Title: The natural diversity and ecology of fission yeast.

Running head: Natural fission yeast diversity and ecology

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Abstract

While the fission yeast is a powerful model of eukaryote biology, there have been few studies of quantitative genetics, phenotypic or genetic diversity. Here I survey the small collection of fission yeast diversity research. I discuss what we can infer about the ecology and origins of *Schizosaccharomyces pombe* from microbiology field studies and the few strains that have been collected.

Introduction

Schizosaccharomyces pombe research began in the 1940s (Fantes and Hoffman 2016) and is now a potent model of eukaryote biology, with a well-annotated curated genome (Wood *et al.* 2002; McDowall *et al.* 2015), an extensive battery of technical methods and genome-scale tools (Hoffman, Wood and Fantes 2015; Hagan *et al.* 2016) and regular international meetings devoted to its study. Part of the important utility of fission yeast as a model is that it contains many vertebrate orthologs that are not present in budding yeast (Hoffman, Wood and Fantes 2015), so it provides a complement for studies of cell biology.

The majority of fission yeast research has used the strains described by Leupold with its three mating types (Leupold 1949), and mutants derived from these strains. Studies of diversity or quantitative genetics have been few and far between. By contrast there is an extensive literature describing diversity and quantitative genetics in the budding yeast *Saccharomyces cerevisiae* and its wild relative *Saccharomyces paradoxus,* and a range of related species (Peter and Schacherer 2016). These include QTL studies

(Swinnen, Thevelein and Nevoigt 2012; Liti and Louis 2012; Fay 2013; Bloom *et al.* 2013; Märtens *et al.* 2016), genome-scale analysis of diversity (Liti *et al.* 2009; Schacherer *et al.* 2009) and analysis of diversity and evolution in the natural environment (Robinson, Pinharanda and Bensasson 2016; Leducq *et al.* 2016). In this review, I survey fission yeast diversity research, and I discuss what little is known about the origins and natural ecology of this species.

Defining fission yeast species

Collections of *Schizoaccharomyces* strains were classified into three groups based on crossing and protoplast fusion (Sipiczki *et al.* 1982), phenotypic characters (Bridge and May 1984), DNA optical reassociation and physiological characters

(Vaughan Martini 1991), simplifying the rather complex list of potential 'species' into three (*Schizoaccharomyces pombe*, *S. japonicus*, *S. octosporus*). *Schizosaccharomyces cryophilus* was identified much later as a contaminant of a *S. octosporus* strain (CBS7191) from Denmark, and the species description was accompanied by a draft genome (Helston *et al.* 2010).

The genomes and transcriptomes of *S. japonicus*, *S. octosporus* and an improved *S. cryophilus* genome were described in 2011, showing that the *Schizosaccharomyces* genus is as divergent on the protein level as the human-amphioxus divergence (~55% amino acid identity) (Rhind *et al.* 2011). This analysis described the conservation of orthologous groups, conservation of transcription, the evolution of mating type regions and transposons. It also features the first sequencing of a non-reference strain of *S. pombe*, concluding that the within-species diversity was < 1% (confirmed later with studies of more strains (Fawcett *et al.* 2014; Jeffares *et al.* 2015)). The current clade of only four highly divergent fission yeast species is a limitation for evolutionary studies, since evolutionary constraints can be estimated only inaccurately, and non-coding sites that are in general subject to weaker purifying selection tend to be saturated (Rhind *et al.* 2011). None of the *Schizosaccharomyces* species is sufficiently closely related to *S. pombe* to reliably determine ancestral nucleotide states.

Early (pre-genome sequence) diversity studies

An early field study of this species was conducted by Florenzano *et al.*, who showed that *S. pombe* was frequently present on grapes in Sicilian vineyards (Florenzano, Balloni and Materassi 1977). Phenotypic characterization began with analysis of xerotolerance (resistance to high solute concentrations) in 27 *S. pombe* strains (Ganthala, Marshall and May 1994). One the first genetic analysis of diversity within *S. pombe* described the intron content of mitochondrial genomes in 26 strains, showing presence/absence polymorphisms in group I and group II introns (Zimmer *et al.* 1987). Interestingly, there appears to be no intron presence polymorphisms in the nuclear genomes of sequenced strains (Mourier & Jeffares, unpublished analyses), though on the longer scale fission yeasts have certainly undergone intron gain and loss (Mourier and Jeffares 2003; Jeffares, Mourier and Penny 2006; Rhind *et al.* 2011).

In a prelude to genome-scale analyses, three studies began to explore genetic and phenotypic diversity on a larger scale. Gomes *et al.,* collected 27

strains from seven Brazilian cachaça distilleries, and characterised osmotolerance, trehalose accumulation and ethanol tolerance, showing that these strains could grow in 50% glucose and 10% ethanol (Gomes *et al.* 2002). They also explored population structure using RAPD-PCR (random amplified polymorphic DNA PCR), demonstrating local population structure in Brazilian cachaça strains. RAPD-PCR was a useful method to characterise diversity prior to next generation sequencing, but the development of 26 primers for microsatellite PCR now provide a simple method to genotype strain collections (Patch and Aves 2007). Brown *et al.* assembled 81 natural isolates of *S. pombe* including samples from all continents (except Antarctica), and measured a large assembly of phenotypic characters, including growth parameters in 42 liquid media and cell length (Brown *et al.* 2011). This analysis also described diversity at three locations, and estimated that the global effective population size of this species is 10^7 (a figure that remained after genome-wide analysis (Farlow *et al*. 2015)). Most interestingly, this work described extensive karyotype diversity within this collection, including reciprocal translocations, duplications and inversions, showing that the ribosomal repeats were located on different chromosome ends in different strains.

Genome-wide sequence analyses

The creation and analysis of the only fission yeast recombinant strain library was published in 2014 (Clément-Ziza *et al.* 2014). This study used a two-parent segregant panel and described expression QTLS (eQTLs) from both protein-coding and non-coding transcripts, during growth and stress conditions. Interestingly this study discovered a larger proportion of associations between genetic variants and non-coding transcripts than coding transcripts. The most significant variant, that affected 44% of eQTL associations and growth rate, was a frameshift in the *swc5* gene - part of a complex that affects histone deposition. Detailed analysis showed that this frameshift caused increased antisense transcription and decreased sense transcription, providing an example of the molecular events that influenced a complex trait such as growth. Further analyses of segregant panels are in progress, describing positive selection and the genetic control of RNA and protein levels (Clément-Ziza, pers. comm.).

An analysis of segregant pool based mapping (bulk segregant analysis) from a two-parent cross showed that this method was feasible in fission yeast (Hu, Suo

and Du 2015). Hu *et al.* localised the probable causal allele of maltose deficiency by sequencing pools grown with and without maltose. The analysis was complicated by an inversion in the reference strain, but few other wild strains (Jeffares *et al.* 2017), which reduces the local recombination rate (Clément-Ziza *et al.* 2014).

Two genome-wide analyses of genetic diversity in *S. pombe* were published soon afterwards (Fawcett *et al.* 2014; Jeffares *et al.* 2015). Both analyses described recombination rate and population structure, and showed that exons, UTRs and introns were the main targets of purifying selection. Estimates of diversity (π) were $\sim 3 \times 10^{-3}$ (pairwise comparison have an average of 3 SNPs/kb), slightly higher than the budding yeast *Saccharomyces cerevisiae* (1×10^{-3}) (Liti *et al.* 2009). From the genetic diversity and mutation rates, the effective population size of *S. pombe* has been estimated to be 12 million, on a similar scale to budding yeast (3 million) (Farlow *et al.* 2015).

The analysis of Fawcett *et al.* (32 strains) described some unusual patterns of diversity that were likely due to soft selective sweeps, and either balancing selection or introgression from some unknown fission yeast outgroup (Fawcett *et al.* 2014). Jeffares *et al.* (161 strains) described transposon insertions and included analysis of quantitative traits, their heritability and quantitative genetics using the genome-wide association study (GWAS) approach (Jeffares *et al*. 2015). This study located 1,400 variants that were significantly associated with traits despite the very small sample size, showing that the combination of simple tractable genetics with the capability to measure traits accurately with abundant repeat measurements in well-controlled environments, is a powerful combination. Further analysis with the same strain collection described structural variants showing that they are both transient and contribute considerably to quantitative traits and reproductive isolation (Jeffares *et al.* 2017). Interestingly the variance in wine-making traits, such as malic acid accumulation and glucose/fructose ultilisation (Benito *et al.* 2016), appeared to be caused entirely by structural variants.

Two genome-scale analyses of the mutation rate estimated the point mutation rate to be 1.7×10^{-10} (or 2.0×10^{-10}) per base per generation (Farlow *et al.* 2015; Behringer and Hall 2015), very similar to estimates for the budding yeast *Saccharomyces cerevisiae* (estimates at 3 and 1.67×10^{-10}) (Lynch *et al.* 2008; Zhu *et al.* 2014). Both studies noted a strong bias towards small insertions,

over deletions, which occur primarily in the non-protein regions of the genome, a pattern that is retained in natural genetic diversity (Jeffares *et al*. 2015).

Reproductive isolation

One topic that has received particular attention is the study of mating types and reproductive isolation. Since the outset of fission yeast research, it was clear homothallic strains could mutate to more or less stable heterothallic genotypes (h^+ or h^-) (Leupold 1949). Natural isolates also vary genetically at mating type regions and in their mating behavior, with some strains mutating more frequently from h^+ to h^- and vice versa (Schlake and Gutz 1993). In an interesting demonstration that reproductive isolation could evolve via pre-zygotic mechanisms, Sieke *et al.* created three novel reproductive groups with different pheromone-receptor pairs (Seike, Nakamura and Shimoda 2015). Given these changes it is likely that pre-zygotic reproductive isolation occurs within some populations.

Several studies described the low spore viability that results from many inter-strain matings (Kondrat'eva and Naumov 2001; Teresa Avelar *et al.* 2013; Zanders *et al.* 2014; Naumov and Kondratieva 2015; Jeffares *et al.* 2015). Viability ranges from pairs showing < 1% viable offspring to strains with 90% viable, similar a range observed for *species* of budding yeast with that have much higher genetic divergence than fission yeast strains (Liti, Barton and Louis 2006), consistent with *S. pombe* strains being 'on the verge of speciation' (Naumov and Kondratieva 2015) (**Figure 1A**). Some homothallic strains are also ineffective at mating with their own genotype (Kondrat'eva and Naumov 2001; Jeffares *et al.* 2015).

Since most crosses do produce mating bodies and asci (Xavi Marsellach, pers. comm.), the isolation is generally post-zygotic (intrinsic reproductive isolation). The accumulation of genetic factors that reduce mating success withinin these relatively closely related strains is probably due to the low frequency of outbreeding in fission yeast. Based on the decay in linkage between wild isolates Farlow *et al.* estimated that *S. pombe* mate with a genetically dissimilar individual on average every 800,000 generations (Farlow *et al.* 2015), far less frequently than the estimates 50,000 generation for *S. cerevisiae* (Ruderfer *et al*. 2006). Given this frequency of, it is not surprising that the existing strains

have accumulated genetic factors that preclude interbreeding in the ~2300 years since these strains have drifted apart (Jeffares *et al.* 2015).

There are at least three (non-exclusive) genetic causes for the reproductive isolation of fission yeasts. Spore killing (meiotic drive), has been proposed to be a mechanism (Kondrat'eva and Naumov 2001; Zanders *et al.* 2014; Naumov and Kondratieva 2015). Many of the crosses analysed by Kondratieva *et al.* from genetically divergent strains and produced strong deviations from expected Mendelian ratios (Kondrat'eva and Naumov 2001; Naumov, Kondratieva and Naumova 2015) (Figure 1B), while the analyses of Zanders *et al.* concluded that there were meiotic drive elements on all three chromosomes (Zanders *et al.* 2014).

Two recent analyses have demonstrated that members of the *wtf* gene family mediate drive with a spore killer-antidote system (Hu *et al.* 2017; Nuckolls *et al.* 2017). Hu *et al.* demonstrate that *wtf9* and *wtf27* genes from the non-reference strain (CBS5557/JB4) drive segregation distortion in when mated to the reference strain, that this drive is independent of genomic location. Nuckolls *et al.* show that *wtf4* promotes distortion in crosses between the reference strain and the kombucha strain (SPK1820/YFS276/JB1180, as initially sequenced by the Broad Institute (Rhind *et al.* 2011)). Other strains analysed by Kondratieva *et al.* also show very biased segregation (Figure 1B).

Collectively, these analyses show that the spore killer (or poison) and antidote functions can be separated by mutations. In the natural state, there are two transcripts that mediate killer/antidote functions (Nuckolls *et al.* 2017). While the killer protein variant is distributed in all four spores of the asci, the antidote remains only within cells with the relevant *wtf* genotype. Since *wtf* genes encode membrane-spanning domains they may travel between asci. The genetics of the poison-antidote systems are complex, in that there are multiple *wtf* genes in different strains that have degenerated to contain the poison and antidote functions, antidote only, or no function. Both analyses show that *wtf* genes are particularly genetically diverse (Figure 1C). However, they do not show an excess of high Tajima's D values (Tajima 1989)(Figure 1C), a genetic diversity parameter which is one of the expected signatures of balancing selection.

Reproductive isolation may also be the result of the aneuploidy that occurs when parents differ in chromosomal inversions and translocations. For example, engineered inversions and translocations reduce spore viability by ~40% (Teresa Avelar *et al.* 2013). *S. pombe* strains do have extensive karyotype differences

(Brown *et al.* 2011; Naumov, Kondratieva and Naumova 2015; Jeffares *et al.* 2017), including a strain that maintains four (rather than the usual three) chromosomes (Brown *et al.* 2014). There is a significant association between viability and the SV-distance between parents (Jeffares *et al.* 2017), though viability declines at less than 40% viability per variant. This is probably because natural structural variants are biased to chromosome ends that do not contain essential genes (Jeffares *et al.* 2015), due to selection for those that do not cause lethal aneuploidies. Structural variants may also contribute to drive (Zanders *et al.* 2014).

Formally, reproductive isolation may also be due to Bateson-Dobzhansky-Muller interactions (BDMIs) or any of the other genetic mechanisms of negative epistasis (Nei and Nozawa 2011). However segregation data from random spores (Kondrat'eva and Naumov 2001; Naumov and Kondratieva 2015) and dissected tetrads is inconsistent with simple two-locus BDMIs, which are expected to produce small deviations from expected segregation patterns (even when the affected alleles were strongly linked to markers) (Hou and Schacherer 2016). Ultimately meiotic drive, epistasis and structural variants may have interacting effects on viability, since locally adapted haplotypes are predicted to develop within areas of reduced recombination (Kirkpatrick and Barton 2006).

With all these studies of population genetics (reproductive isolation, divergence dating, diversity measures, population size *etc.*) the analyses are based on a small collection of strains that are a worldwide sample of mostly human commensals (see below), so conclusions may not represent natural populations.

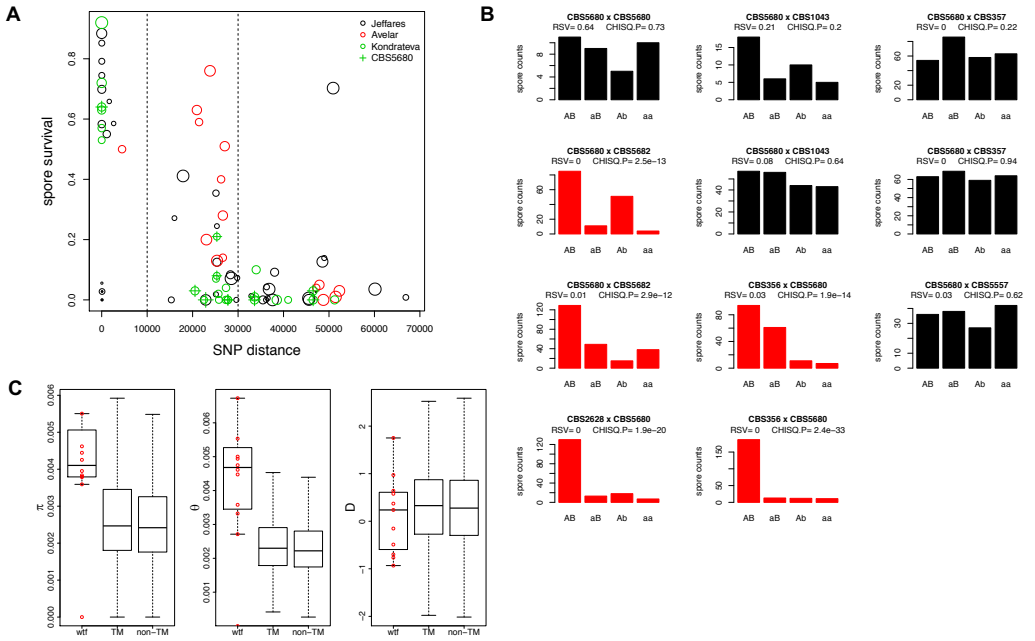

Figure 1. Intrinsic reproductive isolation in *S. pombe*.

A) Random spore viability from three studies shows a decline in spore survival with genetic distance (SNP distance) between parents. The size of circles indicates the lowest self-mating viability of parents. Data from (Kondrat'eva and Naumov 2001; Teresa Avelar *et al.* 2013; Jeffares *et al.* 2015). Crosses involving the strain CBS5680 (as in part B) are indicated with cross hairs. The range of genetic differences that have highly variable effects on viability (10,000 – 30,000 SNPs) is indicated with vertical dashed lines. The outlier at top right is JB848/CBS10475 (Brazil) x JB870/CBS10499 (South Africa), which appears to be real (Xavier Marsellach, pers. comm.). **B)** segregation of control markers in random spore analysis show strong deviations from the expected 1:1:1:1 ratio, data from (Kondrat'eva and Naumov 2001). For one strain (CBS5680/JB873, from Poland) we show the counts of control markers (aB and Ab are parental types, AB, ab are recombinants, see Kondrateva *et al.* for details). Segregation counts whose χ^2 test P-values were < 0.05 are plotted with red bars. Plot text shows the parents of the cross, the random spore viability (RSV) and the χ^2 test P-value (CHISQ.P). **C)** *wtf* genes have high pairwise diversity within strains compared to all other transmembrane domain containing and non-TM genes (π, left panel), high numbers of segregating sites (θ, middle panel), but are not outliers for Tajima's D (which is calculated from the ratio of the two, D, right panel). Plots show diversity estimators from 57 strains, red circle indicate individual values for *wtf* genes.

Predicted transmembrane proteins were collected from a query of Pombase (www.pombase.org), diversity data from (Jeffares *et al.* 2015).

Genetics and the reference strain

The fission yeast community has worked almost exclusively with one reference strain, and spontaneous mutants generated from this strain (Fantes and Hoffman 2016). This laboratory strain is a natural isolate, and is not an unusual strain phenotypically. It does not appear to be adapted to the standard rich or minimal media, since it does not grow particularly rapidly in these media compared to wild strains. There are several important discoveries that are relevant to the fission yeast researcher. Firstly, Wild strains can differ from the reference by up to 68,000 SNPs and up to 24 structural variations, which contribute to phenotypic variation between strains (Clément-Ziza *et al.* 2014; Jeffares *et al.* 2015; Hu, Suo and Du 2015; Jeffares *et al.* 2017). I summarise the structural differences between strains in Supplementary Figure 1. Secondly, the structural differences and meiotic drive elements that wild strains contain complicate crosses between strains, by reducing spore viability and skewing the proportions of alleles that are produced in the offspring (Kondrat'eva and Naumov 2001; Kondrateva and Naumov 2011; Clément-Ziza *et al.* 2014; Hu, Suo and Du 2015; Nuckolls *et al.* 2017; Hu *et al.* 2017).

The ecology of fission yeast

There have been few published attempts to systematically collect fission yeast strains (Gomes *et al.* 2002; Benito *et al.* 2013; Hellberg 2013). However, fission yeasts have been serendipitously discovered in a variety of microbiological studies (Table 1, Figure 2). Sources have generally been traditional non-industrialised fermentations, produced without any intentional inoculation from substrates that contain high concentrations of sugars. When quantitative estimates of species abundances are included *Schizosaccharomyces* yeasts were generally minor components of these fermentations, with the exceptions of kombucha, some cachaça fermentations and baijiu (from tea, sugar cane and sorghum respectively) (Pataro, Guerra and Peixoto 2000; Teoh, Heard and Cox 2004; Wu, Xu and Chen 2012).

Perhaps more informative for fission yeast ecology, are the cases where

fission yeasts have been discovered in natural substrates such as palm wine (a fermentation of palm sap) (Theivendirarajah and Chrystopher 1987; Amanchukwu, Obafemi and Okpokwasili 1989; Ouoba *et al.* 2012). Fission yeast are also present in natural fermentations of fruits such as *Coffea arabica* and *Theobroma cacao* (from which coffee and cocoa beans are harvested respectively) (Silv *et al*. 2000; Schwan and Wheals 2004). Collectively, the field studies show that fission yeasts are a component of natural microbial communities that ferment botanical sugars in several geographic regions.

Including the strains present in stock collections and in field studies the most common substrates for fission yeast have been palm wine, grape wine, high-sugar substrates (molasses, cane sugar, honey) and fruits (Figure 2). Three selective media to have been described to enrich for fission yeast (Florenzano, Balloni and Materassi 1977; Hellberg 2013; Benito *et al.* 2013), so further systematic collections from similar locations and substrates should be possible in the future.

Table 1. *Schizosaccharomyces* in field microbiology

Substrate	**Location**	**Reference**
Grape must	Sicily	(Florenzano, Balloni and Materassi 1977)
Grapes	Ukraine	(Bayraktar 2014)
Palm wine	Sri Lanka	(Atputharajah, Widanapathirana and Samarajeewa 1986; Theivendirarajah and Chrystopher 1987)
Palm wine	Nigeria	(Sanni and Lönner 1993; Amanchukwu, Obafemi and Okpokwasili 2006)
Palm wine	Burkina Faso	(Ouoba *et al.* 2012)
Rum	Haiti	(Fahrasmane, Ganou-Parfait and Parfait 1988)
Molasses, raisin	Japan/Thailand/Taiwan	(Ishitane 1985)

Tequila	Mexico	(Lachance 1995)
Coffee cherries	Brazil Madagascar	(Silv *et al.* 2000) (Ravelomanana *et al.* 1984)
Cachaça (from sugar cane)	Brazil	(Pataro, Guerra and Peixoto 2000; Gomes *et al.* 2002)
Kombucha (fermented tea)	Australia**	(Teoh, Heard and Cox 2004)
Cocoa pulp	Belize	(Schwan and Wheals 2004)
Baijiu (distillate of fermented sorghum)	China	(Wu, Xu and Chen 2012)
Traditional breweries	China	Fen-Yang Bai, pers. comm.
Honey	Fiji	(Ponici and Wimmer 1986)
Honey	Spain	(Benito *et al.* 2014)

322 * Not microbiological study itself, refers to earlier work.

323 ** From commercial kombucha brewers.

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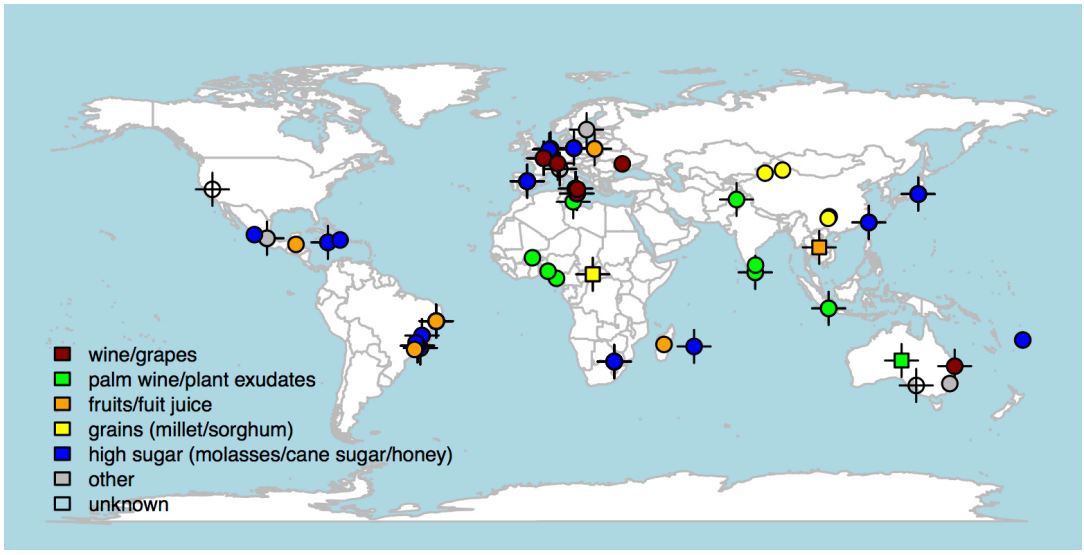

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Figure 2. Fission yeast locations and substrates. The locations and substrates where fission yeast have been discovered, including all strains that have been sequenced from stock centers (Fawcett *et al.* 2014; Jeffares *et al.* 2015), and reports from field studies (Table 1). Sequenced strains are marked with cross-hairs, and strains isolated from uncertain locations are marked with a square.

The origin of fission yeast

S. pombe is now globally distributed (Figure 2), but we know little about its origin and dispersal. We have estimated that these strains began to spread globally in from ~340 BCE (95% confidence interval 1875 BCE–1088 CE), and that the current collection of strains from Brazilian cachaça originated from the remainder in about ~1620 CE (confidence interval 1422–1752 CE) (Jeffares *et al.* 2015), a hint that like budding yeast and *C. elegans*, this model has probably been dispersed as a commensal (most likely in fermented beverages).

The reference strain originated from French grapes (Osterwalder 1924). The common belief is that *S. pombe* originated from Africa, perhaps because the initial species description was from an African millet beer isolate (Lindner 1893; Vorderman 1894). While genetic analysis is consistent with exchange between African and European stocks (Jeffares *et al.* 2015), and some strains have been collected from traditional African fermentations, there is no scientific evidence for an African origin of this species. There are very few studies of the microbial constituents of millet beer from Africa (I could fine none than specifically mentioned *S. pombe*, and one description of sorghum beer that did not mention *S. pombe* (Kayode *et al.* 2011)). Since fission yeasts can be major components of kombucha, which has been traditionally produced in China (Sreeramulu, Zhu and Knol 2000; Teoh, Heard and Cox 2004), palm wine which is widely produced in Asia (Table 1, Figure 2), and in traditional Chinese breweries (Fen-Yang Bai, pers. comm.), China is an equally good candidate for the initial origin of *S. pombe.*

Why study diversity in fission yeast?

The small genomes of budding yeasts enabled the early development of population genomics methods (Liti *et al.* 2009; Schacherer *et al.* 2009), and now large scale accurate quantitative genetics analyses (Bloom *et al.* 2013; Märtens *et al.* 2016). The continuing advance of sequence throughput, analysis software and

laboratory methods (eg: RAD-seq) have now made population genomics approaches available to any species. However, the abundance of genome-scale data and technical tools and the small non-redundant genomes of yeasts make them attractive models for systems biology, including approaches to understanding genetic diversity and traits (Parts 2014). Fission yeast has the benefit of being haploid (so that F1 generations need not be intercrossed). As with budding yeast, fission yeast has abundant heritable phenotypic diversity in growth, stress responses, cell morphology, and cellular biochemistry that is yet to be explored with powerful quantitative genetics (Brown *et al.* 2011; Clément-Ziza *et al.* 2014; Jeffares *et al.* 2015; 2017). Yeasts are also powerful tools for detailed study of evolutionary processes using pooled time-series sequencing and other high-throughput approaches that would be expensive or unfeasible in other species (Cubillos *et al.* 2011; Hou *et al.* 2015). Finally, studies by Benito *et al.* show that some non-reference *S. pombe* strains have potential in the winemaking industry (Benito *et al.* 2014; 2016), so diverse strains could well have potential elsewhere in biotechnology.

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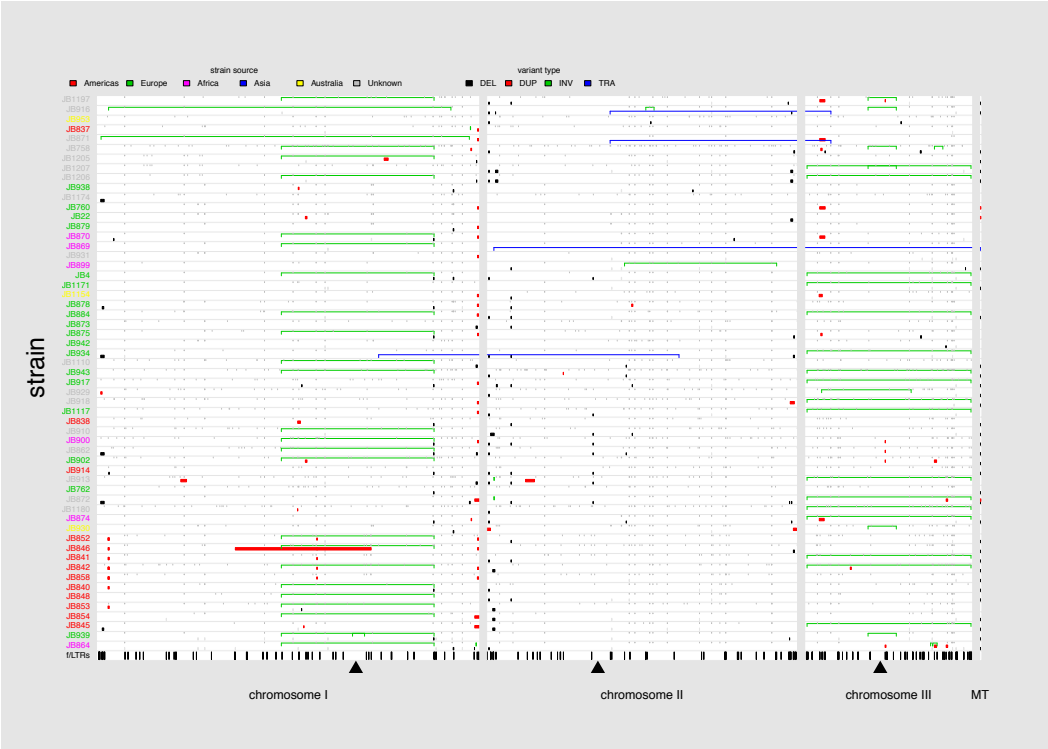
Supplementary data

All used for plots is available at figshare at:
https://figshare.com/projects/The_natural_diversity_and_ecology_of_fission_yeast_/21761



Supplementary Figure 1. Structural variants present in wild fission yeast strains. Using predictions from short read data (Jeffares *et al.* 2017), I show the genomic location of structural variants (SVs) in wild strains contain that differ from the standard laboratory isolate (Leupold's 972) . I show deletions (black), duplications (red), inversions (green) and translocations (blue). SVs present in each of the 57 non-clonal strains are shown within the white horizontal bars, with strain names coloured according to their continent of origin. Tf1-type retrotransposon insertions that are present in some, but not all strains are shown at grey ticks at the tops of bars. The positions of fixed Tf1-type retrotransposon insertions are indicated on the last row (f/LTRs). Centromeres are indicated with black triangles.

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