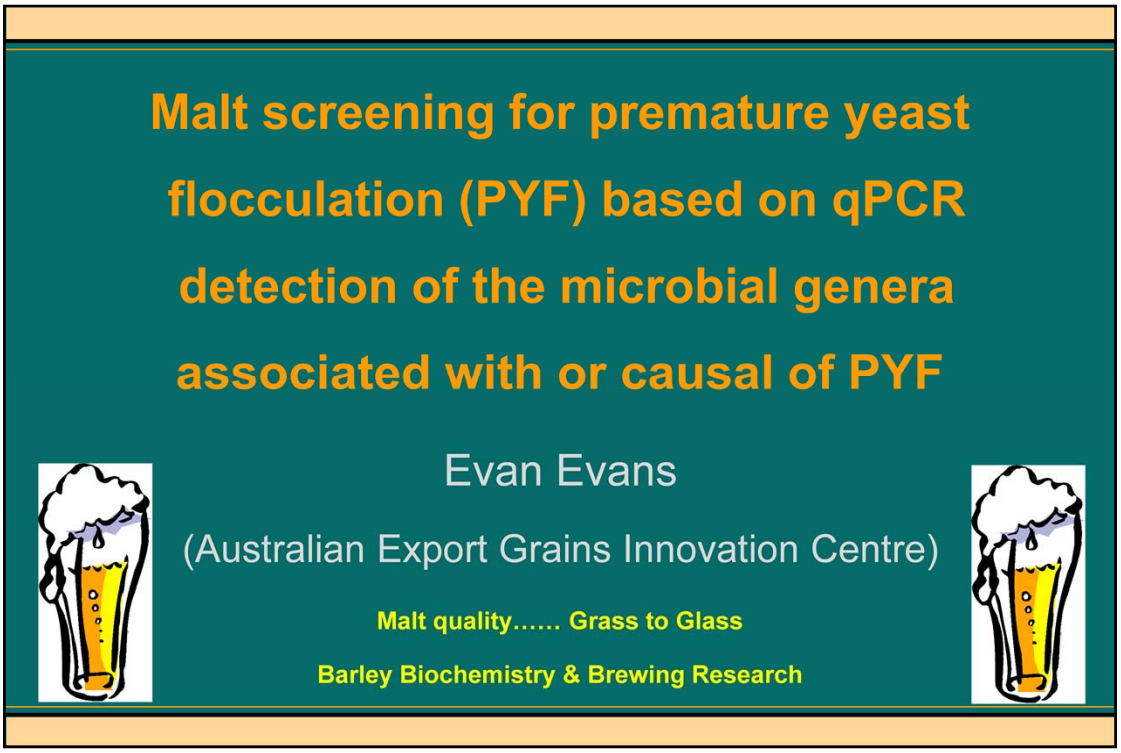


Today I would like to talk about screening for PYF.
This work has largely been undertaken by Mandeep Kaur

Introduction

- Premature yeast flocculation (PYF) is an intermittent fermentation problem.
- PYF results in incomplete wort fermentation.
- PYF occurrence appears to be related to certain malt batches.
- However detection of problem batches is problematic.
- Is a significant problem for some breweries.
- Development an efficient and reliable test for PYF.

PYF or premature yeast flocculation is a sporadic fermentation problem in the brewing industry.

Whereas, yeast flocculation is a desirable phenomenon during beer fermentation early or premature flocculation of yeast cells hampers complete fermentation of a wort's sugars and results in a final product with undesirable flavour characteristics

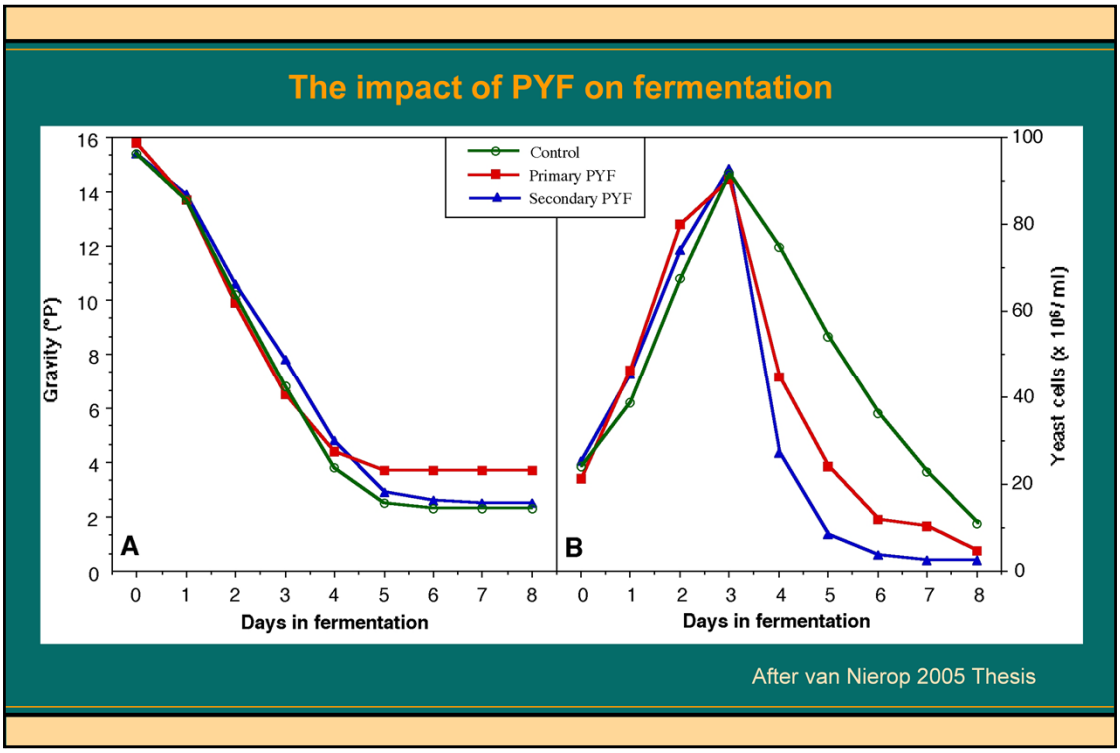
Consequently, PYF results in financial losses to brewers, as the beer requires additional blending or processing, and in severe cases disposal.

Further brand identity may be compromised, potentially resulting in negative consumer reactions.

PYF has been related to certain malt batches.

However detection of these batches is difficult.

This presentation will show the progress towards the development of an efficient and reliable test for PYF.



We understand that there are two different forms of PYF

Primary – incomplete fermentation (red)

Secondary – results in insufficient yeast being in suspension after fermentation to efficiently complete maturation (blue).

Some tests of PYF potential

Test	Description	Brewing Group/ Citation
Kirin test	Seven day fermentation to compare turbidity with control – absorbance measured at 800 nm.	Fujino and Yoshida ***1976***
Improved Kirin test	Eight day fermentation to compare turbidity with control - absorbance measured at 800 nm	Inagaki *et al* ***1994***
Nakamura barley PYF test	4 day test, 50g barley mashing coupled with enzymes followed by 48hr fermentation and absorbance measured at 800 nm	Nakamura *et al* ***1997***
SAB-Miller test	Four day small scale fermentation in separating funnel apparatus to measure excess flocculation against control	van Nierop *et al* ***2004***
Rapid Kirin test	Uses 5g malt or 50g barley extract and ethanol precipitate. Approx 3 hr, Compare absorbance ratio @ 600 nm with control	Koizumi and Ogawa ***2005***
Asahi test	48 hr fermentation with Compare absorbance ratio @ 600 nm with control	Jibiki *et al* ***2006***
Lake and Speers test	<72 hr, 15ml test tube fermentation at 21°C with 4% added glucose, measure turbidity (absorbance ratio @ 600 nm) °Plato and shear rate	Lake *et al* ***2008***
Anti yeast assay	Micro titre plate scale, (absorbance r @ 600 nm) measurement after 24 hr but purification of wort required	van Nierop et al ***2008***

To date, no physicochemical or biological analysis has been developed to routinely detect the presence of PYF in barley or malt.

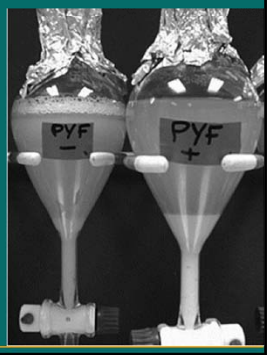
Thus the brewing industry relies on small scale fermentation assays to detect PYF positive malts.

A selection of the more widely used tests are shown in this table.

Perhaps the best in my view is the Lake-Speers test which was recently accepted as an ASBC method.

Problems with PYF tests

- Mostly applicable to malt and not barley
- Time consuming ie 2-8 days fermentation
- Yeast strain dependant
- Very specific conditions required
- Reproducibility and transferability?



There are however a number of problems with these assays:

Broadly they are expensive, time consuming and inconsistent.

They are applicable mostly to malt and not barley thus giving no warning sign to maltsters before malting a particular barley batch.

The tests appear to be yeast specific, so no universal test has yet been developed.

Further, a positive test in the lab may not translate into a problem in the brew house or vice versa.

Hypothesis:

Microbes, when present in high enough numbers or being of the wrong type in the field and perhaps during storage, can grow under favourable malting conditions to produce PYF causing components

Due to the lack of success with identifying a universal and efficeint fermentation test we decided to take another approach.

Our working hypothesis was:

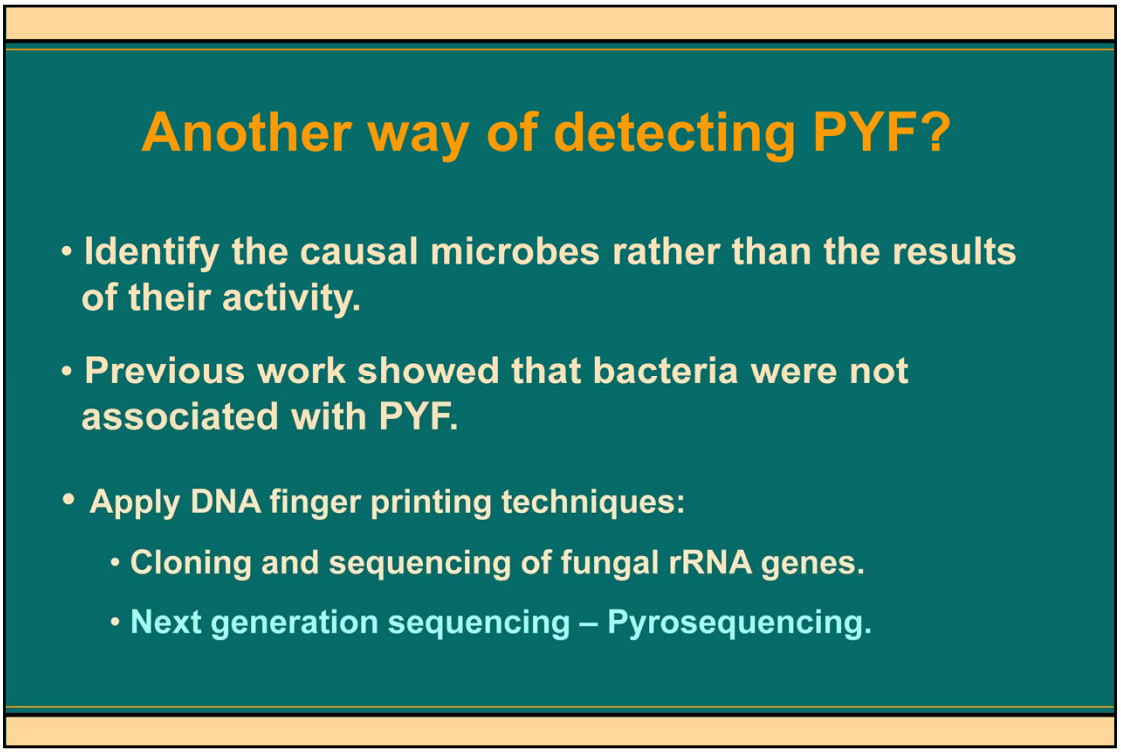
"Microbes, when present in high enough numbers or being of the wrong type in the field or perhaps storage, can grow under favourable malting conditions to produce PYF causing components."

Microbes indicated or associated with premature yeast flocculation in literature.

Fungi/Bacteria	Effect	Citation
Lactobacillus fermentum	Not specifically associated with PYF	Zarattini *et al* 1993
Aspergillus aculeatus, *Aspergillus ficuum*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus*, and *Fusarium culmorum*	Associated with PYF	van Nierop *et al* 2004
Unspecified fungi	Associated with PYF	van Nierop *et al* 2004
Fusarium culmorum and *Fusarium graminearum*	Associated with PYF	Blechová *et al* 2005
Aspergillus fumigatus, Fusarium sp., and *Rhizopus* sp.	Associated with PYF	Yang *et al* 2007
Aspergillus candidus, *Cladosporium cladosporiodes*, *Penicillium cyclopium*, *Penicillium melanconidium,* and *Penicillium viridicatum*	Associated with PYF	Sasaki *et al* 2008

Previous investagators have suggested a number of different fungal species were responsible for PYF.

Typically these are genera that include Fusarium and Aspergillus.



This begs the question, is there another way of detecting PYF?

That is to identify the microbes that cause PYF in the first place using DNA fingerprinting techniques for the conserved fungal rRNA genes using cloning and sequencing, or modern pyrosequencing techniques.

Pyrosequencing has an advantage as many more sequences are captured by this technique.

That is 10,000's of sequences rather than 100's of sequences.

Pyrosequencing of malts (15 PYF +ve and 17 PYF –ve) with two gene markers (LSU and ITS regions of rRNA fungal gene)

Sample No.	Supplier	PYF designation
1	Japan brewery 1	Negative
2	Japan brewery 1	Negative
3	N. America malting	Negative
4	N. America malting	Negative
5	N. America malting	Negative
6	N. America malting	Negative
7	N. America malting	Negative
8	N. America malting	Negative
9	Asia Pacific malting	Negative
10	Asia Pacific malting	Negative
11	Asia Pacific malting	Negative
12	Asia Pacific malting	Negative
13	China brewery	Negative
14	Japan brewery 2	Negative
15	Japan brewery 2	Negative
16	Japan brewery 2	Negative
17	Japan brewery 2	Negative
18	European malting	Positive
19	European malting	Positive
20	European malting	Positive
21	European malting	Positive
22	Asia Pacific malting	Positive
23	Asia Pacific malting	Positive
24	Asia Pacific malting	Positive
25	Asia Pacific malting	Positive
26	Asia Pacific malting	Positive
27	Asia Pacific malting	Positive
28	Asia Pacific malting	Positive
29	China brewery	Positive
30	N. America malting	Positive
31	N. America malting	Positive
32	N. America malting	Positive

Conserved Nuclear Ribosomal DNA genes

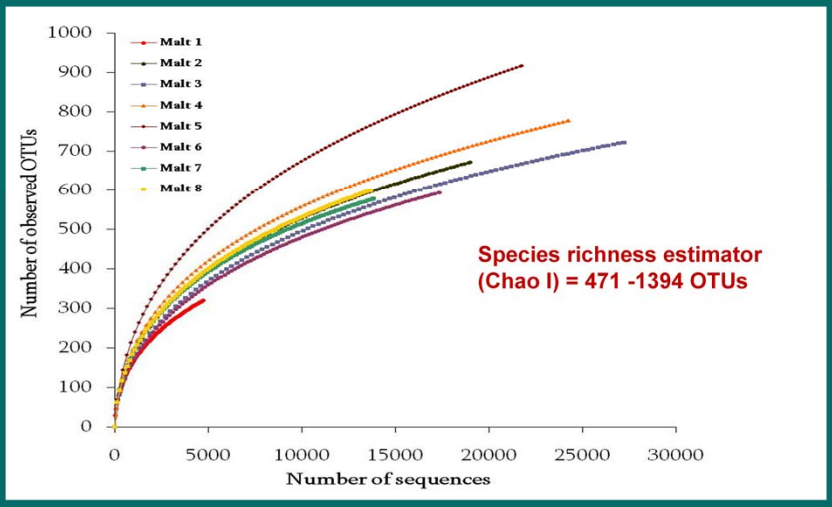
- **LSU** = large sub-unit rRNA gene, 28S subunit
- **ITS** = Internally transcribed spacer region, 5.8S region

Pyrosequencing analysis was conducted on the microbiota of 32 malt samples from a range different international suppliers.

Pyrosequencing was used to target the highly conserved large subunit and the internally transcribed spacer region fungal rRNA genes.

Point out LSU and ITS terminology

Rarefaction curves illustrating the effect of LSU rRNA gene partial sequence number on the number of operational taxonomic units (OTUs) identified from the eight barley malt samples



Rarefaction is a technique to compare species richness computed from samples of different sizes. Rarefaction allows the calculation of the species richness for a given number of sampled individuals and allows the construction of so called rarefaction curves.

This curve is a plot of the number of operational taxinomic units or OTUs, as a function of the number of individuals sampled.

A steep slope indicates that a large fraction of the species diversity remains to be discovered. If the curve becomes flatter to the right, a reasonable number of individuals are sampled therefore more intensive sampling is likely to yield only few additional species.

The rarefaction curves of the observed OTUs and sequences did not approach a plateau but they are close for the 8 samples shown.

The non-parametric Chao 1 estimator predicted the maximum number of OTUs required to capture full fungal richness in these 8 samples ranged from 471 – 1394 depending upon the malt sample with a mean around 952.

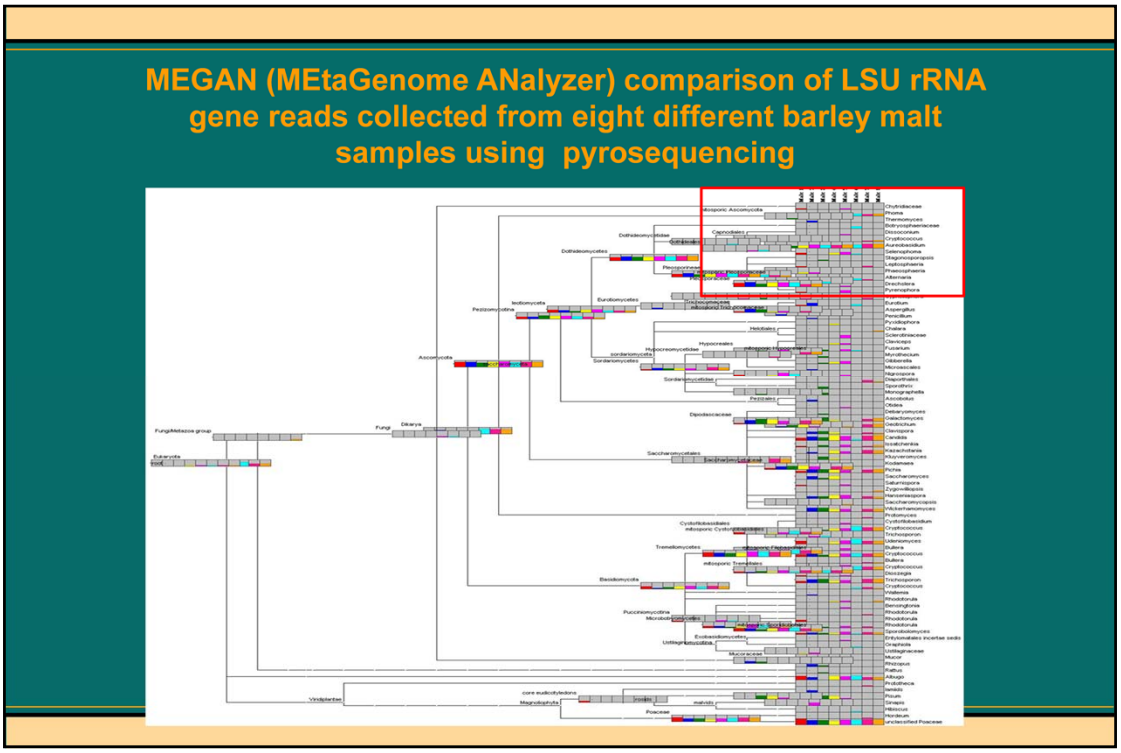
On an average 20,000 seqs per malt sample were obtained confirming that we had sufficient sequences to capture full fungal richness.

The 20,000 seqs per malt sample obtained also indicated the presence of 111-118 fungal genera. This was double the previouly estimated number for barley.

This indicates there is greater fungal diversity associated with barley malts

than has been previously understood.

This is contrary to the traditional view, that the microbiota of different barleys are remarkably similar to each other, other cereals, and they are generally dominated by the same limited number of species.

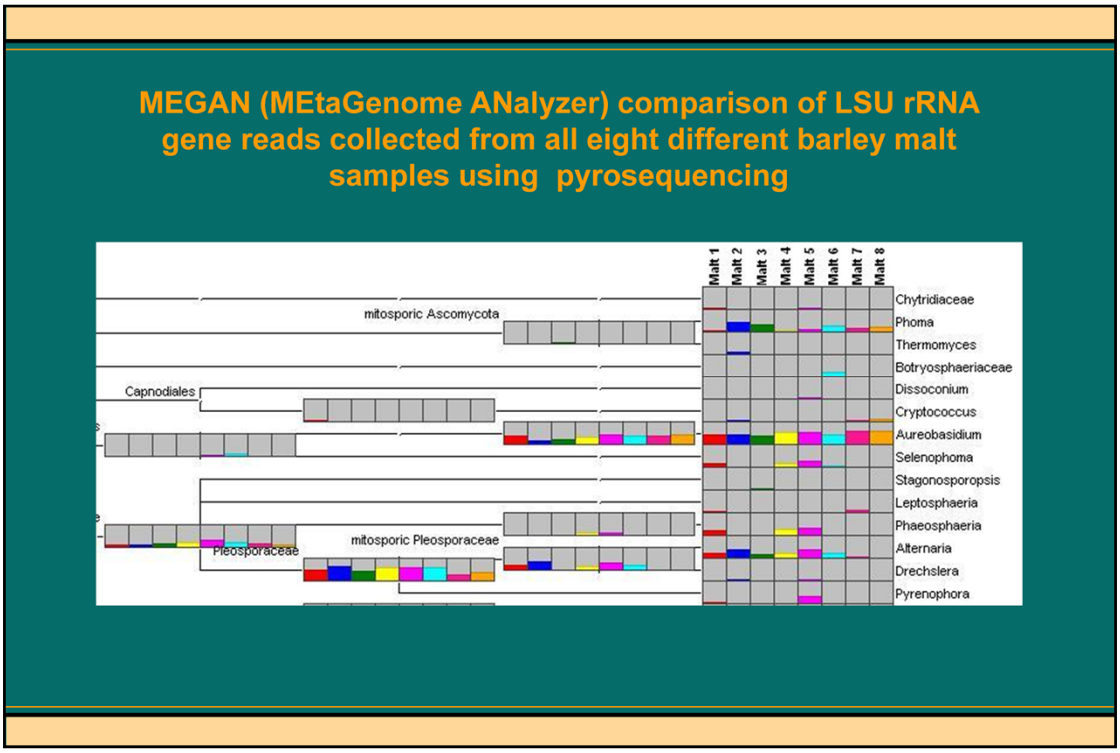


The individual DNA reads were compared against the non redundant GenBank database of known LSU and ITS fungal sequences using the BLASTn algorithm.

MEGAN software was used to compute the taxonomic content of the data set, employing NCBI taxonomy.

MEGAN out put is shown on this slide for 8 malt samples

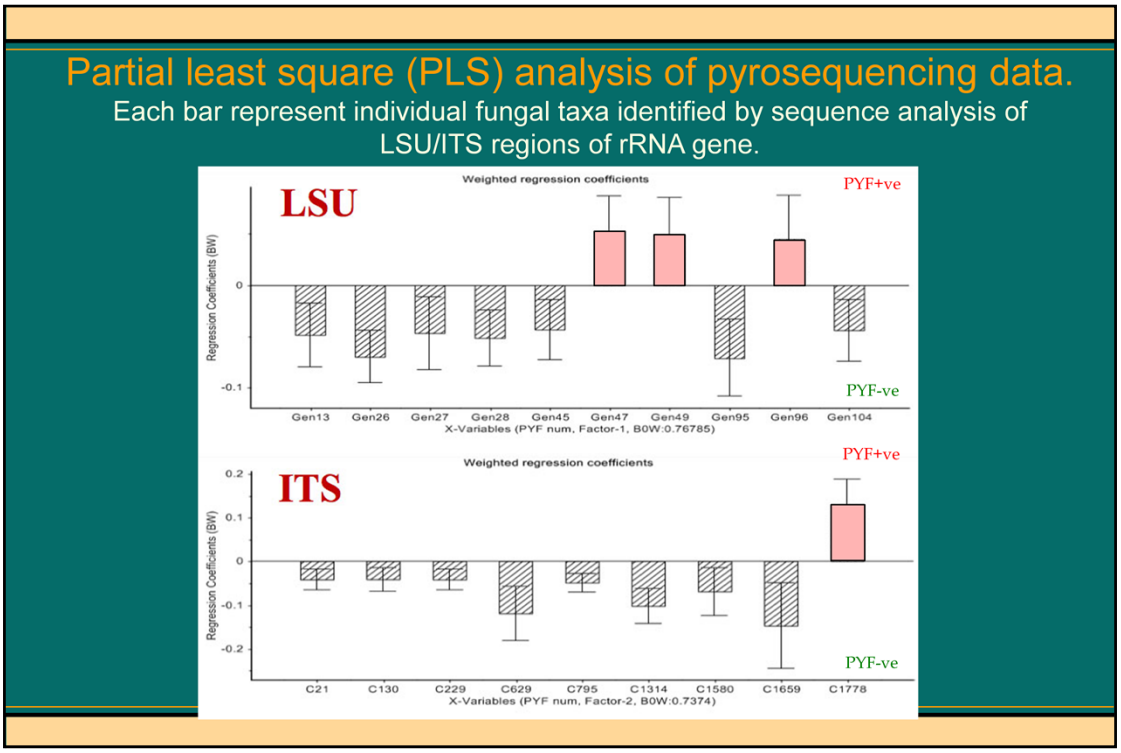
This phylogenetic analysis revealed a distinct distribution of fungal taxa associated with these malts.



To give you a better look at this complexity, this slide shows a snapshot of the distinct fungal distributions between samples.

Here only 8 samples are displayed, not the full 32 samples used to identify the PYF associated genera.

I think you now understand the bioinformatic challenge that Mandeep had to overcome.



We undertook partial least squares regression analysis on the pyrosequencing data to predict PYF.

The results are as shown.

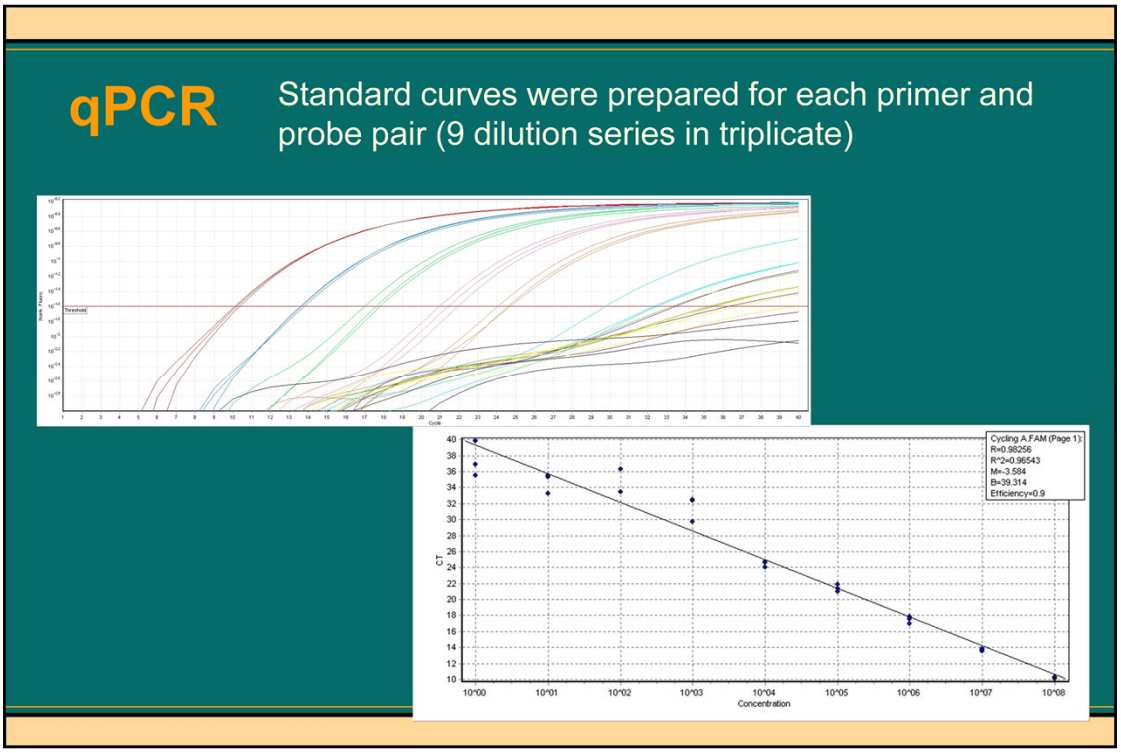
Each bar represents individual fungal taxa identified by sequence analysis of the fungal LSU/ITS regions.

The bars above the X axis are positively correlated with PYF and those below the axis are negatively correlated.

The height of the bar represent the strength of correlation between specific fungal taxa and PYF.

Note that there are both negative and positive relationships with PYF.

This is the first time negative and perhaps antagonistic relationships have been observed for PYF.



Based on PLS analysis - 19 taxa were chosen for further qPCR primer and Taqman probe design.

Mandeep also included genera which were mentioned in the literature as being associated with PYF.

These were Fusarium, Apergillus and two PYF +ve genera identified in Mandeep's PhD thesis.

In total Mandeep was able to design 25 unique primer and probe pairs.

The PCR conditions were optimized and clone libraries were probed, with the products sequenced for all the primer pairs to ensure their specificity.

Clones were also selected, and after plasmid extraction, the DNA was lineralized and used for standard curve construction.

The specificity of the primers and probes was further tested by doing qPCR, cloning and sequencing of qPCR products.

Only the primer and probe pairs which were specific were studied further.

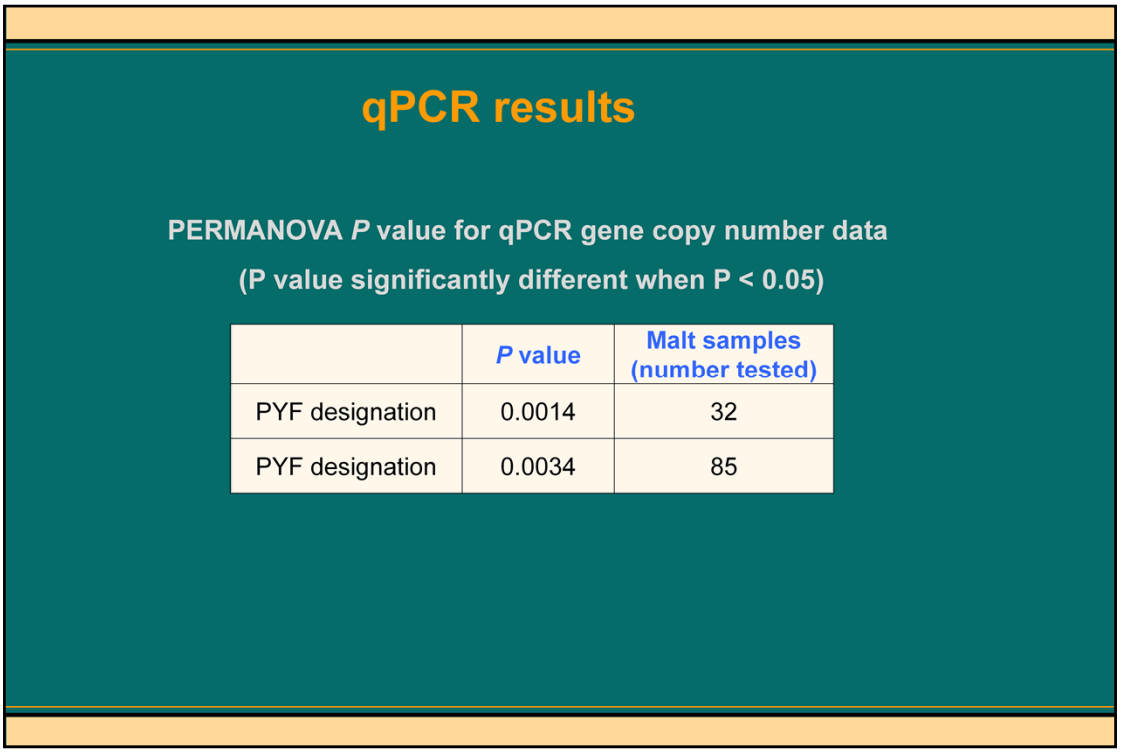
The initial 25 primers were whittled down to 15 primers.

Serial dilutions were prepared for the each lineralised plasmids, and standard curves were prepared to determine qPCR reaction efficiency, detection limit and to calculate DNA copy number in unknown samples.

An example of the dilutions for one primer is shown.

After optimizing qPCR conditions malt samples were run in triplicate with 15 different primers and probes.

The DNA copy no. of each specific taxa was calculated for each malt sample.



The qPCR results were statistically analysed by PERMANOVA.

PERMANOVA is a statistical routine for testing the simultaneous responses of one or more variables to one or more factors in an analysis of variance design on the basis of any resemblance, using permutation methods.

Based on the combined results of all 15 probes the PERMANOVA results indicated highly significant differences between the PYF positive and negative malts.

This held for either the qPCR of the core 32 malt samples or a wider set of 85 malt samples.

qPCR results (counts) for putative PYF negative and positive primers based on the ITS and/or LSU conserved fungal DNA regions

Primer LSU conserved region | Primer ITS conserved region | Primer selected for verification

Malt sample	PYF-ve 1	PYF -ve 2	PYF -ve 3	PYF -ve 4		PYF -ve 5		PYF +ve 1		PYF +ve 2	PYF +ve 3	PYF +ve 4	PYF +ve 5	PYF +ve 6	PYF +ve 7
PYF status Suplier												*Thesis*	*Thesis*	*Fusarium - literature*	*Aspergillus - literature*
neg 1	2.2	71	1	0.8	545	1	0	0	63	1	13	30	1347	55	4
neg 2	0.4	182	5	3.7	866	20	0	0	0	0	60	3	1315	210	1
neg 3	0.5	43	49	0.0	610	0	0	0	123	0	0	9	1011	73	8
neg 4	12.6	126	97	0.0	2371	0	0	0	215	2	25	116	191	36	84
neg 5	32.3	147	301	0.0	1110	0	0	565	1674	0	0	1198	16	1260	2
neg 6	65.2	174	640	0.0	1323	0	0	0	162	0	0	211	64	1040	27
neg 7	14.3	61	73	0.0	615	0	0	0	87	0	0	29	6	585	10
neg 8	26.2	206	1385	0.0	1543	4	0	106	1214	3	65	1300	125	6772	3
neg 9	0.0	34	115	0.0	142	0	0	0	0	0	56	4	2	155	8
neg 10	0.2	49	27	0.0	375	0	0	269	1727	0	7	2519	393	62	4
neg 11	11.9	101	45	0.7	695	0	0	254	1476	1	30	1631	569	113	11
neg 12	1.5	121	18	2.3	435	0	0	115	771	0	47	643	1063	96	2
neg 13	2.8	0	172	0.0	340	7	0	0	16	0	0	13	45	539	0
neg 14	50.6	172	296	3.0	614	0	0	0	6	6	0	55	66	2147	20
neg 15	1.9	111	113	0.0	449	5	0	0	306	0	0	37	47	1177	2
neg 16	7.0	96	316	0.0	580	0	0	0	1187	0	0	18	514	1237	7
neg 17	44.9	220	551	0.0	1509	4	0	0	38	1	19	16	21	2198	22
pos 1	6.9	74	187	0.0	626	0	0	0	84	0	0	8	3	430	7
pos 2	7.3	57	18	0.0	73	391	54	1098	6544	1	15	7346	37	1144	346
pos 3	23.9	350	531	0.0	902	10	0	189	9922	67	10	1045	5170	3772	8
pos 4	0.5	27	16	0.0	486	0	0	615	1868	25	88	7971	256	94	4
pos 5	0.9	10	4	0.0	122	0	0	710	1548	0	12	10	185	62	5
pos 6	1.4	51	38	0.0	255	0	0	25	911	0	121	615	1000	93	4
pos 7	0.0	48	56	0.0	296	0	0	45	2395	0	142	948	1523	75	1
pos 8	4.5	23	74	0.0	465	0	0	0	56	0	42	282	54	45	4
pos 9	0.7	26	699	0.0	1620	0	0	1298	3089	0	607	387	357	1389	5
pos 10	2.2	33	159	0.0	672	0	0	0	45	0	51	68	39	16	11
pos 11	3.3	113	439	0.0	94	2880	997	32792	103028	94	0	420	4	14269	2
pos 12	5.1	136	210	0.0	49	5646	5101	15586	45123	269	132	822	1	3722	11
pos 13	14.9	236	230	0.0	44	2067	836	9581	30341	112	34	153	2	1878	10
pos 14	18.6	129	631	0.0	195	6211	4600	17752	60320	208	0	610	3	18764	5
pos 15	21.5	54	234	0.0	486	264	90	165	1325	50	0	7317	190	1367	301
t test (P < 0.05)	0.437	0.517	0.703	0.037	0.006	0.013	0.004	0.001	0.001	0.005	0.104	0.027	0.399	0.543	0.407

t test = signigificant difference between pos & negative PYF

Traffic light code: PYF +ve = >2x negitive average | PYF ? = +/- 2x negative average | PYF -ve = <2x negitive average

The qPCR results with indicative copy number counts, are displayed for the 15 primer pairs with the 32 malt samples are presented.

Different colors indicate the different malt suppliers.

The green probes associated with the LSU while the blue probes relate to the ITS region.

They are related to 5 PYF negative genera and 3 PYF positive genera identified from pyrosequencing analysis

In addition, 2 PYF+ve genera identified in Mandeep's thesis, and the Fusarium and Aspergillus that were identified in the literature were assessed by qPCR.

The bottom row indicates if the probe can statistically discriminate between PYF+ve and –ve.

Two PYF-ve and three PYF+ve genera primers produced significant results that were indicative of PYF status.

The traffic light colors in the body of the table indicate my putative guess as to PYF +ve, -ve and intermediate results.

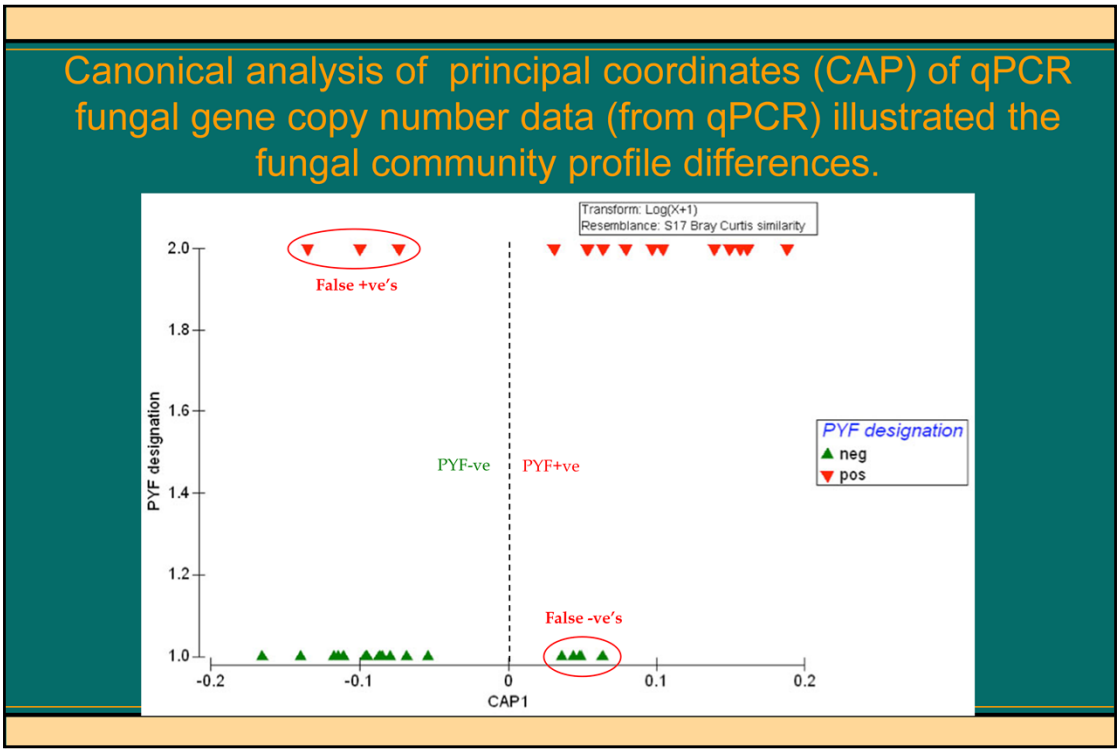
This presentation is on the Summit CD and I recommend its purchase to study this table at your leisure.

In summary, it is likely that PYF status is the result of the interaction between two or more fungal genera.

Clearly this interaction differs between suppliers-brewers and is perhaps a yeast specificity effect?

The qPCR assessment has indicated potential false positives and negatives from the supplier fermentation test PYF designations.

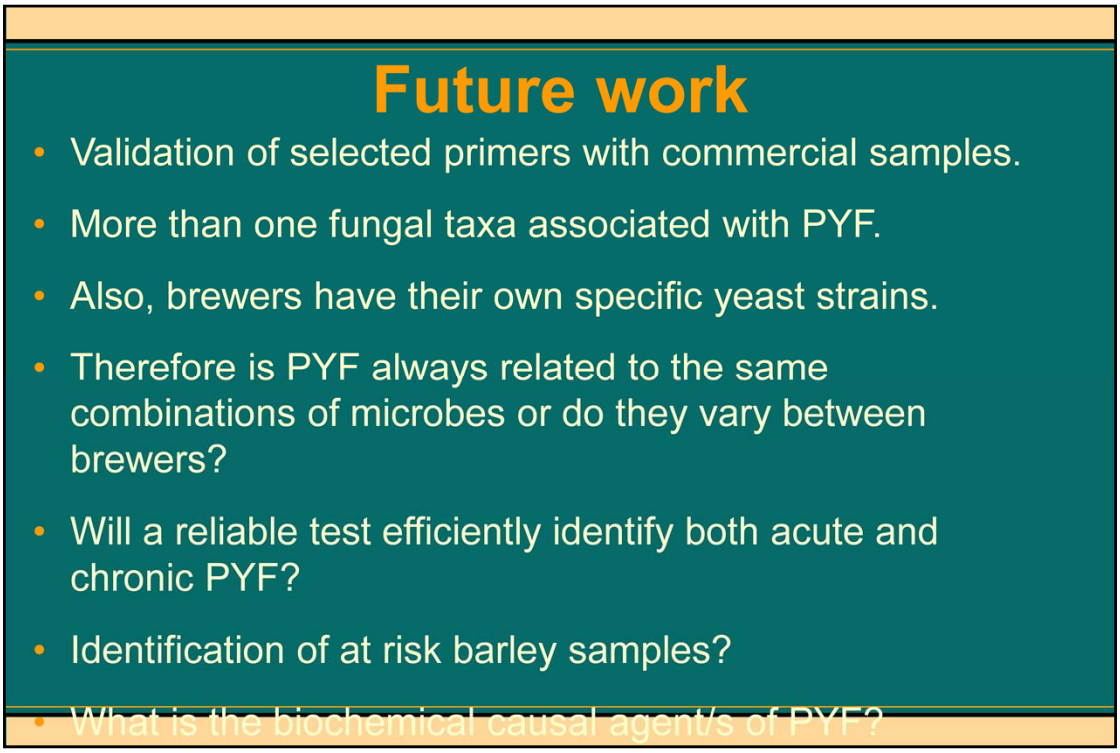
From this analysis, 5 primers (bright yellow) have been selected for validation with commerical malt samples to develop an accurate and reliable qPCR PYF test.



Canonical analysis of principal coordinates of qPCR fungal gene copy number data illustrated the fungal community profile differences among PYF positive and negative malt samples.

The first canonical axis (CAP1) clearly separates PYF positive and PYF negative malts in two distinct groups.

Note the presence of potential false positives and negatives that are commonly result from fermentation style tests.



As per slide

Perhaps with a more sensitive and reliable test malt screening may indicate the presence of chronic PYF problems, the avoidance of which may result in more consistent and trouble free fermentation.

Conclusions

- PYF is a problem associated with certain malt batches that afflicts some breweries intermittently.
- Modern molecular techniques like pyrosequencing have been applied to explore barley malt microbial population ecology.
- The occurrence of PYF has been linked significantly with the presence of certain fungal genera.
- Genera positively and negatively associated with PYF.
- An efficient and reliable qPCR assay is expected to result from commercial validation.

As per slide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