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RESEARCH ARTICLE

Exploring the northern limit of the distribution of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* in North America

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Abstract

We examined the northern limit of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* in northeast America. We collected 876 natural samples at 29 sites and applied enrichment methods for the isolation of mesophilic yeasts. We uncovered a large diversity of yeasts, in some cases, associated with specific substrates. Sequencing of the *ITS1, 5.8S* and *ITS2* loci allowed to assign 226 yeast strains at the species level, including 41 *S. paradoxus* strains. Our intensive sampling suggests that if present, *S. cerevisiae* is rare at these northern latitudes. Our sampling efforts spread across several months of the year revealed that successful sampling increases throughout the summer and diminishes significantly at the beginning of the fall. The data obtained on the ecological context of yeasts corroborate what was previously reported on *Pichiaceae*, *Saccharomycodaceae*, *Debaryomycetaceae* and *Phaffomycetaceae* yeast families. We identified 24 yeast isolates that could not be assigned to any known species and that may be of taxonomic, medical, or biotechnological importance. Our study reports new data on the taxonomic diversity of yeasts and new resources for studying the evolution and ecology of *S. paradoxus*.

Introduction

Budding yeasts of the genus *Saccharomyces*, and particularly the model species *Saccharomyces cerevisiae*, are the most studied group of fungi in the laboratory. *Saccharomyces cerevisiae*, also known as brewer's yeast, has been used for several decades as a model in genetics, cell biology and genomics. Additionally, *S. cerevisiae* and its closely related species have also served as models in ecological genetics and genomics (reviewed in Landry *et al.*, 2006; Replansky & Bell, 2009; Hittinger, 2013). Extensive genetic variation has been documented for several traits, including the ability to grow on different media, at different temperatures and under different stress conditions (Gasch & Werner-Washburne, 2002; Liti *et al.*, 2009; Will *et al.*, 2010), gene expression (Landry *et al.*, 2007; Brown *et al.*, 2008) and colony morphology variation in response to different carbon sources (Granek & Magwene, 2010). Recently, the brewer's yeast has also been genetically engineered to produce an array of biofuels, which is of major interest in biotechnology (reviewed in Buijs *et al.*, 2013). Because *S. cerevisiae* populations that are sampled in the wild have been affected by human activities, the distribution of genetic variation in this model species may not reflect natural population dynamics and biogeography (Liti *et al.*, 2009; Hyma & Fay, 2013), which makes it a limited model for the study of population genetics and adaptation. As a consequence, there is a growing interest for the use of other species of the genus in the study of natural populations and natural variation in relation to abiotic and biotic factors, particularly in the use of its sister species *Saccharomyces paradoxus* (Dunham & Louis, 2011).

Natural isolates from the genus *Saccharomyces* have been successfully isolated from a variety of substrates

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such as deciduous tree bark, exudates or associated soils, vineyard grapes, wild fruits and insects such as *Drosophila* (Phaff *et al.*, 1956; Naumov *et al.*, 1996, 1998; Hyma & Fay, 2013). However, the most common substrate for the isolation of natural *S. paradoxus* is the bark from trees of the *Fagaceae* family, especially from the genus *Quercus*. *Saccharomyces* yeasts usually share these habitats with other *Saccharomycetaceae* such as *Torulaspora* ssp. and *Lachancea* ssp. (Sampaio & Goncalves, 2008). *Saccharomyces paradoxus* has been shown so far to be distributed where the deciduous trees with which it is associated are prosperous, namely in North America, Europe, the far East Asia and New Zealand (Sniegowski *et al.*, 2002; Naumov *et al.*, 2003; Liti *et al.*, 2009; Zhang *et al.*, 2010). In all cases, both *S. paradoxus* and *S. cerevisiae* have been isolated in the same regions, suggesting that their distribution areas largely overlap. At the northern limit of deciduous tree distribution in the northeastern America (Mont St-Hilaire; 45°33′N 73°09′W), a single site has been intensively sampled, and this sampling suggests that *S. cerevisiae* is rare or absent at this latitude because only *S. paradoxus* and other distantly related species have been isolated (Replansky & Bell, 2009).

Given the importance of *S. cerevisiae* and *S. paradoxus* in several fields of the life sciences, including evolution and ecology, as well as in biotechnology, it is of prime importance to better understand the ecological factors that determine the distribution of these species. Many regions, particularly regions where we expect to find the margin of their distribution, have not been or have been poorly sampled. The aim of this study was to examine whether the distributions of *S. paradoxus* and *S. cerevisiae* extend further north than what has been previously described and to test whether they could be found on the same substrates as reported before in other regions as well as on other substrates. More than 800 samples were collected in 26 areas of the low St Lawrence valley and throughout the Gaspé Peninsula. Our samples cover an area of about 150 000 km^2. We sampled a large diversity of substrates, and we used an enrichment method commonly applied for the isolation of *S. cerevisiae* and *S. paradoxus* (Sniegowski *et al.*, 2002).

Materials and methods

Sample collection

We collected 876 samples between April and October in 2011 and 2012 on various substrates on sites indicated in Fig. 1 following methods described in the study by Sniegowski *et al.* (2002) (see also Supporting Information, Table S1). Samples mostly included exudate material, fragments of bark and soil associated with trees, insects, slugs, flowers and decaying fruits. Samples were collected using a 14-mL sterile plastic tube (BD Falcon) or 1.5-mL sterile microtubes (Axygen). Tubes were maintained for a maximum of 1 week at 4 °C before enrichment.

Enrichment culture

All enrichments were performed as described in the study by Sniegowski *et al.* (2002). Ten millilitres (14-mL tubes) or 1 mL (1.5-mL tubes) of medium was added to the sample before incubation, and tubes were sealed airtight. In 2011, tubes from samples collected in paired replicates (two samples from the same substrate) were incubated at 18 and 30 °C, respectively, to ensure that the differences in optimal temperature growth between *S. cerevisiae* and *S. paradoxus* did not bias the isolation success (Sweeney *et al.*, 2004). Tubes corresponding to unique samples were only incu-

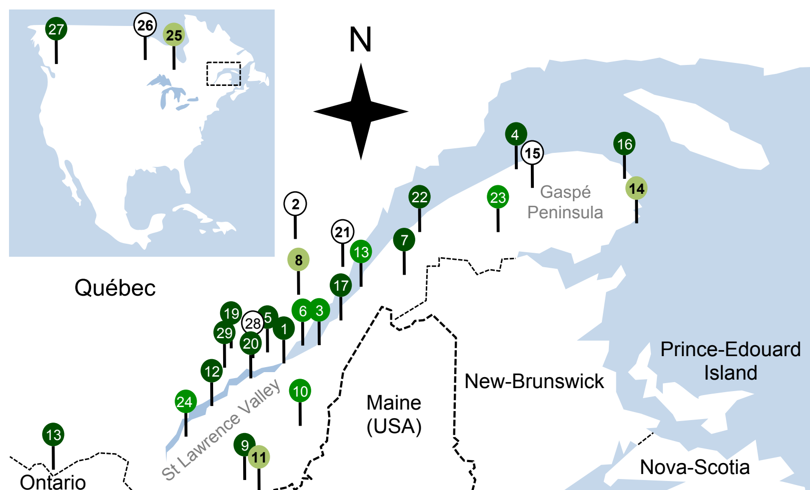

Fig. 1. Map of the 29 sites sampled in this study. The majority of sites (1–24; 28–29) were located in the province of Québec (St Lawrence Valley and Gaspé Peninsula) and three elsewhere in Canada (map on top left; the dotted frame indicates our study area). Sites are represented by circles (see Table S1 for details of locations) and are coloured in dark green when *Saccharomyces* ssp. were found; in green, when *Saccharomycetaceae* yeasts but no *Saccharomyces* sp. were found; in light green, when only non-*Saccharomycetaceae* yeasts were found; and white, when no yeast was isolated.

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bated at 18 °C. Because *Saccharomycetaceae* isolation efficiency was higher at 30 °C (Table S2; Fig. 2), we incubated all samples at this temperature in 2012. All samples were incubated for a period of 10 days. After incubation, tubes were agitated, and 10 μL of each culture was deposited on solid synthetic medium for colony isolation. The medium used was as described in Sniegowski *et al.* (2002), with the following modification: methyl α-D-glucopyranoside was replaced with glucose because some strains could be unable to use methyl α-D-glucopyranoside as a carbon source (Sniegowski *et al.*, 2002). Plates were incubated for 3–4 days at the same temperatures used for enrichment cultures and examined for colony growth. All colonies were considered unless they were mouldy. Cells from all colonies were observed under an optical microscope using phase contrast at 400× to identify clones corresponding to round and budding cells. These colonies were streaked on yeast extract peptone dextrose (YPD) agar plates to obtain isolated clones, after which a single isolated clone for each strain was inoculated into liquid YPD medium, grown to saturation and archived in 25% glycerol at −80 °C.

PCR identification of yeasts

We identified putative yeast isolates using a 850-bp *ITS1-5.8S-ITS2* sequence following Montrocher *et al.* (1998).

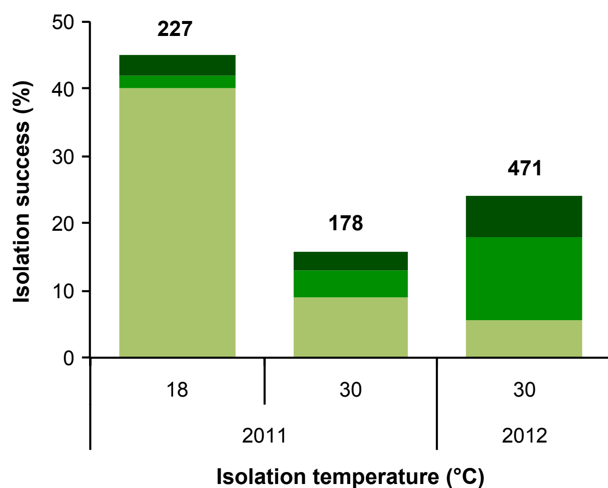

Fig. 2. Effect of temperature on yeast isolation success. Isolation was performed following the methods described in the study by Sniegowski *et al.* (2002) and tested at two different temperatures for the same samples in 2011 (18 and 30 °C) and at 30 °C in 2012. Bars represent proportion of samples that gave at least one strain. Bars are filled in light green when only non-*Saccharomycetaceae* yeasts were found; in green, when *Saccharomycetaceae* yeasts (excluding *Saccharomyces* ssp.) were found; or in dark green, when *Saccharomyces* ssp. were found. The number of samples in each temperature category is shown above the bars.

Genomic DNA was extracted as described in the study by Kaiser *et al.* (1994) from a 5-mL overnight culture for all yeast candidates obtained from the enrichment cultures. PCRs contained 2 μL of 10× Bioshop® buffer, 20 ng of genomic DNA, 1.2 μL of $MgCl_2$ 25 mM, 1.6 μL of dNTP mix 2.5 mM, 1.6 μL of each primer at 2.5 μM and 0.12 μL of Taq Polymerase Bioshop® 5 U μL^{-1}, in a final volume of 20 μL. PCRs were carried out in a thermocycler MasterCycler ProS Eppendorf© with the following steps: 3′ at 95 °C; then, 35 cycles of 30″ at 95 °C, 30″ at 50 °C and 2′ at 72 °C; and a final extension of 10′ at 72 °C. PCR products were then sequenced by Sanger sequencing. Sequences obtained were manually curated according to the original chromatogram and classified using NCBI BLAST against the nucleotide collection (nr/nt). An isolate was identified as a known taxon when it's *ITS1-5.8S-ITS2* sequence identity with the reference was higher than 97% with a longer than 90% coverage (Ciardo *et al.*, 2006). We carefully discarded NCBI BLAST reference strains that matched with our sequences, but for which we suspected identification errors (i.e. strains assigned to a taxon, but matching with uncharacterized strains rather than with the type strains of the taxon). Additionally, we validated our identification using type strains clearly identified in Kurtzman *et al.* (2011). Finally, we identified strains that did not match any clearly named taxon as uncharacterized (identity < 97%). We validated the status of uncharacterized strains after they had been frozen, by repeating *ITS1-5.8S-ITS2* amplification and sequencing.

Statistical analyses of isolation success

We performed statistical analyses on isolation success to test whether our survey was biased with respect to sampling size and to detect the effects of substrate, incubation temperature during enrichment and time of the year on isolation yield. We considered three metrics of efficiency: the proportion of samples giving at least one yeast isolate after enrichment (including *Saccharomycetaceae* yeasts), the proportion of samples that yielded at least one isolate belonging to *Saccharomycetaceae* (including from genus *Saccharomyces*) and the proportion of samples giving at least one *Saccharomyces* isolate. We considered samples from years 2011 and 2012 separately, given the differences in locations, isolation and sampling strategies. For each category and year, we first performed an ANOVA on isolation success rate, estimated as the ratio of the number of strains from the given category to the total number of samples. We considered the nature of the substrate (i.e. decayed fruit vs. insect, tree or associated soil; 2011 only), the temperature of isolation (2011 only), the number of samples and the month of sampling as additive factors in

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a linear model. To investigate more deeply the effect of time of the year on isolation success in each category, we performed a chi-squared test on isolation success at different months. We considered separately years 2011 and 2012 and the nature of substrate. All analyses were performed in R (Chambers & Hastie, 1992).

Results and discussion

Yeast isolation efficiency depends on the temperature of isolation, time of the year and substrate

We sampled 876 substrates in 26 locations in the St Lawrence Valley, Gaspé Peninsula and three other places in Canada (Ontario, Manitoba and British Columbia). Five locations yielded no isolate, despite intensive sampling in two of them (Table S3; Fig. 1). Interestingly, most of these locations were located either at high altitude (Parc de Gaspésie, Grands-Jardins: 600–800 m) or at the northern limit of our sampling area (Saguenay: 40°20′N, 70°18′ W; Manitoba: 52°08′N, 106°40′W), suggesting that cold weather and the scarcity of deciduous trees in these regions could have an effect on yeast diversity. From all the samples obtained, 28% (243 positives samples on 876) yielded putative yeast strains as identified by NCBI BLAST of the *ITS1-5.8S-ITS2* sequence (Table S3). In some rare cases (< 5%), unidentified bacteria were obtained, and in some rarer cases (1%), the filamentous fungus *Syncephalastrum* was isolated (Table S3). For some of the samples, isolation on solid medium gave rise to up to three different yeast taxa. The entire procedure allowed the isolation of 250 yeast strains covering at least 69 taxa (see Table S3).

We carried out our sampling over 2 years (2011–2012) from April to October. In 2011, we systematically isolated our strains at two different temperatures (18 and 30 °C) or only at 18 °C when only one sample was available. Greater isolation efficiency was obtained at 18 °C (45%) with a majority of non-*Saccharomycetaceae* yeast (40%) and few *Saccharomycetaceae* (5%; Fig. 2). At 30 °C, isolation efficiency substantially decreased (16%), but provided *Saccharomycetaceae* (5% and 7%, respectively) and *Saccharomyces* (3% in both cases) in the same proportions as at 18 °C, suggesting that 30 °C is a better temperature to efficiently reduce the isolation of non-*Saccharomycetaceae* species, without affecting the yield of *Saccharomycetaceae* and mesophilic *Saccharomyces* ssp. (Fig. 2). In 2011, we also sampled a wide variety of substrates, including bark and exudate of trees, soil associated with these trees, insects and various decaying fruits. Interestingly, decayed fruits gave the highest yeast recovery (51% on 148 samples; Fig. 3a), which was more than twice the recovery rate observed for other substrates (21% on 250 samples; Fig. 3b). However, almost no *Saccharomycetaceae* were recovered from decaying fruits (Fig. 3a), whereas trees, soil and insects provided 8% of *Saccharomycetaceae* and 4% of *Saccharomyces* (Fig. 3b). Overall, the nature of the substrates appeared to be the more critical factor for *Saccharomycetaceae* and *Saccharomyces* yield efficiency (ANOVA $P < 0.05$, Table S2). Thus, in 2012, we sampled only bark of trees, soil associated with these trees and insects, and we carried out isolation at 30 °C to enhance the yield of *Saccharomyces*. Isolation success was slightly higher than in 2011 for comparable samples isolated in similar conditions (24% on 470 samples) and provided a lower proportion of non-*Saccharomycetaceae* species (6%) and a majority of *Saccharomycetaceae* (18.5%), including *Saccharomyces* (6%; Fig. 2).

We found that isolation success continually and significantly increased from April to August–September and substantially decreased at the end of summer (Fig. 3; Table S2). This effect suggests that yeast abundance could progressively increase during the year, with a peak at the end of summer, and suddenly collapses in the fall. Our survey along the year was, however, conducted on different sites, and this effect has thus to be validated by a survey of the same sites along the year to discard any potential among-site variation. Differences between years could be another source of variation because of difference in isolation temperature and of the higher variety of substrates we sampled in 2011. We controlled for this source of variation and found that the evolution of yeast abundance during the year could be observed independently in 2011 (Fig. 3a and b) and 2012 (Fig. 3c), regardless of the number of samples and the nature of the substratum. This increase was stronger in our sampling of 2012 and for *Saccharomyces* isolation efficiency (ANOVA: $P < 0.1$, Table S2; chi-squared test: $p \ll 0.0001$, Fig. 3).

Non-*Saccharomycetaceae* and new potential taxa

The isolation method we used was not specific to *S. cerevisiae* and *S. paradoxus* and allowed the isolation of yeasts that were not specifically targeted by our sampling. Decaying fruits provided a high proportion (60%) of non-*Saccharomycetaceae* yeasts (Fig. 3a). These included two main families, *Pichiaceae* and *Saccharomycodaceae*, most often represented by the genera *Pichia* and *Hanseniaspora* (Table S3), which supports previous studies providing evidence that yeast diversity on fruits was dominated by these taxa in Europe (Davenport, 1976; Vadkertiova *et al.*, 2012). Additionally to *Saccharomycetaceae*, trees and their associated soils yielded *Debaryomycetaceae* (Table S3). For instance, we found *Debaryomyces*

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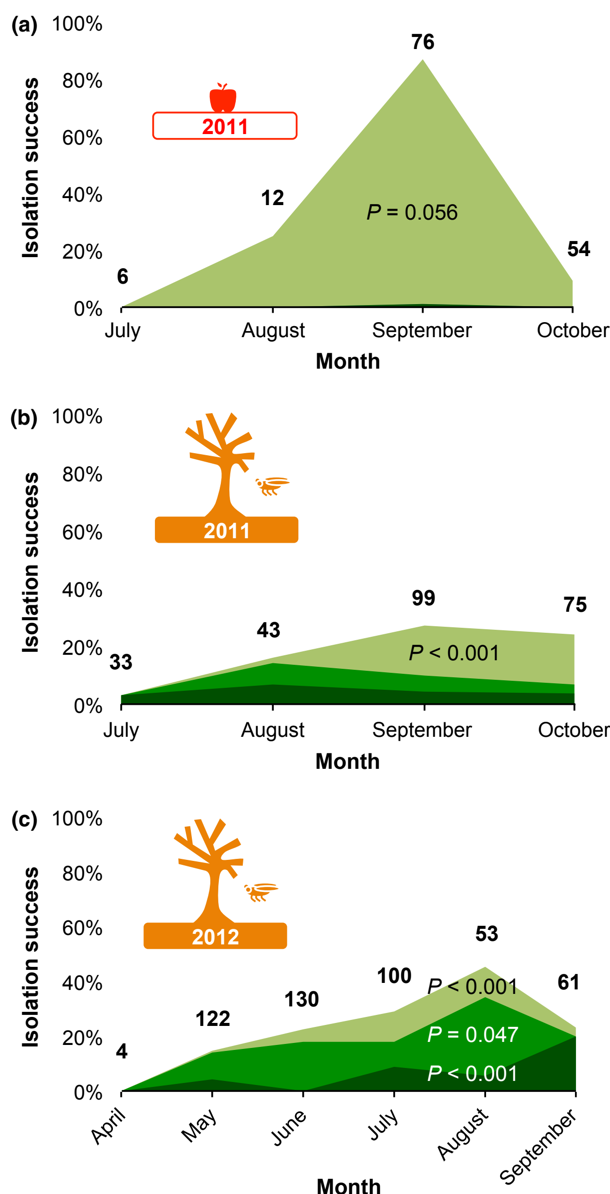

Fig. 3. Season-dependent isolation success. (a) Isolation success from decayed fruits in 2011. (b) Isolation success from trees, associated soils and insects in 2011 and (c) in 2012. In each case, areas indicate the proportion of samples that gave at least one yeast isolate (light green) for each month, one *Saccharomycetaceae* strain (green) or one *Saccharomyces* sp. strain (dark green). The number of samples in each month is represented above distributions. *P*-values indicate whether isolation success significantly varies between months in each category according to a chi-squared test (only significant or marginally significant differences are shown).

hansenii, which has been previously isolated from bark of tree in India (Bhadra *et al.*, 2008). Many *Phaffomycetaceae* (formerly described as *Wickerhamomycetaceae*; Kurtzman & Robnett, 2013; Table S3), mostly represented by *Wickerhamomyces anomalus*, were found to be associated with fruits and trees. This species was previously known to be mainly associated with food products and clinical environment, but some isolates had already been found in the wild on fruits and various plant surfaces (Daniel *et al.*, 2011). It is also known to produce killer agents and is used in the industry as a biocontrol agent against fungal crop pathogens (Lassois *et al.*, 2008) and some bacterial crop pathogens (Wang *et al.*, 2009).

Of these non-*Saccharomycetaceae* yeasts, about 18% (24 of 137) could not be formally identified to the species level. These isolates could be of interest in taxonomic studies as they belong to multiple families in the yeasts current taxonomy such as the *Pichiaceae*, *Saccharomycodaceae* and *Phaffomycetaceae* (Table S4). Two of these are potentially new yeast species, found on bark of trees, and were closely related to yeasts from the genus *Williopsis* (KF057726-7; 88% sequence homology), which are of biotechnological and medical interest (reviewed in Schmitt & Breinig, 2002). Moreover, one of these isolates (KF057493), also found on trees, may be a completely new species, because the closest in homology (85%) sequence belonged to an uncultured compost fungus (Hultman *et al.*, 2008). Our identification of yeast strains was, however, based on only one marker (*ITS1-5.8S-ITS2*), which is likely unavailable for some known species. Hence, the status of potentially new taxa we isolated should be validated with other genetic markers also frequently used in the identification of fungi such as *LSU* rRNA, *SSU* rRNA, EF-1α or cytochrome oxidase II (Kurtzman *et al.*, 2011).

Saccharomycetaceae and *Saccharomyces* diversity

We obtained 113 strains of *Saccharomycetaceae*. The vast majority of them came from bark samples from deciduous trees (92%; Fig. 3c) and were present in most of the sites sampled, excepted northern or elevated sites as discussed above (Saguenay, Grands-Jardins, Parc de Gaspésie; Fig. 1). These *Saccharomycetaceae* strains were represented by the genus *Kazachstania* (6), *Kluyveromyces* (8), *Torulaspora* (21), *Lachancea* (36) and *Saccharomyces* (41; Table S3), as previously found in different regions of the world for instance in Ontario (Maganti *et al.*, 2012) and British Columbia in Canada, in Germany and in Portugal (Sampaio & Goncalves, 2008).

The budding yeast *S. cerevisiae* was not found in any of the samples, while 41 strains of *S. paradoxus* were recovered. This is opposite to what has been observed in other regions of North America, for instance in Pennsylvania (Sniegowski *et al.*, 2002), Oregon and Missouri (Hyma & Fay, 2013). However, as it is the case in Québec, *S. paradoxus*, but not *S. cerevisiae* was recovered in Ontario

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(Maganti *et al.*, 2012). The scarcity or absence of *S. cerevisiae* in these regions could tentatively be explained by the difference in optimal growth temperature for these species (Sweeney *et al.*, 2004), as the climate in the provinces of Québec and Ontario is significantly colder than in the other areas where isolation of *S. cerevisiae* and *S. paradoxus* on the same substrates was reported (Sniegowski *et al.*, 2002; Sampaio & Goncalves, 2008; Liti *et al.*, 2009; Zhang *et al.*, 2010). Our survey also suggests that mesophilic *Saccharomyces* ssp. are absent or rare in cooler regions for instance in the mountains and the north of the St Lawrence River (Fig. 1). However, further sampling and isolation at lower temperature should be used in future investigations of *S. paradoxus* in these regions (Sampaio & Goncalves, 2008). For instance, *Saccharomyces uvarum* was reported in southern British Columbia and was only isolated at 10 °C (Sampaio & Goncalves, 2008).

Regarding the effect of substrates on the taxa isolated, trees belonging to the *Fagaceae* family (mostly oak species) yielded a threefold higher percentage of *S. paradoxus* isolates (12%) than other trees, including maple (4%). These rates remain lower than *Saccharomyces* isolation success in British Columbia, Germany, Portugal (33% on *Fagaceae* and 9% on other trees; Sampaio & Goncalves, 2008) and Pennsylvania (23% on *Fagaceae*; Sniegowski *et al.*, 2002), but are equivalent to what was observed in UK (8% on *Fagaceae*; Johnson *et al.*, 2004) and Ontario (10% on *Fagaceae* and 4% on other trees; Maganti *et al.*, 2012). The low recovery rates that were observed in cooler regions support the hypothesis that the northern distribution of mesophilic *Saccharomyces* ssp. could be limited by low temperatures. Insects, mostly from the genus *Musca* and *Bombus*, yielded some isolates in the same proportion as trees not belonging to the *Fagaceae* family (4%). This low recovery rate suggests that insects represent a poor substrate for the isolation of *S. paradoxus*. This is in contrast with several studies that successfully isolated *S. cerevisiae* from *Drosophila* and suggested that insects could be a major vector of yeast dispersal (Phaff *et al.*, 1956; Naumov *et al.*, 1996; Coluccio *et al.*, 2008; Stefanini *et al.*, 2012). Our result could suggest either that, unlike *Drosophila*, *Musca* and *Bombus* are only incidental vectors of *Saccharomyces* or that contrary to *S. cerevisiae*, *S. paradoxus* is not or rarely associated with insects. However, *S. paradoxus* was widespread across our sampling area, and its distribution was continuous among sites, suggesting that there is no limit to its dispersal at the scale of our study area (Fig. 1).

Conclusion

Our sampling of the northeast of North America unveiled a portion of the cultivable yeast diversity that resides along the St Lawrence valley and within the Gaspé Peninsula in the province of Québec. Our results suggest that this diversity comprises many species that form dynamic communities that prosper and decline with the cycling of the seasons. Some of the yeasts isolated could not be assigned to known species based on ITS sequences, which suggests that there may be some indigenous yeasts in the province of Québec that have not been reported elsewhere in the world. The biotechnology industry uses non-*Saccharomyces* ascomycetous yeasts in diverse applications such as environmental biotechnology, biomedical research, food industry and many other areas (reviewed in Johnson, 2013). The more yeast species or strains are uncovered in studies like this one, the more likely we are to find sources of enzymatic activities or species for biological applications that fulfil our needs. Our study therefore represents a useful resource for this community. Finally, our study supports previous reports suggesting that climatic variables are major factors in shaping the ecological distribution of *Saccharomyces* yeasts (Sweeney *et al.*, 2004; Sampaio & Goncalves, 2008; Liti *et al.*, 2009; Goncalves *et al.*, 2011).

Saccharomyces cerevisiae isolates could not be obtained with methods that were successful in isolating it in other regions of the world, which indicates that the overlap of its distribution with *S. paradoxus* is limited at northern latitudes, at least in North America. Finally, several *S. paradoxus* isolates were obtained and could be used in the context of ecological genomics studies that will provide more information on the ecology, phylogeography and biology of this species.

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Authors' contribution

G.C. and J.-B.L. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of sampling sites.
Table S2. ANOVA analysis of yeast isolation success.
Table S3. List of strains isolated in this study.
Table S4. Uncharacterized yeasts isolated in this study.

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