.a.
<i>Candida tropicalis</i>	WM 1087	CBS 94 T			
<i>Candida tropicalis</i>	WM 30	DUMC 29-3711	1	99.8	n.a.
<i>Clavispora lusitanae</i>	WM 35	CBS 6936 T			
<i>Candida lusitanae</i>	WM 18	CBS 4413 T	1	99.8	94 (4)
<i>Kodamae ohmeri</i>	WM 807	CBS 5367 T			
<i>Candida guilliermondii</i> var. <i>membranifaciens</i>	WM 806	CBS 1950 T	1	99.8	n.a.
<i>Pichia farinosa</i>	WM 803	CBS 185 T			
<i>Candida cacaoi</i>	WM 802	CBS 2020 T	1	99.8	87 (5)
<i>Pichia membranifaciens</i>	WM 46	CBS 107 T			
<i>Candida valida</i>	WM 32	CBS 638 T	1	99.8	114 (6), 84 (7)
<i>Pichia mexicana</i>	WM 805	CBS 7066 T			
<i>Candida veronae</i>	WM 804	CBS 5815 T	1	99.8	n.a.
<i>Yarrowia lipolytica</i>	WM 17	CBS 6124 T			
<i>Candida lipolytica</i>	WM 290	CBS 599 T	1	99.8	n.a.
<i>Pichia anomala</i>	WM 824	CBS 5759 NT			
<i>Candida pelliculosa</i>	WM 825	CBS 605 T	2	99.7	81 (8)
<i>Candida viswanathii</i>	WM 33	CBS 4024 T			
<i>Candida lodderae</i>	NRRL Y-17317	CBS 1924 T	2 (+5) ^b	99.7	89–91 (9)
<i>Candida intermedia</i>	WM 811	CBS 572 T			
<i>Kluyveromyces cellobiovorus</i>	WM 812	CBS 7153 T	3	99.6	>90 (10)
<i>Kluyveromyces marxianus</i>	WM 39	CBS 712 T			
<i>Candida kefyr</i>	WM 13	CBS 834 T	3	99.6	92 (8)
<i>Pichia norvegensis</i>	WM 236	CBS 6564 T			
<i>Candida norvegensis</i>	WM 235	CBS 1922 T	3	99.6	n.a.
<i>Candida norvegensis</i>	WM 808	CBS 8403	1	99.8	n.a.
<i>Pichia jadinii</i>	WM 45	CBS 1600 T			
<i>Candida utilis</i>	WM 31	CBS 621 T	3	99.6	85 (11)
<i>Metschnikowia pulcherrima</i>	WM 80	CBS 2255			
<i>Candida pulcherrima</i>	WM 25	CBS 610 NT	4	99.5	n.a.

Table 4 (continued)

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Pichia fermentans</i>	WM 43	CBS 187 T			
<i>Candida lambica</i>	WM 16	CBS 1876 T	4	99.5	99 (12)
<i>Candida humilis</i>	WM 800	CBS 5658 T			
<i>Candida milleri</i>	WM 801	CBS 6897 T	5 (+5) ^b	99.4	n.a.
<i>Saccharomyces exiguus</i>	WM 789	CBS 379 T			
<i>Candida holmii</i>	WM 788	CBS 135 NT	5 (+17) ^b	99.4	75 (13)
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	WM 36	CBS 767 T			
<i>Candida famata</i> var. <i>famata</i>	WM 280	CBS 1795 T	6	99.3	75 (14)
<i>Candida guilliermondii</i> var. <i>carphophila</i>	WM 829	CBS 5256 T			
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	8	99.1	n.a.
<i>Torulasporea delbrueckii</i>	WM 821	CBS 1146 NT			
<i>Candida colliculosa</i>	WM 820	CBS 133 T	8	99.1	n.a.
<i>Citeromyces matritensis</i>	WM 1095	CBS 864			
<i>Candida globosa</i>	WM 1094	CBS 162 T	8 (+2) ^b	99.1	n.a.
<i>Candida parapsilosis</i> Type 2	WM 1089	CBS 8825			
<i>Candida parapsilosis</i> Type 2	WM 1.57	PL 452	10	98.9	n.a.
<i>Candida spec.</i> ^c	WM 1169	CBS 6857 ^c			
<i>Candida lodderae</i>	NRRL Y-17317	CBS 1924 T	4 (+5) ^b	99.5	n.a.
<i>Candida viswanathii</i>	WM 33	CBS 4024 T	11	98.8	n.a.

Multiple comparisons relate to the first strain listed. Taxa listed in order of decreasing similarity.

n.a., not available.

^a Key to the reference for DNA–DNA reassociation values: (1) Kurtzman et al., 1980; (2) O'Neill and Meyer, 2000; (3) Vaughan-Martini and Martini, 1987; (4) Lachance et al., 1986; (5) Lee et al., 1992; (6) CBS database; (7) I. Spencer-Martins, unpublished, from CBS database; (8) Bak and Stenderup, 1969; (9) Lee et al., 1998; (10) Martini and Martini, 1992; (11) Kurtzman et al., 1979; (12) Smith et al., 1989; (13) Vaughan-Martini and Kurtzman, 1985; (14) Nishikawa et al., 1996; (15) M. Smith, personal communication; (16) Kurtzman and Robnett, 1998; (17) Nakase and Suzuki, 1985; (18) Daniel, in press; (19) Roy and Meyer, 1998; (20) Fuson et al., 1987; (21) Meyer et al., 1975.

^b Possible heterogeneities caused by ambiguous base assignment.

^c This strain does not represent *Pichia segobiensis*, as listed in the CBS catalogue.

* For abbreviations of strain designations see Table 1.

electrophoresis in 0.8% agarose (Amresco, Solon, OH, USA) gels containing 0.4 µg/ml ethidium bromide (Sigma) in Tris–borate–EDTA buffer (Sambrook and Russell, 2001) and visualised under UV-light. Amplification products of the same strain were pooled from two to six reactions, purified using the Wizard™ PCR preparation system (Promega, Madison, WI, USA), assayed by electrophoresis and quantified spectrophotometrically.

Oligonucleotide primers for amplification and sequencing are listed in Tables 2 and 3 and their positions shown in Figs. 1 and 2. Their sequences were obtained from the cited sources or designed manually. The universal actin primers CA21 and CA22R were designed using the program GPrime (Armstrong et al., 1997) to identify conserved binding sites among 20 species of ascomycetous yeasts. The universal primers or the combination of one of them

with CA1 or CA5R produced an amplicon of approximately 1 kb from about 80 species. Nucleotide sequences were obtained by direct cycle sequencing of PCR products for both DNA strands of the actin gene fragments and the D1/D2 large subunit rDNA fragments (LSU) or for one DNA strand in the small subunit rDNA gene (SSU). All actin and LSU gene sequences were determined using an ABI 377 DNA sequencer (Applied Biosystems, Fostercity, CA, USA). The sequencing reactions contained 75 ng (LSU), 100–150 ng (actin) of amplified fragment, 10 pmol/µl of the respective primer and the ABI Big Dye™ Terminators cycle sequencing kit (Applied Biosystems) with AmpliTaq® DNA Polymerase FS. SSU sequences were obtained using the standard LiCor protocol and a Licor LongreadIR 4200 automated sequencer (LiCor Biosciences, Lincoln, NE, USA). The sequencing reactions contained approxi-

Table 5

Interspecies sequence similarity in the actin gene and DNA relatedness for the 30 most similar taxa that were considered to belong to different species

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Candida fukuyamaensis</i>	WM 1091	CBS 7921 T			
<i>Candida guilliermondii</i> var. <i>carpophila</i>	WM 829	CBS 5256 T	17	98.2	n.a.
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	19	98.0	n.a.
<i>Pichia guilliermondii</i>	WM 827	CBS 2030 T			
<i>Candida fukuyamaensis</i>	WM 1091	CBS 7921 T	18	98.1	56 (2)
<i>Candida guilliermondii</i> var. <i>carpophila</i>	WM 829	CBS 5256 T	17	98.2	47 (2)
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	21	97.8	55 (2)
<i>Candida dubliniensis</i>	WM 602	CBS 7987 T			
<i>Candida albicans</i>	WM 2	CBS 562 NT	21	97.8	35 (2)
<i>Debaryomyces hansenii</i> var. <i>fabryi</i>	WM 66	CBS 789 T			
<i>Candida famata</i> var. <i>flareri</i>	WM 60	CBS 1796 T	23	97.6	63 (14)
<i>Candida steatolytica</i>	WM 816	CBS 5839 T			
<i>Candida inositophila</i>	WM 818	CBS 6736 T	30	96.9	73 (15)
<i>Pichia segobiensis</i>	NRRL Y-11571	CBS 6857 T			
<i>Pichia stipitis</i>	WM 810	CBS 5773 T	32	96.6	38 (16)
<i>Debaryomyces hansenii</i> var. <i>fabryi</i>	WM 66	CBS 789 T			
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	WM 36	CBS 767 T	34	96.5	52 (17)
<i>Pichia novogensis</i>	WM 236	CBS 6564 T			
<i>Candida inconspicua</i>	WM 11	CBS 180 T	37	96.2	n.a.
<i>Pichia sydowiorum</i>	WM 81	CBS 5995 T			
<i>Candida nitrativorans</i>	WM 76A	CBS 6152 T	40+(2) ^b	95.9	4 (18)
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T			
<i>Candida parapsilosis</i> Type 2	WM 1.57	PL 452	44	95.5	10 (19)
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T			
<i>Candida parapsilosis</i> Type 3	WM 1.56	PL 448	44 (+9) ^b	95.5	10 (19)
<i>Pichia burtonii</i>	WM 794	CBS 2352 T			
<i>Trichosporon fenicum</i>	WM 792	CBS 5928 T	47	95.1	n.a.
<i>Candida parapsilosis</i> Type 2	WM 1.57	PL 452			
<i>Candida parapsilosis</i> Type 3	WM 1.56	PL 448	48 (+9) ^b	95.0	21 (19)
<i>Kluyveromyces lactis</i>	WM 67	CBS 683 NT			
<i>Kluyveromyces marxianus</i>	WM 39	CBS 712 T	49	94.9	10 (20)
<i>Pichia anomala</i>	WM 824	CBS 5759 NT			
<i>Candida nitrativorans</i>	WM 76A	CBS 6152 T	49	94.9	n.a.
<i>Candida tropicalis</i>	WM 1087	CBS 94 T			
<i>Candida maltosa</i>	WM 603	CBS 5611 T	52	94.6	32 (21)
<i>Saccharomyces cerevisiae</i>	WM 48	CBS 1171 NT			
<i>Saccharomyces bayanus/pastorianus</i>	WM 1168	CBS 2440	53	94.5	n.a.
<i>Arxiozyma telluris</i>	WM 784	CBS 2685 T			
<i>Candida pintolopesii</i>	WM 785	CBS 1787 T	54	94.4	21 (18)
<i>Arxiozyma telluris</i>	WM 784	CBS 2685 T			
<i>Candida bovina</i>	WM 786	CBS 2760 T	54	94.4	7 (18)
<i>Trichosporon hellenicum</i>	WM 817	CBS 4099 T			
<i>Candida steatolytica</i>	WM 816	CBS 5839 T	57	94.1	21–25 (15)
<i>Pichia anomala</i>	WM 824	CBS 5759 NT			
<i>Pichia sydowiorum</i>	WM 81	CBS 5995 T	57 (+3) ^b	94.1	n.a.
<i>Candida tropicalis</i>	WM 1087	CBS 94 T			
<i>Candida dubliniensis</i>	WM 602	CBS 7987 T	60	93.8	n.a.
<i>Candida tropicalis</i>	WM 1087	CBS 94 T			
<i>Candida parapsilosis</i> Type 3	WM 1.56	PL 448	61 (+9) ^b	93.7	n.a.

Table 5 (continued)

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Candida pintolopesii</i>	WM 785	CBS 1787 T			
<i>Candida bovina</i>	WM 786	CBS 2760 T	64	93.4	22 (18)
<i>Candida tropicalis</i>	WM 1087	CBS 94 T			
<i>Candida albicans</i>	WM 2	CBS 562 T	65	93.3	8 (8)
<i>Kluyveromyces delphensis</i>	WM 791	CBS 2170 T			
<i>Candida glabrata</i>	WM 53	CBS 138 T	65	93.3	n.a.
<i>Clavispora opuntiae</i>	WM 58	CBS 7068 T			
<i>Clavispora lusitanae</i>	WM 35	CBS 6936 T	67	93.1	8 (4)
<i>Metschnikowia reukaufii</i>	WM 798	CBS 5834 T			
<i>Candida rancoensis</i>	WM 799	CBS 8174 T	68 (+5) ^b	93.0	<35 (18)
<i>Lodderomyces elongisporus</i>	WM 68	CBS 2605 T			
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T	72 (+3) ^b	92.6	n.a.
<i>Lodderomyces elongisporus</i>	WM 68	CBS 2605 T			
<i>Candida parapsilosis</i> Type 3	WM 1.56	PL 448	73 (+12) ^b	92.5	n.a.

Multiple comparisons relate to the first strain listed. Taxa listed in order of decreasing similarity.

n.a., not available.

^a Key to the reference for DNA–DNA reassociation values: (1) Kurtzman et al., 1980; (2) O'Neill and Meyer, 2000; (3) Vaughan-Martini and Martini, 1987; (4) Lachance et al., 1986; (5) Lee et al., 1992; (6) CBS database; (7) I. Spencer-Martins, unpublished, from CBS database; (8) Bak and Stenderup, 1969; (9) Lee et al., 1998; (10) Martini and Martini, 1992; (11) Kurtzman et al., 1979; (12) Smith et al., 1989; (13) Vaughan-Martini and Kurtzman, 1985; (14) Nishikawa et al., 1996; (15) M. Smith, personal communication; (16) Kurtzman and Robnett, 1998; (17) Nakase and Suzuki, 1985; (18) Daniel, in press; (19) Roy and Meyer, 1998; (20) Fuson et al., 1987; (21) Meyer et al., 1975.

^b Possible heterogeneities caused by ambiguous base assignment.

* For abbreviations of strain designations see Table 1.

mately 400 ng (PCR product) template DNA, 1 pmol/ μ l labelled primer and buffer and polymerase from the SequiTherm EXCEL™ II DNA sequencing kit-LC (Epicentre, Madison, WI, USA).

3. Results

The range of pair-wise sequence similarity among all taxa was determined to visualize the separation of taxa (Fig. 3). They showed the largest overall decrease of similarity with decreasing relatedness for the LSU gene and the smallest for actin amino acids. SSU gene sequences were only available for a smaller subset of taxa and the graphs are therefore not directly comparable. The actin gene showed a steep slope at the beginning, for closely related taxa and, at the end, for comparisons involving outgroup taxa. The section between these extremes was characterised by a very slow decrease in similarities. The most informative region for the separation at the strain and species level, which is the region with the highest similarities,

showed the steepest decline for the actin gene, followed by the LSU gene, and a very slow decline for the inferred actin amino acid sequence (Fig. 3).

The effectiveness of species separation for the different sequences is summarized in Table 8. Partial actin gene sequences comprising 979 bp coding sequence showed generally up to 11 nucleotide differences in intraspecies comparisons (Table 4). Interspecies comparisons started with 17 nucleotide differences (Table 5). A high number of 30 nucleotide differences relative to their 75% DNA relatedness were detected for the comparison of *Candida steatolytica/Candida inositophila*, both named currently *Zygoascus hellenicus* and are under taxonomic revision (M. Smith, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, personal communication). The LSU data showed coinciding similarities for comparisons among and within species (Table 8). Of the 35 intra-species comparisons, 31 showed up to two nucleotide differences and one showed four, five and six differences, respectively (Table 6). A detailed study of the exceptionally high number of 32 different nucleotides in the

Table 6

Intraspecies sequence similarity in the D1/D2 region of the LSU and DNA relatedness for all multiple strains that were considered to belong to the same species

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Candida guilliermondii</i> var. <i>carpophila</i>	WM 829	CBS 5256 T			
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	0	100	n.a.
<i>Candida haemulonii</i> Type II	WM 823	CBS 7798			
<i>Candida haemulonii</i> Type II	n.a.	CBS 6915	0	100	n.a.
<i>Candida parapsilosis</i> Type 2	WM 1089	CBS 8825			
<i>Candida parapsilosis</i> Type 2	WM 1.57	PL 452	0	100	n.a.
<i>Citeromyces matritensis</i>	n.a.	CBS 2764 T			
<i>Citeromyces matritensis</i>	WM 1095	CBS 864	0	100	n.a.
<i>Candida globosa</i>	WM 1094	CBS 162 T	0	100	n.a.
<i>Kluyveromyces lactis</i>	WM 67	CBS 683 NT			
<i>Candida sphaerica</i>	WM 28	CBS 141 T	0	100	n.a.
<i>Kluyveromyces marxianus</i>	WM 39	CBS 712 T			
<i>Candida kefyr</i>	WM 13	CBS 834 T	0	100	92 (8)
<i>Kodamae ohmeri</i>	WM 807	CBS 5367 T			
<i>Candida guilliermondii</i> var. <i>membranifaciens</i>	WM 806	CBS 1950 T	0	100	n.a.
<i>Pichia anomala</i>	WM 824	CBS 5759 NT			
<i>Candida pelliculosa</i>	WM 825	CBS 605 T	0	100	81 (8)
<i>Pichia farinosa</i>	WM 803	CBS 185 T			
<i>Candida cacaoi</i>	WM 802	CBS 2020 T	0	100	87 (5)
<i>Pichia guilliermondii</i>	WM 828	CBS 2031			
<i>Pichia guilliermondii</i>	WM 827	CBS 2030 T	0	100	n.a.
<i>Candida guilliermondii</i> var. <i>guilliermondii</i>	WM 826	CBS 566 T	0	100	100 (3)
<i>Pichia membranifaciens</i>	WM 46	CBS 107 T			114 (6)
<i>Candida valida</i>	WM 32	CBS 638 T	0	100	84 (7)
<i>Pichia mexicana</i>	WM 805	CBS 7066 T			
<i>Candida veronae</i>	WM 804	CBS 5815 T	0	100	n.a.
<i>Saccharomyces barnettii</i>	WM 813	CBS 5648 T			
<i>Saccharomyces barnettii</i>	n.a.	CBS 6946	0	100	100 (13)
<i>Saccharomyces cerevisiae</i>	WM 48	CBS 1171 NT			
<i>Saccharomyces bayanus/pastorianus</i>	WM 1167	CBS 2165	0	100	n.a.
<i>Saccharomyces carlsbergensis</i>	WM 1080	RH 6136	0	100	n.a.
<i>Candida robusta</i>	WM 604	CBS 1907 T	0	100	86 (3)
<i>Torulaspota delbrueckii</i>	WM 821	CBS 1146 NT			
<i>Candida colliculosa</i>	WM 820	CBS 133 T	0	100	n.a.
<i>Yarrowia lipolytica</i>	WM 17	CBS 6124 T			
<i>Candida lipolytica</i>	WM 290	CBS 599 T	0	100	n.a.
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	WM 36	CBS 767 T			
<i>Candida famata</i> var. <i>famata</i>	WM 280	CBS 1795 T	0 (+1) ^b	100	n.a.
<i>Candida ciferrii</i>	WM 814	CBS 4856 T			
<i>Candida ciferrii</i>	n.a.	CBS 5295	1	99.8	n.a.
<i>Candida humilis</i>	WM 800	CBS 5658 T			
<i>Candida milleri</i>	WM 801	CBS 6897 T	1	99.8	n.a.
<i>Candida albicans</i> ST A	WM 2	CBS 562 NT			
<i>Candida albicans</i> ST B	WM 231	CBS 5983	2	99.6	n.a.
<i>Candida fukuyamaensis</i>	WM 1091	CBS 7921 T			
<i>Candida fermentati</i>	WM 1092	CBS 2022 T	2	99.6	68 (2)
<i>Trichosporon appendiculare</i>	WM 1.26	CBS 5265 T	2	99.6	n.a.
<i>Candida viswanathii</i>	WM 33	CBS 4024 T			
<i>Candida lodderae</i>	NRRL Y-17317	CBS 1924 T	2	99.6	89–91 (9)

Table 6 (continued)

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Issatchenkia orientalis</i>	WM 37	CBS 5147 T			
<i>Candida krusei</i>	WM 14	CBS 573 T	2	99.6	99 (1)
<i>Pichia norvegensis</i>	WM 260	CBS 6564 T			
<i>Candida norvegensis</i>	WM 235	CBS 1922 T	2	99.6	n.a.
<i>Pichia fermentans</i>	WM 43	CBS 187 T			
<i>Candida lambica</i>	WM 16	CBS 1876 T	2	99.6	99 (12)
<i>Pichia jadinii</i>	WM 45	CBS 1600 T			
<i>Candida utilis</i>	WM 31	CBS 621 T	2 (+1) ^b	99.6	85 (11)
<i>Candida intermedia</i>	WM 811	CBS 572 T			
<i>Kluyveromyces cellobiovorus</i>	WM 812	CBS 7153 T	4	99.2	>90 (10)
<i>Metschnikowia pulcherrima</i>	WM 80	CBS 2255			
<i>Candida pulcherrima</i>	WM 25	CBS 610 NT	5	98.9	n.a.
<i>Saccharomyces exiguus</i>	WM 789	CBS 379 T			
<i>Candida holmii</i>	WM 788	CBS 135 NT	6 (+1) ^b	98.9	75 (13)
<i>Clavispora lusitaniae</i>	WM 35	CBS 6936 T			
<i>Candida lusitaniae</i>	WM 18	CBS 4413 T	32 ^c	94.0 ^c	94 (4)

Multiple comparisons relate to the first strain listed. Taxa listed in order of decreasing similarity.

n.a., not available.

^a Key to the reference for DNA–DNA reassociation values: (1) Kurtzman et al., 1980; (2) O'Neill and Meyer, 2000; (3) Vaughan-Martini and Martini, 1987; (4) Lachance et al., 1986; (5) Lee et al., 1992; (6) CBS database; (7) I. Spencer-Martins, unpublished, from CBS database; (8) Bak and Stenderup, 1969; (9) Lee et al., 1998; (10) Martini and Martini, 1992; (11) Kurtzman et al., 1979; (12) Smith et al., 1989; (13) Vaughan-Martini and Kurtzman, 1985; (14) Nishikawa et al., 1996; (15) M. Smith, personal communication; (16) Kurtzman and Robnett, 1998; (17) Nakase and Suzuki, 1985; (18) Daniel, in press; (19) Roy and Meyer, 1998; (20) Fuson et al., 1987; (21) Meyer et al., 1975.

^b Possible heterogeneities caused by ambiguous base assignment.

^c Exceptionally high values caused by divergence of continuous 92 bp representing part of a helix in the secondary structure of the LSU.

* For abbreviations of strain designations see Table 1.

LSU of *Clavispora lusitaniae* CBS 6936 T and the corresponding anamorph *C. lusitaniae* CBS 4413 T will be published elsewhere. Inter-species comparisons also showed up to two nucleotide differences in 14 cases and three to six nucleotide differences in six cases (Table 7).

The derived actin amino acid sequences do not provide reliable resolution at the species level due to high sequence similarities. Detailed comparisons of intra- and inter-species variation were not performed for SSU sequences because of the lack in accuracy of multiple database sequences for identical strains. For example, the sequence of *Trichosporon fennicum* CBS 5928 T as determined in this study differed in 16 nucleotides from the database entry AB018162 for the same strain (Table 1). Different sequences were also found for *Candida albicans* CBS 562 NT with up to nine different residues (M60302, AF114470, A013586, X53497, E15168) and *Candida glabrata*: X51831, M60311, *Candida holmii*: X97808, X78601,

Kluyveromyces polysporus: X83825, X69845. In cases of multiple database entries, one sequence was selected arbitrarily to represent the species in the analysis.

The following examples of almost complete SSU sequences indicated the correspondence of substantial sequence variability in the actin and SSU genes in contrast to partial LSU gene sequences: (I) *Z. hellenicus* (CBS 6736, type strain of *C. inositophila*) compared to *Z. hellenicus* (CBS 5839, type strain of *C. steatolytica*) showed no nucleotide differences in the D1/D2 LSU, 13 differences in the SSU gene and 30 differences in the actin gene; (II) *Z. hellenicus* (CBS 4099, type strain of *Trichosporon hellenicum*) compared to *Z. hellenicus* (CBS 5839) showed 3–4 differences in the D1/D2 LSU, 22–25 differences in the SSU gene and 57 differences in the actin gene.

The alignment of about 110 partial actin gene sequences representing 80 species revealed an even

Table 7

Interspecies sequence similarity in the D1/D2 region of the LSU and DNA relatedness for the 30 most similar taxa that were considered to belong to different species

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Candida fukuyamaensis</i>	WM 1091	CBS 7921 T			
<i>Candida guilliermondii</i> var. <i>carpophila</i>	WM 829	CBS 5256 T	0	100	n.a.
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	0	100	n.a.
<i>Candida steatolytica</i>	WM 816	CBS 5839 T			
<i>Candida inositophila</i>	WM 818	CBS 6736 T	0	100	73 (15)
<i>Kluyveromyces lactis</i>	WM 67	CBS 683 NT			
<i>Kluyveromyces marxianus</i>	WM 39	CBS 712 T	1	99.8	10 (20)
<i>Pichia guilliermondii</i>	WM 827	CBS 2030 T			
<i>Candida fukuyamaensis</i>	WM 1091	CBS 7921 T	1	99.8	56 (2)
<i>Candida guilliermondii</i> var. <i>carpophila</i>	WM 829	CBS 5256 T	1	99.8	47 (2)
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	1	99.8	55 (2)
<i>Debaryomyces hansenii</i> var. <i>fabryi</i>	WM 66	CBS 789 T			
<i>Candida famata</i> var. <i>flaveri</i>	WM 60	CBS 1796 T	1	99.8	63 (14)
<i>Pichia segobiensis</i>	NRRL Y-11571	CBS 6857 T			
<i>Pichia stipitis</i>	WM 810	CBS 5773 T	1 (+2) ^b	99.8	38 (16)
<i>Trichosporon appendiculare</i>	WM 1.26	CBS 5265 T			
<i>Candida fukuayamaensis</i>	WM 1091	CBS 7921 T	2	99.6	n.a.
<i>Candida guilliermondii</i> var. <i>carpophila</i>	WM 829	CBS 5256 T	2	99.6	n.a.
<i>Candida xestobii</i>	WM 1090	CBS 5975 T	2	99.6	n.a.
<i>Candida mucifera</i>	WM 815	CBS 7409 T			
<i>Candida cijferrii</i>	WM 814	CBS 4856 T	2	99.6	<17 (18)
<i>Debaryomyces hansenii</i> var. <i>fabryi</i>	WM 66	CBS 789 T			
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	WM 36	CBS 767 T	2 (+1) ^b	99.6	52 (17)
<i>Pichia guilliermondii</i>	WM 827	CBS 2030 T			
<i>Candida fermentati</i>	WM 1092	CBS 2022 T	3	99.4	n.a.
<i>Trichosporon appendiculare</i>	WM 1.26	CBS 5265 T	3	99.4	n.a.
<i>Trichosporon hellenicum</i>	WM 817	CBS 4099 T			
<i>Candida steatolytica</i>	WM 816	CBS 5839 T	3 (+1) ^b	99.4	21–25 (15)
<i>Candida parapsilosis</i> Type 2	WM 1.57	PL 452			
<i>Candida parapsilosis</i> Type 3	WM 1.56	PL 448	4	99.2	21 (19)
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T			
<i>Candida parapsilosis</i> Type 2	WM 1.57	PL 452	5	99.1	10 (19)
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T			
<i>Candida parapsilosis</i> Type 3	WM 1.56	PL 448	6	98.9	10 (19)
<i>Pichia anomala</i>	WM 824	CBS 5759 NT			
<i>Candida nitrativorans</i>	WM 76 A	CBS 6152 T	8	98.6	n.a.
<i>Saccharomyces barnettii</i>	WM 813	CBS 5648 T			
<i>Saccharomyces exiguus</i>	WM 789	CBS 379 T	8 (+1) ^b	98.5	<30 (13)
<i>Pichia sydowiorum</i>	WM 81	CBS 5995 T			
<i>Candida nitrativorans</i>	WM 76 A	CBS 6152 T	9	98.4	4 (18)
<i>Pichia stipitis</i>	WM 810	CBS 5773 T			
<i>Candida shehatae</i>	WM 809	CBS 5813 T	10 (+1) ^b	98.2	n.a.
<i>Saccharomyces cerevisiae</i>	WM 48	CBS 1171 T			
<i>Saccharomyces bayanus/pastorianus</i>	WM 1168	CBS 2440	12	97.9	n.a.
<i>Pichia anomala</i>	WM 824	CBS 5759 NT			
<i>Pichia sydowiorum</i>	WM 081	CBS 5995 T	12	97.9	n.a.
<i>Pichia segobiensis</i>	NRRL Y-11571	CBS 6857 T			
<i>Candida shehatae</i>	WM 809	CBS 5813 T	12 (+1) ^b	97.8	n.a.
<i>Candida albicans</i> ST A	WM 2	CBS 562 NT			
<i>Candida dubliniensis</i>	WM 602	CBS 7987 T	13	97.7	35 (2)

Table 7 (continued)

Species	Strain designation*		Number of nucleotide differences	Similarity in %	DNA–DNA reassociation in % ^a
	Lab no.	Collection no.			
<i>Saccharomyces barnettii</i>	WM 813	CBS 5648 T			
<i>Candida milleri</i>	WM 801	CBS 6897 T	13	97.7	6 (13)
<i>Arxiozyma telluris</i>	WM 784	CBS 2685 T			
<i>Candida pintolopesii</i>	WM 785	CBS 1787 T	14	97.5	21 (18)
<i>Arxiozyma telluris</i>	WM 784	CBS 2685 T			
<i>Candida bovina</i>	WM 786	CBS 2760 T	16	97.2	7 (18)
<i>Candida tropicalis</i>	WM 1087	CBS 94 T			
<i>Candida maltosa</i>	WM 603	CBS 5611 T	16	97.2	32 (21)
<i>Pichia capsulata</i>	WM 796	CBS 1993 T			
<i>Candida molischiana</i>	WM 795	CBS 136 T	16	97.1	n.a.
<i>Lodderomyces elongisporus</i>	WM 68	CBS 2605 T			
<i>Candida parapsilosis</i> Type 1	WM 1088	CBS 604 T	17	97.0	n.a.
<i>Lodderomyces elongisporus</i>	WM 68	CBS 2605 T			
<i>Candida parapsilosis</i> Type 2	WM 1089	CBS 8825	17	97.0	n.a.
<i>Candida pintolopesii</i>	WM 785	CBS 1787 T			
<i>Candida bovina</i>	WM 786	CBS 2760 T	18	96.8	22 (18)

Multiple comparisons relate to the first strain listed. Taxa listed in order of decreasing similarity.

n.a., not available.

^a Key to the reference for DNA–DNA reassociation values: (1) Kurtzman et al., 1980; (2) O'Neill and Meyer, 2000; (3) Vaughan-Martini and Martini, 1987; (4) Lachance et al., 1986; (5) Lee et al., 1992; (6) CBS database; (7) I. Spencer-Martins, unpublished, from CBS database; (8) Bak and Stenderup, 1969; (9) Lee et al., 1998; (10) Martini and Martini, 1992; (11) Kurtzman et al., 1979; (12) Smith et al., 1989; (13) Vaughan-Martini and Kurtzman, 1985; (14) Nishikawa et al., 1996; (15) M. Smith, personal communication; (16) Kurtzman and Robnett, 1998; (17) Nakase and Suzuki, 1985; (18) Daniel, in press; (19) Roy and Meyer, 1998; (20) Fuson et al., 1987; (21) Meyer et al., 1975.

^b Possible heterogeneities caused by ambiguous base assignment.

* For abbreviations of strain designations see Table 1.

distribution of the substitutions over the analysed region. Those in the LSU and SSU sequences were concentrated in their respective divergent regions.

4. Discussion

It was our aim to detect the degree of taxon separation in the actin gene and LSU datasets. The sampled taxa allowed 35 and 37 intra-species comparisons with 2–3 strains per species for LSU and actin genes, respectively. LSU sequences remained highly similar for more than these 35 intra-species comparisons (Fig. 3, Tables 6 and 7). The actin gene similarities decreased earlier and faster than the LSU similarities, providing more variability for closely related species and for strains of the same species. This apparent conflict to the higher overall variability in the LSU alignment can be explained by the tolerance of protein coding genes to synonymous nucleotide and conservative amino acid changes.

The function of the rRNA relies on its secondary structure and therefore on the conservation of longer and uninterrupted sequence sections. Substitutions in rDNA sequences take place in locations that are less conserved features in the secondary structure. They often affect a number of nucleotides (compensatory

Table 8
Sequence similarity and number of differences within (intraspecies) and among (interspecies) species

Dataset	Intraspecies		Interspecies	
	Similarity in %	Residue differences	Similarity in %	Residue differences
Actin DNA	100–98.8	0–11	≤ 98.2	≥ 17
Actin AA	100–99.0	0–2 (+1) ^a	≤ 100	≥ 0
LSU	100–98.9	0–6 (+1) ^a	≤ 100	≥ 0
SSU	99.8–98.7	2–23 (+1) ^a	≤ 100	≥ 0

Only the highest percent similarity and the lowest number of differing residues are provided for interspecies comparisons.

^a Numbers in brackets indicate possible heterogeneities caused by ambiguous base assignment.

base changes in double-stranded regions) leading to high sequence variability in these regions.

Pair-wise comparisons of actin gene sequences resolved all investigated species and some strains (Table 8). The number of nucleotide differences for the intra-species comparisons is, with up to 11 residues (Table 4), larger than the generally accepted differences of up to three nucleotides for D1/D2 LSU sequences (Kurtzman and Robnett, 1998). The correlation of sequence similarities with DNA relatedness data facilitated the differentiation of intra-species from inter-species variation (Tables 4–7). Actin and LSU sequences showed deviations from linearity of substitutions relative to DNA relatedness. Possible explanations include differing evolutionary rates and substitutional saturation. However, they remain difficult to understand based on the few sequences available for multiple comparisons within species. Because species have to be understood as continuous entities rather than distinct units that could be separated by clear boundaries and because of the limited data, no cut-off value for the number of nucleotide differences in the actin gene can be defined. However, the higher variability of the actin gene compared to the D1/D2 region of the LSU gene in sibling species allowed for better differentiation of closely related taxa.

Inter-species comparisons showing low levels of divergence in LSU sequences represent species that were only recently recognised to be heterogenous: *Pichia guilliermondii* (Bai, 1996; San Millan et al., 1997; Bai et al., 2000), *Candida parapsilosis* (Scherer and Stevens, 1987; Lehmann et al., 1992; Meyer et al., 1998), *Z. hellenicus* (M. Smith, personal communication) and *Stephanoascus ciferrii* (Ueda-Nishimura and Mikata, 2002). This study included two species and one variety pair of low level interspecies variability that are well established (*Kluyveromyces lactis*/*Kluyveromyces marxianus*, *Pichia segobiensis*/*Pichia stipitis*, *Debaryomyces hansenii* var. *fabryii*/*D. hansenii* var. *hansenii*). Two strains of one of these species, *D. hansenii* var. *fabryii*/*Candida famata* var. *flareri*, established as an anamorph–teleomorph pair, showed 23 nucleotide differences in the actin gene and 63% DNA relatedness. This would justify a classification at variety level (Table 5). However, as nomenclatural stability is the preceding criterion in taxonomy (Greuter et al., 2000), it would not be recommended

to carry out name changes without a more comprehensive revision of the genus *Debaryomyces*. These examples illustrate that methods of increased sensitivity will result in the recognition of new species from within taxa currently accepted as species.

The value of almost complete SSU sequences for the separation of closely related taxa (James et al., 2001) is strengthened by comparisons within the *Z. hellenicus* species complex, which is currently under revision (M. Smith, CBS, Utrecht, The Netherlands, personal communication). This is partially due to the larger total number of characters that were utilised for the SSU (about 1700 nucleotides) sequence comparisons in contrast to the LSU D1/D2 sequences (about 670 nucleotides). The lack of SSU sequence accuracy in database entries and the restricted number of available sequences prevented a detailed analysis of taxon separation.

We conclude that D1/D2 LSU sequences are not necessarily specific for closely related taxa. Protein coding genes, such as the actin gene, and possibly also the SSU gene, might be better markers for identification purposes. As this conclusion depends on taxon sampling, more data are needed to infer more general implications. The use of more than one gene from more than a single linkage group would be recommended as any single gene sequence might fail to resolve some taxa. This practice would also facilitate our ability to recognise potential hybrid species. The availability of a large database of fungal D1/D2 LSU sequences allows the placement of over 700 yeasts with their nearest relatives by sequence comparisons (Kurtzman and Robnett, 1998; Fell et al., 2000; Boekhout et al., 2002). The most commonly utilised LSU D1/D2 region is also very accessible as it can be easily amplified and the sequence can be determined in one experiment. Therefore, this gene region is the currently preferred target for sequence-based identification. This may change as sequences from other parts of the genome accumulate to substantial databases.

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