A phylogenetic study on galactose-containing *Candida* species based on 18S ribosomal DNA sequences

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Phylogenetic relationships of 33 *Candida* species containing galactose in the cells were investigated by using 18S ribosomal DNA sequence analysis. Galactose-containing *Candida* species and galactose-containing species from nine ascomycetous genera were a heterogeneous assemblage. They were divided into three clusters (II, III, and IV) which were phylogenetically distant from cluster I, comprising 9 galactose-lacking *Candida* species, *C. glabrata*, *C. holmii*, *C. krusei*, *C. tropicalis* (the type species of *Candida*), *C. albicans*, *C. viswanathii*, *C. maltosa*, *C. parapsilosis*, *C. guilliermondii*, and *C. lusitaniae*, and 17 related ascomycetous yeasts. These three clusters were also phylogenetically distant from *Schizosaccharomyces pombe*, which contains galactomannan in its cell wall. Cluster II comprised *C. magnoliae*, *C. vaccinii*, *C. apis*, *C. gropengiesseri*, *C. etchellsii*, *C. floricola*, *C. lactiscondensi*, *Wickerhamiella domercqiae*, *C. versatilis*, *C. azyma*, *C. vanderwaltii*, *C. pararugosa*, *C. sorbophila*, *C. spandovensis*, *C. galacta*, *C. ingens*, *C. incommunis*, *Yarrowia lipolytica*, *Galactomyces geotrichum*, and *Dipodascus albidus*. Cluster III comprised *C. tepae*, *C. antillanae* and its synonym *C. bondarzewiae*, *C. ancudensis*, *C. petrohuensis*, *C. santjacobensis*, *C. ciferrii* (anamorph of *Stephanoascus ciferrii*), *Arxula terrestris*, *C. castrensis*, *C. valdiviana*, *C. paludigena*, *C. blankii*, *C. salmanticensis*, *C. auringiensis*, *C. bertae*, and its synonym *C. bertae* var. *chiloensis*, *C. edax* (anamorph of *Stephanoascus smithiae*), *Arxula adeninivorans*, and *C. steatolytica* (synonym of *Zygoascus hellenicus*). Cluster IV comprised *C. cantarellii*, *C. vinaria*, *Dipodascopsis uninucleata*, and *Lipomyces lipofer*. Two galactose-lacking and Q-8-forming species, *C. stellata* and *Pichia pastoris*, and 5 galactose-lacking and Q-9-forming species, *C. apicola*, *C. bombi*, *C. bombiicola*, *C. geochares*, and *C. insectalens*, were included in Cluster II. Two galactose-lacking and Q-9-forming species, *C. drimydis* and *C. chiropterorum*, were included in Cluster III.

Key Words—galactose-containing *Candida* species; molecular phylogeny; 18S ribosomal DNA sequences

The ascomycetous anamorphic genus *Candida* Berkhout is the largest yeast genus (Barnett et al., 1990; Meyer et al., 1998). In the recent fourth edition of "The Yeasts: A Taxonomic Study," Meyer et al. (1998) recognized 163 species in the genus *Candida* and described their morphological and physiological characteristics, ecological data, ubiquinone systems, DNA base compositions, DNA-DNA reassociation, and other molecular data available. Over the years, various chemotaxonomic methods have been applied

to the classification of this genus as exemplified by serological classification based on cell surface antigens (Tsuchiya et al., 1965), proton magnetic resonance spectra of cell wall mannans and mannose-containing polysaccharides (Spencer and Gorin, 1969, 1971), DNA base compositions (Nakase and Komagata, 1971), ubiquinone (coenzyme Q) analysis (Montrocher et al., 1990; Yamada and Kondo, 1972), immuno-electrophoretic analyses (Montrocher, 1980, 1984), cellular carbohydrate compositions (Von Arx and Weijman, 1979; Weijman and Rodrigues de Miranda, 1988; Weijman et al., 1988), and cellular long-chain fatty acid compositions and electrophoretic karyotypes (Viljoen and Kock, 1989). In spite of their considerable contributions, however, other approaches more amenable to phylogenetic analysis are required for classification of the genus *Candida*.

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Kurtzman and Robnett (1997, 1998) carried out a comprehensive phylogenetic study on all species of ascomycetous yeasts, including members of *Candida* and other anamorphic genera based on partial sequences of the 5' end of the large subunit ribosomal RNA gene (26S rDNA). Kurtzman and Robnett (1998) suggested that additional gene sequences needed to be analyzed before most genera could be phylogenetically circumscribed. Barns et al. (1991), Hendriks et al. (1991, 1992), Wilmotte et al. (1993), and Cai et al. (1996) reported phylogenetic relationships of several medically and industrially important *Candida* species and the related ascomycetous yeasts based on complete or almost complete sequences of the small subunit ribosomal RNA gene (18S rDNA). However, the 18S rDNA sequences of almost all *Candida* species remain undetermined.

Suzuki and Nakase (1998) reported cellular neutral sugar compositions and ubiquinone systems of almost all species of the genus *Candida* and showed that these species were divided into six groups based on the presence or absence of galactose in cellular neutral sugars and from types of the ubiquinone systems, i.e., Group Ia (glucose-mannose, Q-6), Group Ib (glucose-mannose, Q-7), Group Ic (glucose-mannose, Q-8), Group Id (glucose-mannose, Q-9), Group IIa (glucose-mannose-galactose, Q-8), and Group IIb (glucose-mannose-galactose, Q-9). They suggested that an analysis of 18S rDNA sequences was needed to verify phylogenetic relationships among *Candida* species within and between the six groups.

In this study, we examined complete or almost complete sequences of 18S rDNA from 33 species of *Candida* belonging to Groups IIa and IIb containing galactose in the cells to clarify their phylogenetic relationships among the genus *Candida* and related ascomycetous yeasts.

Materials and Methods

Strains employed. The type strains of 33 species of galactose-containing *Candida* species, *Arxula adenivorans*, *Arxula terrestris*, *Wickerhamiella domercqiae*, and *Yarrowia lipolytica* were used in this study (Table 1) as well as two strains of a synonym of *Candida bertae*, *C. bertae* var. *chiloensis*, and a synonym of *C. antillarum*, *C. bondarzewiae*.

Preparation of DNA. The nuclear DNAs of these strains were extracted and purified by the method of Nakase and Suzuki (1985).

Sequencing. For strains of *Candida azyma* and *C. magnoliae*, the small subunit ribosomal RNA gene (18S rDNA) sequences were determined by the direct method, using radioisotopes as described by Takashima et al. (1995). For strains of other species,

the following direct and cloning methods were used. The 18S rDNA was amplified by using the polymerase chain reaction (PCR) with *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Otsu, Japan) and two primers, P1 (5'-ATCTGGTTGATCCTGCCAGT-3') and NS8 (5'-TCCGCAGGTTACCTACGGA-3'). The cycling parameters for direct sequencing were as follows: an initial denaturation step of 95°C for 4 min followed by 30 cycles of 94°C for 1 min (denaturation), 55°C for 2.5 min (annealing), and 72°C for 2.5 min (extension). The PCR products were purified by using the UltraClean PCR Clean-up Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, U.S.A.). The cycling parameters for cloning sequencing were as follows: 25 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 2 min (extension), and the final 7 min extension step for 3'A-overhangs of PCR products. The PCR products were directly cloned with a TA original Cloning Kit (Invitrogen Corporation, Carlsbad, CA, U.S.A.) as recommended by the manufacturer. Plasmid DNA with 18S rDNA (cloned 18S rDNA) was purified by using the Wizard *Plus* Minipreps DNA Purification System (Promega, Madison, WI, U.S.A.). Purified 18S rDNA and cloned 18S rDNA were sequenced by the dideoxy method by using the AutoCycle Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) or the SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, U.S.A.) and the ALFexpress DNA sequencer (Pharmacia Biotech). The primers used for sequencing were 5'-TGGAAT-TACCGCGGCTGCTGGCACC-3', 5'-CCGTCAATTCC-TTAAAGTTTCAGCC-3', 5'-GACGGGCGGTGTGTAC-AAAGGGCAG-3', 5'-TCTGGGCGGCACGCGCGCT-ACACTG-3', 5'-AAGGTCTCGTTTCGTTATCG-3', 5'-ACGGCTACCACATCCAAGGAAGG-3' and 5'-CCTTC-CTTGGATGTGGTAGCCGT-3'. M13 (-40) and M13 Reverse primers contained in the AutoCycle Sequencing Kit were also used for cloned 18S rDNA.

Phylogenetic analysis. Generated sequences and reference sequences from the nucleotide sequence libraries (EMBL, GenBank, and DDBJ) were aligned by using the multialignment program CLUSTAL W (Thompson et al., 1994) (version 1.74). Reference 18S rDNA sequences were as follows: *Candida albicans* (X53497), *C. glabrata* (X51831), *C. holmii* (X78601), *C. krusei* (M55528), *C. lusitaniae* (M55526), *C. maltosa* (D14593), *C. parapsilosis* (AB013588), *C. tropicalis* (M55527), *Debaryomyces hansenii* (X58053), *Dekkera anomala* (X83820), *D. bruxellensis* (X58052), *Dipodascopsis uninucleata* (U00969), *Dipodascus albidus* (X69840), *Galactomyces geotrichum* (X69842), *Hanseniaspora uvarum* (X69844), *Kluyveromyces lactis* (X51830), *K. polysporus* (X69845), *Lipomyces lipofer* (X69848), *Lodderomyces elongisporus* (X78600), *Metschnikowia bicuspidata* (X69846), *Pichia*

Table 1. List of strains used in this study.

Species	Strain ^a	Other designation ^b	Accession No. ^c
<i>C. ancudensis</i> C. Ramírez & A. González	JCM 8915 ^T	CBS 8184	AB018126
<i>C. antillancae</i> C. Ramírez & A. González	JCM 9581 ^T	CBS 9170	AB018134
	JCM 9584 ^d	CBS 8171	AB018137
<i>C. apis</i> (Lavie ex van Uden & Vidal-Leiria) S. A. Meyer & Yarrow	JCM 8256 ^T	IFO 10262	AB018127
<i>C. auringiensis</i> Santa María	JCM 9593 ^T	ATCC 58430	AB018136
<i>C. azyma</i> (van der Walt, E. Johannsen & Yarrow) S. A. Meyer & Yarrow	JCM 1691 ^T	CBS 6826	AB018128
<i>C. bertae</i> C. Ramírez & A. González	JCM 9582 ^T	CBS 8169	AB018155
	JCM 9583 ^e	CBS 8168	AB018140
<i>C. blankii</i> H. R. Buckley & van Uden	JCM 8259 ^T	IFO 10230	AB018125
<i>C. cantarellii</i> (van der Walt & van Kerken) S. A. Meyer & Yarrow	JCM 8260 ^T	IFO 10269	AB018138
<i>C. castrensis</i> C. Ramírez & A. González	JCM 9585 ^T	CBS 8172	AB018139
<i>C. ciferrii</i> Kreger-van Rij	JCM 9551 ^T	IFO 10192	AB018141
<i>C. edax</i> van der Walt & E. E. Nel	JCM 9470 ^T	IFO 10273	AB018129
<i>C. etchellsii</i> (Lodder & Kreger-van Rij) S. A. Meyer & Yarrow	JCM 8066 ^T	IFO 1592	AB018142
<i>C. floricola</i> Tokuoka, Ishitani, S. Goto & Komagata	JCM 9439 ^T	IAM 13115	AB018143
<i>C. galacta</i> (Golubev & Bab'eva) F.-L. Lee, C.-F. Lee, Okada, Komagata & Kozaki	JCM 8257 ^T	IFO 10031	AB018131
<i>C. gropengiesseri</i> (F. C. Harrison) S. A. Meyer & Yarrow	JCM 8255 ^T	IFO 0659	AB018132
<i>C. incommunis</i> Y. Ohara, Nonomura & T. Yamazaki	JCM 8258 ^T	IFO 10228	AB018133
<i>C. ingens</i> van der Walt & van Kerken	JCM 9471 ^T	IFO 10057	AB018130
<i>C. lactiscondensi</i> (B. W. Hammer) S. A. Meyer & Yarrow	JCM 9472 ^T	IFO 1286	AB018144
<i>C. magnoliae</i> (Lodder & Kreger-van Rij) S. A. Meyer & Yarrow	JCM 1446 ^T	CBS 166	AB018145
<i>C. paludigena</i> Golubev & Blagodatskaya	JCM 9614 ^T	NRRL Y-12697	AB018146
<i>C. pararugosa</i> Nakase, Komagata & Fukazawa	JCM 1512 ^T	AJ 4645	AB018147
<i>C. petrohuensis</i> C. Ramírez & A. González	JCM 8922 ^T	CBS 8173	AB018148
<i>C. salmanticensis</i> (Santa María) van Uden & H. R. Buckley ex S. A. Meyer & Ahearn	JCM 8896 ^T	IFO 10242	AB018149
<i>C. santjacobensis</i> C. Ramírez & A. González	JCM 8924 ^T	CBS 8183	AB018150
<i>C. sorbophila</i> (Nakase) S. A. Meyer & Yarrow	JCM 1514 ^T	AJ 4995	AB018151
<i>C. spandovensis</i> (Henninger & Windisch) S. A. Meyer & Yarrow	JCM 9562 ^T	IFO 10249	AB015908
<i>C. steatolytica</i> Yarrow	JCM 1698 ^T	CBS 5839	AB018152
<i>C. tepae</i> Grinbergs	JCM 10265 ^T	NRRL Y-17670	AB018153
<i>C. vaccinii</i> Tokuoka, Ishitani, S. Goto & Komagata	JCM 9446 ^T	IAM 13117	AB015909
<i>C. valdiviana</i> Grinbergs & Yarrow	JCM 9565 ^T	IFO 10317	AB015910
<i>C. vanderwaltii</i> (Vidal-Leiria) S. A. Meyer & Yarrow	JCM 9615 ^T	CBS 5524	AB018154
<i>C. versatilis</i> (Etchells & T. A. Bell) S. A. Meyer & Yarrow	JCM 8065 ^T	IFO 10056	AB018156
<i>C. vinaria</i> Y. Ohara, Nonomura & Yunome ex M. Th. Smith	JCM 1813 ^T	AJ 4676	AB018135
<i>Axula adenivorans</i> (Middelhoven, Hoogkamer-Te Niet & Kreger-van Rij) van der Walt, M. Th. Smith & Y. Yamada	JCM 8914 ^T	CBS 8244	AB018123
<i>Axula terrestris</i> (van der Walt & E. Johannsen) van der Walt, M. Th. Smith & Y. Yamada	JCM 8913 ^T	CBS 278.86	AB018124
<i>Wickerhamiella domercqiae</i> van der Walt	JCM 9478 ^T	IFO 1857	AB018157
<i>Yarrowia lipolytica</i> (Wickerham, Kurtzman & Herman) van der Walt & von Arx	JCM 2320 ^T	AJ 14101	AB018158

^aT, Type strain.^bAJ, Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan; ATCC, American Type Culture Collection, Manassas, VA, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; IAM, IAM Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Osaka, Japan; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, IL, U.S.A.^cDDBJ accession numbers.^dThe type strain of *C. bondarzewiae*, a synonym of *C. antillancae* (Meyer et al., 1998).^eThe type strain of *C. bertae* var. *chiloensis*, a synonym of *C. bertae* (Meyer et al., 1998).

anomala (X58054), *P. membranifaciens* (X58055), *Saccharomyces cerevisiae* (M27607), *Saccharomyces ludwigii* (X69843), *Saccharomycopsis capsularis* (X69847), *S. fibuligera* (X69841), *Schizosaccharomyces pombe* (X58056), *Torulaspora delbrueckii* (X-53496), *Zygosaccharomyces mrakii* (X90757), and *Z. rouxii* (X58057). For this comparative study, we also

used our unpublished sequence data from 11 species, *Candida guilliermondii*, *C. viswanathii*, *C. apicola*, *C. bombi*, *C. bombicola*, *C. geochares*, *C. drimydis*, *C. chiropterorum*, *C. insectalens*, *C. stellata*, and *Pichia pastoris* (Sugita, T. and Nakase, T., submitted; Suzuki, M. and Nakase, T., unpubl.). Distances between pairs of sequences were calculated by using the two-param-

eter model of Kimura (1980). Sites where gaps existed in any of the sequences were excluded. A phylogenetic tree was constructed by using the neighbor-joining method of Saitou and Nei (1987). Bootstrap analysis (Felsenstein, 1985) was performed with 1,000 replicates for the neighbor-joining tree.

Results and Discussion

Complete or almost complete 18S rDNA sequences were determined for the type strains of 33 species of the genus *Candida*, two strains of a synonym of *C. berta* (*C. berta* var. *chiloensis*) and a synonym of *C. antillarum* (*C. bondarzewiae*), and the type strains of *Arxula adenivorans*, *Arxula terrestris*, *Wickerhamiella domercqiae*, and *Yarrowia lipolytica*. From sequencing results, nucleotide base lengths of 18S rDNA were either 1,650–1,730 bases or 1,750–1,790 bases. Our 39 strains sequence data were aligned together with known sequences of 30 species and our unpublished sequences of 11 species. A phylogenetic tree was constructed by the neighbor-joining (NJ) method (Fig. 1), using *Schizosaccharomyces pombe* as an outgroup. *S. pombe* contains galactomannan in its cell wall (Gorin and Spencer, 1970) and is a representative member of the order Schizosaccharomycetales (Kurtzman, 1993). This species is also a member of "Archiascomycetes" proposed by Nishida and Sugiyama (1994). In the tree, *Candida* species and related ascomycetous yeast species were divided into four phylogenetically distinctive clusters, I, II, III, and IV. Galactose-containing *Candida* species and nine ascomycetous genera, containing galactomannans in their cell walls (Gorin and Spencer, 1970) or galactose in the cells (Weijman, 1977, 1979; Weijman and van der Walt, 1989) were a heterogeneous assemblage and classified into three clusters (II, III, and IV) between *S. pombe* (outgroup) and the *Clavispora-Metschnikowia* clade in Cluster I.

Thirty-one species have Q-9 and 3 have Q-8 (*C. galacta*, *C. lactiscondensi*, and *C. incommunis*) as the major ubiquinone (Table 2). The three species with Q-8 did not form a single cluster, but were scattered among the species with Q-9.

Species of Cluster I

This cluster comprised 9 galactose-lacking *Candida* species, *C. glabrata*, *C. holmii*, *C. krusei*, *C. tropicalis* (the type species of *Candida*), *C. albicans*, *C. viswanathii*, *C. maltosa*, *C. guilliermondii*, and *C. lusitanae*, and 17 species of teleomorphic ascomycetous yeasts that do not contain galactose in their cell wall mannans (Gorin and Spencer, 1970) or in their cell walls (Prillinger et al., 1993). Recently in our laboratory, 18S ribosomal DNA sequences have been deter-

mined for about 100 species of the genus *Candida* belonging to Groups Ic (glucose-mannose and Q-8) and Id (glucose-mannose and Q-9) of Suzuki and Nakase (1998) and several species of related teleomorphic ascomycetous genera (Sugita, T. and Nakase, T., submitted; Suzuki, M. and Nakase, T., submitted). Only 9 species were outside Cluster I. They were *C. apicola*, *C. bombi*, *C. bombicola*, *C. geochares*, *C. drimydis*, *C. chiropterorum*, *C. insectalens*, *C. stellata*, and *Pichia pastoris*. In this study, we revealed that these 9 species were included in Cluster II or in Cluster III as discussed below.

Species of Cluster II

This cluster is an assemblage of phylogenetically diverse species with large interspecific divergences and is further divided into four subclusters, IIA, IIB, IIC, and IID.

Subcluster IIA was composed of two groups, IIA-1 and IIA-2, and *C. geochares*, *C. bombicola*, and *C. bombi*. Subcluster IIA-1 comprised four galactose-containing species, *C. magnoliae*, *C. vaccinii*, *C. apis*, and *C. gropengiesseri*, and was connected to Subcluster IIA-2, which is comprised of galactose-containing *C. etchellsii*, *C. floricola*, and *C. lactiscondensi*, together with two galactose-lacking species, *C. stellata* and *C. apicola*. These subclusters were connected to galactose-lacking *C. geochares* (soil isolate) and subsequently joined with galactose-lacking *C. bombi* and *C. bombicola* (bumblebee and honey isolates, respectively). The species of Subcluster IIA-1 were isolated from flowers and several kinds of bees (Table 2). The species of Subcluster IIA-2 were isolated from various sources, including flowers and bee's gut as well as cucumber brine, soy mash, condensed milk, and wine grapes (Table 2). *C. vaccinii*, *C. etchellsii*, *C. floricola*, *C. lactiscondensi*, *C. apicola*, *C. bombi*, and *C. bombicola* are sugar- or halo-tolerant (Suzuki et al., 1992; Tokuoka et al., 1985, 1987), but appear to be separate species. Five galactose-lacking *Candida* species, *C. apicola*, *C. bombi*, *C. bombicola*, *C. geochares*, and *C. stellata*, were included in this subcluster. The former 4 species have Q-9 as the major ubiquinone and the latter has Q-8. Spencer and Gorin (1970) grouped *C. apicola*, *C. bombi*, *C. bombicola*, *C. magnoliae*, *C. apis*, and *C. etchellsii* based on similarities of proton magnetic resonance (PMR) spectral patterns of their cell wall mannans and galactomannans. Kurtzman and Robnett (1997) showed the same results as ours, using 26S ribosomal DNA partial sequence analysis.

Subcluster IIB comprised 7 galactose-containing *Candida* species and the teleomorphic species *Wickerhamiella domercqiae* (van der Walt et al., 1973), which contains galactomannan in its cell wall (Gorin and Spencer, 1970; Spencer and Gorin, 1970). *C.*

Fig. 1. A phylogenetic tree of *Candida* species and related ascomycetous yeasts with and without galactose in their cells, their cell walls, or their cell wall polysaccharides.

The tree was constructed by the neighbor-joining method. *Schizosaccharomyces pombe* was used as the outgroup species. The numerals represent the confidence level from 1,000 replicate bootstrap samplings (frequencies less than 50% are not indicated). Species in bold type contain galactose in their cells, their cell walls, or their cell wall polysaccharides.

Table 2. Main source of isolation, major ubiquinone, pseudo- and true hyphae formation, and inositol assimilation of galactose-containing *Candida* species.

Cluster	Subcluster	Species	Main source of isolation ^a	Major ubiquinone ^d	Pseudo-hyphae ^a	True hyphae ^a	Inositol assimilation ^a
II	IIA	<i>C. magnoliae</i>	flower of <i>Magnolia</i> sp., gut of bees, etc.	Q-9	—	—	—
		<i>C. vaccinii</i>	blueberry flower	Q-9	—	—	—
		<i>C. apis</i>	trachea of a bee	Q-9	—	—	—
		<i>C. gropengiesseri</i>	an insect	Q-9	—	—	—
		<i>C. etchellsii</i>	cucumber brine, soy mash, etc.	Q-9	—	—	—
		<i>C. floricola</i>	dandelion flower	Q-9	—	—	—
	IIB	<i>C. lactiscondensi</i>	condensed milk, cucumber brine, etc.	Q-8	—	—	—
		<i>C. versatilis</i>	cucumber brine, soy mash, etc.	Q-9	—	—	—
		<i>C. azyma</i>	lichen	Q-9	+	—	—
		<i>C. vanderwaltii</i>	winery equipment, South Africa	Q-9	—	—	—
		<i>C. pararugosa</i>	human feces	Q-9	+	—	—
		<i>C. sorbophila</i>	washings of ion-exchange resin, Japan	Q-9	primitive	—	—
		<i>C. spandovensis</i>	Pilsner beer	Q-9	+	—	—
		<i>C. galacta</i>	cocoon of red ants	Q-8	—	—	—
		<i>C. ingens</i>	brewery material	Q-9	+	—	—
		<i>C. incommunis</i>	grape must, Japan	Q-8	+	—	+
III	IIC	<i>C. tepae</i>	rotten wood, Chile	Q-9	+	—	—
		<i>C. antillancae</i>	rotten wood, Chile	Q-9	+	—	—
	IID	<i>C. ancudensis</i>	rotten wood, Chile	Q-9	primitive	—	—
		<i>C. petrohuensis</i>	rotten wood, Chile	Q-9	—	—	—
		<i>C. santjacobensis</i>	rotten wood, Chile	Q-9	+	—	+
		<i>C. ciferrii</i>	cow	Q-9	+	+	+
		<i>C. bertae</i>	rotten wood, Chile	Q-9	+	+	+
		<i>C. edax</i>	insect tunnel	Q-9	+	+	+
		<i>C. steatolytica</i>	mastitic bovine udder	Q-9	+	+	+
		<i>C. castrensis</i>	rotten wood, Chile	Q-9	+	+	+
		<i>C. paludigena</i>	high-moor peat near Moscow, Russia	Q-9	+	+	+
		<i>C. valdiviana</i>	sputum, rotten wood ^b , Chile	Q-9	+	+	+
		<i>C. blankii</i>	organs of a mink, soil, horse	Q-9	+	+	+
		<i>C. salmanticensis</i>	alpechin ^c in Spain	Q-9	+	—	—
		<i>C. auringiensis</i>	alpechin in Spain	Q-9	+	—	+
IV		<i>C. cantarellii</i>	grape must, South Africa	Q-9	—	—	—
		<i>C. vinaria</i>	grape must, Japan	Q-9	primitive	—	—

^aFrom Meyer et al. (1998).^bFrom Grinbergs and Yarrow (1970).^cAlpechin: the aqueous solution separating during the manufacture of olive oil.^dFrom Suzuki and Nakase (1998).

azyma, *C. pararugosa*, and *C. vanderwaltii* were reportedly related to one another, but clustered together. *C. versatilis* was connected to *W. domercqiae*. Spencer and Gorin (1970) reported that the galactomannan of *C. versatilis* showed a similar PMR spectrum to *W. domercqiae*. *C. versatilis* and *C. etchellsii* (included in Subcluster IIA) are known as important halo-tolerant yeasts in the maturation of soy sauce fermentation in Japan (Asao et al., 1967; Onishi, 1957). Since these two species were phylogenetically different from each other, it is possible to establish an identification and detection system by using species-specific primers based on 18S rDNA sequences. *C. galacta* was associated with *C. spandovensis* and *C. sorbophila*, but was phylogenetically distant from *C. apis*, which is included in Subcluster IIA-1. *C. galacta* was at one time considered a variety of *C. apis*

(Meyer et al., 1984). However, Lee et al. (1993) proposed a new combination, *Candida galacta*, because *C. galacta* had Q-8 as the major ubiquinone, whereas *C. apis* had Q-9, and because these two species showed significant differences in DNA base composition. Our results showed that these two species were phylogenetically different from each other, as was also suggested by 18S and 26S rRNAs partial sequence analysis (Yamada et al., 1994) and by 26S rDNA partial sequence analysis (Kurtzman and Robnett, 1998). Although Viljoen and Kock (1989) reported that these two species were similar to each other in cellular fatty acid compositions, the similarity seems to be biochemically convergent.

Subcluster IIC comprised galactose-containing *Candida ingens* (synonym of *Dipodascus ingens*) and one galactose-lacking *Candida* species, *C. insectal-*

ens, and the teleomorphic species *Pichia pastoris*, which does not contain galactose in its cell wall mannan (Gorin and Spencer, 1970). *C. ingens* was associated with *P. pastoris*, but the distance between the two species was very large. This association is still unstable because we sometimes encountered other cases of association with the *Dipodascus-Galactomyces* clade, with *C. incommunis*, or with *Yarrowia lipolytica* when new sequences were added to a tree generated with version 1.6 of the CLUSTAL W program. When van der Walt and van Kerken (1961) originally described *C. ingens*, no statement concerning arthroconidium formation was in its description. De Hoog et al. (1986) found that cells of this species were strongly coherent and later disarticulated into separate cells. They transferred it to the anamorphic genus *Geotrichum* and after mating reactions found that its teleomorph belonged to the genus *Dipodascus*. This species was nomenclaturally corrected as *Dipodascus ingens* by De Hoog et al. (1997). Kurtzman and Robnett (1995, 1998) showed that *Dipodascus* species were divided into two phylogenetic groups based on 26S rDNA partial sequences and that *Galactomyces geotrichum* and *Dipodascus albidus* belonged to one group, whereas *Dipodascus ingens* belonged to the other group. The galactose-lacking and Q-9-forming *C. insectalens* was connected to galactose-lacking and Q-8-forming *P. pastoris*, but the distance between the two species was large.

Subcluster IID comprised galactose-containing *C. incommunis* and three teleomorphic ascomycetous species, *Yarrowia lipolytica*, *Dipodascus albidus*, and *Galactomyces geotrichum*, which contain galactomannans in their cell walls (Gorin and Spencer, 1970). *C. incommunis* was connected to *Y. lipolytica*, although these two species appeared to be phylogenetically remote to each other. *C. incommunis* had Q-8 as the major ubiquinone and was isolated from grape must in Japan. This species was phylogenically removed from Q-9-possessing *C. vinaria* included in cluster IV, although both species were isolated from grape must in Japan. In this study, we reexamined the 18S rDNA sequence of the type strain of *Y. lipolytica* because a sequence of this species registered in GenBank contained a great many unidentified bases that lead to tree instability. Kurtzman (1998) recognized *Y. lipolytica*, which was at one time included in the genus *Saccharomycopsis*. *Y. lipolytica* is phylogenetically quite different from *Saccharomycopsis*, as suggested from our results and data on partial sequences of 18S and 26S ribosomal RNA/DNA sequences (Kurtzman and Robnett, 1994, 1995, 1998; Yamada and Nogawa, 1990) and almost complete sequences of 18S ribosomal DNA (Wilmotte et al., 1993). These two genera are also chemotaxonomically different from each other.

Namely, *Yarrowia* has Q-9 as the major ubiquinone and contains galactose in the whole cells, whereas *Saccharomycopsis* has Q-8 as the major ubiquinone and does not contain galactose in the whole cells (Gorin and Spencer, 1970; Goto and Takami, 1986; Suzuki and Nakase, unpubl.; Yamada et al., 1976).

Species of Cluster III

Nine species (including two varieties of one species) described by Ramírez and González (1984a–g), *C. antillancae*, *C. bondarzewiae* (synonym of *C. antillancae*), *C. tepae*, *C. ancudensis*, *C. petrohuensis*, *C. drimydis*, *C. santjacobensis*, *C. castrensis*, *C. bertae* var. *bertae*, and *C. bertae* var. *chiloensis* (synonym of *C. bertae*) were found in the *Stephanoascus-Zygoascus* lineage. Interestingly, all these species were isolated from rotten wood in Chile, as shown in Table 2. These species were divided into two groups by the ability or inability to assimilate inositol (Table 2). The group with the inability to assimilate inositol consisted of 6 species. Of these, 5 were galactose-containing species, *C. antillancae*, *C. bondarzewiae*, *C. tepae*, *C. ancudensis*, and *C. petrohuensis*, and 1 was galactose-lacking (*C. drimydis*). These species are phylogenetically closely related. Meyer et al. (1998) considered *C. bondarzewiae* to be a synonym of *C. antillancae* based on their morphological and physiological similarities. Furthermore, Kurtzman and Robnett (1997, 1998) showed *C. tepae*, *C. antillancae*, and *C. bondarzewiae* to be conspecific from 26S rDNA partial sequence analysis. Moreover, Kurtzman and Robnett (1997, 1998) showed *C. petrohuensis*, *C. ancudensis*, and *C. drimydis* to be conspecific from 26S rDNA partial sequence analysis. Consequently these species are predicted to reduce to only 2 species, *C. tepae* and *C. petrohuensis*, but DNA-DNA reassociation experiments are needed to confirm this prediction. The group with the ability to assimilate inositol was divided further into three subgroups. The first consisted of *C. bertae* and its synonym, *C. bertae* var. *chiloensis* (Meyer et al., 1998). They were related to *C. edax* (anamorph of *Stephanoascus smithiae*) (Giaménez-Jurado et al., 1994), and linked to *C. steatolytica* (synonym of *Zygoascus hellenicus*) (Smith, 1986) and *Arxula adenivorans*. The second subgroup consisted of *C. santjacobensis*, and it was connected to *A. terrestris*, the type species of the anamorphic genus *Arxula* (van der Walt et al., 1990), which contains galactose in the cells (Weijman, 1979). These had a linkage with *C. ciferrii* (anamorph of *S. ciferrii*). A phylogenetically close relationship between *A. terrestris* and *S. ciferrii* was also suggested on the basis of partial sequences of 18S and 26S rRNAs (Yamada and Nogawa, 1990) and partial sequences of 26S rDNA (Kurtzman and Robnett, 1995). The third subgroup

consisted of 3 species, *C. castrensis*, *C. paludigena*, and *C. valdiviana*. *C. castrensis* had a phylogenetically close relationship with *C. paludigena*, which was isolated from high-moor peat near Moscow in Russia (Golubev et al., 1981). Kurtzman and Robnett (1997, 1998) showed these 2 species to be conspecific from 26S rDNA partial sequence analysis. They were associated with *C. valdiviana* described by Grinbergs and Yarrow (1970). A cluster comprised of these subgroups was connected to the galactose-lacking species *C. chiropterorum*, an isolate from the liver of a bat. *C. chiropterorum* can also assimilate inositol and form true-hyphae.

C. blankii, which assimilates inositol and forms true-hyphae, was associated with the *Stephanoascus-Zygoascus* lineage. Middelhoven (1993) reported that *C. blankii* assimilated *n*-alkanes, butylamine, and putrescine, but not certain benzene compounds, and that *Arxula terrestris* and *Stephanoascus ciferrii* assimilated *n*-alkanes, butylamine, putrescine, and benzene compounds. It is interesting, from a phylogenetic point of view, that these species share the ability to assimilate three of these compounds. *C. auringiensis* and *C. salmanticensis* constituted a cluster associated with *C. blankii* and with the *Stephanoascus-Zygoascus* lineage. These two species were originally isolated from the same source (alpechin, an aqueous solution separating during the manufacture of olive oil).

Species of Cluster IV

C. vinaria and *C. cantarellii* were linked and connected to *Lipomyces lipofer* and *Dipodascopsis uninucleata* (members of the family Lipomycetaceae), which contain galactose in the cells (Weijman and van der Walt, 1989). *C. vinaria* was isolated from grape must in Japan, whereas *C. cantarellii* was isolated from grape must in South Africa. The connection of these species with genera *Lipomyces* and *Dipodascopsis* was also suggested on the basis of partial sequences of 26S ribosomal DNA (Kurtzman and Robnett, 1997), but bootstrap support is weak.

Kurtzman and Robnett (1998) showed phylogenetic relationships among all the nearly 500 currently accepted species of ascomycetous yeasts, including members of *Candida* and other anamorphic genera, from analysis of 26S rDNA partial sequences. Galactose-containing *Candida* species, with the exception of *Candida ingens* (a synonym of *Dipodascus ingens*) in the *Dipodascus* clade, were included in the *Stephanoascus/Metschnikowia* clade, which was divided into two groups, *Sporopachydermia-Stephanoascus-Wickerhamiella-Zygoascus* and *Clavispora-Cyniclomyces-Metschnikowia-Yarrowia*. The former group included our Subcluster IIB, Cluster III except *C. blankii*, *C. auringiensis*, and *C. salmanticen-*

sis, and Cluster IV. The latter one included our Subcluster IIA, *C. blankii*, *C. auringiensis*, and *C. salmanticensis* in Cluster III, and *C. incommunis* in Subcluster IID.

In conclusion, galactose-containing species of *Candida* were phylogenetically heterogeneous and were divided into three clusters. These clusters were phylogenetically quite different from a group of galactose-lacking *Candida* species, which includes the type species of the genus *Candida*. However, eight galactose-lacking *Candida* species were included in these clusters. Furthermore, 18S ribosomal DNA sequences from galactose-lacking *Candida* species that have Q-6, Q-7, Q-8 or Q-9 as the major ubiquinone need to be analyzed for a better understanding of phylogenetic relationships between galactose-containing *Candida* species and galactose-lacking *Candida* species.

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