SHORT COMMUNICATION

Yeasts associated with the New Zealand *Nothofagus* honeydew system

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Abstract: New Zealand honeydew beech forests are the only natural ecosystem where large quantities of above-ground labile carbon are added year-round. Microbes can potentially play an important role in the flux of nutrients through food webs; because of their potential for specialisation to sugar-rich niches we tested for the presence of yeasts in the honeydew system. We found at least two abundant species (*Hanseniaspora osmophila* and *Candida railenensis*), as well as two species (*Zygosaccharomyces cidri* and *Z. rouxii*) likely present at lower frequencies. Only *Candida railenensis* is known to be associated with *Nothofagus*, but the other species are associated with fruits and winemaking/fermentation. These other species found in honeydew may be indigenous, but it is also possible that they represent microbial examples of invasive species and consequently add to the considerable list of human-introduced species that have invaded New Zealand's ecosystem.

Keywords: beech tree, honeydew ecology, invasive species, New Zealand indigenous yeasts

Introduction

Honeydew is the sugary exudate of sap-sucking insects such as aphids (Aphidae) and scale insects (Coccidae) found in the *Nothofagus* (beech) forests on the South Island of New Zealand. Although honeydew may be produced by a variety of sap-sucking insects, in these forests honeydew is produced by endemic sooty beech scale insects (*Ultracoelostoma* sp.; Morales et al. 1988; Beggs & Wardle 2006; Wardhaugh et al. 2006). These scale insects are most densely concentrated in the northern half of the South Island (Morales et al. 1988), where they are commonly found on black beech (*Nothofagus solandri* var. *solandri*), mountain beech (*N. s.* var. *cliffortioides*), red beech (*N. fusca*) and hard beech (*N. truncata*; Gaze & Clout 1983; Wardle 1984). The blackened appearance of the bark is due to the growth of unrelated ascomycetes and imperfect fungi on the honeydew (Hughes 1981; Reynolds 1999).

Honeydew beech forests are ecologically unique because they are the only natural ecosystem worldwide where such large quantities (up to 3500–4500 kg^{-1} ha^{-1} annually) of above-ground labile carbon (honeydew) are added year-round (Beggs & Wardle 2006). It is not surprising that a variety of trophic levels access the abundant resources that honeydew represents, and this food web is reasonably well characterised (Gaze & Clout 1983; Morales et al. 1988; Beggs & Wardle 2006). Even though they comprise a relatively small biomass, scale insects play a pivotal role in the flow of energy through these ecosystems and are therefore considered to be a keystone species (Beggs & Wardle 2006). While many of the components of this particular system are known (Beggs & Wardle 2006), there are few reports examining the microbial community. Yeasts are known to be present in coniferous aphid honeydew systems (Stadler et al. 1998); and yeasts (mainly *Sporobolomyces* sp. and *Cryptococcus* sp.) appear to prevent the detrimental accumulation of aphid honeydew on wheat (Dik et al. 1992). Due to their potential specialisation to sugar-rich niches, we were interested in determining whether there are *Saccharomyces* and other yeasts associated with the honeydew system in New Zealand.

Materials and methods

Sample sites

Three separate black beech trees were selected near Pelorus Bridge, northern South Island, New Zealand (41º17′ S, 173º34′ E), all of which were infested with *Ultracoelostoma* sp. (the sooty beech scale). Three areas on or near each tree were sampled using a sterile scalpel: the trunk, the 'splash zone' underneath the tree, and the

honeydew droplets directly. Samples were collected on 7 December 2006 and immediately placed in sterile containers and transported on ice to the laboratory for analysis.

Yeast isolation procedures

We chose to use a plating method to examine some of the yeasts associated with this system, but due to possible culturability issues we in no way claim that this method exhaustively enumerates or accurately reflects the diversity and number of yeasts in these niches. In the laboratory sterile water was added to the samples and mixed thoroughly. Each sample was then processed by two methods. First 100 µl of 10^{-2} and 10^{-3} dilutions were directly plated onto YPD agar (1% yeast extract, 2% peptone, 2% glucose and 2% agar); YPD is a permissive medium that generally allows many microbes to grow. To expedite analyses, these plates were incubated at 30ºC (which undoubtedly selected against certain yeasts, but is permissive for many) and subsequently inspected. Second, the procedure described by Mortimer and Polsinelli (1999), designed to select for fermentative yeast species, was employed. We were interested to test for the presence of *Saccharomyces* sp. and so determined the efficacy of this method to isolate *S. cerevisiae* from populations mixed with *Torulaspora delbrueckii* when *S. cerevisiae* was at a frequency of one in a thousand, ten thousand, or ten million cells. We were able to reliably recover *Saccharomyces cerevisiae* from all mixed artificial populations (data not shown), suggesting this method may be able to recover *S. cerevisiae* from samples where it is as rare as one in ten million culturable cells. For our environmental samples an equivalent sized piece of bark or 400 µl of solution was inoculated into 10 ml of SelMed medium (1% yeast extract, 2% peptone, 10% glucose and ethanol to a final concentration of 5% added after autoclaving). The vials were sealed and incubated at 30ºC for 8 days without shaking. Each day the samples were checked for evidence of fermentation, which is indicated by the evolution of gas. After 8 days 0.5 ml were removed from each vial and used to inoculate a further 10 ml of SelMed and these were again incubated at 30ºC for another 8 days and checked for evidence of fermentation. At days 8 and 16, 100 µl each of a 10^{-2} and 10^{-3} dilution were directly plated onto YPD agar and incubated at 30ºC for 2 days and the resulting cultures examined. Controls were included to preclude contamination.

Microbial identification

DNA was extracted from candidate colonies by incubating approximately 1 mm^3 of each sample in 150 µl of a 5% Chelex solution (Walsh et al. 1991) at 100ºC for 10 min. In order to identify and discriminate among the microbial specimens obtained we employed the polymerase chain reaction (PCR) to amplify the internal transcribed spacer region (ITS1 – 5.8S rRNA – ITS2), and the D1–D2 region of the 26S rRNA of the specimens (Kurtzman & Robnett 1998). These areas were amplified using the ITS1 and ITS4, and NL1 and NL4 primers respectively described by White et al. (1990) and Kurtzman & Robnett (1998). PCR products were electophoresed through 1% agarose in order to determine their size and both strands of the amplified products were sequenced using the dye terminator method. We sequenced the PCR products from four different colonies, KS1, KS2, KS3 and KS4; see Table 1. The DNA sequences were then compared with those in GenBank, using the BLASTn tool in the nucleotide Blast facility available on NCBI's website.

Sugar utilisation tests

The four colonies (KS1–KS4) were streaked onto SD (yeast nitrogen base 1.7 g L^{-1}, Sigma no. Y1251; 5 g L^{-1} ammonium sulphate), supplemented with either 20 g L^{-1} of glucose, fructose or sucrose, incubated for 3 days at 30°C and then visually inspected.

Results

Direct plating method

Microbial diversity and identification

Visual inspection of the agar plates after incubation showed they were infected with a diversity of microbes, which appeared to include a range of bacterial-like colonies, filamentous fungi, and yeast. Samples that exhibited yeast-like colonies were selected for molecular analyses.

The direct plating method produced yeast-like colonies from two of the three beech trees. Six colonies from the honeydew droplets, five colonies from the splash zone, and eight colonies from the trunk produced ITS and 26S PCR products. The 26s D1–D2 PCR product size was uniform for all the samples, but the ITS region differed. The samples from the honeydew and trunk produced an ITS PCR product of approximately 700 basepairs (bp) in length as did four of the colonies from the splash zone. However, one colony from the splash zone produced an ITS product of approximately 800 bp. A conservative estimate is that there are at least two species of microbial eukaryote associated with the New Zealand honeydew system.

We determined the sequence of the ITS and 26S regions of the two differently sized ITS PCR products. These sequences have been deposited in GenBank (Table 1). The sample we chose to represent the 700 bp ITS PCR product cohort (designated KS1) originated from a splash zone sample, as did the 800 bp ITS PCR product sample (designated KS4). Although it is not an exact match, the sequences from KS1 most closely match those from *Candida railenensis* and the sequences from KS4 are an exact match to those from *Hanseniaspora osmophila*.

Selective method

Many of the samples showed evidence of fermentation, and there proved to be at least two differently sized ITS PCR products present: one size emanated from a splash zone sample (700 bp; designated KS2), and another from a trunk sample (800 bp; KS3). Since this method is selective for microbes that can ferment in high sugar and ethanol it gives a much skewed representation of the microbial diversity from these samples.

In order to identify these microbes more accurately, and to see if they were the same two species that we recovered using the direct method, we again sequenced their PCR products and then compared them with the sequences deposited in GenBank. The ITS and 26S sequences from KS2 were an exact match to those of *Zygosaccharomyces cidri* (Table 1). The isolate KS3 matches well, but not exactly, to sequences for *Zygosaccharomyces rouxii*. Since the selective method was able to recover *Saccharomyces cerevisiae* when initially present at very low frequencies, at least in artificially constructed communities, its absence in the screen of the honeydew samples allows us to reasonably claim that it is not present in this niche at high frequencies.

Discussion

These data provide, as far as we are aware, the first direct evidence that yeasts are associated with scale insect honeydew systems in general, and is certainly the first evidence for this system in New Zealand. From just nine environmental samples we were able to unambiguously differentiate four different species of yeasts. While we did not detect any *Saccharomyces* species, it seems that some of these yeasts are so abundant within these niches that they may be isolated directly (*Candida railenensis* and *Hanseniaspora osmophila*), while others are only apparent if one selects for them (*Zygosaccharomyces cidri and Z. rouxii*) and are therefore presumably present at lower frequencies. Whether these organisms are active participants of the honeydew system, or are transients that have merely been vectored there is unknown. The recovery of *Candida railenensis* is interesting since it correlates with species recovered from *Nothofagus* from other continents in the Southern Hemisphere: two strains of *C. railenensis* in the CBS culture collection (CBS8164T and CBS8165) were isolated from *Nothofagus* in Chile (Ramirez & Gonzalez 1984; Barnett et al. 1990). Although *Candida railenensis* has been isolated from other plant

Table 1. Yeasts isolated from the *Nothofagus* honeydew system, South Island, New Zealand, and carbon sources they can utilise.

Isolate	Isolation method[a]	Growth on glucose, fructose and sucrose[b]	Region sequenced	Accession number	Best match in GenBank[c]	Origin of match
					AY528672.1 (99%)	*Candida railenensis*
KS1	Direct	+, +, +	ITS	EF620028	AY528671.1 (99%)	*Candida oleophila*
			26S	EU302821	EF653933 (98%)	*Candida railenensis*
KS4	Direct	+, +, +	ITS	EF620031	AJ271030 (100%)	*Hanseniaspora osmophila*
			26S	EU302824	U84228.1 (100%)	*Hanseniaspora osmophila*
KS2	Selective	+, −, +	ITS	EF620029	AY046205 (100%)	*Zygosaccharomyces cidri*
			26S	EU302822	U84236.1 (100%)	*Zygosaccharomyces cidri*
KS3	Selective	+, +, +	ITS	EF620030	AY046189 (98%)	*Zygosaccharomyces rouxii*
			26S	EU302823	AJ966531.1 (98%)	*Zygosaccharomyces rouxii*

[a] The mode of isolation is described in the Methods section.
[b] 'Direct' growth was defined by visual inspection after 3 days at 30°C on solid minimal medium where the listed sugars were the only source of carbon.
[c] The best matches (percent identity), using the Blastn tool, to the ITS1–ITS2 and D1–D2 regions of the 26S rRNA sequences present in GenBank.

sources (Ramirez & Gonzalez 1984; Barnett et al. 1990), it is possible that this species is well adapted to inhabit niches provided by *Nothofagus*. *Hanseniaspora osmophila* seems distributed widely, and has been isolated from, among other places, tree bark, soil and *Drosophila* (Barnett et al. 1990; Lachance et al. 1995; Mills et al. 2002; Cadez et al. 2003); however, *H. osmophila,* and *Hanseniaspora* in general, are often found associated with grapes, fruit ferments and winemaking (Mills et al. 2002; Xufre et al. 2006). The two *Zygosaccharomyces* species seem to be present at lower frequencies in the honeydew system. Among other places, as its name indicates, *Zygosaccharomyces cidri* has been isolated from cider ferments and it is able to ferment various hexoses (Barnett et al. 1990). The type strain of *Zygosaccharomyces rouxii* was originally isolated from concentrated black-grape must, and in general has been isolated from, and is adapted to, a variety of sugar-rich niches, including wine (Barnett et al. 1990), and for that reason may be considered a spoilage organism.

If these species are established members of the honeydew ecosystem then their role has yet to be elucidated, but it is not unreasonable to assume they have a function in decomposing the sugar-rich honeydew. The honeydew from the South Island comprises only a small proportion of glucose, but larger amounts of fructose, sucrose and other unidentified oligosaccharides (Beggs et al. 2005). While the strains of *Candida railenensis* and *Zygosaccharomyces cidri* examined by Barnett et al. (1990) are able to utilise sucrose aerobically, the strains of *Hanseniaspora osmophila* they examined are unable to utilise sucrose either aerobically or aerobically, and *Z. rouxii* shows delayed growth on sucrose. Barnett et al. (1990) present no data on the ability of these strains to utilise fructose. We tested the ability of the KS1–KS4 honeydew isolates to grow in an aerobic environment on minimal medium where glucose, fructose and sucrose were the sole carbon sources. All isolates grew on the glucose plates, showing that, as was expected, they are all able to utilise glucose; additionally, growth on this minimal medium showed that they are all prototrophic. All these isolates were also able to grow on a medium where sucrose was the sole carbon source; while our data agree with the growth capabilities of *Candida railenensis* and *Zygosaccharomyces cidri* reported by Barnett et al. (1990) they disagree for *Hanseniaspora osmophila* and *Zygosaccharomyces rouxii*. All the isolates, except the one we identified as *Z. cidri* (KS2), were able to use fructose as a sole source of carbon (see Table 1).

The isolates from the honeydew are highly likely to be genotypically different from those strains examined by Barnett et al. (1990), and it is entirely feasible that there is variation for the ability to utilise various sugar sources within and among the various yeast species. Clearly, natural selection will operate on those organisms able to utilise the abundant resources in a particular niche, and if fructose and sucrose are abundant in the honeydew it not surprising that some of the organisms that inhabit this niche are able to utilise these sugars. However, the fact that *Zygosaccharomyces cidri* was unable to utilise fructose (at least when placed on minimal medium at 30°C) is of note. It would be interesting to elucidate the remaining unidentified oligosaccharides in the honeydew, which comprise 40–80% of the sugars (Beggs et al. 2005), and to compare these to the carbon sources which these isolates are adapted to use.

New Zealand is well known for its unique flora and fauna, which evolved due to the country's distinctive geographic and ecological isolation. It seems that some species we found associated with this system, namely *Candida railenensis*, are also found associated with *Nothofagus* in other areas in the Southern Hemisphere. However, the discovery of the other species in the honeydew system raises some questions. There are at least two possible explanations for their presence: either they represent species indigenous to New Zealand (i.e. their presence pre-dates human arrival) or these species have invaded after initial human introduction. Although humans have been in New Zealand only a relatively short time (c. 800 years), anthropogenic impacts have been great and New Zealnd's fragile ecosystems are among the most highly invaded on earth (Clout 1999). The invasion explanation has some worth since the large Marlborough vine-growing region is only 25 km away, and *Hanseniaspora osmphila, Zygosaccharomyces cidri* and *Z. rouxii* have all been isolated from fruits and fermenting environments (Barnett et al. 1990). Indeed, at least two *Hanseniaspora* species are known to be present in New Zealand (Pennycook & Galloway 2004), and one of these, *H. vineae*, is on New Zealand's unwanted organisms register (http://www.biosecurity.govt.nz). More pertinently, the authors have isolated *H. osmophila* from grape juice in Marlborough, and *Hanseniaspora* yeasts from vineyards and wineries in the Auckland and Marlborough regions (unpublished data, and see GenBank accession number AY796120). Although it could be that these yeasts were naturally present in New Zealand before humans arrived, it seems at least possible that yeasts associated with the winemaking process were vectored to New Zealand by humans, and then invaded indigenous niches to which they were pre-adapted, such as the honeydew systems.

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