

Supplementary data: Morphology

Mrakia frigida

CBS 5270^T

Growth on YEP agar for 3 weeks at 6°C: Colonies were a dull yellow-white cream colour, had a pasty or membranous texture, margin was raised and lobate with a smooth, raised, convex center giving each colony the appearance of a small flower surrounded by tiny petals, this was generally fringed with hyphae that extended into and along the agar (Fig 1).

Growth in YEP after 1 week at 15°C, cells were generally ovoid-elongate, pseudo- and true hyphae were regularly observed, measured 1-2.5 x 5-50 µm and budding was polar (Fig 2).

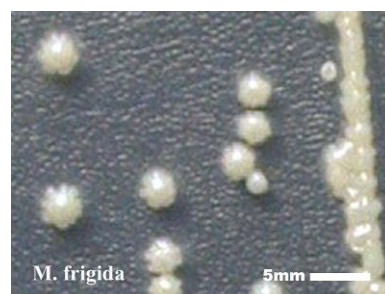


Fig. 1 CBS 5270 colonies

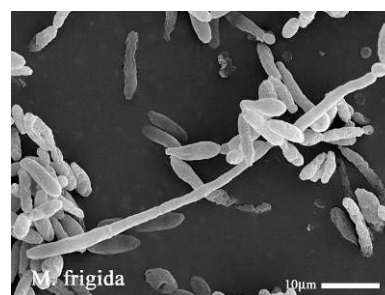


Fig. 2 SEM of CBS 5270

CBS 8907

Growth on YEP agar after 3 weeks at 15°C: Colonies were white cream, smooth, butyrous, convex and circular, the margin was entire with occasional mycelial growth which extended from colonies into and along the agar (Fig 3).



Fig. 3 CBS 8907 colonies

Growth in YEP after 1 week at 6°C: cells were generally ovoid-elongate, measured 1.5-3 x 2-8 µm and budding was polar (Fig 4). Short hyphae (10-20 µm) were occasionally observed, long mycelia (>20µm) were observed very infrequently. Fig 5 (a scanned image) reveals what may be forming pseudo-hyphae (a linear arrangement of budding cells that remain attached to each other), or it may be a forming hypha tip.



Fig. 4 SEM of CBS 8907

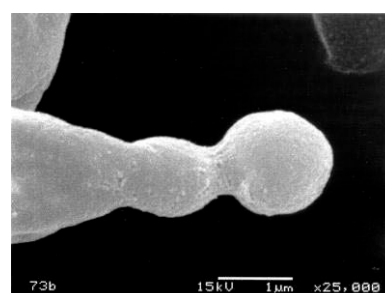


Fig. 5 SEM of CBS 8907

Mrakia robertii

CBS 8918 and CBS 8919

Growth on YEP agar after 3 weeks at 6°C: Colonies were white cream, smooth, butyrous, convex and generally circular. Occasional mycelial growth extended from colonies into and along the agar (Fig 6). Ten-day-old colonies with emerging hyphae are shown in Fig 8.

Growth in YEP after 1 week at 15°C, cells were generally round to ovoid-elongate, measured 2-4 x 2-7 µm and budding was polar (Fig 7 & 9). Hyphae were occasionally observed.

CBS 8912^T

Growth on YEP agar after 3 weeks at 6°C: Colonies were white-yellow cream, smooth, butyrous, convex and circular. Mycelia surrounded the colony within one week, this extends into the agar giving the colony a halo-appearance (Fig 10); hyphae may also extend along the top of the agar and become raised so that finger-like projections extend from the colony giving it a sun-like appearance (Fig 12).

Growth in YEP after 1 week at 15°C, cells were generally ovoid-elongate, pseudo and true mycelium were regularly observed, measured 1.5-4 x 3-50 µm and budding was polar (Fig 11).

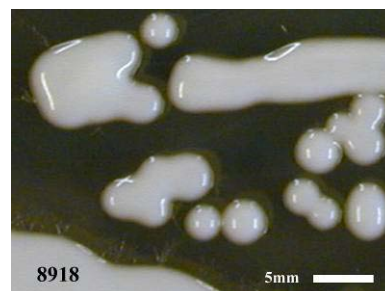


Fig. 6 CBS 8918 colonies



Fig. 7 SEM of CBS 8918



Fig. 8 CBS 8919 colonies



Fig. 9 SEM of CBS 8919

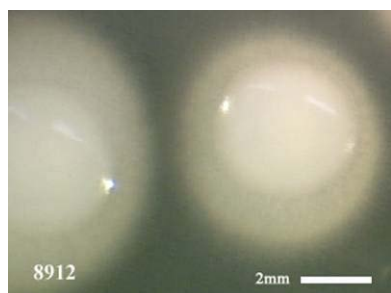


Fig. 10 CBS 8912 colonies

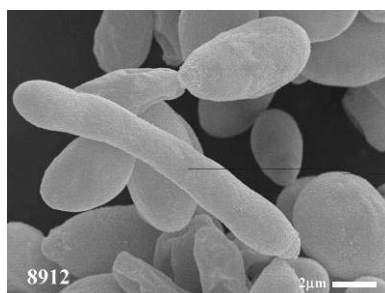


Fig. 11 SEM of CBS 8912

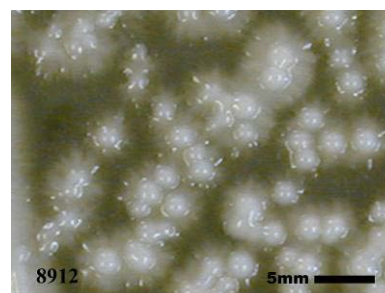


Fig. 12 CBS 8912 colonies

Mrakia blollopis

CBS 8921^T, CBS 8909 and CBS 8910

Growth on YEP agar after 3 weeks at 6°C: colonies were a white cream colour and mature (4-5 weeks) into dull yellow-white cream colour. Butyrous colonies were smooth, convex and circular with surrounding membranous mycelia. Pseudo- and true hyphae extended into and along agar to produce the appearance of a diffuse halo at the margins. The extent of hyphae varied between each strain; CBS 8921^T (Fig 13) had the furthest reaching mycelia, CBS 8909 (Fig 14) had the shortest extending mycelia, 8910 (Fig 15) had the strongest mycelial growth, which build up on top of each other resulting in many raised fingerlike projections extending from each colony.

Growth in YEP after 1 week at 15°C, CBS 8921^T (Fig 16 & 17) cells were generally ovoid (2-4 x 3.5-7) µm or round (5-7µm in diameter) and occurred singly or in budding pairs, budding was polar, true hyphae that extend from some cell were observed to be 25 µm and longer. CBS 8909 (Fig 18) cells were generally ovoid-elongate, measured 2.5-4 x 3.5-7 µm, occurred singly or in parent-bud pairs, budding was polar, pseudo- and true hyphae were observed in excess of 15 µm. CBS 8910 (Fig 19) cells were generally ovoid-elongate, measured 2.5-5 x 4-14 µm, occurred singly or in parent-bud pairs, budding was polar, pseudo- and true hyphae were observed regularly.

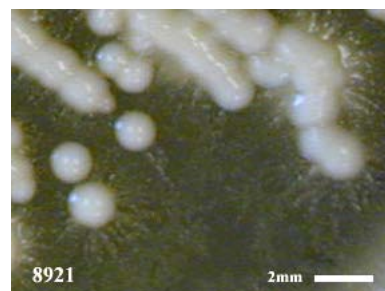


Fig. 13 CBS 8921 colonies

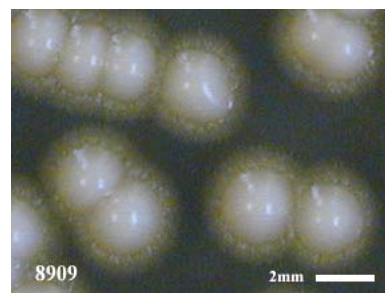


Fig. 14 CBS 8909 colonies

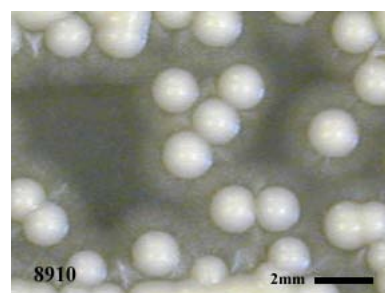


Fig. 15 CBS 8910 colonies

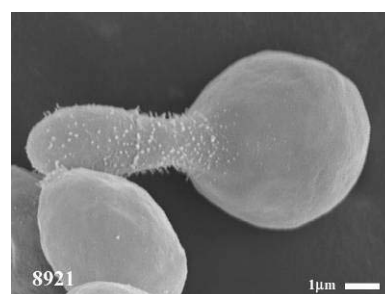


Fig. 16 SEM of CBS 8921



Fig. 17 SEM of CBS 8921

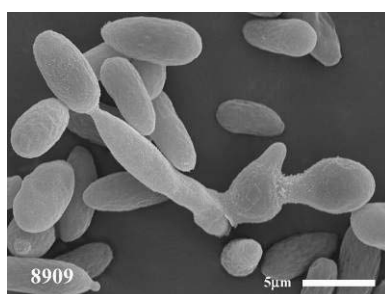


Fig. 18 SEM of CBS 8909

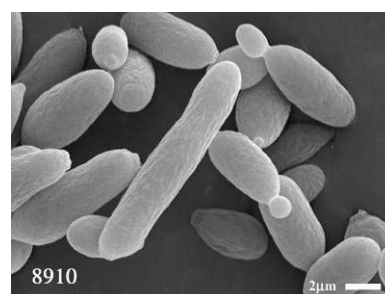


Fig. 19 SEM of CBS 8910

Sexual states

Each strain was streaked out on malt agar (MA), corn meal agar (CM), nitrogen base agar (NB) and carbon base agar (CB). These were incubated at 6°C and observed for sexual structures after 1, 2, 3, 6, 9 and 14 months. The following descriptions were from 14-month observations. The extent of hyphal growth was described as some, numerous, abundant or copious amounts. When teliospores were observed, they were transferred to dH₂O and incubated at 6°C for 3 weeks to allow germination on nutrient-free agar (NF) at 6°C.

CBS 8912^T

MA- Large mucoid colonies with numerous spreading hyphae, intercalary teliospores were present.

CM- Small butyrous colonies with numerous spreading hyphae.

NB- Tiny colonies with copious amounts of hyphae, small cells.

CB- Small butyrous colonies with numerous long, thin spreading hyphae, often with terminal teliospores (Fig. 20).

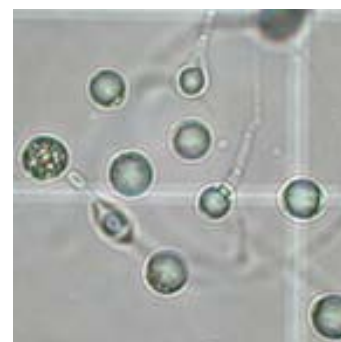


Fig. 20 CBS 8912. slide of terminal teliospore from CB.

CBS 8918 no teliospores were observed in this strain.

MA- Large butyrous colonies with some spreading hyphae.

CM- Small butyrous colonies with numerous spreading hyphae.

NB- Tiny colonies with copious amounts of spreading hyphae.

CB- Small butyrous colonies with an abundance of hyphae spreading into and along agar.

CBS 8919

MA- Large mucoid colonies with numerous spreading hyphae, teliospores were present.

CM- Small butyrous colonies with numerous spreading hyphae.

NB- Tiny colonies with copious amounts of spreading hyphae.

CB- Small butyrous colonies with copious amounts of spreading hyphae, terminal and intercalary teliospores were observed, often in pairs (Fig. 21).

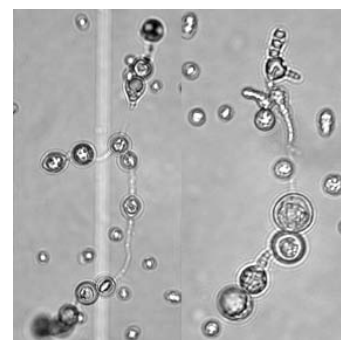


Fig. 21 CBS 8919. Intercalary teliospore on CB.

CBS 8907

MA- Large mucoid colonies with some spreading hyphae, intercalary teliospores were observed (Fig. 22).

CM- Small butyrous colonies with some spreading hyphae.

NB- Tiny colonies with some hyphae spreading into the agar.

CB- Small butyrous colonies with some spreading hyphae, teliospores were present though not connected to hyphae.



Fig. 22 CBS 8907. Slides of teliospores from dH₂O.

CBS 8909

MA- Large mucoid colonies with abundant spreading hyphae, teliospores were present.

CM- Small butyrous colonies with numerous spreading hyphae.

NB- Tiny colonies with numerous far-reaching thin hyphae.

CB- Small butyrous colonies with numerous far-reaching thin spreading hyphae, teliospores were present. Teliospores were incubated in dH₂O and germinated on NF agar (Fig. 23)



Fig. 23 CBS 8909. Germinated teliospore on NF.

CBS 8910

MA- Large mucoid colonies with copious amounts of spreading hyphae, terminal teliospores were present on hyphae.

CM- Small butyrous colonies with numerous spreading hyphae.

NB- Tiny colonies with numerous spreading hyphae, small cells.

CB- Small butyrous colonies with numerous spreading hyphae, terminal teliospores were regularly observed. Teliospores were incubated in dH₂O and germinated on NF agar (Fig. 24).

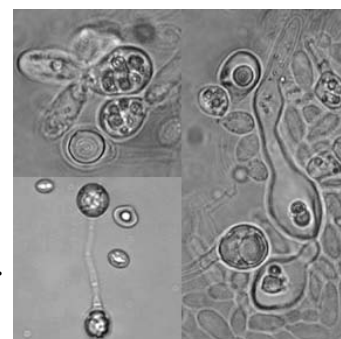


Fig. 24 CBS 8910. Differentiated cells and teliospore on NF.

CBS 8921^T

MA- Large mucoid colonies with numerous spreading hyphae.

CM- Small butyrous colonies with numerous spreading hyphae.

NB- Tiny colonies with copious amounts of spreading hyphae.

CB- Small butyrous colonies with numerous spreading hyphae, terminal and intercalary teliospores were observed (Fig 25).



Fig. 25 CBS 8921^T. Slide of intercalary teliospores, MA.

Species	Strain	Antarctic location	Sample	Conspecific strain	GenBank accession numbers		
					D1/D2	ITS	IGS
<i>M.frigida</i>	CBS 5266	Scott Base	Soil		AF189849	AF144484	AF144415
<i>M.frigida</i>	CBS 5270 ^T	Scott Base	Snow and Soil		AF075463	AF144483	AF144414
<i>M.gelida</i>	CBS 5272 ^T	Scott Base	Soil		AF189831	AF144485	AF144416
<i>M.frigida</i>	CBS 5688	(Japan)	Frozen fish		AF189847	AF144482	AF144413
<i>M.gelida</i>	CBS 5917	Bouvet Island	Snow and Soil		AF189830	AF144486	AF144417
<i>M.frigida</i>	CBS 8907	Lichen Valley	Snow Petrel Carnage		AY038806	AY038836	AY038815
<i>M.frigida</i>	125e	Marine Plain	Soil	CBS 8907			
<i>M.frigida</i>	133a	Lichen Valley	Soil and Lichen	CBS 8907			
<i>M.frigida</i>	146c	Lichen Valley	Dried Algal Mat	CBS 8907			
<i>M.frigida</i>	157b	Lichen Valley	Dried Algal Mat	CBS 8907			
<i>M.robertii</i>	CBS 8912^T	Mossell Lake	Soil and Lichen		AY038811	AY038829	AY038822
<i>M.robertii</i>	CBS 8918	Moss Cirque	Soil		AY038805	AY038834	AY038817
<i>M.robertii</i>	CBS 8919	Lichen Valley	Soil and Lichen		AY038804	AY038835	AY038816
<i>M.robertii</i>	46a	Lichen Valley	Soil	CBS 8919			
<i>M.robertii</i>	56	Teat Lake	Lichen	CBS 8919			
<i>M.robertii</i>	110c	Marine Plain	Soil and Lichen	CBS 8919			
<i>M.robertii</i>	149a	Mossell Lake	Soil and Lichen	CBS 8919			
<i>M.robertii</i>	154a	Mossell Lake	Lichen	CBS 8919			
<i>M.robertii</i>	165d	Lichen Valley	Stromatolite and Soil	CBS 8919			
<i>M.robertii</i>	170c	Moss Cirque	Soil and Lichen	CBS 8919			
<i>M.gelida</i>	3	Partizan Island	Soil	5	Not submitted to GenBank		
<i>M.gelida</i>	5	Rookery Lake	Red mat and feathers		Not submitted to GenBank		
<i>M.gelida</i>	8	Ekho Lake	Soil	5			
<i>M.gelida</i>	30	Gardener Island	Soil	5			
<i>M.gelida</i>	53b	Watts Lake	Stromatolite	5			
<i>M.gelida</i>	66	Marine Plain	Soil	5			
<i>M.gelida</i>	113	Moss Cirque	Soil	5			
<i>M.gelida</i>	CBS 8911	Organic lake	Soil		AY038808	AY038832	AY038819
<i>M.gelida</i>	CBS 8914	Lichen Valley	Soil and Lichen		AY038807	AY038833	AY038818
<i>M.gelida</i>	CBS 8916	Moss Cirque	Soil		AY038810	AY038830	AY038821
<i>M.gelida</i>	CBS 8922	Moss Cirque	Soil and Lichen		AY038809	AY038831	AY038820
<i>M.gelida</i>	97a	Moss Cirque	Soil	CBS 8911			
<i>M.gelida</i>	102d	Marine Plain	Soil	CBS 8916			
<i>M.gelida</i>	116d	Moss Cirque	Soil and Lichen	CBS 8911			
<i>M.gelida</i>	130a	Moss Cirque	Soil, benthic, algal mat	CBS 8911			
<i>M.gelida</i>	174d	Moss Cirque	Soil	CBS 8916			
<i>M.blollopis</i>	CBS 8909	Moss Cirque	Soil		AY038812	AY038828	AY038823
<i>M.blollopis</i>	CBS 8910	Moss Cirque	Soil, benthic, algal mat		AY038813	AY038827	AY038824
<i>M.blollopis</i>	CBS 8921^T	Marine Plain	Soil		AY038814	AY038826	AY038825
<i>M.blollopis</i>	45a	Lichen Valley	Soil	CBS 8909			
<i>M.blollopis</i>	109b	Moss Cirque	Soil, Moss and Lichen	CBS 8909			
<i>M.blollopis</i>	116e	Moss Cirque	Soil and Lichen	CBS 8909			
<i>M.blollopis</i>	160b	Lichen Valley	Soil and Lichen	CBS 8909			
<i>M.blollopis</i>	163	Moss Cirque	Soil and Lichen	CBS 8909			
<i>M.blollopis</i>	174c	Moss Cirque	Soil	CBS 8909			
<i>M.blollopis</i>	177b	Moss Cirque	Soil	CBS 8909			
<i>M.blollopis</i>	179a	Moss Cirque	Soil	CBS 8909			

Supplementary Table 1. List of *Mrakia* strains identified in the study of Antarctic yeasts. Phylogenetic group also corresponds to the groups identified by the protein fingerprints.

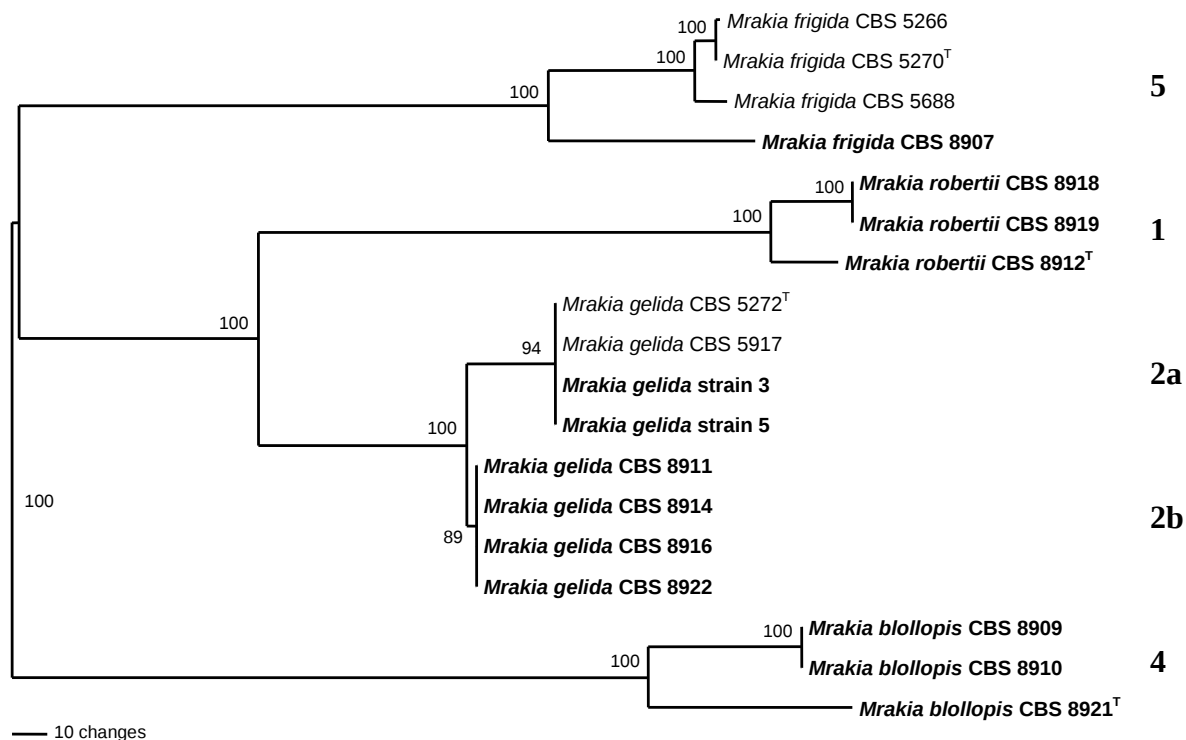


Fig. 26 *Mrakia* consensus tree of the IGS region. One parsimony tree, TL = 881, NC = 793, CC = 239, PUC = 35, PIC = 519, CI = 0.893, RI = 0.955. Bootstrap values from 1000 full heuristic replications. Outgroup = *Mrakia blollopis* clade.

To further delineate differences in the *Mrakia* clade and build on the work done by Diaz and Fell (2000) phylogenetic analysis of the IGS region of their DNA was performed (Fig. 26). The analysis was hindered by extreme variation that occurs within the region; for example, between any two of the four species this ranged from 26% to 50% sequence divergence. This meant that the alignment had very few positions of agreement, the majority of these were at the 5'-end of the sequence, corresponding to the 3'-end of the 26S ribosome. In order to determine the best cut-off point, all sequences were trimmed to 800 nt and then each group aligned to each of the other groups and cut at the region of highest homology at the 3'-end (the longest sequence was 765 and the final alignment length was 793). The level of variation within each species was relatively low, the most extreme being 12.5% between CBS 8907 and CBS 5688. High inter-species and low intra-species variation meant that the phylogenetic analysis had perfect bootstrap support (100%) between each of the four species analyzed.

Substrate	CBS 5272 ^T <i>Mrakia gelida</i>	CBS 8912 ^T <i>Mrakia robertii</i>	CBS 5270 ^T <i>Mrakia frigida</i>	CBS 8921 ^T <i>Mrakia blollopis</i>	AS 2.1971 ^T <i>Mrakia psychrophila</i>	CBS 9136 ^T <i>Mrakia curviuscula</i>	CBS 5443 ^T <i>Mrakiella aquatica</i>	CBS 8917 ^T <i>Mrakiella niccombsii</i>	CBS 10834T <i>Mrakiella cryoconiti</i>
Carbon assimilation									
D-Glucose	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	w	w
L-Sorbose	+	w	d	w	w	-/d	+	w/+	+
D-Glucosamine	d	+	+	w/+	+	+	-	w/+	v
D-Ribose	+	w	-	w/+	w	w	w,d,-	w/+	+
D-Xylose	+	w/+	+	w/+	+	+	+	+	+
L-Arabinose	+	w/+	+	+	+	+	+	w	+
D-Arabinose	+	w/-	d	w/-	+	v	-	w	+
L-Rhamnose	+	w/+	-	w	+	+	-	w/+	+
Sucrose	+	+	+	+	+	-	+	+	+
Maltose	+	+	-	+	+	+	+	+	+
α , α -Trehalose	+	+	d	+	+	+	+	w/+	+
Me α -D-Glucoside	-	w/-	-	w/-	+	-	-	w	-
Cellobiose	+	+	+	+	+	+	+	w/+	+
Salicin	+	w/+	+	w/+	+	+	+	w/+	+
Melibiose	+	+	-	+	+	-	+	w	+
Lactose	-	-	+	w	+	+	w, d, +	w	+
Raffinose	+	+	+	+	+	-	+	+	+
Melezitose	+	+	-	+	+	+	+	w/+	+
Inulin	-	w	-	-	+	-	-	w	+
Soluble starch	+	w	-	v	+	v	+	w	+
Glycerol	w,d,-	-	-	v	+	-	d	w/+	+
Erythritol	-	-	-	w/-	-	-	-	v	-
Ribitol/Adonitol	+	+	+	+	+	s	+	-	+
D-Glucitol/D-Sorbitol	+	+	+	+	+	+	+	w	+
D-Mannitol	+	+	+	w/+	+	+	+	w	+
Galactitol/Dulcitol	-	-	-	w/-	-	-	-	w/+	-
<i>myo</i> -Inositol	-	w/+	d	w/+	+	-	-	w	+
D-Gluconate	+	+	+	+	+	+	+	+	+
D-Glucuronate	+	+	+	+	w	+	+	w/+	+
DL-Lactate	-	-	d	w/-	-	w	-	-	-
Succinate	+	w	+	v	+	+	+	w	+
Citrate	+	w/+	+	w/+	+	+	+	w	-
Methanol	-	-	-	w/-	w	-	-	-	-
Ethanol	+	w	+	+	+	v	+	w/+	+
N-Acetyl-D-glucosamine	-	w/+	-	w	+	+	+	w	+
Hexadecane	-	w/-	-	w/-	-	-	-	-	-
Nitrogen assimilation									
Nitrate (KNO ₃)	+	+	+	+	+	-	+	+	+
Nitrite (NaNO ₂)	+	v	+	v	-	-	+	-	+
Ethylamine	-	-	-	-	+	-	-	-	-
L-Lysine	+	+	+	+	+	-	+	-	+
Cadaverine	-	-	+	-	nd	-	-	-	-
Creatine	-	v	-	-	+	-	-	-	-
Creatinine	-	v	-	-	+	-	-	-	-
Fermentation									
Glucose	w,d	+	w,d	+	d	-	-	-	-
Galactose	-	-	-	-	-	-	-	-	-
Sucrose	w,d	+	w,d	+	d	-	-	-	-
Maltose	-	d	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-
Raffinose	-	d	-	-	-	-	-	-	-
Growth without vitamins									
Vitamin-free	-	w/+	-	w	+	-	-	-	-
Biotin-free	-	+	-	+	+	-	-	+	-
Thiamine-free	-	w/+	-	w/+	+	-	-	-	-
Other tests									
Starch formation	+	+	w	+	+	+	+	+	+
Urease	+	+	-	+	+	+	+	+	+
Diazonium Blue B reaction	+	+	+	+	+	+	+	+	+
50% (w/w) glucose-	-	-	-	-	+	w/-	-	-	+
10% NaCl - 5% glucose	-	w/+	-	w/+	-	-	-	+	-
Gelatin liquefaction	+	v	v	+	-	-	-	+	-
Growth at 25°C	-	-	-	-	-	-	-	-	-
Max growth Temperature	17	18	17	18	18	25	20	18	20

Supplementary Table 2: Comparison of all phenotypic characteristics between the type strains of the species belonged to the genera *Mrakia* and *Mrakiella*.

Note: "+", "-", "v", "w", "d" stand for positive, negative, variable, week and delayed.

The data obtained from CBS data base (www.cbs.knaw.nl) for *M. gelida* CBS 5272T, *M. frigida* CBS 5270T and for *Mr. aquatica* CBS 5443T, and from Bab'eva et al. (2002), Xin and Zhou (2007) and Margesin & Fell (2008) for *M. curviuscula* CBS 9136T, *M. psychrophila* AS 2.1971T and *Mr. cryoconiti* CBS 10834T, respectively.

1D-SDS-PAGE of whole cell protein profiles

Protein fingerprints of the *Mrakia* working strains (Fig. 27 and 28) were arranged according to their phylogenetic groups and these groups were run side-by-side for closer analysis.

In order to maximize the resolution of the protein fingerprints, two polyacrylamide gels were run; a 12% gel, optimal for separating low molecular weight proteins 50 kDa and below (Fig. 27) and a 7% gel optimal for separating high molecular weight proteins 50 kDa and above (Fig. 28). The majority of variations in the protein fingerprint were observed above 50 kDa, however, some clear differences were also evident in protein bands < 50 kDa in Group 1 compared to the other three groups. Vancanneyt *et al.* (1992) created a dendrogram based on the variation in protein banding pattern and through this they were able to determine correlation levels between all species analysed. However, at the time of this study no computer software was freely available for the correlation analysis, hence all variations were identified by eye. Although this was not optimal it was sufficient to identify isolates with very similar protein fingerprints for classifying homologous strains. Isolates found to have virtually identical fingerprints (conspecific strains) are listed in table 1, three isolates from each cluster of conspecific strains were chosen to represent the group for further analyses. The preliminary PAGE screen was later validated by the sequence analysis. Further interpretation of figures 27 – 29 is presented in the manuscript under the section “PAGE analysis of proteins”.

Fig 27. Comparison by 1D-PAGE of whole cell protein extracts from the *Mrakia* strains. Silver stained 12% polyacrylamide gel. Low molecular weight standards (kDa) as indicated on the left. Group numbers as defined by phylogenetic analyses, are indicated at the bottom.

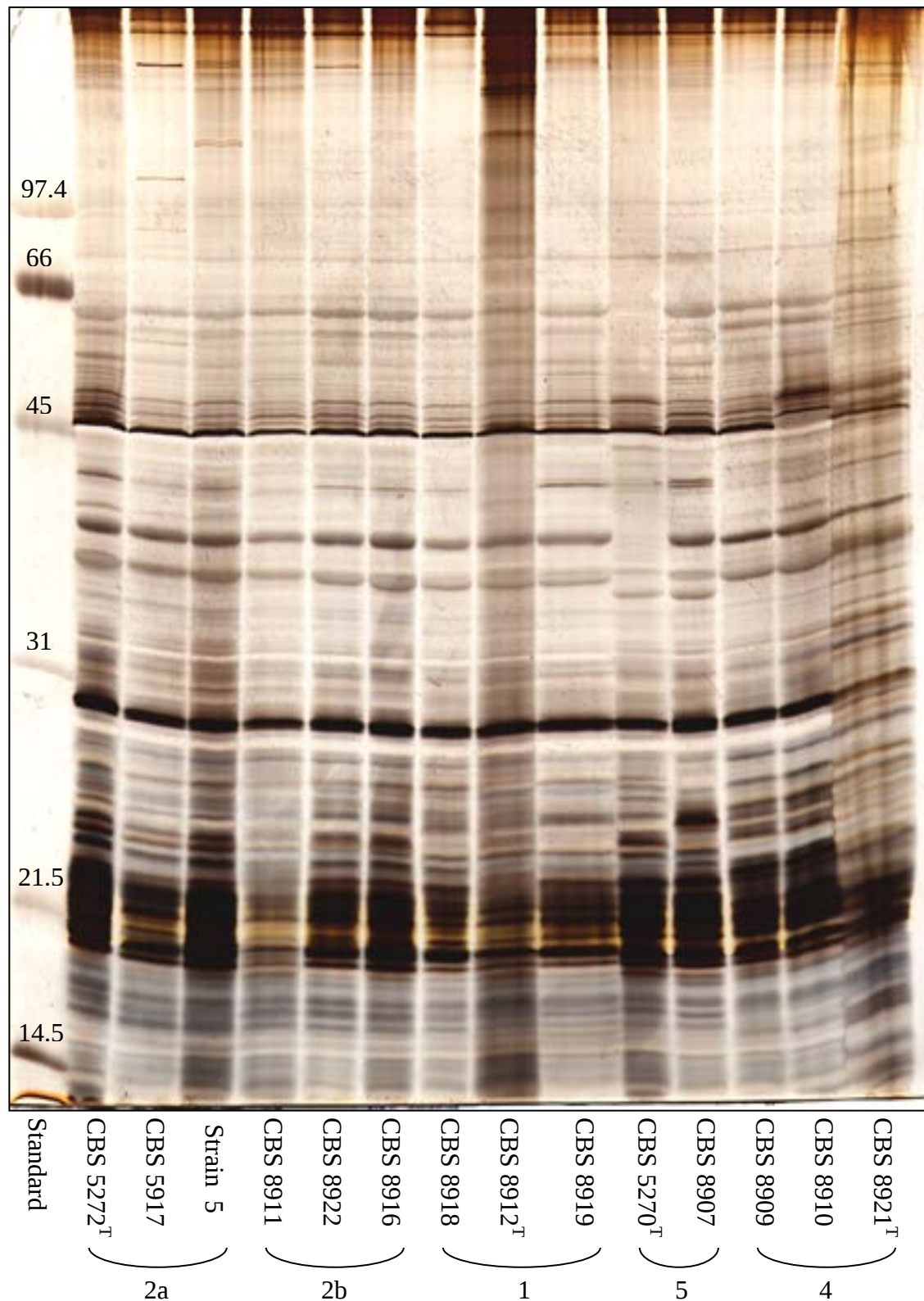
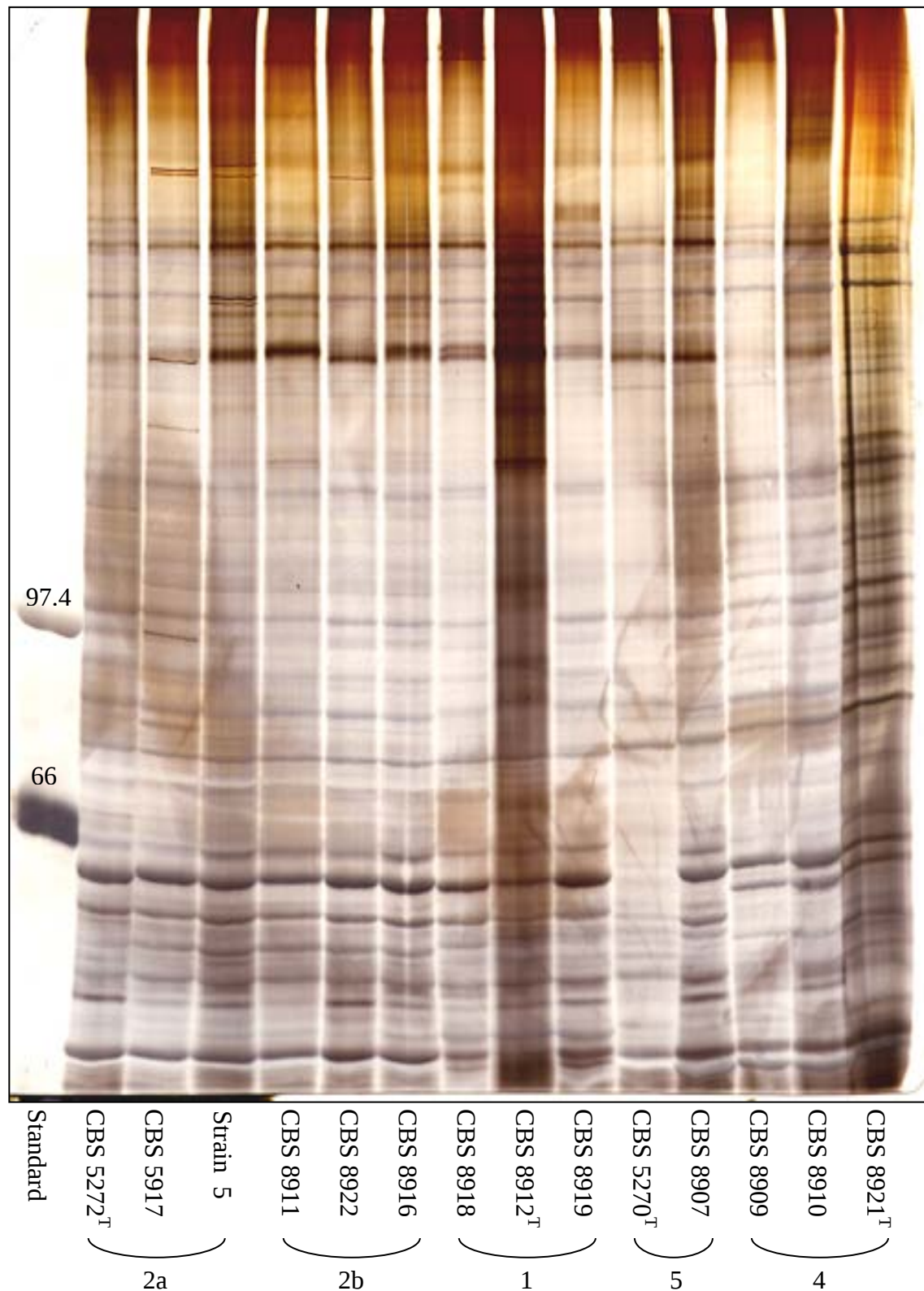


Fig 28. Comparison by 1D-PAGE of whole cell protein extracts from the *Mrakia* strains. Silver stained 7% polyacrylamide gel. Low molecular weight standard (kDa) as indicated on the left. Group numbers as defined by phylogenetic analyses, are indicated at the bottom.



Protein profiles of isolates CBS 5443^T, CBS 8917^T and CBS 8924 were obtained by 1D-PAGE for comparative proteome fingerprinting. Comparison of the three fingerprints (Fig 29) showed a significant level of variation between *Mr. aquaticus* and *Mr. niccombsii*, thus supporting the classification of these two as distinct species. On the other hand, comparison between CBS 8917^T and CBS 8924 also revealed a surprising level of variation, with an excess of ten protein bands that differed. This observation would indicate that there were physiological differences between the two strains that are not apparent in the cell morphology or sequence analysis. Alternatively, cultures may have been in different growth stages at the time of harvest for protein fingerprinting. However, due care was taken to ensure that protein extractions were performed on cultures that were in the same growth phase, namely logarithmic phase, though sometime a cultures' growth would be latent and this would delay the batch processing.

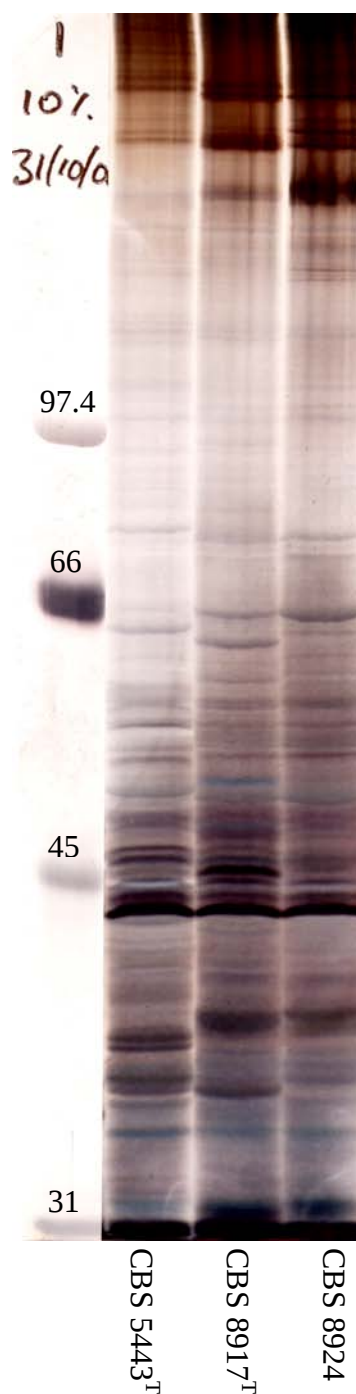


Fig 29. Protein comparison by 1D-PAGE of whole cell extracts from CBS 5443^T, CBS 8917^T and CBS 8924. Silver stained 10% polyacrylamide gel. Lane 1, low molecular weight standard (kDa).

Cold fermentation and Antarctic beer

Following the observation that the *Mrakia* strains fermented efficiently at low temperatures, attention turned to the commercial potential of these strains in cold beer fermentation. Potential advantages that these strains may have over the traditional brewing strains of *Saccharomyces cerevisiae* is; the ability to ferment more efficiently at lower temperatures, the possibility that the omega-3 fatty acids would be imparted to the beer and in effect make it healthier overall, and that a variation in the flavor would naturally result from brewing with a new variety of yeast. The *Mrakia* strains also utilized xylose which is a major carbon source in grapes and a strain that can ferment or utilize xylose is a desirable trait in the next generation of wine yeasts (Pretorius 2000). Sinclair and Stokes (1965) also noted that CBS 5917 could ferment more efficiently at 10 to 15°C than the traditional brewing strains of *S. cerevisiae*.

Results from the initial experiments were encouraging; all four species fermented at 6°C in a Wanders malt beer mix containing 2% or 5% sucrose w/v. Trace quantities of omega 3 fatty acids found in the filtered ferment product (Fig 31). However the absolute gravity of the ferment, originally at 1.04, would not progress past 1.03 – 1.02, to the desired 1.01 – 1.006, thus the sucrose fermentation was stuck after 2 weeks, with the alcohol content ranging between 1-2%. This was bottled at 6 weeks without additional sugar and stored at 15°C. Bottles were opened 2 months later (a normal maturity time for beer) and the alcohol content was found to have increased up to a maximum of 2.7%. The beer was also exceptionally carbonated from the significant CO₂ pressure that had built up in the bottles. It was clear that fermentation had been re-initiated by the bottling process that would have re-oxygenated the liquid to some degree and by the increase in temperature to 15°C.

In a second batch, the fermentation temperature was raised to 15°C, which increased the speed of fermentation for all strains but here again all the *Mrakia* strains were stuck at 2% alcohol. The conventional Wanders brewing yeast (*S. cerevisiae*) was added after 2 weeks to complete the fermenting. Fermentation was completed after a further week's incubation at 15°C and the beer was bottled with the standard addition of sugar. The control wort, fermented with conventional Wanders brewing yeast, completed the fermentation after 2 weeks at 15°C. Bottles were opened 2 months later and the alcohol content was between 5 and 6% for each of the five batches according to a Sigma-Aldrich alcohol testing kit.

Additional tests were performed on the effects of ethanol on the growth rate of the *Mrakia* strains. In YEP broth plus 2% ethanol v/v, growth was latent but uninhibited at 15°C. In YEP broth plus 5% ethanol v/v, growth was very slow and weak, thus increasing ethanol percentage inhibited the growth of these strains. Anaerobic growth in the *Mrakia* strains and the brewing strain *S. cerevisiae* was also tested. Appropriate strains were inoculated into degassed (N₂) sealed bottles containing YEP 2% w/v glucose and incubated at 15°C. No growth was observed in any of the bottles, thus *Mrakia* strains are unable to grow under strict anaerobic conditions.

There was the possibility that the metabolites produced by the *Mrakia* strain during fermentation could be toxic. In order to test for this an experiment was set up using rats as test subjects. Six female rats from the same litter were paired up in three cages. One cage was given access to the fermented product from CBS 8907 or CBS 8918. The second experimental cage was given access to the fermented product from CBS 8910 or CBS 8911 and the control cage was feed beer produced by Wanders brewing yeast. For the first 2 months (April and May), the fermented product was swapped for the water bottle for 8 hr every second day. As no adverse effect was observed in any of the rats the experiment continued but with unlimited access to both the fermented product and water for the remainder of the experiment. There was a three-month break in the experiment between the rats being fed product from the first batch of beer (*Mrakia* fermented only) to the second batch (*Mrakia* and *S. cerevisiae* combined fermentation). Rates of consumption are displayed in Fig 30. The rate of consumption for the *Mrakia* fermented product was double that for water or the control ferment product. This may be attributed to the higher sugar content, which the rats appeared to prefer, due to the incomplete fermentation. It was note worthy that preference for the fermented product stopped when in November the second batch, with fermentation completed, replaced the first batch.

At the completion of the experiments an autopsy was performed on each of the rats. The livers and hearts were examined for abnormalities and the body weight of each rat was compared with the liver weight. No abnormalities were observed in any of the rats and all organs were found to be healthy. Livers were deep maroon with brown specs on the larger half. These were more prominent in the control experiment rats. Liver weight to body weight did not vary greatly between rats or experimental groups.

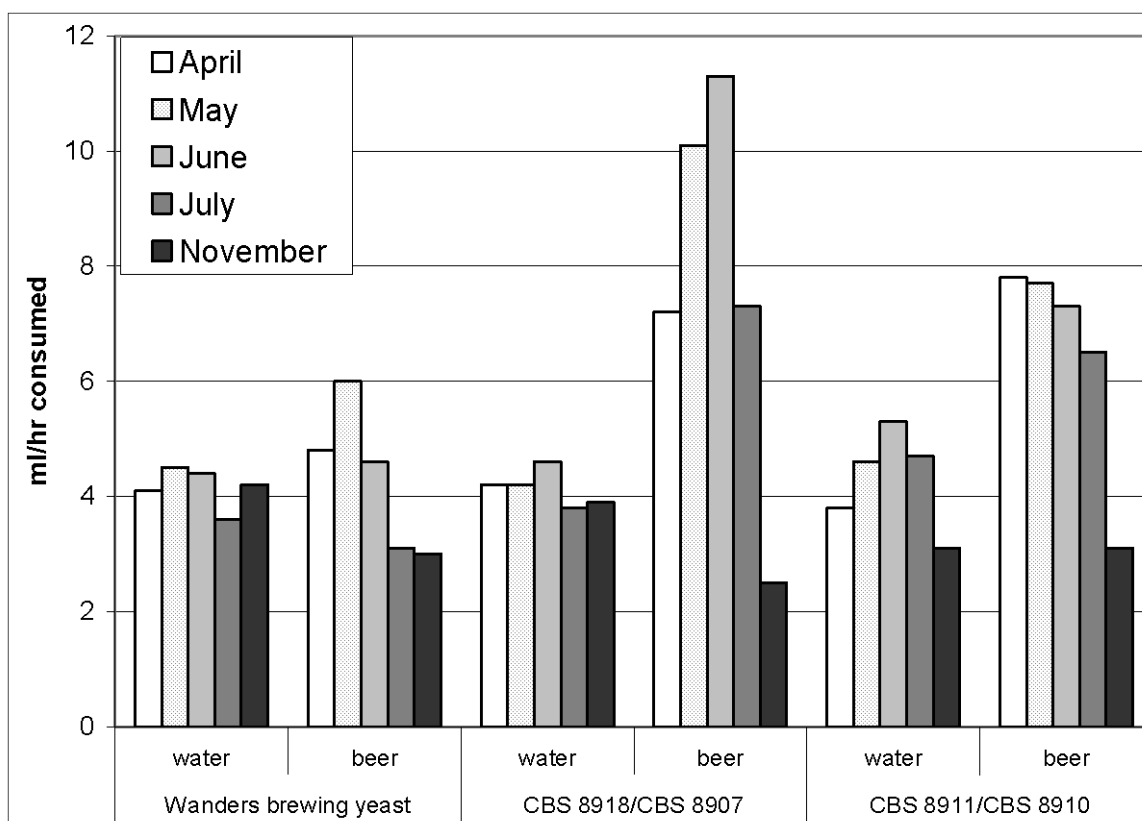


Fig 30. Graph of the average water and beer consumed each month by each cage.

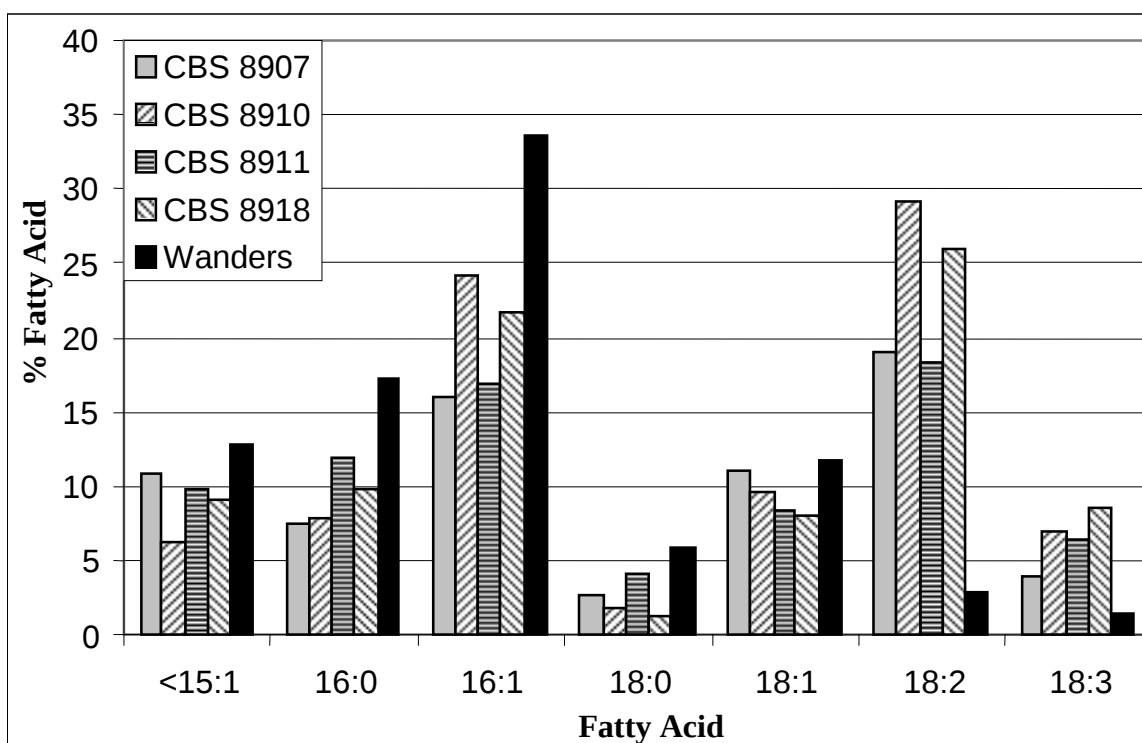


Fig 31. Fatty acid profile of fermented product from 5 different yeast strains