Saccharomyces sensu stricto as a model system in ecology and evolution

Taissa Replansky

McGill University, Montreal

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Contribution of Authors

I thank Dr. Graham Bell for his guidance and support throughout my thesis. I would also like to acknowledge Dr. Vassiliki Koufopanou's extensive contribution to the second chapter of this thesis. She has graciously provided primers and protocols for the genetic identification of *sensu stricto* isolates, as well as identified the specimens collected previous to my own research but included here. Her guidance in the methods and population genetics of natural yeasts has been invaluable.

Abstract

Baker's yeast, *Saccharomyces cerevisiae*, is a well-studied model system in genetics and molecular biology. It is also a promising system for experimental ecology, evolution, and epidemiology, and is very important in the fermentation industry. The large amount of information generated by studies using this organism cannot be fully exploited until sufficient ecological data is gathered. Only when the natural environment of *S. cerevisiae* is well characterized can research using this yeast as a model system be put into context. The lack of information about the natural environment of *S. cerevisiae* is what prompted this work. First, I review the current available data on the ecology and evolution of *S. cerevisiae* and its sister species (the *sensu stricto* species complex). I then report results from fieldwork in an old growth forest. Finally, I report a community ecology experiment carried out using three naturally coexisting yeasts from this forest.

Résumé

La levure boulangère, *Saccharomyces cerevisiae* est reconnue comme un organisme modèle en génétique et en biologie moléculaire. Cette levure, reconnue comme un excellent système pour l'étude de la biologie évolutive, d'écologie expérimentale, et de l'épidemiologie, est également importante pour l'industrie de la fermentation. Par contre, puisque très peu d'informations sont disponibles quant à l'environnement et l'écologie de *S. cerevisiae*, il est impossible d'interpréter les résultats de ces études dans un contexte naturel. C'est ce vide important qui a motivé cette recherche. Premièrement, l'étendue des connaissances entourant l'écologie et l'évolution de *S. cerevisiae* ainsi que les espèces faisant partie du complexe *sensu stricto* sont examinées. Deuxièmement, des données obtenues lors d'un échantillonage en forêt sont présentées. Finalement, je présente une expérience concernant l'écologie communautaire de trois espèces de levure qui coexistent naturellement dans cette forêt.

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Introduction

The yeast *Saccharomyces cerevisiae* has been used for decades in genetic and molecular research, creating an extensive knowledge of its genome. The ease of assaying yeast in the laboratory, as well as its fast generation time, easily-manipulated sexual system, close relationship to higher eukaryotes, and extremely large ecological range also makes yeast an attractive organism for studies in ecology and evolution (Zeyl 2000). Research involving model organisms is often criticized with respect to its limited applicability to natural systems and populations, however. A successful model organism must be well characterized both in nature and in the laboratory, which is rarely seen.

In order to perform extensive experiments with *S. cerevisiae* as a model organism, its basic ecology and environment must be well characterized. This will allow us to construct a null model of yeast ecology, which will serve as a baseline for future ecological and evolutionary research. The lack of ecological information for this yeast is what prompted this work. First, I review the current knowledge of the evolution and ecology of *S. cerevisiae* and its most closely related sister species that together form the *Saccharomyces sensu stricto* complex. I then present data obtained by sampling in an old growth forest. As previous studies have identified oak trees as a major habitat (Sniegowski 2002), I examined oak trees in detail to determine the microhabitat of *sensu stricto* yeasts (bark, leaves, surrounding soil, tree interior). Trees were sampled repeatedly and at several ecologically distinct sites in order to determine any ecological or temporal variation in wild yeast growth. Details of the spatial structure of wild yeast

growth have been obtained by sampling tree bark using a grid. In order to identify other possible environments, I also sampled the canopy of several different species of tree, as well as roadside and understory plant growth. Insects were collected in order to identify possible yeast vectors. Finally, I investigated the relationship of environmental heterogeneity, biological diversity,and productivity, using naturally coexisting yeast isolates from Mont Saint Hilaire.

Saccharomyces sensu stricto as a model system for evolution and ecology.

Taissa Replansky and Graham Bell

Department of Biology, McGill University, Montreal, Canada

Corresponding author: Taissa Replansky

Department of Biology, McGill University, 1205 Dr. Penfield, Montreal, QC, H3A 1B1, Canada

E-mail address: taica.replansky@mail.mcgill.ca

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Abstract

Baker's yeast, *Saccharomyces cerevisiae*, is not only an extensively used model system in genetics and molecular biology, but is an upcoming model for research in ecology, evolution, and epidemiology. It is also of great commercial importance due to its use in various fermentation industries, and is of emerging clinical importance. As ecological information remains scarce for this species, the vast amount of data that is being generated using *S. cerevisiae* as a model system remains difficult to interpret in an evolutionary context; only when the environment of *S. cerevisiae* is fully characterized can we interpret this data in a meaningful context. We review the current knowledge of the evolution and ecology of *S. cerevisiae* and suggest future research directions that will help define its natural environment.

Introduction

Research involving microbial model systems is often criticized for its limited applicability to natural populations. One way of addressing this criticism would be to use natural populations of model organisms to conduct experiments and to estimate parameters. This is rarely done, though a handful of studies comparing laboratory and natural isolates exist (Ziemer *et al* 2000; Marchi *et al* 2004; Collins & Bell 2006). A promising system recently proposed for research in ecology and evolution is the yeast *Saccharomyces cerevisiae*, which has been used for decades in genetic and molecular research, resulting in an extensive knowledge of its genetics including a fully sequenced genome (Goffeau *et al* 1996). The ease of assaying this yeast in the laboratory, as well as its short generation time, readily manipulated sexual system, close relationship to higher eukaryotes, and extremely large ecological range further adds to its attractiveness as a model system (Zeyl 2000). Until recently, it was argued that no natural strains of *S. cerevisiae* existed (Martini 1993; Vaughan-Martini & Martini 1995). Any strain found at a natural source was thought to have escaped from a vineyard despite evidence to the contrary (Naumov *et* al 1992). If this were the case, then the association of *S. cerevisiae* with humans may have altered its geographic distribution, as well as selected for novel genetic and phenotypic properties, rendering *S. cerevisiae* unsuitable for ecological and evolutionary research (Johnson *et al* 2004). Its sister species, *S. paradoxus* and *S. cariocanus*, were suggested as a more appropriate model as they are nearly indistinguishable from *S. cereviviae* (Barnett 2000; Sweeney *et al* 2004), coexist with it (Naumov *et al* 1998; Sniegowski *et al* 2002; unpublished data), but are not associated

with humans. While recent sequencing of *S. cerevisiae* strains isolated from oak trees has demonstrated that wild *S. cerevisiae* is significantly differentiated from domesticated strains (Fay and Benadives 2005; Aa *et al* 2006), the historical relationship of wild *S. cerevisiae* with human activity will never be clearly known. As this human association will always remain a possibility with any strain of *S. cerevisiae*, it is important to develop its wild relative, *S. paradoxus*, as a parallel system for work in natural environments.

If results from experiments using *S. cerevisiae* and its siblings as a model system are to be interpretable in a natural, real-world context, their basic ecology and biogeography must be well characterized. This knowledge is necessary to provide a null model for experiments in yeast ecology and evolution, which would serve as a baseline comparison for future research where model systems are used in order to draw conclusions about natural populations. Here we review the current knowledge about the ecology and evolution of *S. cerevisiae* and its closest relatives which form the *Saccharomyces sensu stricto* species complex.

Saccharomyces genome evolution

The ancestor of *S. cerevisiae* first underwent a loss of transposons and a reduction in number of introns, perhaps due to genome-size constraints that remain to be elucidated (summarized in Dujon *et al* 2004). The appearance of centromeres in their current form

may have facilitated segmental duplication (Dujon *et al* 2004). Such duplication may have created the *HMR/HML* silent mating-type cassette pair, which appeared around the same time. This was followed by the acquisition of the *HO* endonuclease from a mobile genetic element (Butler *et al* 2004). Such changes allowed *Saccharomyces* yeasts to switch from an ancestral obligate heterothallic system to a mating type switching system, and, as such, greatly changed the sexual capacity of these yeasts. A subsequent whole genome duplication is believed to have occurred, followed by a loss of nearly 90% of the redundant genes by deletion; comparison of the *S. cerevisiae* genome to those of the pre-duplication species *K. waltii* reveals approximately 500 paralogs in a genome of 5,500 genes, which suggests that an ancestor of 5,000 genes underwent duplication, with a subsequent loss of the extra copies at 90% of the loci (Kellis *et al* 2004). Kellis *et al* (2004) show that these remaining 500 paralogs fall into three major groups, one of which has striking asymmetries in evolutionary rates between copies. Most of the paralog pairs fall into the first group (numbering 321), where there are similar rates of evolution between copies. The second group numbers around 60 pairs, and exhibits decelerated protein evolution. This group includes cytosolic ribosomal genes and protein translation genes that have hardly deviated from their original form. There is likely a strong evolutionary advantage in retaining two copies of this set of genes, which is strengthened by evidence of their homogenization by gene conversion (Langkjaer 2003; Kellis 2004). The third group is composed of 115 gene pairs in which one paralog has evolved at least 50% faster than the other. It is possible that, in a pair, the more slowly evolving copy has a function more similar to that of the pre-duplication gene, which allows us to elucidate a gene's ancestral function. The faster-evolving paralog attains a derived function, and

tends to be specialized in its localization, expression, and function (Kellis 2004). The degree of differentiation between paralog pairs can be quite extreme; for example, a small subset have similarities as low as 13% in a Clustal W alignment, and do not even hit each other in a BLAST search (Wolfe 2004).

Genome duplication has provided many new opportunities for the evolution of the *Saccharomyces* yeasts. For example, the ability to grow anaerobically may be a consequence of genome duplication, since transcription of each gene of a paralogous pair is differentially controlled by oxygen availability (Kwast *et al* 2002). The low- and high-affinity glucose systems in *S. cerevisiae* are also likely differentiated following the creation of redundant genes by duplication (Geladé *et al* 2003). This new flexibility in glucose and oxygen use may have coincided with the radiation of fruit-bearing plants, 100-200 mya (Piskur & Lankjaer 2004). It has been suggested that the ability to grow anaerobically and to produce ethanol may provide a competitive advantage against bacteria and other microorganisms, as has been observed in grape-wine ecosystems (Fleet 2003). It is likely that gene duplication also fuelled the development of a bipolar budding pattern in *Saccharomyces* yeasts; *Bud8* and *Bud9* are paralogous genes that have been shown to differentially mark the poles of yeast cells (Harkins 2001). Such differentiation allows *Saccharomyces* yeasts to bud asymmetrically (producing small daughter cells from large mothers) from either pole, in contrast to pre-duplication species such as *K. waltii*, which bud symmetrically (mitosis is delayed until the daughter cell reaches the size of the mother) from the end opposite the previous mother-daughter junction.

Radiation of the sensu stricto species complex

The *Saccharomyces sensu stricto* group was first proposed by van der Walt (1970) on the basis of morphological and physiological properties. Recent advances in molecular identification techniques have divided the *sensu stricto* complex into six species; *S. cerevisiae*, *S. paradoxus*, *S. cariocanus*, *S. bayanus*, *S. mikatae*, and *S. kudriavzevii*, with *S. pastorianus* as a sterile hybrid species that results from crosses between *S. bayanus* and *S. cerevisiae* (Naumov *et al* 2000).

Recent sequencing of several of the *sensu stricto* species estimates that *S. cerevisiae* diverged from the common ancestor of *S. paradoxus* and *S. cariocanus* around 5-10 million years ago (Mya) (Kellis *et al* 2003). *S. cariocanus* subsequently diverged from *S. paradoxus* (Goddard & Burt 1999; Naumov *et al* 2000). Estimates of divergence of *S. cerevisiae* from *S. mikatae*, *S. kudriavzevii*, and *S. bayanus* suggest that these siblings are much older (divergence from *S. cerevisiae*= 10-15Mya, 15-20Mya, and 20Mya respectively; Kellis *et al* 2003). The *sensu stricto* complex is fairly young, as is further demonstrated by its lack of pre-zygotic reproduction barriers; species within this complex can mate with each other. Such pairings result in largely sterile hybrids, however (1% fertility; Naumov 1996; Greig *et al* 2003), and interactions between the nuclear and the mitochondrial genome may also be impaired (Sulo *et al* 2003). This indicates that post-zygotic barriers are responsible for the maintenance of the species complex, and several

mechanisms that contribute to these barriers have been identified. For instance, alteration of the mismatch repair system has been shown to cause sterility in F1 hybrids of *S. cerevisiae* and *S. paradoxus* (Hunter *et al* 1996). Greig *et al* (2003) further demonstrated that when genetically isolated populations of either *S. cerevisiae* or *S. paradoxus* are crossed, they are partially sterile, but that fertility is greatly increased by deleting the mismatch repair system. Recent work by Delneri *et al* (2003) shows that chromosomal rearrangements are also involved in the maintenance of species diversity. Delneri *et al* created strains of *S. cerevisiae* whose chromosomes are collinear with the other *sensu stricto* yeasts, but otherwise identical to the wild-type strain. The crosses resulted in fertile interspecific hybrids, suggesting that chromosomal incompatibility may be largely responsible for low F1 hybrid viability. However, Greig *et al* (2002) note that yeast populations can be very large ($>10^8$), thus fertile hybrids may be found in appreciable amounts even with low F1 viability. Greig *et al* (2002) show that when these fertile F1 hybrids self-fertilize, they produce highly viable F2 offspring (84.40% fertility) that are genetically isolated for the parental species. If F2 offspring mate with each other, then the offspring (F3) viability is much lower (10.64%). Furthermore, there is an interaction between parental (F2) genotypes and F3 viability. Should the more viable of the F3 offspring autofertilize, however, then they produce offspring that are as fertile as the original *S.cerevisiae* or *S. paradoxus* parents, and this fertility is independent of the genotype of the F3 parents. Inbreeding is very common in wild yeast populations (Johnson *et al* 2004), and hybrid winemaking and brewery strains have been identified (Masneuf *et al* 1998; Groth *et al* 1999). Thus it is possible that wild *S. cerevisiae* and *S. paradoxus* can form genetically isolated and perfectly viable hybrids. If postzygotic

reproduction barriers can be so easily overcome then ecological barriers may play an important role in the maintenance of diversity of the *sensu stricto* yeasts. For example, lower temperatures favour the growth of *S. paradoxus* and *S. bayanus* (*S. bayanus* can tolerate temperatures as low as 4°C) over *S. cerevisiae* (Giudici *et al* 1998; Greig *et al* 2002), and *S. cerevisiae* has a faster growth rate than *S. paradoxus* at higher temperatures (37°C; Sweeney *et al* 2004).

Ecology, genetic structure and dispersal

One of the first comprehensive descriptions of a natural *Saccharomyces* environment is given by Yoneyama (1957), who successfully isolated *S. cerevisiae* var. *tetrasporus* (= *S. paradoxus*) year-round from the bark and surrounding soil of *Quercus* species, as well as from soil surrounding *Pinus* species. He found that total *S. paradoxus* abundance is equal between bark and soil, and that *S. paradoxus* abundance is greater on the northern face of trees, suggesting a preference for shady, wet environments. Examinations of the cross-section of bark samples revealed that yeasts were found only on the outermost layer of the bark. Other studies in Japan found *S. paradoxus* growing on the bark of many different tree genera (Kodama 1974), and S. *cerevisiae* were found in high abundance on decayed leaves and dung, while isolates from mushrooms and flowers were rare (Banno & Mikata 1981).

Knowledge of *sensu stricto* yeast habitats has grown very little in the intervening years; recent studies have confirmed *Quercus* species as a dominant habitat of *S. cerevisiae* and *S. paradoxus* (Naumov *et al* 1998; Sniegowski *et al* 2002). *S. cariocanus* has been isolated in a Canadian woodland (Replansky and Bell, unpublished). Repeated sampling revealed that *Saccharomyces sensu stricto* remain at a low and constant abundance throughout the summer season. In addition to *Quercus* species, they are found on other broad leaf trees such as *Acer saccharum* and *Fagus grandifolia*, while understory, meadow and roadside plants do not appear to be habitats. More specifically, *sensu stricto* yeasts are most abundant in soil surrounding broad leaf trees, and have also been isolated from their canopy, though rarely. Colony growth on oak bark is structured vertically; yeast may grow preferentially in the cooler, more protected microclimate of bark troughs, or be washed down by distinct rivulets of water during rainfall (Replansky and Bell, unpublished). Aside from this recent work, habitat descriptions for the *sensu stricto* yeasts are rare; *S. cariocanus* has been isolated from *Drosophila* spp. in Brazil (Morais *et al* 1992; characterized in Naumov *et al* 1995a), and the two Japanese strains have been found on decayed leaf and soil (characterized in Naumov *et al* 1995b). *S. bayanus* has been isolated from fruiting bodies of the *Amanita citrina* mushroom, exudates of the broad-leaf trees *Carpinus betulus* and *Ulmus pumila*, as well as from *Mesophylax adopterus* and *Drosophila* spp. It is commonly used in low-temperature viniculture, and its niche is likely determined by both its cryophilic nature and its ability to ferment melibiose (Naumov *et al* 2003). Given how little is known about where wild yeast live, it is possible that there are many more species within the *sensu stricto* complex that have yet to be described. An incomplete species group has implications for any research that

uses this group as a model system, such as studies of the mechanisms of speciation, ecological interactions, microbial biogeography, and so on.

The lifecycle of the *Saccharomyces* yeasts is well documented in the laboratory (Herskowitz 1988). Yeast normally grows as a diploid that reproduces clonally, but will undergo meiosis in response to nitrogen starvation (a common cue for gamete production in many microorganisms). Starved cells produce asci containing four haploid spores, two of each mating-type. Opposite mating types usually mate within the ascus upon germination, but spores can reproduce mitotically as haploids as well. Haploid spores can either outcross or undergo a mating-type switch, which allows them to mate with their clonemates. There is a common assumption that yeast growth in nature is similar to that in the lab, and especially that the diploid phase predominates. This assumption has never been directly tested, because the growth of natural isolates cannot be directly observed in nature; they are collected by lengthy isolation procedures. However, Johnson *et al* (2004) found evidence that all three modes of reproduction (outcrossing, clonal, inbreeding) occur in a natural population of *S. paradoxus* ranging over an area of 10 km^2. Repeated isolation of the same genotype gave evidence of clonal growth. High levels of homozygosity, indicative of high levels of inbreeding, were also found, with only a few rare instances of outcrossing. As expected, auxotrophic mutants were found at extremely low frequencies; only one case was documented. Similarly, natural vineyard populations of *S. cerevisiae* are thought to be predominantly diploid, and carry no auxotrophic mutations (Mortimer *et al* 2000). Some notable differences between these species may have arisen due to human association, however. Outcrossing is much more common in

vineyards, as indicated by moderate levels of heterozygosity (10%; Mortimer *et al* 2000). The majority of vineyard strains are also homozygous for the homothallism gene (*HO/HO*) (Mortimer *et al* 1994).

Yeast dispersal mechanisms both in vineyard and non-vineyard environments are poorly known. Phaff (1986) observed that *Saccharomyces* yeasts were rare outside of the fermentation industry. Despite this, the *Saccharomyces* genus was found to be the most widely represented of the yeasts isolated from intestinal tracts of wild *Drosophila* species, and included one of the *sensu stricto* species; *S. cerevisiae* var. *tetrasporus* (= *S. paradoxus*) (Phaff *et al* 1956). Insect-mediated dispersal of *Saccharomyces* also receives support from evidence in vineyard environments, where yeast is present on the grapes for only a few weeks out of the year, and cannot be found a few weeks prior to, or after harvest (Kunkee & Amerine 1970). As grapes become nearly ripe, insects begin to feed on damaged berries (Mortimer & Polsinelli 1999). These insects may be inoculating the grapes with yeasts and other microorganisms, which they may be transporting from their nests. Recent work has reinforced the role of *Drosophila* spp. in *Saccharomyces* ecology and dispersal. Reuter *et al* (2007) demonstrated that outcrossing rates increase 10-fold when *S. cerevisiae* spores pass through the intestinal tract of *D. melanogaster*. The ascus protects the spores during their passage through the insect's digestive tract; only asci, not vegetative yeast cells, were shown to survive after excretion. Asci are partly digested by enzymes during this passage, which facilitates outcrossing by liberating spores from their tetrad partners (Reuter *et al* 2007). As yeast populations have been found to be clonal over the kilometer scale (Johnson *et al* 2004), it is likely that insects facilitate long

distance dispersal. The increased the rate of outcrossing mediated by insect dispersal may thus play an adaptive role for the transmitted yeast population; increasing the amount of genetic variation in transmitted spores increases their chances of survival in more distant (diverse) habitats (Reuter et al 2007). While the role of *Drosophila* spp. in dispersing *sensu stricto* yeasts appears pivotal, studies that surveyed *Drosophila* feeding sites failed to detect a natural *Saccharomyces* environment; yeasts isolated from the crop of *Drosophila* species differed repeatedly and markedly from yeasts found on suspected feeding sources of adult flies (Shehata *et al* 1955; Carson *et al* 1956). Studies of bark beetles (genus *Dendroctonus* and *Ips*), which deposit yeasts inside tree bark while creating egg galleries, also found that these insects vector yeast flora that is completely unrelated to that found in the crops of *Drosophila* species (Shifrine and Phaff 1955). Fungus-feeding *Drosophila* species, as well as Hymenopterans, were found to be vectors of filamentous fungi rather than of yeasts (Gilbert 1980).

A limited number of surveys of the population genetic structure of the *sensu stricto* species have been undertaken, mostly involving *S. paradoxus*. Genetic similarity of isolates was found to decrease with increasing distance both within and between trees for a European population of this species. However, populations of *S. paradoxus* within Europe are well mixed, and have been shown to have high levels of gene flow acting on a kilometer scale. The European populations differ from Far East strains by 1.5% sequence divergence, and even more so from Canadian strains (5%), suggesting at least three independent lineages of *S. paradoxus* on a worldwide scale (Koufopanou *et al*, 2006).

S. cerevisiae, however, does not display such divergence of strains, which implies a more recent common ancestor than for *S. paradoxus* (Sniegowski *et al* 2002), or more thorough population mixing. At the global scale, *S. cerevisiae* and *S. paradoxus* are ubiquitous, *S. mikatae* and *S. kudriavzevii* are endemic to Japan, *S. bayanus* has been isolated in Europe and the Far East, and *S. cariocanus* has not been found outside of the Americas. As is expected from this distribution and from species divergence dates, *S. cerevisiae*, *S. paradoxus*, and *S. cariocanus* are more closely related to each other than they are to the two geographically isolated Japanese strains, and *S. bayanus* is the most divergent species (Naumov *et al* 2000). In vineyard populations, diversity is surprisingly high (reviewed in Pennisi, 2005). This level of diversity may vary with location; higher variation was found in certain regions of Italy (Mortimer 1994; Cavalieri 1998) as compared to a more limited number of ubiquitous strains found in certain French vineyards (Versavaud *et al* 1995). While such differences in diversity could be the result of selection in or adaptation to different environments, it is important to note that the molecular identification techniques used differ between these two studies and may bias this result. No relationship between distance and genetic similarity has yet been found for vineyard populations, but as diversity studies of wine yeasts continue to increase in number, it should become possible to describe their large-scale genetic structure, should one exist.

Domestication

Since the production of wine does not require inoculation with yeast, the domestication of yeast likely began with winemaking as opposed to beer or bread making (McGovern *et al* 1996). Fay and Benavides (2005) show that it is likely that yeast was first domesticated in Africa, and that sake and wine yeasts diverged around 11,900 years ago. This date of domestication coincides with the earliest evidence of winemaking 9,000 years ago in China (McGovern *et al* 2004). Based on analysis of DNA found in ancient wine containers, yeast has been used in winemaking in Egypt since 3150 BC (Cavalieri *et al* 2003), and further molecular analysis of jars finds evidence of wine-making in the Near-East as early as 5400-5000 BC, around the time when the first permanent human settlements were though to occur (McGovern et al 1996). Subsequent divergence of strains within the vineyard and sake groups is though to have taken place 2,700 and 3,800 years ago respectively (Fay & Benavides 2005). Domestic yeast is important for a variety of industries, and has led to selection for baking (Rose & Vijayalakshmi 1993; Bell *et al* 2001), brewing (Hammond 1993), and wine-making (Kunkee & Bisson 1993). This has resulted in specialized strains of *S. cerevisiae*, which are not readily interchangeable. Several strains have also been selected for laboratory use, where S288c is the progenitor of many of the mutant and segregant strains currently used to study yeast genetics (Mortimer & Johnston 1986). It was derived from EM93, a strain that was isolated from a rotting fig in California (Mortimer & Johnston 1986). That EM93 is heterothallic, while a large majority of natural strains (70%) are homothallic (Mortimer 2000), illustrates the importance of drawing distinctions between assays using laboratory versus natural strains.

Ecology of killer yeasts.

S. cerevisiae faces intense resource competition from other yeasts and microflora, as well as bacteria. This competition may be intensified in high-sugar environments (fruit and vineyard environments). Yeast species that are commonly found in these types of habitats display a very interesting characteristic; they produce toxins (in the form of small extracellular proteins or glycoproteins) that are lethal to sensitive yeasts and bacteria, while themselves remaining immune. Toxin-producing strains are known as killer yeasts, strains susceptible to the toxin are described as sensitive, while neutral strains neither produce nor are affected by toxins. While the phenomenon was first documented in *S. cerevisiae* (Bevan & Makower 1963), it has since been found in a limited number of other yeasts. Killer yeasts have been classified into groups, depending on the spectrum of killing activity or cross-reactivity of the killer toxin. *S. cerevisiae* is the sole member of the *sensu stricto* group in which killer activity has been documented, and its activity has been grouped into five classes: K1, K2, K3, KT28, and K3GR1 (Woods *et al* 1974; Young & Yagiu 1978; Extremera *et al* 1982; Pfeiffer & Radler 1982). The killer toxin is produced by double-stranded virus-like particles in the cytoplasm (as in the case of *S. cerevisiae*), or plasmids. The peptides (toxins) created by virus-like particles kill sensitive cells by damage to their plasma membrane or by disruption of the membrane's permeability, while strains secreting plasmid-made proteins bring about G1 arrest in sensitive cells (Starmer *et al* 1987). It has been suggested that the killer phenomenon

provides a competitive advantage against bacteria and other yeasts by preventing competitors from gaining access to resources (Ganter & Starmer 1992; Starmer *et al* 1992). Killer strains have been shown to exclude sensitive strains both in laboratory conditions (Bussey *et al* 1988) and in industrial culture conditions (Hammond & Eckersley 1984). Marked environmental differences between vineyard and non-vineyard environments have led to a difference in killer dynamics in these two habitats.

Killer activity in non-vineyard populations has been well studied by Starmer *et al* (1987). They found that killer activity was limited to a few species (notably from the genus *Pichia*, but excluding *S. cerevisiae*) while sensitivity was much more widespread. Immunity to all toxin classes was rare. Most communities support only one killer species; distinct killer strains were isolated more frequently from different localities than from within the same habitat. Appearance of killer yeasts is seasonal, with a marked increase during cooler periods (Radler *et al* 1985; Starmer *et al* 1987). The incidence of killer yeasts has been linked to specific environments and conditions, suggesting that it is an important feature of some natural habitats. Low incidences of killer activity (11/140 isolates) have been documented on the slime flux of oak (*Q. emoryii*) trees (Starmer *et al* 1987), however none of the *S. cerevisiae* strains isolated exhibited killer activity. Most yeasts, including *S. cerevisiae*, produce toxins that are stable at low pH (Young & Yagiu 1978). Low pH is common in fruit environments, and killer activity is seen at highest frequencies in natural fruit communities (30/112 isolates), where resistant types are more common than susceptibles. None of the killer strains were *Saccharomyces* yeasts, however (Starmer *et al* 1987). Killer toxins are most often produced in exponentially

growing populations, which suggests that the killer ability confers an advantage during early stages of population growth. This may be ideal for species that colonize environments that are undergoing early stages of necrosis, such as fruit, where abundant resources and competition between exponentially growing populations is likely to favour killer types.

Yeast community composition has been shown to be affected by the presence of a killer strain: killer strains reduce the overall population densities of the susceptible yeasts in the community, while themselves growing to higher densities as compared to communities where the non-killer counterpart was present (Ganter & Starmer 1992). There is evidence that variation in killer activity is uniformly distributed within localities, regions, and continents (Starmer *et al* 1992). Killer phenotype is largely determined by the particular habitat of a strain or species rather than by phylogeny, and sensitivity to a particular killer type is correlated with the probability of interaction with that strain (Ganter & Starmer 1992).

S. cerevisiae strains are largely absent from studies of non-vineyard killer ecology. Perphaps the phenomenon is rare in this species, however it is likely that current screening procedures for killer activity underestimate the frequency of killer strains (*Saccharomyces* and others) in nature. A killer strain could fail to be identified as such if the correct susceptible strain is not used, or if the strains killer activity occurs at an unusual pH. Conversely, classification of susceptible strains may also be underestimating their true numbers; the number of sensitive species affected is

proportional to the strength of killer activity, thus the results from sensitivity tests depend on strength of the killer strains used (Magliani *et al* 1997).

Microbial dynamics play a very important role in the fermentation industry, as changes in composition and densities of yeast and bacterial cultures in fermentation practices can drastically alter the flavour of fermented beverages (Fleet 2003). Much more information on the killer phenomenon in *S. cerevisiae* is available in wine environments, perhaps because of this marked economic and cultural interest. Several differences exist between killer systems in vineyard and non-vineyard environments. First, the total incidence of *S. cerevisiae* is much higher, as is the frequency of *S. cerevisiae* killer strains (from 1.9-95.6% of the population; (Vagnoli *et al* 1993). The diversity of killer types is much lower, and predominantly of the K2 type (Vagnoli *et al* 1993). Low pH values (3 to 3.5) are encountered in fermentations, and these minimize the activity of toxins other than the *S. cerevisiae* K2 type. K2 types are thus most commonly isolated from vineyards (Magliani *et al* 1997).

Heterogeneous populations of both killer and sensitive strains of *S. cerevisiae* coexist within the same fermentation and winery (Vagnoli *et al* 1993). Killer abundance shows a temporal pattern, like that in non-vineyard communities; killer frequencies are initially low but increase during fermentation and with successive vintage periods (Vagnoli *et al* 1993). Higher frequencies of killer strains in vineyard environments can be expected, because the total density of *S. cerevisiae* (killer and non-killer) is much higher than in other environments, and thus there would be a greater advantage to killing conspecifics.

The distribution of killer phenomenon also appears to be worldwide; it has been isolated from vineyards of many different countries (Vagnoli *et al* 1993; Vazquez & Toro 1994; Versavaud *et al* 1995; daSilva 1996).

As killer strains have a potentially important role for control of microbial contaminants in the fermentation industry, much interest exists for what initial ratio of killer to susceptible yeast cells is needed for killers to predominate. One experimental study demonstrated that killer yeast predominated only when the ratio of killer to susceptible cells exceeded 1:2. Susceptible strains were never completely eliminated, even at the highest ratio of killer to susceptible strains tested (2:1, Petering *et al* 1991). Killer activity has been found at ratios as low as 1:500 (reviewed in Magliani *et al* 1997). It is possible that killer and sensitive types may coexist at such low ratios, as no difference was found between pure culture growth rates of the killer and sensitive strains in one assay (Petering *et al* 1991). Other studies have shown a lack of killer activity at ratios of 1:10 and 1:7, however (Heard & Fleet 1987). Such large discrepancies may be due to differences in strength of killer activity between the different strains as well as to differences in culture media and growth conditions used between studies.

S. cerevisiae as a human pathogen.

Very few people think of baker's yeast as a human pathogen. It is uncertain whether it exists as a true commensal of the human digestive system, or if its colonization is simply transient, however there is strong evidence of *S. cerevisiae* as an emerging human pathogen, albeit of low virulence (Nyirjesy *et al* 1995; McCullough *et al* 1998; Murphy & Kavanagh1999). Very little is known about the epidemiology of *S. cerevisiae*, although clinical strains are very diverse (Zerva *et al* 1996; Hennequin *et al* 2000; Malgoire *et al* 2005), and are though to exist as diploids (García-Martos *et al* 1996). Clinical cases involving *S. cerevisiae* usually involve chronic vaginal infections in women, where *S. cerevisiae* was responsible for less than 5% of the cases (Nyirjesy *et al* 1995). *S. cerevisiae* may also cause a wide array of diseases in immunocompromized patients, including pulmonary, systemic, and blood infections (Eng *et al* 1984; Aucott *et al* 1990), and is responsible for 3.6% of all fungemias (Piarroux *et al* 1999). Furthermore, the use of *S. boulardii* (a strain of *S. cerevisiae*) as an oral probiotic in Europe has been linked to yeast sepsis (Piarroux *et al* 1999; Lherm *et al* 2002). *S. cerevisiae* is also a promising vaccine delivery vector (Stubbs *et al* 2001), and ß-glucan, a predominant cell wall molecule in yeasts, has potential clinical applications as an immunostimulatory molecule (Sutherland 1998). Clinical isolates of *S. cerevisiae* are thus likely to be important both because of the potential use of yeast in therapy and because it may be an emerging pathogen.

Clinical isolates of *S. cerevisiae* have a higher thermal tolerance (ability to grow at 41°C) than laboratory strains, a characteristic that is thought necessary for their survival in mammalian hosts (McCusker *et al* 1994). This tolerance is linked to a mutation in the

gene SSD1. The SSD1 mutation also alters the yeast cell wall composition in a manner that increases flocculence and hydrophobicity, properties that have been positively associated with increased virulence in the well-known human pathogen *Candida albicans* (Wheeler *et al* 2003). These properties may also aid in the formation of yeast biofilms (complex, surface-attached microbial aggregates). Biofilms have been found on inert, plastic surfaces such as catheters, and increase resistance to antimicrobial treatment in bacteria (Costerton *et al* 1999; O'Toole *et al* 2000). Colonization of the digestive system may occur through use of *S. cerevisiae* in the human diet (Nyirjesy *et al* 1995; McCullough *et al* 1998; Hennequin *et al* 2001). Increased adhesive properties may play important roles in transmission of clinical yeasts from patient to patient, and may be especially important for *S. boulardii*, which is increasingly implicated in blood infections and whose transmission is though to occur by improper handling of biotherapeutic *S. boulardii* treatments (Hennequin *et al* 2000). Clinical isolates of *S. cerevisiae* have also developed the ability to adhere to epithelial (buccal) cells (Murphy & Kavanagh 2001), and have higher incidences of invasive, pseudohyphal morphologies than do laboratory yeasts (McCusker *et al* 1994). Furthermore, our innate immune system recognizes specific structures of fungal cell walls (Underhill & Ozinsky 2002; Brown *et al* 2002), thus any changes to these properties are likely to increase fungal virulence (Wheeler *et al* 2003).

There are limited tests evaluating the resistance of clinical *S. cerevisiae* to antifungal drugs. Several studies have noted high resistance in clinical yeast strains to azole fungicides (Barchiesi *et al* 1998; Sobel *et al* 1993). The minimum inhibitory

concentration (MIC) for many fungicides from this family were more than 10-fold higher than those of *C. albicans*, and clinical strains were resistant to two widely-used fungicides, fluconazole and itraconazole (Zerva *et al* 1996; Sobel *et al* 1993). While clinical *S. cerevisiae* strains remain sensitive to several other treatments, their resistance to a group of widely used antifungal drugs may promote the emergence of *S. cerevisiae* as an opportunistic human pathogen, especially in immunocompromised patients (Zerva *et al* 1996; Barchiesi 1998). Clinical *S. cerevisiae* is increasingly used as an important model system for fungal survival in mammalian hosts, both because of its close relationship to *C. albicans*, and for its ability to survive in immunocopromised mouse hosts (Goldstein & McCusker 2001; Wheeler *et al* 2003).

Discussion

The rate of increase of genetic and molecular knowledge currently exceeds by far the rate at which we are able to interpret it in an ecologically meaningful context. *Saccharomyces cerevisiae*, the best-characterized organism in genetics and molecular biology, is no exception. Given this yeast's great importance in industrial and medical applications, as well as its underdeveloped potential as a model system in ecology, evolution, and epidemiology, it is unfortunate that so little is known about its natural history. The vast amount of molecular and genetic information that is being gathered cannot be put into an ecological or evolutionary context without this knowledge, and

consequently remains of limited use. Fortunately, increased awareness of *S. cerevisiae*'s undervalued potential as a model system is stimulating renewed work on the ecology and biogeography of the *sensu stricto* species complex. Similarly, efforts to characterize natural and industrial yeast strains are increasing as ever more refined technologies allow industries to fine tune fermentation dynamics and processes. These efforts will allow scientists to engineer commercial strains to be used as starter cultures for fermentation, and increase control of community composition during the fermentation process. Increased information on natural vineyard and non-vineyard populations would also allow comparative studies on these two environments to be performed, which could generate interesting data on the effects of human association on yeasts. Yeast can also be used as a model system in epidemiology because it is an occasional human pathogen, and thus would benefit from increased efforts in characterization of clinical strains.

Future studies on *Saccharomyces* ecology need to be carefully thought out. Exploration of habitats other than oak trees are necessary, as there is no reason to assume that these trees are the sole (or indeed primary) habitat of *sensu stricto* yeasts. Repeated sampling of habitats, new and old, will allow us to distinguish between environments where yeasts can grow and where they merely survive. This data will also allow us to discern the frequency of *sensu stricto* species in nature, and detect whether there are ecological differences between what are otherwise indistinguishable species. Isolation of other yeasts and bacteria that coexist with *Saccharomyces* would provide natural microbial communities that could be used in a variety of community and microbial ecology studies. Information on the seasonal abundance and dispersal mechanisms of yeast would give a

more dynamic picture of natural yeast survival. More studies of population genetic structure at various spatial scales are also needed; not only will these help in defining the evolutionary history and radiation of the *sensu stricto* clade, but they will benefit microbial ecology in general by showing to what extent microbes follow distinct biogeographical and environmental patterns. Systematically testing new isolates for killer activity and susceptibility would determine how widespread this phenomenon is in nature, and would define what habitats favour communities with killer strains.

The *Saccharomyces sensu stricto* yeasts can be used as a general microbial model system once we have investigated their ecology, distribution and abundance more thoroughly. The wealth of genetic knowledge available for baker's yeast presents a unique opportunity where laboratory research can be effectively related to natural field studies and vise versa. The ability to contextualize genetic, ecological and evolutionary research, as well as the importance of the *Saccaromyces sensu stricto* complex to so many fields, is perhaps what makes it the most exciting model system to work with.

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While research into the ecology of the *sensu stricto* yeasts appears to be increasing, there is an obvious paucity in data for natural, non-vineyard environments. Little is known of the seasonal abundance and dispersal of the *Saccharomyces sensu stricto* yeasts, or how their survival changes depending on forest conditions. A principal habitat for these yeasts has yet to be definitively stated and characterized. In light of its increased use as a model system in ecology and evolution, this lack of information prompted me to investigate the occurrence and growth of natural yeast populations in a natural, old-growth forest.

Ecology of *Saccharomyces sensu stricto* in an old-growth Canadian woodland.

Taissa Replansky and Graham Bell

Department of Biology, McGill University, Montreal, Canada

Corresponding Author: Taissa Replansky

Department of Biology, McGill University, 1205 ave Dr. Penfield, Montreal, QC,

H3A 1B1 Canada

E-mail address: taica.replansky@mail.mcgill.ca

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Abstract

The yeast *Saccharomyces cerevisiae* is increasingly used as a model system in a wide range of fields. However, if results from experiments using *S. cerevisiae* and its siblings as a model system are to be interpretable in a natural, real-world context, their basic ecology and biogeography must be well characterized. The scarcity of ecological information about the *Saccharomyces sensu stricto* complex provided us with the motivation for this study, whose goal was to further define their natural habitats by sampling in an old growth forest. The association of *sensu stricto* yeasts with oak, *Q. rubra*, was verified and refined; these yeasts were most abundant in surrounding soil, half as abundant on bark, and a quarter as abundant on leaves. None were found to inhabit a wide range of understory, roadside and meadow plants, and none were isolated from insects. Total abundance of these yeasts remained low and constant throughout the entire sampling season, and did not vary between ecologically distinct sites. *Sensu stricto* yeasts grow in a vertical, clustered pattern on the bark of *Q. rubra*. A novel environment for these yeasts was identified; yeasts were isolated from the leaves of *Q. rubra*, *Q. saccharum*, *F. grandifolia*, and *A. pensylvaticum*. We speculate that the leaf surface may be the main site for the growth of *Saccharomyces sensu stricto* at our study site.

Introduction

Research into microbial population genetics, ecology, and biogeographical distribution has faced many obstacles. Studies of the microbial kingdom could not even begin until relatively recently, with the invention of the microscope. Early identification techniques were hampered by the fact that metabolic profiles and phenotypic traits did not accurately represent evolutionary relationships among microbes, and even more so by the fact that many microbial species simply cannot be cultivated in the lab (Pace, 1997). With the development of molecular identification techniques, these problems have been greatly reduced. The development of microbial ecology has been strongly influenced by the "everything is everywhere" hypothesis, first advanced by Baas-Becking (Quispel, 1998), and later championed by Fenchel and Finlay (Finlay, 2002; Fenchel & Finlay, 2004). Debate and criticism of Baas-Becking's hypothesis (Cho & Tiedje, 2000; Coleman, 2002; Whitaker *et al*, 2003) has led to a renewed interest in microbial ecology and biogeography. This effort is long overdue, considering the importance of microbial ecology in many fields such as medicine, epidemiology, agriculture, and the fermentation industry. Research involving microbial model systems is also in need of ecological information, as the copious genetic, molecular, and experimental data produced in recent years greatly exceeds information about their ecology. This is an unfortunate shortcoming, as genomic and proteomic data cannot be interpreted in evolutionary terms without knowing where and how an organism grows. Although it is one of the most extensively studied microbial eukaryotes, *Saccharomyces cerevisiae* is no exception to this rule. A member of the *sensu stricto* species complex (which includes *S. cariocanus*,

S. paradoxus, *S. bayanus*, *S. mikatae*, and *S. kudriavzevii*), *S. cerevisiae* and its sister species are increasingly recognized as a powerful model system for experimental ecology and evolution (Zeyl, 2000). Unfortunately, these experiments will remain difficult to interpret until more detailed ecological information becomes available.

The few descriptions of *Saccharomyces sensu stricto* ecology that we have come mostly from research before the mid-1980s. Natural isolates of *sensu stricto* yeasts have often been reported, albeit at low abundance (Phaff, 1986). They have been repeatedly isolated from the bark and surrounding soil of oak trees (Yoneyama, 1957; Naumov *et al*, 1998; Sniegowski *et al*, 2002), but have also been found in association with other trees (Yoneyama, 1957; Kodama, 1974). *Saccharomyces sensu stricto* has also been found at high abundance on decayed leaves and dung, but have seldom been isolated from flowers and mushrooms (Banno & Mikata, 1981). The mechanisms of *Saccharomyces* dispersal are still poorly known. Many studies have focused on *Drosophila* and beetles as possible vectors, as these insects are important for the dispersal of other yeast microflora (Starmer *et al*, 1980). However, while high levels of *S. paradoxus*, sister species to *S. cerevisiae*, have indeed been found in the intestinal tracts of *Drosophila* species (Phaff *et al*, 1956), these yeasts consistently fail to be found at suspected feeding sites of adult *Drosophila* (Shehata *et al*, 1955; Carson *et al*, 1956). Beetles do not vector these yeasts (Shifrine & Phaff, 1955). Recent work has confirmed the role of *Drosophila* spp. in yeast dispersal. Reuter *et al*, (2007) demonstrated that outcrossing rates increase 10-fold when *S. cerevisiae* spores pass through the intestinal tract of *D. melanogaster*. The ascus protects the spores during their passage through the insect's digestive tract; only asci, not

vegetative yeast cells, were shown to survive after passage through the gut. Asci are partly digested by enzymes during this passage, which facilitates outcrossing by liberating spores from their tetrad partners. As yeast populations have been found to be clonal at a scale of kilometres (Johnson *et al*, 2004), it is likely that insects facilitate long distance dispersal. The increased the rate of outcrossing mediated by insect dispersal may thus play an adaptive role for the transmitted yeast population; increasing the amount of genetic variation may increase the probability that some spores survive in more distant habitats (Reuter *et al*, 2007). Encouragingly, a picture of global *Saccharomyces* distribution is also growing; *S. cerevisiae* and *S. paradoxus* are found worldwide, *S. cariocanus* is found in North and South America, and *S. kudriavzevii* and S. *mikatae* are endemic to Japan (reviewed in Naumov *et al*, 2000). Concurrently, studies of natural populations of wine yeasts are also increasing, and hint at high levels of yeast diversity (reviewed in Pennisi, 2005).

The lack of ecological information about the *Saccharomyces sensu stricto* complex provided us with the motivation for this study, whose goal was to characterize their natural habitats in more detail by sampling in an old growth forest. As previous work (Yoneyama, 1957; Naumov *et al*, 1998; Sniegowski, 2002) suggested that oak trees are a natural yeast habitat, we examined red oak (*Quercus rubra*) trees in detail to identify the precise habitat (bark, leaves, soil, or tree interior) of the yeasts. Taken together, the habitat description and growth rate comparisons allow us to begin to evaluate how phenotypes described in the lab may correspond to those seen in natural populations.

Methods

Field site information – Mont Saint Hilaire

Mont Saint Hilaire ((45°N, 75°W; 415 m) is the least disturbed of the Monteregian Hills found in the St. Lawrence River Valley, Quebec, Canada. Situated approximately 40 km south-east of Montreal, this nature reserve protects 10 km^2 of the only remaining old-growth forests in the region, which is otherwise heavily disturbed by tourism, agriculture, and real estate development. The reserve is divided into a strict preservation sector ($4.5km^2$) and a $5.5km^2$ area that is open to the public. It has been designated as a world heritage site under the UNESCO Biosphere Reserve Program since 1978. The forest is transitional between northern boreal forests and southern deciduous forests, and is dominated by stands of sugar maple (*Acer saccharum)* and American beech (*Fagus grandifolia*). The reserve is bordered by extensive apple orchards, which may contribute to the yeast flora found in the forest.

Seasonal sampling

There are several locations at Mont Saint Hilaire where red oaks (*Quercus rubra*) can be found in abundance, and two such sites, chosen on the basis of distinctive environmental characteristics, were repeatedly sampled at two-week intervals, starting 21 June 2005 and ending 18 September 2005, for a total of five sampling dates (see Figure 1). The Pré site is situated near a meadow created by the removal of an orchard, which is now overgrown with wildflowers and roadside flora. The Sunrise site is an oak stand that is undisturbed

by human activity and lies slightly above the base of one of Mont Saint Hilaire's peaks. A third site, Dieppe, was sampled only twice, 29 July and 18 September 2005. The Dieppe site is located on the top of one of Mont Saint Hilaire's peaks (altitude of 381m). The oak trees at this site are dwarfed and surrounded by flora that is typical of nutrient-poor environments, with red pine (*Pinus resinosa*) and members of the Heath family (Ericaceae) predominating.

For each site, the same ten trees were repeatedly sampled for soil, inner bark, outer bark, and leaves. Four replicates of each sample type were collected. Soil samples were collected at four different points around the foot of each tree, using a metal scoop that was sterilized with ethanol between the collection of each sample. Outer bark was sampled by applying pieces of sterile Blue Tac™ (plastic strips) onto the tree bark and then removing these strips using sterile tweezers. Inner bark samples were collected at four regular intervals around each tree, using a Suunto Increment Hammer (Forestry Suppliers Inc, Jackson, MS). All samples were stored in sterile eppendorf tubes. Leaves were harvested using a ladder and extendable hedge trimmers, and were stored separately in sterile test tubes. Gloves were worn and sprayed with ethanol before handling each leaf.

Non-seasonal sampling

Table 1 provides an overview of all samples collected, excluding the seasonal sampling described above.

Spatial sampling

Blue Tac was applied to the bark of two oak trees, one from the Pré site and one from the Sunrise site, in a grid of 15 x 20 tac pieces (80 x 140 cm, starting 30 cm from the ground, Tac pieces placed approximately 1cm apart). The Pré tree was sampled on its northwestern side, while the Sunrise tree was sampled on the eastern side. Each piece of Tac was applied and removed using sterile tweezers, and incubated in sterile eppendorf tubes.

Core sampling

Cores were sampled several times in the spring, summer, and fall. A total of 100 cores, each from a different tree, were collected. The bark layer was removed by drilling using a wide bit, after which the area was sterilized by ethanol. Cores were removed using a Haglof Increment Borer (Forestry Suppliers Inc; Jackson, MS) and placed in sterile test tubes. The increment borer was sterilized with ethanol between each use. Core samples were cut up into smaller pieces in the laboratory, and incubated in eppendorf tubes.

Canopy, meadow, insect, and roadside and understory plant sampling

The upper canopy of two trees from each of three species, *Q. rubra*, *A. saccharum*, and *F. grandifolia* (25, 24, and 20 leaves per species, respectively) was sampled from a canopy crane, using the sterile methods described above. In addition to the seasonal sampling of leaves, the lower canopy of these three tree species was intensively sampled (100 leaves for each species, each leaf from a different tree). Sixty insect samples (mostly Coleoptera and Diptera) and 40 meadow plant samples were collected from the Pré area. Eighty

understory/roadside plants were sampled throughout Mont Saint Hilaire (Table 1). All leaf, meadow, roadside, understory, and insect samples were handled in the same manner as canopy samples.

Isolation and identification

Samples were incubated in Paradoxus Isolation Medium 1 (PIM 1): 3g yeast extract, 3g malt extract, 5g peptone, 10g sucrose, 76mL EtOH, 1mg chloramphenicol, and 1mL of 1-N HCl per litre (Sniegowski *et al* 2002). After ten days of incubation at 28°C, a 10μL aliquot of each sample was spread onto selective agar plates (PIM 2), containing 15g of agar, 10g methyl-α-D-glucopyranoside, 6.7g yeast nitrogen base with amino acids and ammonium sulfate (Difco, USA), and 4mL 1-M HCl per litre (Sniegowski, 2002). Plates were incubated for several days, and examined for glossy white yeast colonies. Such colonies were streaked onto YPD plates (Rose *et al* 1990), from which one clone per sample was picked and re-streaked on the same medium, grown to stationary phase, and frozen at -80°C for long-term storage. Species that resembled *Saccharomyces* were identified by growth and visual inspection on YPD plates. The internal transcribed spacer region (ITS1-5.8rRNA-ITS2) was amplified by PCR using primers ITS1 and ITS4 (White *et al*, 1990) for all isolates resembling *Saccharomyces* species. This region is highly conserved within eukaryotes, and yields a characteristic 800bp region for the *Saccharomyces* genus. Isolates with ITS fragments matching that of a control *S. cerevisiae* strain were sequenced at the Cen9 region in order to distinguish between *Saccharomyces* species. Centromere primer sequences were obtained from Vassiliki Koufopanou (Imperial College, UK). Centromere sequences were edited in Bioedit

(Hall, 1998) along with sequences for *S. cerevisiae*, *S. paradoxus*, and *S. cariocanus* for species identification. The sequence alignment and neighbour-joining tree was generated in MEGA v.4 (Tamura *et al*, 2004) to compare the relationship of isolated strains. *S. cerevisiae* was designated as the outgroup for the neighbour-joining tree.

Data Analysis

The overall pattern of yeast grow on bark was first analyzed by comparing the variance of rows and columns of the grid separately to a null distribution where yeasts are equally abundant in each row and column (variance ratio test). Clustering of *sensu stricto* isolates was analyzed by assigning co-ordinates to the grid locations that yielded positive *sensu stricto* isolates, and then calculating Euclidean distances for each positive isolate to all of its neighbours. Averages were taken for each distance class; thus, the distances of each positive isolate to its nearest-neighbour were averaged to give the test statistic (g_1) for distance class 1, the distances of each positive isolate to its second-nearest neighbour were averaged to give the test statistic (g_2) for the second distance class, and so on. For each tree, a randomization of the distribution of positive isolates was run over 1000 iterations. 95% confidence levels were calculated from these randomizations.

Results

Previous sampling

A subset of yeast isolates from previous sampling by the lab were sequenced first at the ITS region, and then at fragments of 6 genes involved in the mating reaction, following previously established protocols (Johnson *et al*, 2004; Koufopanou *et al*, 2006). Of the 33 isolates sequenced, 7 did not belong to the *sensu stricto* clade, while the rest were *S. cariocanus*. No *S. cerevisiae* or *S. paradoxus* was found. *S. cariocanus* strains were predominantly isolated from the bark and surrounding soil of *Q. rubra*, but were also isolated from different tree species (Table 1).

Identification

Sequence analysis shows that all Mont Saint Hilaire isolates are *S. cariocanus*, although three of these isolates appear to be more differentiated than the rest. These differences are a result of nucleotide variation mostly in the regions where the three control strains differ from each other. While this variation matches the sequence of either *S. cerevisiae, S. cariocanus*, or *S. paradoxus* in several instances, at least half of the nucleotide differences are shared uniquely between these three genotypes. These three isolates were all collected at the same study site (Dieppe). No clustering by environment or sample type was detected in general, however, thus we have no evidence of genetic structure.

Seasonal sampling

S. cariocanus remain at a constant, low abundance throughout the entire sampling term (Table 2). Strains were most commonly isolated from the soil (40%); bark and blue tac samples retrieved half the amount of samples as from soil (25%). Leaf isolates were rarest (4%). The three sites did not differ in abundance; an average of three *S. cariocanus*

isolates were found per sampling date. Over the entire sampling period, *S. cariocanus* strains were isolated from only 0.7% of the total oak grove samples (40 out of 5,760 samples).

Spatial sampling

Figure 3 depicts the pattern of all *S. cariocanus* isolates and other (non-*Saccharomyces*) yeasts found on the bark of two *Q. rubra* trees. Variance of the columns is significantly larger than if the yeasts had been randomly distributed among cells ($F_{0.05(2)15,115}$= 256 and 289, [P<0.001] for the first and second tree respectively), as was the row variance ($F_{0.05(2)15,115}$= 18.78 and 30.25 [P<0.001] for the first and second tree respectively). Note that in both cases, column variance is much larger than that of the rows, which reflects the vertical growth pattern that is evident in the distribution maps (Figure 3). Growth on the first tree displays some degree of clustering. Weak clustering occurs at the first nearest neighbour distance class, although the position of *S. cariocanus* isolates becomes random, then more dispersed than expected by chance at subsequent (higher) distance classes (Figure 4).

Core samples

No yeast was retrieved from any of the core samples, suggesting that *sensu stricto* yeasts grow exclusively on the outer structures of trees.

Canopy, meadow, insect, and roadside and understory plant samples

No *sensu stricto* isolates were recovered from insect samples, and only one isolate was obtained from the understory sampling, from an *Acer pensilvaticum* seedling (Table 3). No *Saccharomyces sensu stricto* were found in the upper canopy, although several *S. cariocanus* isolates were found in the lower canopy of *Q. rubra* (4/100) and *A. saccharum* (4/100) and *Fagus graldifolia* (1/100) (Table 1).

Discussion

From the phylogenetic relationships of the centromere sequences, it appears that only strains of *S. cariocanus* were isolated. This is consistent with previous sampling work at Mont Saint Hilaire, where *S. cariocanus* have been isolated exclusively. Three of the isolates are slightly more differentiated from the rest. Examination of these sequences shows that the differences are a result of nucleotide variation found mostly in regions where the three species strains differ from each other. This variation is often unique to these three strains, that is, many short base pair segments are identical between these three strains, yet different from all other *S. cariocanus* isolates and species consensus sequences. Furthermore, these three strains were all isolated from the same study site (Dieppe; the most ecologically extreme of the three). In general, the isolates do not exhibit any pronounced spatial structure, however, as they do not appear to cluster by site or sample type.

While fermentative yeasts are known to peak in abundance mid-summer (reviewed in Kinkel, 1997), *S. cariocanus* remained at a constant, low abundance throughout the entire sampling period. This discrepancy may have arisen from the choice of sampling location; most previous work has focused on fruit environments, where fermentation is a key process. Our work centered on an environment with few fermentable fruits, where fermentative microorganisms may be naturally kept at low levels. Interestingly, no difference was found between the three oak groves. It may be that differences between different forest environments are not very large for these yeasts, so that only drastically different environments such as vineyards would generate any differences in abundance when compared to the forest environment.

Saccharomyces sensu stricto has previously been reported from the bark of broad-leaf trees, especially oak, and our observations support the conclusion that tree surfaces are a principal habitat of these yeasts. A previous study (Koufopanou *et al*, 2006) has shown a weak tendency for neighbours to be clone-mates, and our survey likewise shows a weak degree of spatial aggregation on bark. Clustering occurs at the smallest distances, which is indicative of clonal growth. That colonies were more dispersed than randomly expected at intermediate distances may be due to the topology of tree bark. A distinctive vertical growth pattern of all isolated yeasts was clearly observed. It is possible that yeasts grow preferentially in the crevices of bark, where humidity is higher, temperatures are less extreme, and nutrient deposition is more likely. Thus the lowest distances show clustering within ridges, while large growth gaps at intermediate distances correspond to ridges where little growth occurs. When yeast is cultured in aqueous extracts of bark and

soil it will grow feebly (Graham Bell, unpublished), perhaps also explaining the low degree of spatial aggregation on bark that has been reported (this study; Koufopanou *et al*, 2006). Finally, several different genotypes may be co-inhabiting the tree bark, thus the larger than expected distances between *S. cariocanus* isolates at intermediate scales could also imply repulsion between genotypes. The weak clustering of *sensu stricto* yeasts on bark (cf Koufopanou *et al*, 2006) may indicate clonal growth, but bark is a very poor substrate, especially for organisms that are best known for their ability to ferment fruit juices with high concentrations of sugars. The main source of sugar in trees is the xylem sap, which may comprise 1-2% sugar by weight in species such as *A. saccharum*. Several types of yeasts have been found to inhabit tree xylem (Young, 1949; Wilson, 1961; Wilson, 1965; Zhao *et al*, 2002), but we have failed to find yeast in the interior of trees, which with our sampling methods seems to be almost completely devoid of organisms. It has often been suggested that yeast feed on the sap exudates that run down the trunk when the bark is disrupted, for example by a branch breaking off (Sniegowski *et al*, 2002). This will undoubtedly be the case, but we have never seen exudates on our study trees and doubt that they can provide the only or even the principal source of nutrients. The remaining possibility is that yeasts live on the leaf surface, where they can metabolize sugar that leaks out of the leaf or is pumped out by aphids (Charles Godfray, personal communication). We have also observed extensive pseudohyphal growth in ageing cultures of *S. paradoxus* and *S. cariocanus* on agar, suggesting that they may be able to gain access to the interior of the leaf through lesions or stomata. Our isolation of *Saccharomyces sensu strico* from oak leaves gives some credence to this theory. Extensive canopy sampling reveals that leaves of *A. saccharum* and *F. grandifolia* may

also be important habitats for the *sensu stricto* yeasts. The frequency of isolation of *S. cariocanus* from *A. saccharum* was equal to that from *Q. rubra* (Table 1), and one strain was also isolated from *A. pensylvaticum*. This suggests that *A. saccharum* and other hardwood species may be a novel and important habitat, which has yet to be fully explored. Rainfall would then wash yeast from the leaves down the trunk, forming the afore-mentioned vertical trains of cells that would accumulate in the soil at the base of the trunk. We tentatively suggest that leaves are the principal source habitat for wild yeasts, with bark and soil being sinks. On the other hand, the abundance of *sensu stricto* species on leaves of *Q. rubra* is low compared to that of soil and bark. More detailed and extensive sampling will be required to confirm or refute this possibility.

Aside from maple and beech trees, the sampling of non-oak environments failed to determine new habitats. No isolates were obtained from the roadside and understory plants, meadow wildflowers, nor insects. The sampling of non-oak environments was intended to identify as many new putative environments of *sensu stricto* yeasts as possible. Without more extensive sampling, however, we cannot exclude the possiblilty that *Saccharomyces sensu stricto* may be able to grow on other plant species.

S. cerevisiae is being increasingly used as a powerful model system in a wide range of fields. Information about this species and its sister *sensu stricto* species will increase as they become even more widely used in research. Without the proper ecological knowledge, however, none of this data will ever be interpretable in a natural, real-world context, so it is imperative that more effort is given to explore this group's ecology.

Once its environment is well characterized, the *Saccharomyces sensu stricto* species complex will become a fully developed model system, which can be used in areas as diverse as brewing, drug development, population biology, community ecology, and evolution. That this group can be used in such diverse applications is perhaps what makes it the most exciting model system to work with.

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Table 1. Sampling overview. All dates are for 2005 unless otherwise indicated. Species abbreviations: An= *Acer nigrum*, Ap= *Acer pensylvaticum*, As= *Acer saccharum*, Ba= *Betula alleghaniensis*, Bp= *Betula papyrifera*, Bpo= *Betula populifolia*, Cc= *Carya cordiformis*, Fg= *Fagus grandifolia*, Fn= *Fraxinus nigra*, Jc= *Juglans cinerea*, Pr= *Pinus resinosa*, Ps= *Pinus strobus*, Qr= *Quercus rubra*, Ta= *Tilia americana*, Tc= *Tsuga canadensis*.

Sample type	Date	Number of samples collected	Number of yeasts isolated	Number of *sensu stricto* positives	Notes
Previous work	Jul/Aug 03	n/a	Subset sequenced=33	23	13 Qr; 1 Ap, As, Ba, Bpa, Fg, Fn, Jc, Pr, Ps, Ta
Cores	Oct 12/04	18	0	0	11 Qr; 2 As, Fg, Ps; 1 Bp
	Nov 2/04	15	0	0	10 Qr; 2 Bp, Fg; 1 As
	Nov 26/04	14	0	0	11 Qr; 2 Bp; 1 Fg
	May 10	15	0	0	6 Qr; 5 As; 2 Bp, Fg
	May 25	20	0	0	5Qr, Fg; 4 As, Bp; 2 An
	Sept 22	18	0	0	18 Qr
Seasonal sampling	June 21-Sept 18	5,760	496	40	1,440 samples each of *Qr* bark, soil and leaves (see Table 2)
Upper canopy	June 29	79	10	0	34 As, 25 Qr, 20 Fg
Lower canopy	Sept 30	300	25 Qr, 16 As, 16 Fg	4 As; 4 Qr; 1 Fg	100 Qr, 100 As, 100 Fg
Meadow plants	June 29	40	6	0	grasses and wildflowers
Roadside & understory plants	Sept 23	80	12	1	see Table 2
Arthropods	June 8 & 29	60	8	0	Acaridae, Araneae, Diptera, Coleoptera, Hymenoptera

Table 2. Summary of *S. cariocanus* isolates from seasonal sampling of three oak groves. P=Pre, S=Sun, D=Dieppe. Dates: Jn=June, Jl=July, Se=September. N=Total. Note how abundance (number of positive isolates) remains constant over the entire sampling season (exception of July 5th).

Day	21Jn	23Jn	5Jl	7Jl	23Jl	23Jl	29Jl	31Jl	31Jl	16Se	16Se	18Se	N
Site	*P*	*S*	*P*	*S*	*P*	*S*	*D*	*P*	*S*	*P*	*S*	*D*	-
Leaf	0	0	1	0	1	0	0	1	1	0	0	0	4
Tac	2	0	1	0	3	0	1	0	0	3	0	0	10
Bark	1	0	5	0	0	0	0	1	1	0	1	1	10
Soil	1	1	3	2	0	3	3	0	0	0	0	1	16
N	4	1	10	2	4	3	4	2	2	3	1	2	40

Table 3. Roadside and understory plants sampled. Names in bold denote plants from which *S. cariocanus* was isolated. Asterisks denote plant species from which where non-*sensu stricto* yeasts were isolated. Samples consisted of leaves unless otherwise indicated.

Latin name	Common name	Number of times sampled
Acer pensylvaticum	**Striped maple**	**seedling**
* *Acer rubrum*	*Red maple	x 3 (seedlings)
Actaea	Baneberry	x 2 (1x berries, 1x leaf)
Ambrosia artemisiifolia	Common ragweed	
Amphicarpa bracteata	Hog peanut	x 3
Aralia nudicaulis	Wild sarsaparilla	x 3
**Arisaema triphyllum*	*Jack-in-the-pulpit	x 3 (2x berries, 1x leaf)
Asclepias syriaca	Common milkweed	
Aster acuminatus	*Whorled or mountain aster	x 2
**Carya cordiformis*	*Bitternut hickory	x 2 (seedlings)
**Circaea lutetiana*	Enchanter's nightshade	x 3 (2x leaf, 1x flower)
Cirsium arvense	Canada thistle	
Diervilla lonicera	Bush honeysuckle	
Epifagus virginiana	Beech drops	
**Fagus grandifolia*	*American beech	x 2 (seedlings)
Fragaria virginiana	Virginia or field strawberry	leaf
Geum canadense	White avens	
Hepatica	Noble liverleaf	
**Lactuca canadensis*	*Wild lettuce	x 3
Lapsana communis	Nipplewort	
**Leodonton autumnalis*	Fall dandelion	x 2 (leaves only)
Leucanthemum vulgare	Oxeye daisy	
Matteucia struthiopteris	Ostrich fern	
Onoclea sensibilis	Sensitive fern	x 2 (1x fertile frond, 1xfrond)
**Parthenocissus quinquefolia*	Virginia creeper	
**Phegopteris connectilis*	*Long beech fern	x 2
**Plantago lanceolata*	Lance-leaved plantain	x 3 (2x leaf, 1xfertile frond)
Polygonatum pubescens	Hairy Solomon's seal	x 3
Polystichum acrosticoides	Christmas fern	x 2
Ranunculus abortivus	Small-flowered buttercup	
Rubus alleghaniensis	Blackberry	x 2
Rubus idaeus	Red raspberry	
Rubus occidentalis	Black raspberry	
Sanguinaria canadensis	Bloodroot	
Sanicula trifoliata	Black sanicle	x 2
Thalictrum dioicum	Early meadow rue	
**Trifolium agrarium*	Hop clover	x 2 (1x leaf, 1x with flower)
Tsuga canadensis	Hemlock	
Trillium	Trillium	
**Tussilago farfara*	Colt's foot	x 2
Uvularia grandiflora	Large-flowered bellwort	
Viburnum cassinoides	Maple-leaved viburnum	
Viburnum alnifolium	Hobble bush	x 3
Viola	Violet	x 2

Figure legends

Figure 1. Field sites for seasonal sampling (Mont Saint Hilaire, Quebec, Canada). Sites are numbered as (1)Pre, (2)Sunrise, and (3) Dieppe. Trails are designated by the think, solid lines.

Figure 2. Phylogeny of natural isolates of *Saccharomyces sensu stricto* yeasts based on centromeric DNA. Controls are designated as Scer= *S. cerevisiae*, Spar= *S. paradoxus*, and Scar= *S. cariocanus*. Key for other isolates: First letter represents sampling site, first number, the sampling date: P=Pré, S=Sunrise, D=Dieppe. Second letter represents the sample type: B=bark, T=tac, S=soil, L= leaf. Last number distinguishes the replicate. Eg. P1B4=Pre sampling date 1, bark sample number 4. Other samples: U=understory, B= *F. grandifolia* leaf, M= *A. saccharum* leaf, O= *Q. rubra* leaf, S9 and P4 without an indicated sample type represent spatial isolates.

Figure 3. Growth of yeast on bark of *Q. rubra*. Yeast was isolated where squares are marked with an X. Shaded squares indicate *sensu stricto* isolates, identified by cen9 sequence alignment. The first tree was sampled at the Sunrise site (a), while the second was sampled at the Pre site (b).

Figure 4. Growth of sensu stricto yeasts on the bark of Q. rubra: Comparison between observed g statistics and the 95% confidence limits generated by

randomization of the observed distance matrix. Observed values are indicated by black diamonds, confidence limits by dashed lines. Values that fall within the confidence limits indicate a random distribution, those that fall below the confidence limits indicate clustering, while those that fall above show more scattering that randomly expected.

Figure 1.

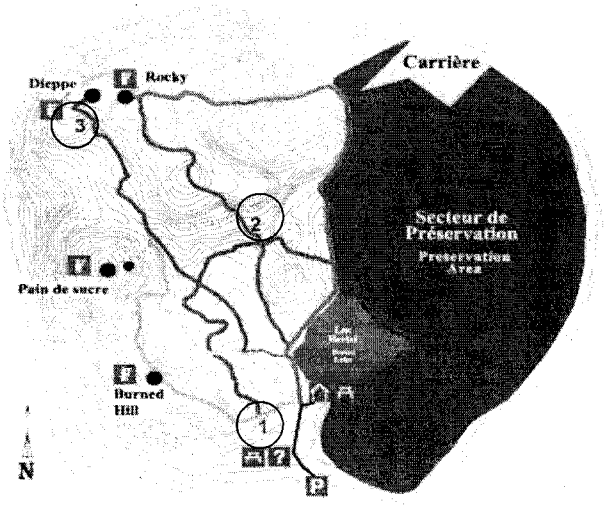

Carrière
Dieppe
Rocky
3
2
Secteur de
Préservation
Preservation
Area
Pain de sucre
Burned
Hill
1
N

Figure 2.

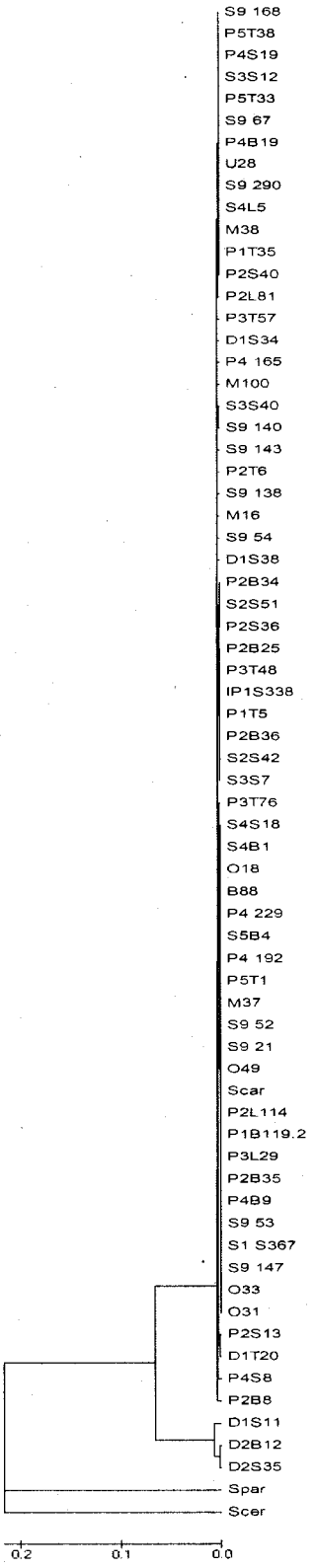

S9 168
P5T38
P4S19
S3S12
P5T33
S9 67
P4B19
U28
S9 290
S4L5
M38
P1T35
P2S40
P2L81
P3T57
D1S34
P4 165
M100
S3S40
S9 140
S9 143
P2T6
S9 138
M16
S9 54
D1S38
P2B34
S2S51
P2S36
P2B25
P3T48
IP1S338
P1T5
P2B36
S2S42
S3S7
P3T76
S4S18
S4B1
O18
B88
P4 229
S5B4
P4 192
P5T1
M37
S9 52
S9 21
O49
Scar
P2L114
P1B119.2
P3L29
P2B35
P4B9
S9 53
S1 S367
S9 147
O33
O31
P2S13
D1T20
P4S8
P2B8
D1S11
D2B12
D2S35
Spar
Scer
0.2
0.1
0.0

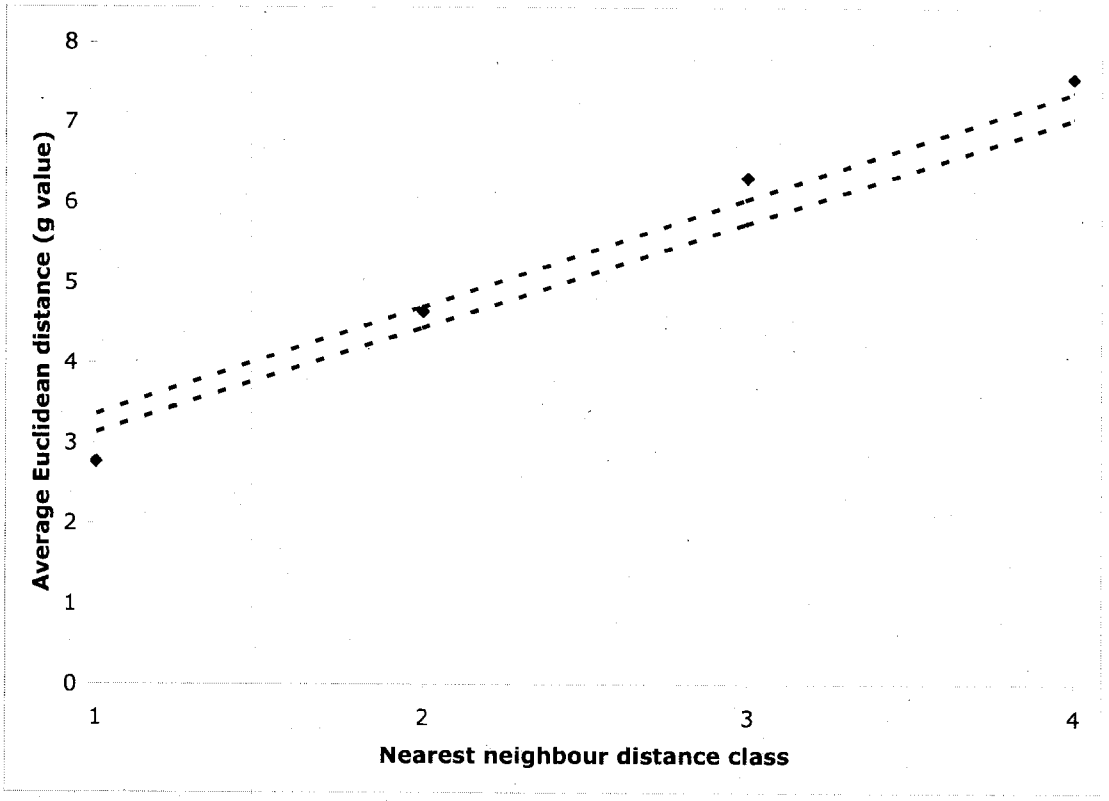
Figure 3 (a)

X	X			X				X	X		X	X	X	X
X	X	X						X					X	X
X	X	X		X			X				X		X	X
X	X	X	X							X	X		X	X
X	X	X	X		X			X			X		X	X
X			X						X		X		X	
X	X	X	X				X				X		X	X
X				X	X			X	X			X	X	X
X		X		X				X			X	X	X	
X				X									X	X
		X				X			X			X	X	
	X	X						X	X		X		X	
X		X								X	X		X	
	X	X	X					X				X	X	
	X	X		X			X	X	X	X	X		X	
X	X	X	X	X				X		X	X	X	X	X
		X	X	X			X	X			X	X	X	X
		X	X	X		X		X	X	X	X	X	X	
		X	X				X	X		X			X	X
	X	X				X	X	X			X	X	X	

Figure 3 (b)

								X	X					
								X	X		X			
								X	X					
				X				X		X				
									X	X	X	X		
									X		X			
								X			X			
								X			X			
	X										X			
	X								X		X			X
								X			X			
	X									X				X
										X				
X								X		X				
X	X									X	X			
X	X	X						X						
										X				
X	X													
X		X						X	X		X			
	X		X	X						X	X			

Figure 4.


8
7
6
5
4
3
2
1
0
Average Euclidean distance (g value)
1
2
3
4
Nearest neighbour distance class

The previous chapter describes the isolation of *sensu stricto* yeasts from many different habitats in an old growth forest. *S. cariocanus* was found to coexist on oak-associated microhabitats (leaves, bark, and surrounding soil), as well as on other hardwood trees, while neither *S. cerevisiae* nor *S. paradoxus* were isolated. Two other non-*Saccharomyces* yeasts had been isolated and identified during previous fieldwork. These yeasts are indistinguishable by eye from the *sensu stricto* yeasts, and were isolated from many of the same environments and individual trees where *S. cariocanus* isolates were collected. This suggests that these three species coexist naturally, and provides an excellent yeast community with which to study ecological dynamics. Previous investigations into community processes using microbial model systems were limited, as it is very difficult to characterize the components of a microbial mixture (species identities and proportions). Without this data, it is impossible to say whether mixtures were most productive due to complementary resource use between the different component species (complementation; Tilman *et al* 1997) or due to the fact that mixtures with more species were more likely to include the type of high productivity, which then excludes all other species in the mixture and drives the positive relationship between diversity and productivity (replacement; Tilman *et al* 1997). Here, a new methodology allows me to identify the mixture components; yeasts all yield bands of different sizes for a region that is highly conserved within eukaryotes (ITS1-5.8rRNA-ITS2; White *et al* 1990), thus species mixtures can be amplified and run on acrylamide gels in order to identify their components, as well as the relative proportions of these components. I thus investigated the relationship between environmental heterogeneity, species diversity, and community productivity using this mixture of naturally coexisting yeasts.

The relationship of environmental heterogeneity, species diversity, and productivity in a natural yeast community.

Taissa Replansky and Graham Bell

Department of Biology, McGill University, Montreal, Canada

Corresponding author: Taissa Replansky

Department of Biology, McGill University, 1205 Dr. Penfield, Montreal, QC, H3A 1B1, Canada

E-mail address: taica.replansky@mail.mcgill.ca

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Abstract

Diversity, whether ecological or genetic, is widely believed to be beneficial to the functioning of ecosystems. Although many studies have investigated relationships between environmental complexity, biological diversity and ecosystem function, few have examined them simultaneously. We propagated combinations of 3 naturally coexisting species of yeast for 200 generations, in environments of different complexity (number of carbon sources). Competitive ability is transitive, and not related to productivity, which is equal among the species. Particular environments had more effect on species diversity than did environmental complexity; certain carbon sources promoted greater species richness than others, and the sole case of coexistence of all three yeasts after 200 generations was documented on a single carbon source, melezitose. Species diversity had a weak but inconsistent positive effect on productivity, and overyielding in mixtures was caused primarily by complementation. Our results suggest that specific environmental properties and species combinations may be important in maintaining diversity independently of heterogeneity. To our knowledge, this is also the first documented instance of multi-species coexistence on a single limiting resource.

Introduction

The recent acceleration of the loss of species diversity and environmental heterogeneity has given rise to a steady accumulation of experiments investigating the effect of these losses on ecosystems. Ecological theory predicts that divergent selection in complex or structured environments may facilitate coexistence (Rosenzweig 1995). This has been demonstrated both in nature (reviewed in Tews *et al* 2004) and in laboratory experiments (eg. Rainey & Travisano 1998; Chao & Levin 1981). The relationship of biodiversity and productivity is believed to be unimodal, although the mechanisms responsible for this relationship remain unclear (Rozensweig 1995, 1992). This suggests that different mechanisms may be involved in particular systems, in which case it would be important to understand the features of ecosystems that determine which mechanism is likely to dominate. Elevated productivity may result from niche differentiation, where species use resources in complementary ways (complementation; Tilman *et al* 1997). Alternatively, higher diversity may increase productivity by increasing the chance that the superior competitor will be present in the mixture (replacement; Tilman *et al* 1997). If growth and competitive ability are negatively correlated, however, diversity may actually reduce productivity (Loreau 2000). The relationship between diversity and productivity may therefore depend on interactions between species that may in turn depend on the state of the environment.

Microbial model systems present an ideal framework to tackle such complex questions (Jessup *et al* 2004). Microbes have short generation times, which allows for easy

investigation of longer time scales. Experimental treatments can be easily replicated, and conditions can be carefully controlled in order to isolate variables of interest. This provides an effective alternative to studying natural populations, which can quickly become complicated, time-consuming, and mechanistically difficult to explain. The relationship between environmental heterogeneity and biodiversity (Halbets *et al* 2006; Rainey & Travisano 1998; Bell 1997; Korona 1996; Korona *et al* 1994; Chao & Levin 1981), and biodiversity and productivity (Bell 2005; Hodgson *et al* 2002; Kassen *et al* 2000; Bell 1991; Bell 1990), has been thoroughly investigated by microbial experiments. This has provided mechanistic descriptions of how diversity and productivity respond when they are determined by a limited number of variables. None of these studies have simultaneously looked at environmental and species diversity effects on productivity, however.

Here we test these relationships using a naturally coexisting yeast community that has previously been isolated from the bark of red oak, *Quercus rubra* (Replansky *et al*, unpublished). Yeasts, particularly *Saccharomyces sensu stricto*, are increasingly used as a model system in experimental ecology and evolution because of their easily manipulable life cycles and genetic relationship to higher eukaryotes, as well as for the vast amounts of genetic data available (Zeyl 2000). In addition, the particular yeasts used in our study co-occur outside the lab, and provide an intermediate model system between highly derived laboratory strains and the natural populations that they are meant to represent. We are also able to disentangle the effects of complementation and replacement in this system; while these yeasts are indistinguishable by eye they can be

differentiated by amplifying and visualizing a genetic locus that differs in length beween different yeast genera.

We propagated three yeasts in all species combinations, over ecologically long timescales and on environments of increasing complexity. Community composition and productivity on all environments were monitored over time, and competitive interactions were determined from the outcome of all pairwise species mixtures. By controlling environmental and species composition, we are able to disentangle the roles that environmental and species complexity play in the functioning of a simple model ecosystem.

Methods

The three species of yeast assayed, *Kluyveromyces lactis*, *Saccharomyces cariocanus*, and *Zygosaccharomyces fermentati*, were all isolated from soil surrounding red oak, *Quercus rubra*, located in an old-growth forest at Mont Saint-Hilaire, Quebec, Canada (45°N, 75°W; altitude 415 m). Eight carbon sources were chosen for the assay, based on the ability of all three species to grown on them (Table 1). Growth was assayed on all single carbon sources, and on a subset of more complex mixtures of these compounds. All of the possible species combinations (pure culture, two- or three-species mixtures) were grown in each of the different environmental treatments (carbon source combinations). Pure cultures were first grown in vials containing 50mL YPD in a

shaking incubator at 28°C, transferred once, and allowed to grow again overnight. The cultures were then washed twice with sterile dH_2O and diluted to equal densities before making up the species mixtures. A total of 50uL of yeast culture was inoculated into vials containing 5mL minimal medium containing one or more of the carbon substrates at a fixed total concentration of 20g/L. 50uL of culture was transferred into 5mL of fresh medium daily for 200 generations, and kept in a shaking incubator at 28°C. Absorbance readings (λ=600nm) were taken daily for one week, using a Universal Microplate Reader (Biotek Instruments Inc). Absorbance values were related to density using a standard curve, and then log transformed for the analysis. At every 50^{th} generation, we extracted DNA from 50uL of each culture using the Quiagen DNAeasy Tissue Extraction Kit. Extracted DNA was stored at -20°C until the end of the experiment, and then amplified to identify species composition. The internal transcribed spacer region (ITS1-5.8rRNA-ITS2) was amplified by PCR using primers ITS1 and ITS4 for all DNA extractions (White *et al.* 1990). This region is highly conserved within eukaryotes, and yields a product of different length depending on the yeast species: 800bp region for the *Saccharomyces* genus, 700bp for *K. lactis*, and 650bp for *Z. fermentati*. PCR products were visualized on a Li-Cor 4300 DNA Analysis System (LiCor Biosciences, USA). For species mixtures, relative species density was obtained by estimating relative band intensity using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, USA). Simpson's Index of Diversity was calculated for all cultures, based on these band intensities. Theory predicts that diversity, as expressed by Simpson's Index, will increase with environmental complexity. Simpson's index is $SI = 1-\sum p_i^2$, where p_i is the proportion of genotype 'i' in the mixture. A value of 0 indicates complete exclusion by

one species, while a value of 0.67 indicates maximum diversity. A secondary assay was carried out using identical methods, over a span of 100 generations. The three species were grown together in glucose, sucrose, and melezitose, where each environment was replicated twelve times. Regressions were performed in SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

Results

The outcomes of pairwise species mixtures followed a competitive hierarchy, whereby *K. lactis* > *Z. fermentati* > *S. cariocanus*. *K. lactis* is a superior competitor in 98% of the cases, and often comprises around 90% of the species mixture. *Z. fermentati* and *S. cariocanus* are more evenly matched. Competitive ability is not related to productivity of the species; all three species grew to similar densities in monoculture ($F_{0.05(1)2,72}$=1.32, P=0.27).

Simpson's index is weakly related to increasing environmental complexity (Figure 1a), although the regression is not significant ($F_{0.05(1)1,23}$=2.45, P=0.13). Coexistence appears to depend more on the inclusion of certain carbon substrates rather than the total number of carbon sources comprising a particular medium (Figure 1b). While species evenness is higher on environments of increased complexity, there is only a single instance where all three species coexist after 200 generations. This occurs on a single carbon source, melezitose (Figure 2). We validated this result by growing the three-species mixture on

each of glucose, sucrose, and melezitose, using twelve replicate vials for each environment. Cultures were assayed after 100 generations of growth, at which point all three species were found to coexist in 9 out of 12 vials containing melezitose. *K. lactis* was the sole species found in all vials containing glucose or sucrose.

Genetic diversity appears to increase productivity in nearly all environmental conditions, though this relationship is weak and not significant ($F_{0.05(1)12,162}$= 1.32, P=0.22; Figure 3). However, mixture tended to produce more than the mean of their components ($t_{0.05(2),56}$= 4.83, P<0.0001; $\chi_{0.05,1}$=24.012, P<0.001). This overyielding is caused primarily by complementation (Figure 4).

Discussion

Ecological theory predicts that at small scales habitat heterogeneity sets the levels of biodiversity, and that this relationship is positive (Rosenzweig 1992). This has been demonstrated in several microbial experiments (reviewed in Rainey *et al* 2000). Although we observed a weak trend for diversity to increase with environmental complexity, the presence of certain substrates appears to favour coexistence more than does absolute substrate number. Substrates such as galactose, sucrose, and raffinose support the coexistence of the two more evenly matched competitors (*S. cariocanus* and *Z.fermentati*) for 200 generations, whereas *K. lactis* outcompetes them both when present. Melezitose proves to be the most interesting substrate, as it is the only

environment (simple or complex) that allows all three species to coexist over the entire span of the experiment. The effect of melezitose and other 'high diversity' substrates became gradually less significant with increasing environmental complexity. This is likely due to their decreasing concentration in increasingly complex substrate mixtures; they become too dilute to have a positive effect on species diversity. The idea that environmental identity may have a larger effect on diversity than does environmental complexity has been previously demonstrated in natural systems (Estades 1997; Welch 1988).

Most mixtures produced more than the mean of their components, and this overyielding was due to complementation. However, the overall positive relationship of increased productivity at higher genetic diversity was not significant. This suggests that the occurrence and extent of overyielding varies depending on particular species combinations in particular environments. Microbial community composition has been previously shown to be important in determining ecosystem functioning (Bell *et al* 2005). It is also possible that the lack of relationship between species diversity and productivity is due to the lack of functional group diversity in our study, as previous work has demonstrated that resource partitioning (and hence increased productivity) is greatest with species differing strongly in functional type, particularly in simple two- or three-way mixtures (McKane *et al* 2002). If the species used in this experiment are functionally divergent, then frequency-dependent selection generated by differential resource depletion could be responsible for maintaining diversity in the two-species mixtures. Such dynamics have been observed in mixtures of two *E. coli* strains, where each strain

was a superior competitor on a different galactoside (Lunzer *et al* 2002). Diversity was only maintained within a narrow range of galactoside ratios, however. Finally, the lack of significant relationship between diversity and productivity could be due to the fact that our experiment contained only one 3-species combination, thus our results may be specific to the particular species combination that we investigated. An ideal experiment would be composed of several groups of different species combinations, in order to replicate the general effects of diversity on productivity. Having only 3 species also limits the experiment to relatively low levels of diversity. If more naturally-coexisting yeast species are found, we would be able to test the unimodal relationship between diversity and productivity that has been previously reported in the literature (Rozensweig 1995, 1992).

The stable coexistence of all three yeasts on a single substrate (melezitose) falsifies the competitive exclusion principle, which states that the number of coexisting genotypes cannot exceed the number of available resources (Hardin 1960; Gause 1934). The presence of a competitive hierarchy (transitivity), and the large difference in competitive ability between *K. lactis* and the other two species marks this coexistence as even more improbable. Competitive hierarchies (transitivity) have been found in nature (Buss & Jackson 1979), though not exclusively (Shipley & Keddy 1994). Certain types of interactions between genotypes may allow coexistence on a single limiting resource, however. Metabolic interactions where one species excretes metabolites that are used by another species as a resource (crossfeeding) may be responsible for the observed coexistence on melezitose. If this substrate were partially hydrolyzed by *K. lactis* into

glucose and turanose (an isomer of sucrose), *S. cariocanus* and *Z. fermentati* might be able to coexist on secondary metabolites, especially as they are of roughly equal competitive ability. Crossfeeding has previously been documented using polymorphic bacteria founded from a single clonal genotype (Treves *et al* 1998; Turner *et al* 1996; Rosenzweig *et al* 1994; Helling *et al* 1987).

The nature of the relationship between diversity and environmental heterogeneity, together with the inconsistent relationship between species diversity and productivity show how specific species and environmental properties may influence ecosystem functions. We found that specific substrates had more effect on species coexistence than did absolute substrate number, so environmental composition may affect diversity more so than environmental complexity itself. The varying effect of diversity on productivity suggests that overyielding occurs for particular species combinations in particular environments. This suggests that predicting ecological opportunity and ecosystem properties based on environmental complexity and species richness alone may not always be sufficient, but that specific components and properties of environments and species must be carefully studied in order to predict the ecological outcomes of mixtures more accurately.

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Table 1. List of environments.

Number of carbon sources	Carbon composition of medium*
1	A
1	B
1	C
1	D
1	E
1	F
1	G
1	H
2	A+E
2	B+C
2	G+H
2	B+G
2	C+F
2	F+H
2	A+D
3	D+F+G
3	A+B+G
3	A+C+H
3	B+D+F
3	D+E+H
4	B+D+E+G
4	A+C+F+H
4	B+E+F+G
6	A+C+D+F+G+H
6	A+B+D+E+F+H
8	ALL

***Key to carbon substrates:** A = glucose, B = galactose, C = sucrose, D = raffinose, E = melezitose, F = inulin, G = melibiose, H = sorbitol.

Figure legends.

Figure 1. Effect of environmental complexity on diversity after 200 generations, for cultures starting with 3 yeast species. a) Overall relationship. Error bars indicate standard error. **b) Variability of diversity indices on different environments.** Closed circles = high diversity environments (containing galactose, melezitose, and raffinose, or a combination of these). Open circles = low diversity environments. Note that the highest diversity index is recorded on a single carbon source (melezitose).

Figure 2. Growth dynamics of the three species mixture on melezitose. Open circles= *K. lactis*; closed circles= *S. paradoxus*, triangles= *Z. fermentati.*

Figure 3. Effect of species diversity on productivity of a mixture. Each data series denotes a particular environmental diversity treatment: Diamonds = one carbon source; squares= 2 carbons; triangles= 3 carbons; x= 4 carbons; asterisks= 6 carbons; circles= 8 carbons.

Figure 4. Observed versus expected cell densities in yeast mixtures. Closed circles = 2 species mixtures, open circle = 3 species mixture. Thin dashed line indicates line of equality, thin dashed lines represent 95% confidence intervals for the slope of the regression $y = 0.2131x + 3.2214$. Expected density was calculated as the sum of the

weighted (by abundance of the species in the mixture) average of the monoculture yield for the component species.

Figure 1a

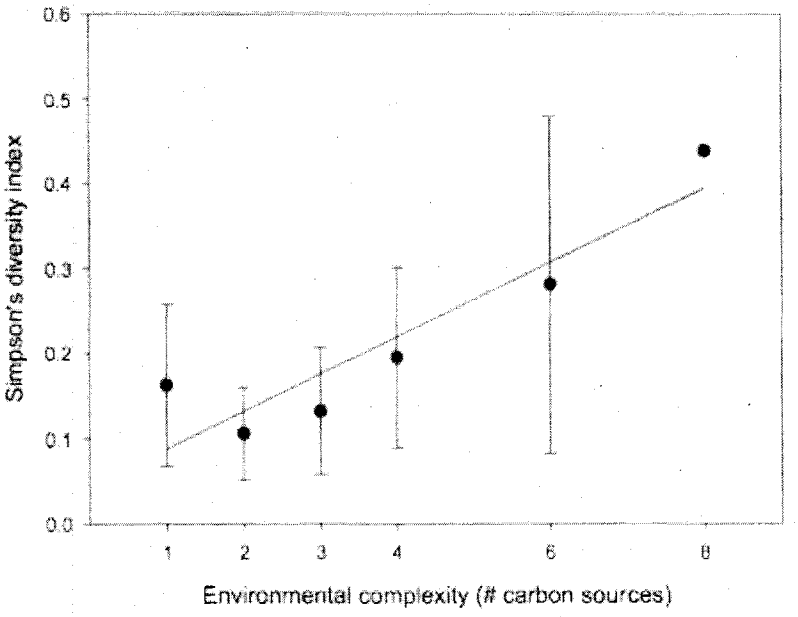

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Simpson's diversity index
1
2
3
4
6
8
Environmental complexity (# carbon sources)

Figure 1b.

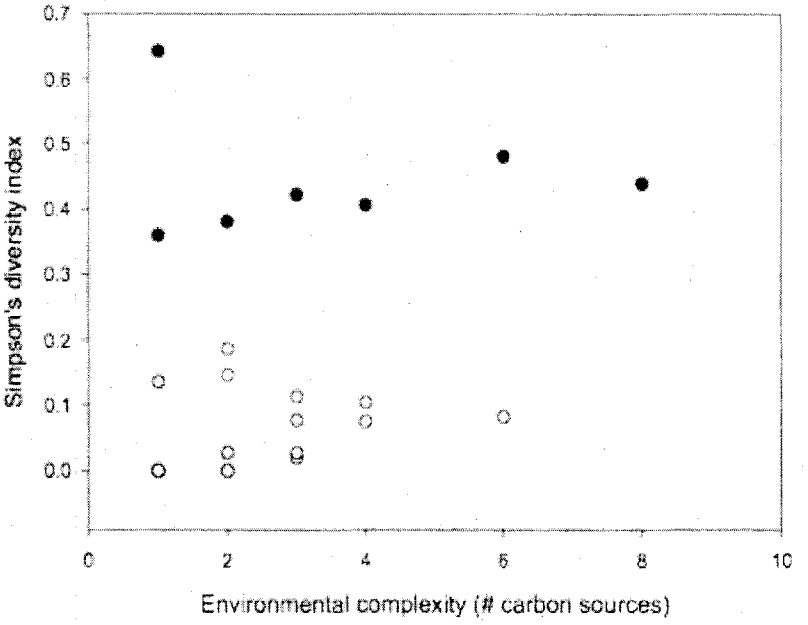

Simpson's diversity index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
Environmental complexity (# carbon sources)

Figure 2.

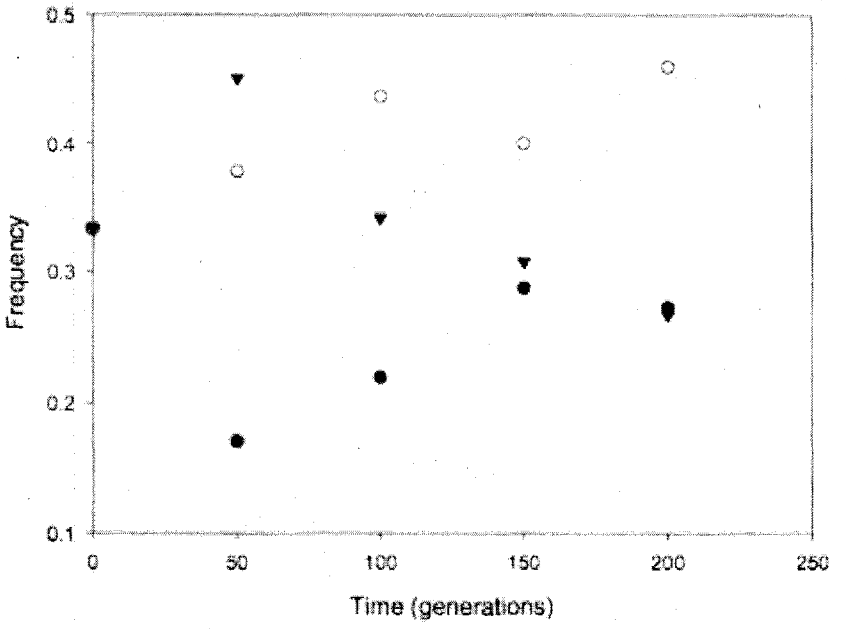


Figure 3.

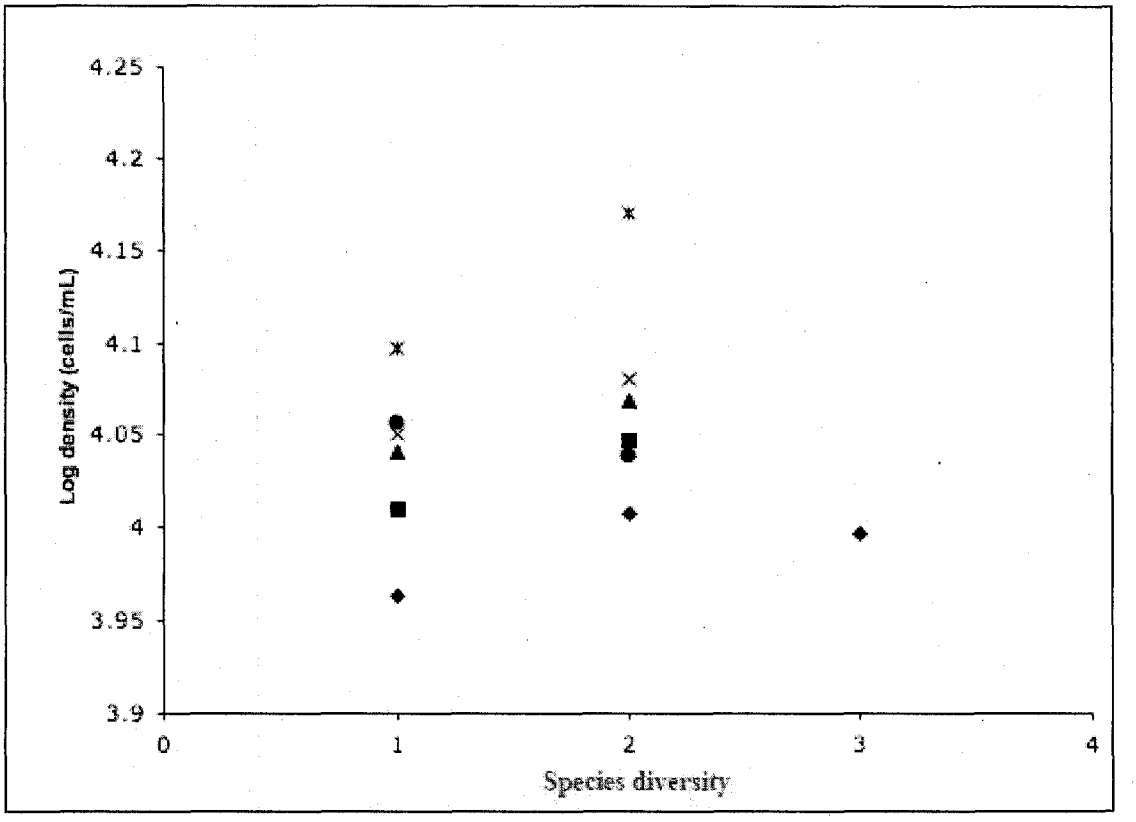
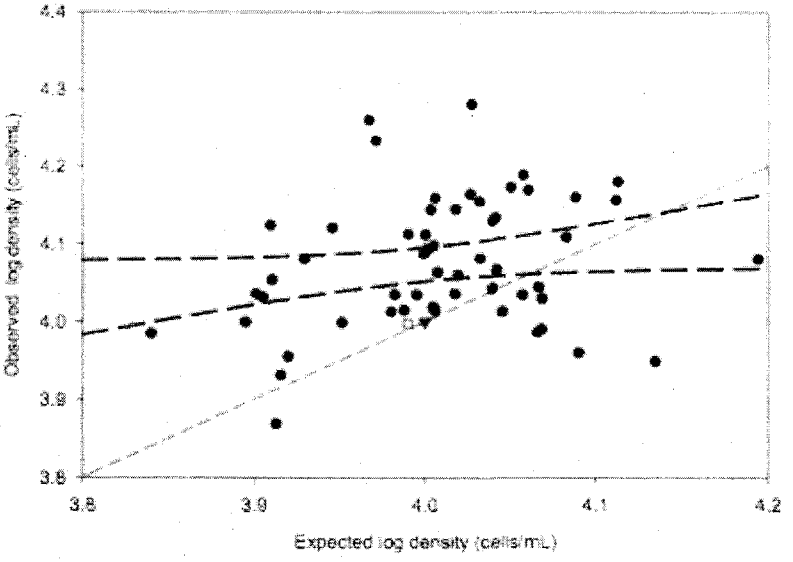

4.25
4.2
4.15
4.1
4.05
4
3.95
3.9
Log density (cells/mL)
0
1
2
3
4
Species diversity

Figure 4.


4.4
4.3
4.2
4.1
4.0
3.9
3.8
Observed log density (cells/mL)
3.8
3.9
4.0
4.1
4.2
Expected log density (cells/mL)

Conclusion

If ecological and evolutionary experiments using *S. cerevisiae* and other *sensu* stricto species a model system are to be interpreted in a meaningful way, sufficient knowledge of the ecology of these yeasts must be available. While the existing literature hints at several habitats for these yeasts, many gaps in knowledge remain to date. The work presented in this thesis synthesizes all of the available data on *Saccharomyces* ecology, and has further expanded this knowledge. My work at Mont Saint Hilaire has confirmed the association of *sensu stricto* yeasts with oak-associated microhabitats (leaf, bark, surrounding soil), and suggests that other trees are also inhabited by these yeasts. Leaves also represent a novel environment, as to my knowledge *Saccharomyces* have never been isolated from the living canopy. It is possible that leaves are the primary habitat of these yeasts, which are then washed down the trunk and deposited onto the soil by rainfall. The pattern of yeast on oak bark supports this hypothesis; growth is restricted to a vertical pattern, with only a mild clustering by genotype (clonal growth). It is also possible that likely that yeast preferentially grow in the crevices of oak bark, which may offer a more protected and permissive microhabitat. Investigation into the seasonal abundance of these yeasts revealed no pattern; *sensu stricto* yeasts are found at a constant, low abundance throughout summer and early fall. I failed to detect yeasts on any understory or roadside plants, which suggest that they may be associated mostly with hardwood trees.

As our understanding of the natural environment of yeasts grows, their potential as a model system increases. Using different yeast species that were previously collected and

identified, I investigated the effect of community and ecological diversity and composition on an important ecosystem process, productivity. It appears that, for this mixture of yeasts, community and ecological composition has a larger impact on productivity than does species or environmental complexity, and an effect of complementation was also detected. While the experiment presented only a community of three species, it demonstrates the ease with which ecological dynamics can be investigated.

While this work has increased our understanding of the ecology of *sensu stricto* yeasts, more extensive surveys are needed to provide us with a detailed description of the types of conditions the *sensu stricto* group is faced with. Broad leaf trees other than oak merit more attention, as this is not the first time that this group has been isolated from trees other than oak (Kodama 1974). As *sensu stricto* yeasts are common in soil, more understory plants should be sampled to confirm or reject them as a possible environment. Knowledge of the dispersal of these yeasts is lacking, but would greatly facilitate the exploration of yeast environments; any associated insects could be tracked and used to find new environments. A complete picture of the ecology of *sensu stricto* yeasts allows them to be widely used as a microbial model system whose results may be interpreted in a meaningful, natural context. Increasing our knowledge of the natural history and ecology of the *Saccharomyces sensu stricto* clade is important to a wide range of fields, from fermentation, to epidemiology, to community ecology and evolution. That *S. cerevisiae* is relevant in such diverse fields is perhaps what makes it the most exciting model system to work with.

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