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# TRFLP analysis reveals that fungi rather than bacteria are associated with premature yeast flocculation in brewing

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**Abstract** Premature yeast flocculation (PYF) is a sporadic fermentation problem in the brewing industry that results in incomplete yeast utilization of fermentable sugars in wort. Culture-independent, PCR-based fingerprinting techniques were applied in this study to identify the associations between the occurrence of the PYF problem during brewery fermentation with barley malt-associated microbial communities (both bacteria and fungi). Striking differences in the microbial DNA fingerprint patterns for fungi between PYF positive (PYF +ve) and negative (PYF −ve) barley malts were observed using the terminal restriction fragment length polymorphism (TRFLP) technique. The presence of terminal restriction fragments (TRFs) of 360–460 bp size range, for fungal *Hae*III restriction enzyme-derived TRFLP profiles appeared to vary substantially between PYF +ve and PYF −ve samples. The source of the barley malt did not influence the fungal taxa implicated in PYF. TRFLP analysis indicates bacterial taxa are unlikely to be important in causing PYF. Virtual digestion of fungal sequences tentatively linked *Hae*III TRFs in the 360–460 bp size range to a diverse range of yeast/yeast-like species. Findings from this study suggest that direct monitoring of barley malt samples using molecular methods could potentially be an efficient and viable alternative for monitoring PYF during brewery fermentations.

**Keywords** Barley malt · Brewery fermentation · Community fingerprinting · Microbial communities · Yeast

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## Introduction

Representing an important process in the fermentation of brewer's wort into beer, yeast flocculation has been intensively studied and is well understood [6, 41, 43–47, 51]. However, the understanding of the phenomenon of premature yeast flocculation (PYF) lags behind, despite it being studied for over 40 years. PYF is an intermittent issue in the brewing industry that results in incomplete utilization of fermentable sugars in the wort, resulting in out of specification beer and disrupted brewing production schedules, leading to significant economic losses for the effected brewer [4].

Several substances in the wort derived from malt, particularly the barley husk, have been implicated in inducing PYF [14, 25, 26]. It is most likely that the responsible factor/s for PYF are produced as a result of microbial contamination of barley grains in the field that lead to an undesirable level of certain microbial taxa in the inocula that proliferate during malting especially under favorable warm and moist steeping, and presumably germination conditions [2–5, 15, 39, 49, 55].

The microbes associated with the barley husk secrete enzymes that break down the cell wall of grains into different substances, some of which are utilized [49]. Certain degradation products, such as acidic high molecular weight polysaccharides, have been proposed to accelerate the cross-linking of the lectin-like proteins on the yeast cell surface, forming large aggregates or flocs of yeast cells. Furthermore, during grain maturation and malting, microbial stress triggers an anti-pathogenesis immunological response by the barley grains, resulting in the accumulation of plant defensins that include antimicrobial peptides. These plant defensins have been suggested to also induce PYF due to impairment of yeast cell metabolism, respiration, and cell membrane integrity, causing irreversible cell injuries [14, 26, 49, 53].

As the specific identity of the causal PYF factor/s has not been achieved, to date no physical or chemical analytical methods have been developed to routinely detect the presence of the PYF factor/s in malt or barley. Consequently, the brewing industry relies on fermentation assays [21, 22, 24, 27, 49], which are expensive, time-consuming, and inconsistent [26]. Some success has been reported in reducing the scale and increasing the speed of these assays, although real problems remain in the transferability and reproducibility of these assays between testing laboratories. The lack of consensus with respect to selecting a universal standard assay for PYF by the brewing industry is a substantial problem, which makes comparison of research reports on PYF difficult, as one assay may deem a particular malt batch PYF-positive, while another assay may not. In addition, a positive result in the test may not necessarily translate into a problem in the brewery, or vice versa.

Despite the consensus of opinion being that PYF stems from microbial contamination of barley/malt, relatively little work has been reported on linking specific barley or malt microbial taxa with PYF malt [5, 39, 49, 54, 56]. Most of these studies have used traditional culture-dependent approaches of microbial detection and identification (except for work done by Sasaki et al. [39]) that often result in an incomplete understanding of the true microbial diversity present in a malting ecosystem. Furthermore, nearly all of the reported work has been directed towards fungal studies with little effort being devoted to bacteria. It is possible that the occurrence of PYF is dependent on the interactions between microbial taxa or could be caused by different taxa.

In this study, terminal restriction fragment length polymorphism (TRFLP) finger printing of barley malt microbes (both bacteria and fungi) was used to identify the structures of microbial communities that were associated with the incidence of PYF by comparing 41 geographically diverse PYF-positive (PYF +ve) and PYF-negative (PYF −ve) commercial barley malts. This identification is anticipated to eventually provide the basis for a diagnostic assay for avoiding malts with high PYF potential. The TRFLP approach is a PCR-based genetic fingerprinting technique that is widely used to monitor spatial and temporal changes in microbial community structure [23, 30, 35, 48]. Furthermore, construction of clone libraries in parallel to the TRFLP analysis was undertaken to assess and interpret the TRFLP profiling data utilizing the same set of PCR primers [31, 40, 52] in order to determine whether specific taxa are responsible for causing PYF.

## Materials and methods

### Barley malt samples

#### *Primary experiment*

A total of 32 kilned barley malt (malt hereafter) samples (included two PYF +ve and one PYF −ve control malts) were obtained from five different commercial maltsters and brewers from three intercontinental locations (Table 1). Due to the commercial sensitivity of the PYF problem, the providers and their locations are not shown. The positive control malt samples had exhibited PYF characteristics during commercial brewery fermentation and their PYF status were determined using the small-scale fermentation assay as described in Lake et al. [27]. For the remaining 29 malt samples, the PYF status was determined by their

**Table 1** Detail of barley malt samples and their PYF status as assessed by providers

| Location | Provider | Number of malt samples (primary experiment) | | Number of malt samples (secondary experiment) | |
|---|---|---|---|---|---|
| | | PYF +ve | PYF −ve | PYF +ve | PYF −ve |
| Location 1 | 1 | 8 | 8 | – | – |
| | 4 | 2 | – | 9 PYF status unknown before TRFLP analysis | |
| Location 2 | 3 | 2 (control) | 1 (control) | 2 (control) | 1 (control) |
| | 5 | 1 | 2 | – | – |
| Location 3 | 2 | 5 | 3 | – | – |

providers using either one of the Asahi [22], Kirin [21], or SAB—Miller [49] fermentation tests for PYF. For statistical analyses, these malt samples were grouped as PYF −ve and PYF +ve malts (based on the providers' designation) and geographical locations 1, 2, and 3.

*Secondary experiment*

A further 12 malt samples (three controls from the above 32 malts and nine new malt samples) from two providers were tested by TRFLP analysis (with only *Hae*III restriction enzyme for fungal analysis) to validate the discrimination between PYF −ve and PYF +ve malts based on the observed TRF peak differences in a partial blind trial (Table 1). For nine of these new malt samples, the PYF status of the samples was unknown before being analyzed with TRFLP assay.

DNA extraction and PCR

Collected samples were stored, no longer than 1 month, in airtight bags at room temperature before grinding. Samples were ground in a Cyclone Sample Mill using a 0.1-mm screen (UDY Corporation, Fort Collins, CO, USA) and stored immediately at −20°C until being used for DNA extraction. Cross contamination between samples was avoided by blowing high-pressure dry air through the grinding mill and collection container in between the samples and taking only the middle portion of the ground sample from the container for analysis.

Genomic DNA from ground samples (0.1 g) was extracted in duplicate with the FastDNA® Spin Kit for Soil (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer's instructions except that the samples were homogenized with a Retsch MM300 bead beater (Retsch GmbH, Haan, Germany) at 30 Hz for 4 min. Immediately after extraction, DNA samples were stored at −20°C until further use.

Extracted DNA was PCR amplified using bacterial 16S rRNA 5′ D3 WellRED dye-labeled 27F (AGA GTT TGA TCM TGG CTC AG) forward and 5′ D4 WellRED dye-labeled 1492R (TAC GGY TAC CTT GTT ACG ACT T) reverse primer pair (Sigma-Proligo, TX, USA) as described by Gurtler and Stanisich [16]. Each 60-µl reaction mixture contained 30 µl of 2 × ImmoMix Red™, 22.5 µl of DNAase/RNAase—free water (Bioline, Sydney, NSW, Australia), 3 µl of each forward and reverse primers (10 pmol) and 1.5 µl genomic DNA template. The PCR consisted of an initial denaturation at 95°C for 10 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The final extension was at 72°C for 10 min.

5′ D3 WellRED dye-labeled NL1 forward (GCA TAT CAA TAA GCG GAG GAA AAG) and 5′ D4 WellRED dye-labeled NL4 (GGT CCG TGT TTC AAG ACG G) reverse primers [11, 37] specific to D1/D2 domain of the 26S rRNA gene were used for PCR amplification of malt-associated fungal communities. The PCR was performed as described above except that the number of cycles was increased to 35. To check the purity and size of PCR amplicons, 5 µl of each reaction mixture was run on a 1.5 % agarose gel (w/v) stained with 500 ng/ml ethidium bromide. The PCR product was purified using UltraClean™ PCR Clean-up Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and visualized again on a 1.5 % agarose gel to determine purification efficiency.

TRFLP analysis of bacterial and fungal communities

Microbial community fingerprint patterns (based on 16S rRNA and D1/D2 domain of 26S rRNA genes for bacterial and fungal communities, respectively) were obtained with the TRFLP approach. Aliquots of purified DNA were digested with *Hae*III, *Msp*I, and *Rsa*I (for bacterial PCR samples) and *Hae*III, *Hinf*I, and *Rsa*I (for fungal PCR samples) restriction enzymes (NewEngland Biolabs Inc., Ipswich, MA, USA) at 37°C for 3 h on a thermocycler and the reactions were stopped by a further incubation at 80 or 65°C, depending upon the restriction enzyme used for 20 min. Three restriction enzymes were used to eliminate false-positives and pseudo-terminal restriction fragments [10], which can occur when using only one restriction enzyme. The digested labeled fragments were desalted and purified by ethanol precipitation using 3 M sodium acetate (pH 5.2) with glycogen as a carrier molecule. Cleaned fragments were mixed in a 30-µl formamide sample loading solution and 0.25 µl of Beckman Coulter size standard 600 (Beckman Coulter Australia Pty Ltd., Sydney, NSW, Australia). Fragments were obtained by capillary electrophoresis on a Beckman Coulter CEQ8000 automated sequencer using modified Frag-4 (injection of 2.0 kV/30 s, run at a capillary temperature 50°C/4.8 kV for 1.5 h). Terminal restriction fragments (TRFs) thus obtained were analyzed using the Beckman Coulter fragment analysis package version 8.0. Profiles were generated for each sample in duplicate based on relative area (abundance) of peaks whereby a peak height threshold was set to 5 % and only TRFs between 60 and 640 bp sizes were used for further analysis.

Statistical analysis of TRF data

Raw fragment data for bacterial and fungal communities obtained from Beckman Coulter CEQ8000 genetic analysis system were imported into Microsoft Excel software. As samples were processed in duplicate, T-Align (Web-based

software) was used to obtain a single fragment data whereby fragments were binned with a 1.0 base confidence that also culled any fragments not present in duplicate samples [42]. The resultant data set was imported into the multivariate statistical software package Primer v6 (Primer-E Ltd, Plymouth Marine Laboratory, Plymouth, UK) and a similarity matrix of relative abundance data was calculated utilizing the Bray-Curtis coefficient [7]. A two-way crossed analysis of similarity (ANOSIM, PERMANOVA) was applied to examine the statistical significance of any relationship present between sample groups (PYF status and sample location). Percent dissimilarity contributions of TRFs between sample sets were determined using SIMPER analysis [1, 8, 38]. TRFs were identified by virtually digesting clone library sequences with restriction enzymes used in this study using BioEdit 7.0.5.3 software (Ibis Biosciences, Carlsbad, CA, USA).

For the above-mentioned statistical analyses for 32 malt samples (primary experiment), fungi and bacteria were treated separately and the relative abundance data of forward and reverse TRFs obtained from all the three restriction enzymes were pooled together. For the other 12 malt samples (secondary experiment), the relative abundance data of forward and reverse TRFs obtained from *Hae*III restriction enzyme (for fungi only) were pooled for statistical analyses.

TRFLP electropherograms of all the malt samples from all restriction enzymes, both for bacteria and fungi, were analyzed visually. The electropherograms for the *Hae*III digests used for fungal analysis showed clear visual differences between PYF +ve and PYF −ve malts; therefore these were assessed and studied in detail. Depending upon number and relative abundance of *Hae*III-derived TRFs peaks in the 360–460 bp range, the PYF +ve and PYF −ve malt samples were scored on a 0–5 scale in duplicate followed by averaging. The score increased with increasing number and intensity of these peaks. In addition, the area under the peaks was also calculated for all the malt samples in duplicate. To account for loading variation the abundance of these peaks was normalized by dividing the peak area with the total peak area obtained for all *Hae*III-derived TRFs. Thereafter, box plots were created based on the TRFLP score, $\log_{10}$ of average peak area, and normalized peak area by using JMP® version 5.1 software (SAS Institute Inc., Cary, NC, USA). ANOVA was performed using Microsoft Excel 2010. Correlation between $\log_{10}$ average peak area and visual electropherograms scores was calculated.

### Small-scale fermentation assay

Twelve malt samples, a subset of the 32 samples from primary experiment, were analyzed by the small-scale fermentation assay as described by Lake et al. [27]. The *t* test was applied to the small-scale fermentation assay parameters using JMP® version 5.1 software.

### Clone library construction, sequencing, and phylogenetic analysis

Three fungal and three bacterial clone libraries were generated from DNA extracted from three control malt samples.

Bacterial 16S rRNA gene and fungal 26S rRNA gene amplicons obtained from selected samples were cloned using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The correct insert size in each clone was checked by vector-targeted PCR (primers M13F and M13 R) and agarose gel electrophoresis. PCR amplicons were purified using the UltraClean™ PCR Clean-up Kit. Clones were sequenced using the BigDye Terminator Ready Reaction mix sequencing reaction kit and reactions were resolved on an automated DNA sequencer (Applied BioSystems). The sequences were edited using BioEdit [17] and compared to then GenBank database using the Blastn algorithm.

The 26S rRNA fungal gene sequences were aligned with sequences from GenBank database using ClustalW alignment application, checked manually and a phylogenetic tree was created using the Neighbour-Joining algorithm in BioEdit. The 26S rRNA sequence for *Chytridium lagenaria* was used as an outgroup in the Neighbor-Joining analysis. Phylogenetic differences among fungal communities associated with three malts were assessed using weighted UniFrac significance and *P* tests for each pair of malt samples based on 100 permutations in UniFrac [32].

### Nucleotide sequence accession numbers

The nucleotide sequences generated in this study have been deposited in the GenBank database under the accession numbers JX005948–JX006033, HQ143267.

## Results

### Malt-associated microbial diversity

Three 26S rRNA gene clone libraries were constructed for the fungal communities from two PYF +ve and one PYF −ve malts. In all, 301 (94 for PYF +ve control 1, 102 for PYF +ve control 2 and 105 for PYF −ve control libraries), good-quality sequences (600–640 bp in length) were obtained and further analyzed. Database comparisons indicated that most of the sequences had 97–100 % homology to known 26S rRNA fungal sequences (Fig. 1). Most of the clones recorded in this study appeared to be

fungal taxa generally found associated with plant and soil-based ecosystems. All sequences were grouped into the phyla *Ascomycota* (81–88 %) and *Basidiomycota* (8–19 %) with the exception of three clones belonging to *Zygomycota* (3 %) in one of the clone libraries of two PYF +ve malts. Comparative abundance of different fungal genera in PYF +ve and PYF −ve malts is presented in Table 2 and showed differences among samples with regard to type of taxa and their abundances. However, phylogenetic differences observed among fungal communities associated with the three control malts based on UniFrac significance or $p$ tests ($p > 0.1$) were non-significant.

Three 16S rRNA gene clone libraries were also constructed for the bacterial community. The majority of the bacterial clones detected were typical of those found in agro-ecosystems as in the case of the fungi. The main genera observed were *Arthrobacter*, *Corynebacterium*, *Curtobacterium*, *Microbacterium*, *Plantibacter*, *Rhodococcus*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Enterococcus*, *Kurthia*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*. No clear differences in bacterial taxa composition could be observed between the PYF +ve and PYF −ve clone libraries (data not shown).

## Similarity of fungal and bacterial communities associated with malts from different locations and of different PYF status.

Two-way crossed ANOSIM analysis with PYF status of malt samples as one factor and location as a second factor was applied to observe variation in the fungal and bacterial communities' structures. To enable statistical analysis, some of the PYF +ve and PYF −ve malts were grouped into geographical locations 1, 2, and 3. As some providers did not have sufficient numbers of samples for valid statistical analysis, samples from more than one provider were amalgamated to perform the required analyses. The samples grouped were from the same country or were from countries that were adjacent to each other.

Combined ANOSIM analysis of the 32 malts, for which the providers had determined PYF status, showed that in fungal and bacterial communities' structures of PYF +ve and PYF −ve malts the differences were not significant (Table 3). The negative value for global R revealed more within group dissimilarities than between groups when comparing PYF +ve and PYF −ve groups. Geographic location of malt samples significantly influenced the fungal and bacterial communities' structures. Pair-wise comparison of geographically different malt groups revealed that malts from location 1 were significantly different from location 2 and 3 malts. There was no significant difference between malts from locations 2 and 3.

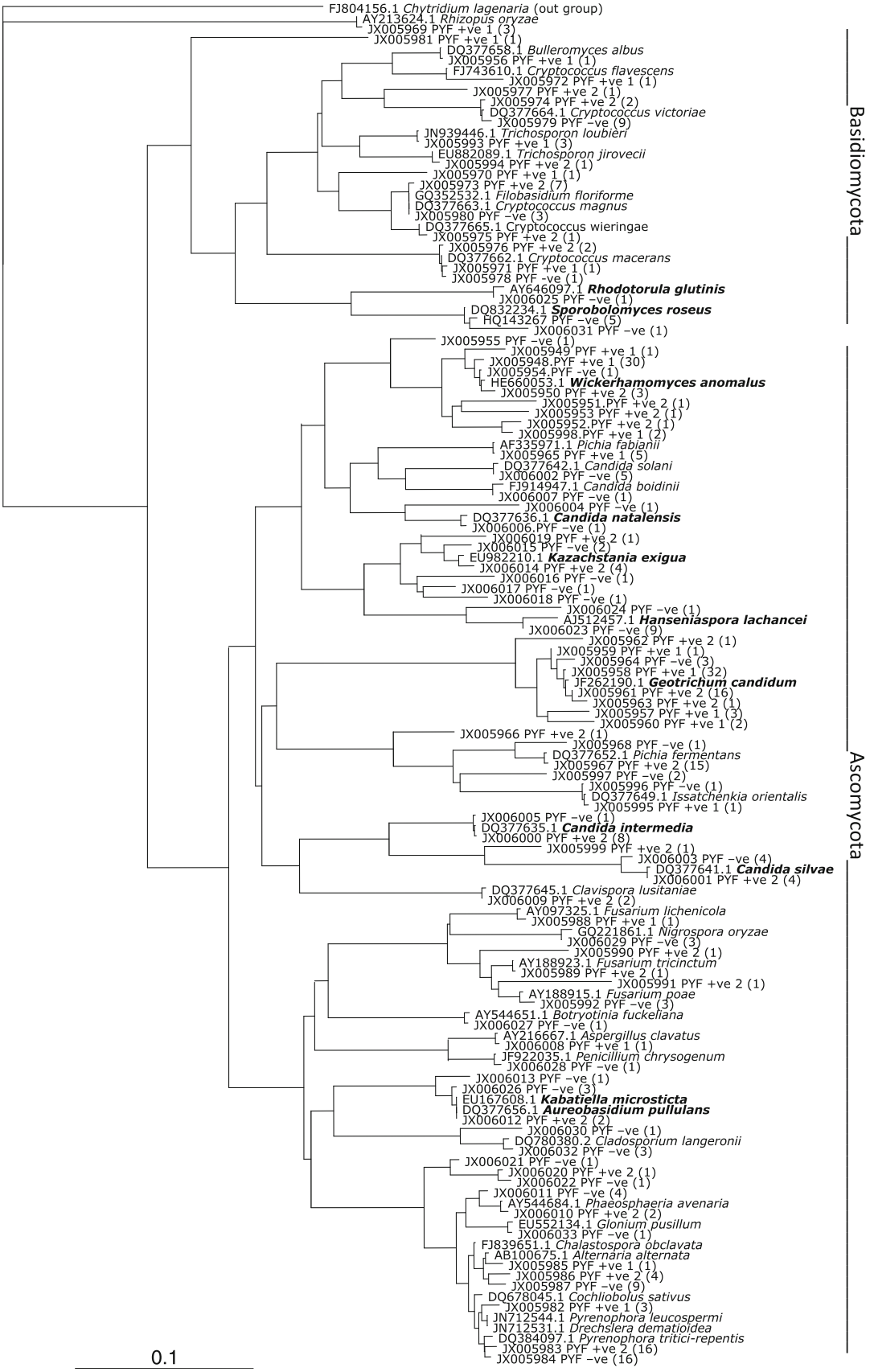
With SIMPER analysis, the average percent dissimilarity and the number of fungal and bacterial TRFs needed to explain 90 % of this dissimilarity within groups was analyzed. When different malt groups were compared between themselves, the PYF +ve malts showed the least dissimilarity from PYF −ve malts (Table 3). The highest value was observed for the location 1 and location 3 malt groups closely followed by the location 1 and location 2 malt groups.

In contrast to the above, when ANOSIM was performed on the 12 malts with only *Hae*III restriction enzyme, significant differences were observed between PYF +ve and PYF −ve malts with regard to fungal community structure (Table 3). Furthermore, PYF +ve malts showed great dissimilarity from PYF −ve malts within SIMPER analysis. Also, the number of fungal TRFs constituting this dissimilarity was lower than those observed in the primary experiment.

## Visual assessment of TRFLP electropherograms

The TRFLP electropherograms of all restriction enzymes, for all malt samples, were visually analyzed in an attempt to clarify the relationship between microbial population composition and the occurrence of PYF. It was questioned that if the causal PYF microbial taxa were minor electropherogram features, the statistical comparison employed might not detect this association. Representative electropherograms are shown to indicate the typical distribution of TRFs with respect to malt PYF status (Figs. S1–S2). It was observed that for the fungal *Hae*III-derived electropherograms, the presence of TRFs of 360–460 bp, appeared to vary substantially between PYF +ve and PYF −ve malt samples regardless of sample location, initially based on three control samples (Fig. S1 A, C, E). The visual assessment of the bacterial TRFLP electropherograms (Fig. S1 B, D, F) did not indicate any obvious differentiation between PYF +ve and PYF −ve malts unlike the fungal electropherograms. As such, bacterial TRFLP profiles were not conducted during the secondary experiment. The fungal *Hae*III 360–460 bp TRFs scored on a 0–5 scale in terms of peak number and relative abundance are shown in Fig. S2. A good correlation ($R^2 = 0.9$) was observed between visual scores of electropherograms and $\log_{10}$ average peak area of the 360–460 bp TRFs (Fig. S3).

The box plots of electropherogram score and average peak area for 360–460 bp TRFs and normalized peak area of *Hae*III electropherograms were produced for all the malt samples segregated into provider groups (Fig. S4). The combined plot for all the PYF +ve and PYF −ve malts based on electropherogram visual score showed that overall PYF +ve malts tended to have a significantly higher score (mean 2.5) than PYF −ve malts (mean 1.2),


FJ804156.1 Chytridium lagenaria (out group)
AY213624.1 Rhizopus oryzae
JX005969 PYF +ve 1 (3)
JX005981 PYF +ve 1 (1)
DQ377658.1 Bulleromyces albus
JX005956 PYF +ve 1 (1)
FJ743610.1 Cryptococcus flavescens
JX005972 PYF +ve 1 (1)
JX005977 PYF +ve 2 (1)
JX005974 PYF +ve 2 (2)
DQ377664.1 Cryptococcus victoriae
JX005979 PYF –ve (9)
JN939446.1 Trichosporon loubieri
JX005993 PYF +ve 1 (3)
EU882089.1 Trichosporon jirovecii
JX005994 PYF +ve 2 (1)
JX005970 PYF +ve 1 (1)
JX005973 PYF +ve 2 (7)
GQ352532.1 Filobasidium floriforme
DQ377663.1 Cryptococcus magnus
JX005980 PYF –ve (3)
DQ377665.1 Cryptococcus wieringae
JX005975 PYF +ve 2 (1)
JX005976 PYF +ve 2 (2)
DQ377662.1 Cryptococcus macerans
JX005971 PYF +ve 1 (1)
JX005978 PYF –ve (1)
AY646097.1 Rhodotorula glutinis
JX006025 PYF –ve (1)
DQ832234.1 Sporobolomyces roseus
HQ143267 PYF –ve (5)
JX006031 PYF –ve (1)
JX005955 PYF –ve (1)
JX005949 PYF +ve 1 (1)
JX005948.PYF +ve 1 (30)
JX005954.PYF -ve (1)
HE660053.1 Wickerhamomyces anomalus
JX005950 PYF +ve 2 (3)
JX005951.PYF +ve 2 (1)
JX005953 PYF +ve 2 (1)
JX005952.PYF +ve 2 (1)
JX005998.PYF +ve 1 (2)
AF335971.1 Pichia fabianii
JX005965 PYF +ve 1 (5)
DQ377642.1 Candida solani
JX006002 PYF –ve (5)
FJ914947.1 Candida boidinii
JX006007 PYF –ve (1)
JX006004 PYF –ve (1)
DQ377636.1 Candida natalensis
JX006006.PYF –ve (1)
JX006019 PYF +ve 2 (1)
JX006015 PYF –ve (2)
EU982210.1 Kazachstania exigua
JX006014 PYF +ve 2 (4)
JX006016 PYF –ve (1)
JX006017 PYF –ve (1)
JX006018 PYF –ve (1)
JX006024 PYF –ve (1)
AJ512457.1 Hanseniaspora lachancei
JX006023 PYF –ve (9)
JX005962 PYF +ve 2 (1)
JX005959 PYF +ve 1 (1)
JX005964 PYF –ve (3)
JX005958 PYF +ve 1 (32)
JF262190.1 Geotrichum candidum
JX005961 PYF +ve 2 (16)
JX005963 PYF +ve 2 (1)
JX005957 PYF +ve 1 (3)
JX005960 PYF +ve 1 (2)
JX005966 PYF +ve 2 (1)
JX005968 PYF –ve (1)
DQ377652.1 Pichia fermentans
JX005967 PYF +ve 2 (15)
JX005997 PYF –ve (2)
JX005996 PYF –ve (1)
DQ377649.1 Issatchenkia orientalis
JX005995 PYF +ve 1 (1)
JX006005 PYF –ve (1)
DQ377635.1 Candida intermedia
JX006000 PYF +ve 2 (8)
JX005999 PYF +ve 2 (1)
JX006003 PYF –ve (4)
DQ377641.1 Candida silvae
JX006001 PYF +ve 2 (4)
DQ377645.1 Clavispora lusitaniae
JX006009 PYF +ve 2 (2)
AY097325.1 Fusarium lichenicola
JX005988 PYF +ve 1 (1)
GQ221861.1 Nigrospora oryzae
JX006029 PYF –ve (3)
JX005990 PYF +ve 2 (1)
AY188923.1 Fusarium tricinctum
JX005989 PYF +ve 2 (1)
JX005991 PYF +ve 2 (1)
AY188915.1 Fusarium poae
JX005992 PYF –ve (3)
AY544651.1 Botryotinia fuckeliana
JX006027 PYF –ve (1)
AY216667.1 Aspergillus clavatus
JX006008 PYF +ve 1 (1)
JF922035.1 Penicillium chrysogenum
JX006028 PYF –ve (1)
JX006013 PYF –ve (1)
JX006026 PYF –ve (3)
EU167608.1 Kabatiella microsticta
DQ377656.1 Aureobasidium pullulans
JX006012 PYF +ve 2 (2)
JX006030 PYF –ve (1)
DQ780380.2 Cladosporium langeronii
JX006032 PYF –ve (3)
JX006021 PYF –ve (1)
JX006020 PYF +ve 2 (1)
JX006022 PYF –ve (1)
JX006011 PYF –ve (4)
AY544684.1 Phaeosphaeria avenaria
JX006010 PYF +ve 2 (2)
EU552134.1 Glonium pusillum
JX006033 PYF –ve (1)
FJ839651.1 Chalastospora obclavata
AB100675.1 Alternaria alternata
JX005985 PYF +ve 1 (1)
JX005986 PYF +ve 2 (4)
JX005987 PYF –ve (9)
DQ678045.1 Cochliobolus sativus
JX005982 PYF +ve 1 (3)
JN712544.1 Pyrenophora leucospermi
JN712531.1 Drechslera dematioidea
DQ384097.1 Pyrenophora tritici-repentis
JX005983 PYF +ve 2 (16)
JX005984 PYF –ve (16)
0.1
Basidiomycota
Ascomycota

◀ **Fig. 1** Neighbor-Joining phylogenetic tree of the D1/D2 domain of 26S rRNA gene sequences from three control barley malt samples (PYF +ve 1, PYF +ve 2, and PYF −ve), including known fungal sequences from GenBank database (sequences with accession no. followed by taxonomic binomial names) for comparison. The tree is rooted using *Chytridium lagenaria* as an outgroup. Representative sequences from three samples are incorporated. Each individual sequence is labeled as PYF +ve 1, PYF +ve 2, or PYF −ve, indicating the malt sample it belongs to followed by *numbers in parentheses*, which indicate the number of times a sequence recorded in the clone library. The *names in bold letters* show the sequences for which the virtual digestion tentatively linked to *Hae*III TRFs in the 360–460 bp size range of TRFLP profiles

**Table 2** Abundance of fungal genera observed in different malt samples

| Fungal genera | PYF +ve malt 1 | PYF +ve malt 2 | PYF −ve malt |
|---|---|---|---|
| | Relative abundance (%) | | |
| *Alternaria* | 1 | 4 | 9 |
| *Aspergillus* | 1 | – | – |
| *Aureobasidium* | – | 2 | 1 |
| *Botryotinia* | – | – | 1 |
| *Bulleromyces* | 1 | – | – |
| *Candida* | 2 | 13 | 12 |
| *Chalastospora* | – | 1 | 2 |
| *Cladosporium* | – | – | 4 |
| *Clavispora* | – | 2 | – |
| *Cryptococcus* | 3 | 13 | 12 |
| *Filobasidium* | 1 | – | – |
| *Fusarium* | 1 | 3 | 3 |
| *Geotrichum* | 40 | 18 | 3 |
| *Glonium* | – | – | 1 |
| *Hanseniaspora* | – | – | 10 |
| *Issatchenkia* | 1 | – | 3 |
| *Kabatiella* | – | – | 3 |
| *Kazachstania* | – | 4 | 5 |
| *Nigrospora* | – | – | 3 |
| *Penicillium* | – | – | 1 |
| *Phaeosphaeria* | – | 2 | 4 |
| *Pichia* | 5 | 16 | 1 |
| *Pyrenophora* | 3 | 16 | 15 |
| *Rhizopus* | 3 | – | – |
| *Rhodotorula* | – | – | 1 |
| *Saccharomyces* | – | 1 | – |
| *Sporobolomyces* | – | – | 6 |
| *Trichosporon* | 3 | 1 | – |
| *Wickerhamomyces* | 33 | 6 | 2 |

($p = 0.002$—TRFLP/$p = 0.027$—providers' PYF analyses) (Fig. S4A). The combined plot for all the PYF +ve and PYF −ve malts based on average peak area showed that overall PYF +ve malts tended to have higher peak area (mean 5.4) than PYF −ve malts (mean 4.7) ($p = 0.0025$—TRFLP analysis/$p = 0.048$—provider's PYF analyses) (Fig. S4B). When normalized peak areas were compared (Fig. S4C), the results were not significant ($p = > 0.29$), which indicated that the putative PYF causing taxa likely constitute relatively minor components of the total micro biota associated with the malt samples studied here.

Comparison of individual box plots for each of the malt provider gave further insight into the identification of PYF malts (Fig. S4). The malts from provider 3 were the control samples, while nine of the 11 malts from provider 4 were those examined in the secondary experiment in terms of *Hae*III TRF patterns. The PYF +ve malts from providers 3 and 4 recorded higher average peak areas than PYF −ve malts from these sources. Provider 1 indicated that the PYF +ve malts they supplied were borderline in terms of their PYF status. Consistent with this description, PYF +ve malt samples from provider 1 had a mean peak area close to 5.0. This observation was consistent with the observation that most PYF +ve malts exceeded an average area of 5.0 and that the PYF −ve samples from this provider had a slightly lower mean peak area of 4.5. Malts from provider 2 tended to possess higher average peak areas consistent with most samples being PYF +ve.

## Relationship between small-scale fermentation assay parameters and TRFLP assay

Twelve malt samples were analyzed using both the TRFLP assay and the small-scale fermentation assay of Lake et al. [27] and their results were compared with that of the malt providers' PYF designations (Table 4). Three different diagnostic parameters from the small-scale fermentation assay: (1) inflection point (M), (2) wort gravity (°Plato), and (3) turbidity ($A_{600}$) were grouped relative to the TRFLP results. Turbidity was negatively correlated with TRFLP electropherogram peak areas (correlation −0.80, $p < 0.05$) and scores derived for 360–460 bp TRFs (correlation −0.82). No significant correlation could be observed with either inflection point and or wort gravity. Overall, malt samples that showed lower turbidity values, indicative of greater flocculation in the small-scale fermentation assay, also produced higher TRFLP peak areas/scores. These samples tended to be PYF +ve, suggesting taxa corresponding to the 360–460 bp *Hae*III TRFs potentially could be responsible for PYF.

## Connection of PYF presumptive *Hae*III TRFs with malt-associated fungi

The fungal sequences were virtually digested using the same three restriction enzymes used to cleave the PCR products from malt DNA. The lengths of these theoretical

**Table 3** ANOSIM pair-wise comparisons of barley malt PYF status and geographical locations based on TRFLP data, % average dissimilarity realized by SIMPER analysis

| Pair-wise comparison | Fungi | | | Bacteria | | |
|---|---|---|---|---|---|---|
| | ANOSIM | | SIMPER | ANOSIM | | SIMPER |
| | R value | *p* value | (% dissimilarity) | R value | *p* value | (% dissimilarity) |
| Primary experiment | | | | | | |
| PYF +ve (18)–PYF −ve (14)[a] | −0.087 | 0.956 | 53.6 (256)[b] | −0.003 | 0.486 | 53.9 (190) |
| Location 1 (18)–Location 2 (6) | **0.613** | **0.001** | 69.4 (278) | **0.685** | **0.001** | 69.0 (210) |
| Location 1 (18)–Location 3 (8) | **0.633** | **0.001** | 70.9 (254) | **0.742** | **0.001** | 70.4 (208) |
| Location 2 (6)–Location 3 (8) | 0.164 | 0.152 | 53.9 (329) | 0.113 | 0.227 | 58.9 (254) |
| Secondary experiment | | | | | | |
| PYF +ve (5)–PYF −ve (7) | **0.602** | **0.016** | 74.3 (63) | | | |
| Location 1 (9)–Location 2 (3) | 0.475 | 0.086 | 66.8 (88) | | | |

The bold values are significant

[a] Figures in parentheses show number of barley malt samples analyzed in each group

[b] Figures in parentheses represent the number of TRFs observed in each group

TRFs were calculated and sequences were assigned to peaks found in the electropherograms. In all, the known clones could be assigned to 38.7 % of the total 256 TRFs, contributing 53.6 % average dissimilarity between PYF +ve and PYF −ve malts (Table 3). Fifteen *Hae*III TRFs (367, 378, 388, 390, 414, 428, 430, 431, 437, 438, 441, 442, 454, 455, 459) of the 360–460 bp TRFLP region were tentatively identified as *Aureobasidium pullulans*, *Candida intermedia*, *Candida natalensis*, *Candida silvae*, *Geotrichum candidum*, *Hanseniaspora* sp., *Kabatiella microsticta, Kazachstania exigua*, *Rhodotorula glutinis*, *Sporobolomyces roseus*, and *Wickerhamomyces anomalus*.

## Discussion

Previous studies on PYF have generally been concentrated on identifying the biochemical components that cause PYF rather than attempting to identify the root cause (i.e., the microbes associated with the PYF phenomenon during brewery fermentation). Invariably, where the microbes associated with PYF are identified, traditional "wet plate" culture techniques have been used. These results might be biased towards the selective enrichment of fast-growing microorganisms adapted to high substrate concentrations that could potentially represent a minor fraction of the resident microbial community. Furthermore, these studies have generally investigated a relatively small number of PYF +ve and PYF −ve malt samples compared with the 41 samples investigated in this study from different providers of intercontinental locations. Cultivation-independent, PCR-based fingerprinting techniques were applied in this study to identify associations between the occurrence of PYF with microbial diversity and structural composition. In addition, the PCR-based fingerprinting approach was also expected to identify the microbial taxa that would most likely be associated with the PYF problem.

TRFLP analysis and cloning and sequencing of bacterial 16S rRNA gene techniques were used to compare bacterial community structures of the malts obtained. This is, to our knowledge, the first study where bacterial communities have been studied to ascertain if they are directly related to the occurrence of PYF. Both similarities (ANOSIM) and community pattern (SIMPER) analyses showed that there were no differences between the TRF patterns of PYF +ve and PYF −ve malts for bacteria in the main set of 32 malt samples (Table 3). Visual assessment of bacterial TRFLP electropherograms also did not reveal obvious differences between PYF +ve and PYF −ve malts (Fig. S1). The conclusion was that bacterial taxa were unlikely to be important in causing PYF. This conclusion was consistent with the views of Lemos et al. [29] and van Nierop et al. [49], who reported that fungi produce far more extracellular polysaccharide hydrolyzing enzymes (attributed to cell wall degradation components that produce PYF factors) than bacteria and thus fungal infection was more important with regard to PYF.

The combined statistical analyses of TRFLP data from 32 malt samples for all the three restriction enzymes resulted in non-significant differences in fungal community structures of PYF +ve and PYF −ve malts. Whereas, the visual observations and computation of 360–460 bp *Hae*III TRFs and statistical analysis of TRFLP data of 12 samples showed overall qualitative and quantitative differences between PYF +ve and PYF −ve malts. The discrepancy between these two results appeared mainly to stem from the proportion of the specific TRFs constituting only a minor fraction of the overall fungal community of a given

sample. Discrepancies between the results could also be due to borderline fermentation test results that were obtained by the sample providers, and in particular, provider 1. The lack of a standard protocol, even after more than 40 years of research, has hindered definitive quantification of PYF malt [26, 27]. One likely factor contributing to inconsistent PYF status determination stems from different nutritional compositions of the worts used for the fermentation tests; generally no effort has been made to standardize basic factors such as wort gravity or the concentration of zinc, which are routinely/often monitored or adjusted in commercial breweries [18, 19, 33].

Despite non-significant differences in the fungal TRF patterns of PYF +ve and PYF −ve malts in the main set of 32 malt samples (Table 3), location was found to have a significant effect on microbial community structure. This geographical influence was not observed for the second set of samples. In addition, the interaction between location and PYF status was found not to be significant when TRFLP data were analyzed using PERMANOVA analysis (data not shown). Furthermore, the pattern of *Hae*III 360–460 bp TRFs varied substantially between PYF +ve and PYF −ve malts regardless of location of the malt sample. Thus, location of the malt did not seem to influence the fungal taxa implicated in PYF.

ANOSIM of the TRFLP data for the *Hae*III enzyme, however, showed clear differences in fungal community structures of PYF +ve and PYF −ve malts (Table 3) and it could be demonstrated that there is a correlation between the abundance of *Hae*III 360–460 bp TRFs and PYF status. The diagnostic value of these TRFs was compared directly with the small-scale fermentation assay developed by Lake et al. [27] (which is gaining reputation in malting and brewing industry/research) as a reference method. This also allowed us to standardize malt PYF designation as positive or negative based on the *Hae*III 360–460 bp TRFs, thus avoiding any inconsistency created with dissimilar PYF fermentation tests used by different malt providers for PYF designation. It was clearly shown that of the three diagnostic statistics produced by the small-scale fermentation test, turbidity correlated strongly the TRFLP determination of PYF (Table 4). That is, the PYF +ve malt samples tended to have less yeast cells in suspension (lower turbidity) in wort, indicating their sedimentation due to flocculation. Lake (pers. comm.) also considered that the turbidity results in the fermentation test [27] were most definitive in identifying PYF malts. The diagnostic value of this conclusion was confirmed because the turbidity measurement is the determining feature of all the widely used PYF fermentation assays [13, 21, 22, 24, 27, 34, 49, 50].

The presence of more than one diagnostic TRF suggests involvement of multiple fungal taxa in the PYF problem. Sasaki et al. [39] have also reported five different fungal species belonging to three different genera being responsible for causing PYF in malts they studied. Similar conclusions were also made by Blechová et al. [5], van Nierop et al. [49], and Yang et al. [54].

Furthermore, the range of average peak area/visual score of electropherogram rather than a discrete value for PYF +ve malts, and also the presence of TRF peaks (although in less number and abundance) in PYF −ve malts, suggested that PYF-responsible microbes were also present in the PYF −ve malts but at lower levels. The PYF phenomenon appeared to occur only when their numbers

**Table 4** Relationship between different parameters of the small-scale fermentation assay, TRFLP assay (360–460 bp TRFs), and barley malt providers PYF assay, sorted according to the small-scale test turbidity ($A_{600}$)

| Location | Inflection point (M) | Wort gravity (°Plato) | Turbidity ($A_{600}$) | Electropherogram score | Av. peak area (log10) | Normalized 360 – 460 bp peak area | TRFLP PYF status | Provider's PYF status |
|---|---|---|---|---|---|---|---|---|
| 2 | 17.2a | 6.8c | 0.109a | 4 | 5.68 | 0.1143 | +ve | +ve |
| 2 | 16.9a | 5.6bc | 0.287b | 2.5 | 5.56 | 0.0955 | +ve | +ve |
| 1 | 23.0b | 6.4c | 0.334bc | 3 | 5.41 | 0.0850 | +ve | +ve |
| 1 | 22.7b | 6.7c | 0.353bc | 5 | 5.87 | 0.1420 | +ve | +ve |
| 2 | 23.2b | 6.3c | 0.457c | 3 | 5.27 | 0.0682 | +ve | +ve |
| 2 | 23.8b | 5.6bc | 0.458c | 3 | 5.20 | 0.0521 | +ve | +ve |
| 1 | 19.2a | 5.5b | 0.554cd | 2.3 | 5.09 | 0.0701 | -ve | +ve |
| 1 | 25.5b | 6.2c | 0.644d | 1 | 4.77 | 0.0780 | -ve | +ve |
| 1 | 22.1b | 5.9bc | 0.694de | 1 | 4.77 | 0.0700 | -ve | -ve |
| 2 | 25.1b | 4.3a | 0.716de | 2 | 5.11 | 0.0676 | -ve | -ve |
| 1 | 21.2ab | 5.7bc | 0.824e | 1.8 | 5.15 | 0.0994 | -ve | -ve |
| 2 | 23.2b | 6.5c | 0.853e | 0.3 | 4.74 | 0.0782 | -ve | -ve |

A *common letter* indicates a non-significant difference. *Non shaded* and *shaded values* indicate PYF −ve and PYF +ve status, respectively

increase above a certain threshold. This might be the reason that Herrera and Axcell [20] found PYF factor (PAS I) in all tested malts, although the concentration of this factor in malts producing normal fermentation patterns was lower than those causing PYF. van Nierop et al. [49] have also suggested that PYF flocculation coincided with heavy infection of barley, resulting in severe degradation of grain and up-regulation of antimicrobial factor synthesis such as defensins or anti-microbial peptides. These antimicrobial factors along with other compounds have been reported to be implicated in PYF, especially the metabolic type PYF or for their anti-yeast behavior [4, 49, 50, 53].

Of the three restriction enzymes used in this study, *Hae*III showed a better ability to differentiate between PYF +ve and PYF −ve malts. This might be because the other two restriction enzymes, *Rsa*I and *Hinf*I used in this study, might have cut the targeted rRNA gene sequences of PYF responsible fungal taxa non-specifically or produced fragments that were not detected within the size limits (60–640 bp TRFs) used here.

Cloning and sequencing showed great fungal diversity associated with malts. Most of the clones recorded in this study appeared to be characterizing fungal taxa already reported to occur in the barley malting and brewing ecosystem [12, 28, 36]. Ascomycetous genera dominated the studied malts as compared to basidiomycetous (Fig. 1, Table 2), consistent with results reported by Laitila et al. [28]. Ascomycetous yeasts are known for their ability to better tolerate higher temperatures prevailing during the final stages of malting (i.e., kilning) than basidiomycetous yeasts [28]. Furthermore, PYF +ve and PYF −ve malts revealed dissimilarities among themselves with regard to types of taxa and their relative abundances. Virtual digestion of fungal sequences tentatively linked 15 *Hae*III TRFs in the 360–460 bp size range to a diverse range of yeast/yeast-like species. This was in contrast with the literature where PYF has mostly been linked to infection of malt with filamentous fungi including *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, or *Rhizopus* spp. [5, 39, 49, 54]. These differences in results might be because of the differences in methods of studying microbial communities' structures, with greater focus on filamentous fungi rather than yeast in the past. Yeasts are an important component of the malt microbial ecosystem; the second most [12] abundant microbes after bacteria in viable counts in pre harvest barley. High numbers of yeasts and yeast-like fungi have been observed throughout the malting process as well [28]. Furthermore, yeasts and yeast-like fungi are known for the production of hydrolytic enzymes, including amylases, $\beta$-glucanases, celluloses, and xylanases [9, 28]. These enzymes help such microbes to degrade the grain cell wall and to penetrate plant cells. However, care should be taken in considering these differences as conclusive, considering that the identification of TRFs was tentative and might not be complete, as the reason being that only three clone libraries were constructed that were able to identify only 1/3rd of the total TRFs, contributing 53.6 % average dissimilarity between PYF +ve and PYF −ve malts (Table 3). There is a need for greater in-depth sequencing data generation to better cover the fungal diversity associated with PYF +ve and PYF −ve malts in future studies, especially considering the relatively large number of samples examined in this study and the relatively low proportion of PYF-associated fungal taxa apparent in the TRFLP profile data. Future monitoring of these PYF-responsible taxa could potentially be an efficient and practical alternative in the detection of PYF-affected malts.

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