Taxonomic Relationships of *Cryptococcus* and *Tremella* Based on Fatty Acid Composition and Other Phenotypic Characters

By E. J. SMIT,¹* J. L. F. KOCK,² J. P. J. VAN DER WESTHUIZEN¹
AND T. J. BRITZ²

¹Department of Dermatology, Mycology Section, Medical Faculty, and ²Department of Microbiology, PO Box 339, University of the Orange Free State, 9300 Bloemfontein, Republic of South Africa

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The cellular long-chain fatty acids present in 33 strains, representing 15 species of *Cryptococcus*, and 4 species of *Tremella*, were determined by gas chromatography. According to the relative amounts of fatty acid methyl esters, the *Cryptococcus* species studied were divided into four main groups. Possible relationships between species representing the two genera are presented in a new model, where cellular long-chain fatty acid compositions and other phenotypic characteristics are included.

INTRODUCTION

Cellular long-chain fatty acids have been found in all bacteria (Tornabene, 1985) and bacterial lipid analyses are now well established in the taxonomy of these organisms (Shaw, 1974; Lechavalier, 1977; Minnikin *et al.*, 1978; Goodfellow & Minnikin, 1982; Collins *et al.*, 1982; Bousfield *et al.*, 1983). However, the first attempt to associate lipid composition with taxonomic classification was made by Abel *et al.* (1963), who illustrated that qualitative fatty acid analyses were useful for distinguishing between heterogeneous bacteria. Primary fatty acids, ranging from C₁₄ to C₂₀, have been detected in ascomycetous yeasts and have been used in the classification and identification of these organisms (Cottrell *et al.*, 1985; Kock *et al.*, 1986; Viljoen *et al.*, 1986) as well as for the basidiomycetous yeasts (Smit *et al.*, 1987*a, b*; van der Westhuizen *et al.*, 1987*a, b*).

Since species of the genera *Tremella* and *Cryptococcus* synthesize starch and polymers consisting of D-mannose, D-xylose and D-glucuronic acid, and are morphologically similar, Slodki *et al.* (1965) suspected a phylogenetic relationship between them. Similarity of assimilation patterns also indicated that *C. laurentii* and its morphological varieties might be closely related to *T. encephala* and *T. aurantia* (Slodki *et al.*, 1965). They concluded that some species of *Cryptococcus* may represent one or more stages of the life cycle of some species of *Tremella*.

This report presents the results of long-chain fatty acid analyses on 33 strains representing 15 species of *Cryptococcus* and 4 species of *Tremella*. Comparisons have been made between their phenotypic characteristics and their long-chain fatty acid compositions.

METHODS

Strains. Cultures used in this study are given in Table 1; they were supplied by the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands (CBS) and Professor F. Oberwinkler, Lehrstuhl Spezielle Botanik, University of Tübingen, FRG (FO).

Abbreviation: FAME, fatty acid methyl ester.

Long-chain fatty acid analyses. The methods used for cultivation of strains and long-chain fatty acid analyses were as described previously (Smit *et al.*, 1987*b*). Comparative retention times of known standard methyl esters of C_{14:0} (myristic acid), C_{16:0} (palmitic acid), C_{16:1} (palmitoleic acid), C_{17:0} (margaric acid), C_{17:1} (*iso*-margaric acid), C_{18:0} (stearic acid), C_{18:1} (oleic acid), C_{18:2} (linoleic acid), C_{18:3} (linolenic acid), and C_{20:0} (arachidic acid) were used as a basis for the identification of the fatty methyl esters. Relative amounts of the fatty acids present in the strains were calculated by integration of the respective methyl ester peak areas.

Phenotypic characteristics. Characteristics were determined as described by Rodrigues de Miranda (1984). The following characters were used: pigmentation of culture; growth at 37 °C; starch formation; and assimilation of nitrate, erythritol, melibiose, raffinose, sucrose, lactose, soluble starch, maltose, L-rhamnose, citric acid, gluconolactone, galactose, succinic acid, sodium glucuronate, D-mannitol, ribitol, D-ribose, L-arabinose, D-xylose, trehalose, cellobiose and inositol.

Phenotypic similarities. Analyses were done by comparing groups of up to 33 strains. Both matched and unmatched pairs were equally weighted, using the simple matching coefficient of association of Sokal and Michener (Sokal & Sneath, 1963). A dendrogram depicting phenotypic similarities (Fig. 1) was prepared by arranging species in clusters, by means of single linkage analyses (Sokal & Sneath, 1963).

RESULTS AND DISCUSSION

The results of fatty acid analyses were reproducible when the strains were cultivated in triplicate (Table 1). The fatty acid methyl ester (FAME) profiles can be arranged into four groups. Group I was characterized by a higher mean percentage of C_{18:1} (45% ± 2.5%) and a lower mean percentage of C_{18:2} (28.9% ± 5.67%) and the presence of C_{18:3} (2.52% ± 1.21%). Group II comprised strains that also produced C_{18:3} (4.05% ± 3.12%) but had a higher mean percentage of C_{18:2} (38.57% ± 9.03%) than C_{18:1} (27.26% ± 5.02%). Groups III and IV comprised strains in which very little or no trace of C_{18:3} could be detected under the experimental conditions. Strains representing group III were characterized by a lower mean percentage of C_{18:1} and a higher mean percentage of C_{18:2}, when compared to group IV (Fig. 1, Table 1).

Within *Cryptococcus*, fatty acid compositions were largely species specific. With few exceptions, strains of the same species contained similar long-chain fatty acid compositions and could be grouped together (Fig. 1). *C. macerans* CBS 2206 and CBS 2425 were allocated to Group I; *C. ater* CBS 4685 and CBS 5809 were both in group II; *C. neoformans* CBS 132, CBS 879 and CBS 5474 were in Group III; and *C. hungaricus* CBS 4214 and CBS 5124 and *C. terreus* CBS 1895 and CBS 6517 were all in group IV. Kwon-Chung (1975) proposed the new genus *Filobasidiella* as the perfect state of *C. neoformans* and it is also interesting to note that FAME profiles obtained for several strains of *F. neoformans* (Smit *et al.*, 1987*a*) were similar to those found for *C. neoformans*.

Concerning the exceptions, *C. albidus* var. *albidus* CBS 1929 was in group II whereas *C. albidus* var. *albidus* CBS 142, *C. albidus* var. *diffluens* CBS 160 and *C. albidus* var. *aerius* CBS 155 were all in Group III. Phaff & Fell (1970) disassociated the variety *diffluens* (isolated from nail material) from the variety *albidus*, using its inability to assimilate lactose as the sole criterion. This variety was rejected by Rodrigues de Miranda (1984) because of the vagueness of the criterion used by Phaff & Fell (1970). The FAME profile of the strain CBS 160 of the variety *diffluens* showed similarities to the profile found for the variety *albidus* strain CBS 142, which strengthens Rodrigues de Miranda's argument to reject the variety *diffluens*. Although there are several characteristics by which the variety *aerius* can be distinguished from *albidus*, the main physiological characteristic used is the consistent inability of the variety *aerius* to form starch and to grow at temperatures above 30 °C. Notwithstanding these facts, the FAME profile of the strain CBS 155 of the variety *aerius* proved to be very similar to that found for strains CBS 142 and CBS 160, showing that this strain at least shares a very close relationship with the others. Strain CBS 1929 had a markedly different FAME profile when compared to the other strains of *C. albidus*, showing a high relative percentage of C_{18:3} (7.45%). This is inexplicable at this stage and further studies seem necessary.

Further anomalies were seen for *C. laurentii* CBS 5595, CBS 942, CBS 139 and CBS 2174. According to our study all the strains representing *C. laurentii* exhibited similar FAME profiles

Fig. 1. Relationships between species representing the genera *Cryptococcus* and *Tremella*, arranged according to long-chain fatty acid compositions (vertical axis, left; the histograms show the percentage fatty acid composition for each group) and phenotypic similarities (horizontal axis, top). The species are positioned according to the results indicated on these axes. Key to organisms: Cal 1, 2, 3 and 4, *C. albidus* CBS 1929, 142, 160 and 155, respectively; Cat 1 and 2, *C. ater* CBS 4685 and 5809, respectively; Cdi, *C. dimenae* CBS 5770; Cel, *C. elinovii* CBS 7051; Cfi, *C. flavus* CBS 331; Cga 1 and 2, *C. gastricus* CBS 1927 and 7016, respectively; Che, *C. heveanensis* CBS 569; Chu 1 and 2, *C. hungaricus* CBS 4214 and 5214, respectively; Cke, *C. kuetzingii* CBS 2289; Cla 1, 2, 3 and 4, *C. laurentii* CBS 5595, 942, 139 and 2174, respectively; Clu 1 and 2, *C. luteolus* CBS 4937 and 943, respectively; Cma 1 and 2, *C. macerans* CBS 2206 and 2425, respectively; Cne 1, 2 and 3, *C. neoformans* CBS 132, 879 and 5474, respectively; Csk, *C. skinneri* CBS 5029; Cte 1 and 2, *C. terreus* CBS 1895 and 6517, respectively; Tau, *T. aurantia* FO 31861; Ten, *T. encephala* FO 23371; Tfo, *T. foliaceae* FO 25113; Tme, *T. mesenterica* FO 25145. Key to FAMES: 1, $C_{14:0}$; 2, $C_{16:0}$; 3, $C_{16:1}$; 4, $C_{17:0}$; 5, $C_{17:1}$; 6, $C_{18:0}$; 7, $C_{18:1}$; 8, $C_{18:2}$; 9, $C_{18:3}$; 10, $C_{20:0}$.

except strain CBS 5595 (Table 1). It is therefore clear that this species does not seem to be homogeneous as proposed by Rodrigues de Miranda (1984).

Although *T. foliaceae* showed a FAME profile similar to *C. kuetzingii*, the fact that their phenotypic similarity is only 79% probably denotes that they are not in fact all that closely related. *C. luteolus* CBS 943, in group II, *C. luteolus* CBS 4937 in group I, *C. gastricus* CBS 7016 in group IV and *C. gastricus* CBS 1927 in group III also represent exceptions. In the case of *C. gastricus* the two strains studied differed significantly only in their $C_{18:1}$ to $C_{18:2}$ ratio, but were otherwise very similar. *C. luteolus* CBS 943 and CBS 4937 differed greatly in their relative percentage $C_{18:0}$ (2.60% for CBS 4937 and 21.64% for CBS 943), at present however, there is no explanation for this observation.

Table 1. *FAME* profiles of strains representing the genera *Cryptococcus* and *Tremella*
The values shown are the means of three or more repetitions. The SD values were about 8% of the mean (range 0.8-39%).

Group	Organism	Strain no.	Relative amount of FAME (%)									
			C _{14:0}	C _{16:0}	C _{16:1}	C _{17:0}	C _{17:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:0}
I	<i>C. luteolus</i>	CBS 4937	0.87	15.58	0.91	1.50	1.42	2.60	46.29	28.63	1.88	0.32
	<i>C. flavus</i>	CBS 331	0.47	12.96	0.76	0.10	0.52	4.97	45.55	32.88	1.77	0.00
	<i>C. macerans</i>	CBS 2206	0.24	17.33	0.28	0.92	0.51	5.31	45.66	28.16	0.99	0.59
	<i>C. macerans</i>	CBS 2425	0.30	19.10	0.42	0.53	0.24	4.55	47.74	25.28	1.46	0.37
	<i>C. dimenmae</i>	CBS 5770	0.68	17.62	0.58	0.30	0.14	8.33	46.67	21.31	3.97	0.38
	<i>C. skinneri</i>	CBS 5029	0.84	17.28	0.64	0.15	0.33	5.97	43.74	25.81	4.29	0.51
	<i>C. kuetzingii</i>	CBS 2289	0.21	10.69	0.44	0.30	0.21	5.26	39.35	40.23	3.27	0.03
	<i>T. aurantia</i>	FO 31861	0.37	15.18	1.03	0.14	0.22	0.92	47.34	33.77	0.51	0.51
	<i>T. foliaceae</i>	FO 25113	1.45	14.00	0.83	0.49	0.28	6.09	50.29	23.18	2.08	1.32
	Mean ...		0.52	15.79	0.58	0.61	0.48	5.28	45.00	28.90	2.52	0.31
	SD ...		0.26	2.75	0.20	0.44	0.41	1.59	2.57	5.67	1.21	0.21
II	<i>C. laurentii</i>	CBS 5595	0.39	13.88	0.36	2.17	0.00	17.50	23.64	41.18	0.20	0.68
	<i>C. luteolus</i>	CBS 943	0.48	15.69	0.25	0.42	0.03	21.64	32.90	27.46	0.39	0.74
	<i>C. albidus</i>	CBS 1929	0.52	13.19	6.10	1.96	1.29	6.83	33.77	28.42	7.45	0.45
	<i>C. ater</i>	CBS 4685	0.37	13.36	0.37	1.07	0.61	7.23	24.03	47.40	5.55	0.00
	<i>C. ater</i>	CBS 5809	0.87	14.30	0.88	1.13	0.38	5.22	21.94	48.38	6.64	0.26
	<i>T. mesenterica</i>	FO 25145	0.40	13.76	0.57	0.77	0.29	3.54	36.59	43.56	0.13	0.38
	Mean ...		0.53	14.08	1.59	1.35	0.46	11.68	27.26	38.57	4.05	0.43
	SD ...		0.18	0.89	2.26	0.64	0.47	6.61	5.02	9.03	3.12	0.27

The organism described in 1921 by Groenewege as *Torula heveanensis* and assigned to *C. laurentii* var. *magnus* by Lodder & Kreger-van Rij (1952) was also studied by Baptist & Kurtzman (1976). They found that strain Y-1510 of *Torula heveanensis* was different from both *C. laurentii* var. *magnus* and *C. laurentii* var. *laurentii* and could not be considered an additional variety of *C. laurentii*. They therefore proposed a new species, *C. heveanensis*, with the culture originally described as *Torula heveanensis* NRRL Y-1510 (CBS 569) as the type strain. Our results (Fig. 1., Table 1) show that the FAME profile of *C. heveanensis* CBS 569 and *C. laurentii* also differed significantly.

On the basis of assimilation patterns, Slodki *et al.* (1965); Slodki & Wickerham (1966) suggested that *C. laurentii* might be closely related to *T. encephala* and *T. aurantia*. Kurtzman (1973), however, was unsuccessful in mating sexually reactive isolates of *C. laurentii* with those of *Tremella* species, while different enzyme patterns were found for *T. encephala*, *T. aurantia* and *C. laurentii* (Baptist & Kurtzman, 1976). This was criticized by Phaff (1984) for including too few enzymes, making interpretation of the results quite subjective and sensitive to the effects of population variation.

Our results show that *T. encephala* FO 23371 appears to be closely related to *C. laurentii*, while *T. aurantia* FO 31861 exhibited a different FAME profile. It would thus seem that the suggestion of Slodki *et al.* (1965), that a possible taxonomic relationship between certain *Tremella* and *Cryptococcus* species exists, may have merit after all. Polyacrylamide gel electrophoresis of total cell proteins is currently being used to introduce a further taxonomic character, to evaluate the results so far obtained.

According to our results the following conclusions can be drawn. The identification of individual *Cryptococcus* species, using long-chain fatty acid composition as the sole parameter, does not seem viable but distinct groupings of the FAME profiles were evident. For example, with few exceptions, strains of the same species of *Cryptococcus* showed similar FAME profiles. Secondly, based on the similarity of their FAME profiles *C. albidus* var. *aerius* and *C. albidus* var. *diffluens* seem to share a very close relationship with *C. albidus* var. *albidus*; this is in accordance with the proposal of Rodrigues de Miranda to reject the variety *diffluens*. Thirdly, the significant difference in the FAME profile of one of the *C. laurentii* strains may imply that this species is not homogeneous. Finally, considering both long-chain fatty acid composition and phenotypic characteristics, *T. encephala* appears to be closely related to *C. laurentii*, while *T. aurantia*, thought to be closely related to *C. laurentii* (Slodki *et al.*, 1965), seems to differ significantly.

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