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Xanthophyllomyces dendrorhous (*Phaffia rhodozyma*) on stromata of *Cyttaria hariotii* in northwestern Patagonian *Nothofagus* forests

DIEGO LIBKIND[1]*, CELIA TOGNETTI[1], ALEJANDRA RUFFINI[1], JOSÉ PAULO SAMPAIO[2], MARÍA VAN BROOCK[1]

[1]*Laboratorio de Microbiología Aplicada y Biotecnología, INIBIOMA, UNComahue-CONICET, Av. Quintral 1250, Bariloche, Río Negro, Argentina;* [2]*Centro de Recursos Microbiológicos, Secção Autónoma de Biotecnologia, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal.*
**Correspondence. E-mail: libkindfd@comahue-conicet.gob.ar*

ABSTRACT

The occurrence and distribution of *Xanthophyllomyces dendrorhous* associated with *Cyttaria hariotii* parasitizing three *Nothofagus* species (*N. dombeyi, N. antarctica* and *N. pumilio*) in northwestern Patagonia (Argentina), as well as the factors that may affect this distribution were herein studied. Between 2000 and 2007, samples were obtained from 18 different locations. Based on physiological tests and morphological characteristics of sexual structures, 72 isolates were identified as *X. dendrorhous*. Representative strains were studied by MSP-PCR fingerprinting and sequence analysis of the ITS region. MSP-PCR fingerprints were similar for the newly isolated strains, and were also identical to the profiles of the strains previously found in this region. Patagonian strains appear to be a genetically uniform and distinct population, supporting the hypothesis that the association with different host species has determined genetically distinct *X. dendrorhous* populations worldwide. *X. dendrorhous* was recovered from *N. dombeyi* and *N. antarctica*. Approximately half the sampling sites and samples were positive for *X. dendrorhous*, but the isolation recovery rate was low. *X. dendrorhous* was absent in the early stages of ascostromata maturation, becoming more abundant in later stages. The present work represents a step forward in the understanding of the natural distribution and ecology of this biotechnologically relevant yeast.

Key words: Astaxanthin, biogeography, molecular phylogeny, South America

RESUMEN

***Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) asociado a estromas de *Cyttaria hariotii* en bosques de *Nothofagus* en el noroeste de la Patagonia**. Se estudió la ocurrencia y la distribución de *Xanthophyllomyces dendrorhous* asociado a *Cyttaria hariotii* en tres especies de *Nothofagus* (*N. dombeyi, N. antarctica* y *N. pumilio*) del noroeste de la Patagonia (Argentina), y los factores que podrían afectar esta distribución. El muestreo se realizó entre 2000 y 2007 en 18 sitios diferentes. Según las pruebas fisiológicas y las características morfológicas de las estructuras sexuales, 72 de los aislamientos obtenidos se identificaron como *X. dendrorhous*. Se estudiaron cepas representativas mediante la técnica de MSP-PCR *fingerprinting* y secuenciación de la región ITS. Los perfiles de MSP-PCR fueron similares, tanto entre los nuevos aislamientos como entre estos y los de cepas previamente obtenidas en la región. Aparentemente, las cepas patagónicas forman una población genéticamente uniforme y distinta de otras poblaciones. Esto apoya la hipótesis de que la asociación con diferentes especies hospedadoras ha determinado la diferenciación genética de *X. dendrorhous* en todo el mundo. *X. dendrorhous* se recuperó de *N. dombeyi* y de *N. antarctica*. Aproximadamente la mitad de los sitios de muestreo y de muestras fueron positivos para *X. dendrorhous*, pero la tasa de aislamiento fue muy baja. *X. dendrorhous* está ausente en estadios tempranos de maduración de ascostromas y se hace más abundante en estadios más tardíos. El presente trabajo contribuye al mejor entendimiento de la distribución natural y la ecología de esta levadura, de relevancia biotecnológica.

Palabras clave: Astaxantina, biogeografía, filogenia molecular, América del sur

INTRODUCTION

Xanthophyllomyces dendrorhous (sexual stage *Phaffia rhodozyma*) is a basidiomycetous yeast that develops pink to red colonies, and has a set of unique features among yeast species. On the one hand, its main carotenoid pigment is astaxanthin, an economically important pigment that is absent in other yeasts (9). On the other hand, it couples the ability to produce this pigment with the ability to ferment simple sugars (17). This combination of characteristics is unique among yeast species.

Original isolations of *X. dendrorhous*, which have served for most of the studies involving this species, were carried out in the 1960s by Phaff *et al* (17). These isolates were obtained from slime exudates of various broadleaved trees in different mountainous regions in the northern

hemisphere, such as Japan and Canada. More recently, isolations of this species have been carried out in Italy (22), Germany (23) and Argentina (13). The isolations carried out in northwestern (NW) Patagonia, Argentina were the first to be reported in the southern hemisphere, and unlike all other previous isolations, they were not from sap flow, but rather from ascostroma of *Cyttaria hariotii* (13) parasitizing *Nothofagus dombeyi*. However, this study was performed in a single location and *Nothofagus* species, thus the widespread occurrence of *X. dendrorhous* in the Patagonian *Nothofagus* forests lacked confirmation.

The Patagonian strains of *X. dendrorhous* bear genetic differences when compared to collection *X. dendrorhous* strains, yet their assignment to that species was supported by a high DNA homology based on DNA-DNA reassociation assays (13). The genetic differences within the species (intraspecific variability) have been hypothetically explained by geographic isolation and habitat specificity (13).

C. hariotii is an ascomycetous fungus, which is endemic of the south hemisphere and exclusively parasites *Nothofagus* spp., the main tree genus of the Andean-Patagonian forests (2) and in particular of Nahuel Huapi National Park. Besides being very abundant, *C. hariotii* is one of the most widely distributed species of the genus *Cyttaria*. Its distribution coincides with that of the genus *Nothofagus* in South America, i.e. from around 33ºS latitude in central Chile to 56º in Tierra del Fuego (Argentina) (2). Five of the South American *Nothofagus* species are susceptible to the parasitic *C. hariotii* fungus: *N. dombeyi*, *N. antarctica, N pumilio, N. betuloides*, and *N. nitida* (5), of which only the first three are present at Nahuel Huapi National Park.

The purpose of the research reported here was to study the distribution of *X. dendrorhous* in Patagonian *Nothofagus* forests (NW Patagonia, Argentina), especially concerning its occurrence and association to *C. hariotii* from the three main species that grow at Nahuel Huapi National Park (*N. dombeyi, N. antarctica* and *N. pumilio*). Methodology was based on *C. hariotii* sample collection, yeast isolation, phenotypic characterization of the isolated yeasts and molecular characterization of suspected *X. dendrorhous* isolates. Factors like maturity of ascostromata and presence of insects/larvae that may affect the distribution of this yeast were also analyzed.

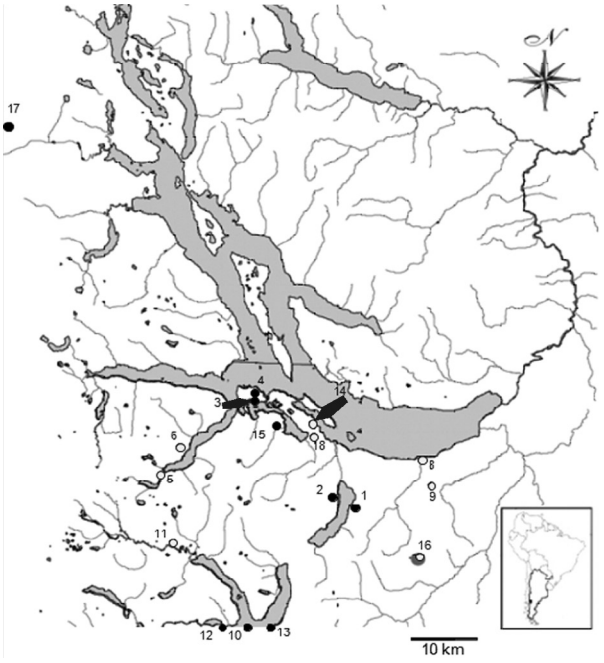

Figure 1. Geographic location of the sampling sites (Nahuel Huapi National Park, NW Patagonia, Argentina). Black circles correspond to *X. dendrorhous* positive sites and light grey circles indicate where *X. dendrorhous* was not found. Grey areas represent waterbodies. Arrows help associate sites 3 and 14 to their respective site number. Site 7 is not included because it was located near the city of Bolson (outside the mapped area).

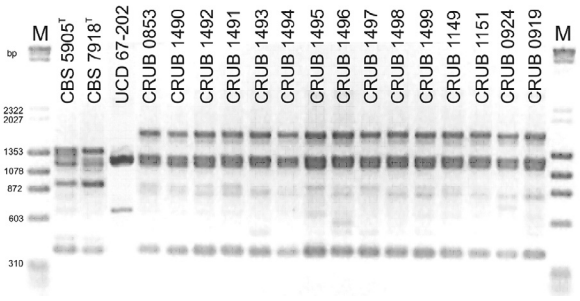

Figure 2. MSP-PCR fingerprints of selected *X. dendrorhous* strains generated with minisatellite primer M13. CBS 5905T, type strain of *Phaffia rhodozyma*, CBS 7918T, type strain of *X. dendrorhous*. UCD 67-202, *X. dendrorhous* strain from *Cornus* spp. Strains with the CRUB acronym correspond to Patagonian isolates. M, molecular size marker (λ DNA cleaved with *Hind*III and ΦX174 DNA cleaved with *Hae*III).

MATERIALS AND METHODS

Sampling area, sample collection, yeast isolation and phenotypic characterization

The sampling area covered 18 locations in the Nahuel Huapi National Park region, in NW Patagonia (41º05`S, 71º30`W), at altitudes of 500-1200 m above sea level (Figure 1, Table 1). *C. hariotii* from these sites grew on tumors of three different *Nothofagus* species: *N. dombeyi, N. antarctica* and *N. pumilio*.

Sampling was carried out in the months of November – December (spring), between 2000 and 2007. One to 10 samples of *C. hariotii*, comprising one to 11 ascostromata each, were aseptically collected at each location. Samples were stored in refrigerated sterile flasks until processing upon arrival at the laboratory (< 48 h). For each sample, the number of ascostromata and the fresh weight of the samples were registered. Three ascostromata maturity stages were considered as follows, (i) immature: ascostroma covered by a tough, elastic skin or cortical layer, which becomes membranous and tightly stretched; (ii) mature: ruptured cortical layer exposing mouths of apothecia; (iii) overmature: ascostroma has lost turgidity, cortex grows old, and the distinct odor of alcoholic fermentation may be perceived. Finally, ascostromata were checked for presence of insects/lar-

vae. Geographic location and altitude of each site were registered using a GPS (Garmin, Legend, USA).

Survey of yeasts present in the samples was carried out as described by Libkind *et al.* (13), with minor modifications. Ascostromata were cut into cubes (approximately 2 x 2 cm), placed in bags with sterile distilled water (1:1 w/v), and crushed manually inside the bags. The bag content was then transferred to Erlenmeyer flasks and shaken at 20 °C for 30 min at 300 rpm. Aliquots of 100 µl of the extracts (diluted so as to obtain no more than 300 colony-forming units per plate) were inoculated on YPD agar (g/l: yeast extract 10, peptone 20, glucose 20, agar 15). Culture medium was adjusted to pH 4.5 – 5 and supplemented with 200 mg/l chloramphenicol. Incubation temperature was 15-18 °C. Depending on the methodology used, isolations obtained could correspond both to the surface or the internal portion of the stromata.

Pigmented colonies that appeared on the plates were transferred to fresh YPD agar plates and were tested for production of amyloid compounds and ability to ferment glucose (25). Sexual stage formation was assessed as described by Kucsera *et al.* (11). Twenty-nine representative isolates were stored both by refrigeration (4 °C) on YMA (g/l, yeast extract 3; malt extract 3; peptone 5; dextrose 10; agar 20) and by deep-freezing (-180 °C) in liquid nitrogen.

Molecular characterization

X. dendrorhous isolates were characterized by the minisatellite primed-PCR technique (MSP-PCR) and then subjected to cycle sequencing of the ITS rDNA region. Pigmented isolates other than *X. dendrorhous* were also characterized by MSP-PCR, but sequencing was performed for the D1/D2 domains of the 26S rRNA gene. The DNA extraction protocol, MSP-PCR and electrophoresis conditions, and gel image analysis procedures were those reported in Libkind *et al.* (14). The primer employed was the core sequence of the M13 phage (5´-GAGGGTGGCGG-TTCT-3´). For sequence analysis, rDNA was amplified using the forward primer ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and the reverse primer ITS4 (5´-TCCTCCGCTTATTGATATGC-3´), as described in Libkind *et al.* (13). Alignments were made with BioEdit v7.0.9.0 (8), and visually corrected. Phylogenetic relationships were estimated using the MEGA program version 4.0.2 (19); the phylogenetic tree was constructed using the neighbor joining (NJ) algorithm, and bootstrap values calculated from 1000 replicate runs. The Kimura two-parameter model (10) was used to estimate evolutionary distance. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Sequences available in the GenBank database were used for comparative purposes.

RESULTS

Over 100 orange-pigmented yeast isolates were recovered from 56 samples of *C. hariotii* collected from 18 different locations at Nahuel Huapi National Park (Figure 1). Seventy-two of these isolates were considered *X. dendrorhous*-like, for their ability to produce amyloid compounds and ferment glucose. Patagonian isolates produced typical sexual structures of *X. dendrorhous*, confirming that they belong to *X. dendrorhous* and suggesting these Patagonian strains constitute a teleomorphic population of fungi. As depicted in Figure 2, DNA profiles obtained from MSP-PCR fingerprinting were highly similar for all isolated

Table 1. Additional information for surveyed sampling sites

Site code[(a)]	Sampling date	Site altitude (m.a.s.l.)	Tree host species	Number of samples obtained
1 a	December 2000	812	*N. dombeyi*	4
1 b	December 2003	833	*N. dombeyi*	5
2	December 2003	805	*N. dombeyi*	10
3	December 2004	816	*N. dombeyi*	4
4	December 2004	949	*N. dombeyi*	8
5	January 2005	811	*N. dombeyi*	2
6	January 2005	800	*N. dombeyi*	1
7	January 2005	501	*N. antarctica*	2
8	November 2005	800	*N. dombeyi*	2
9	December 2005	1091	*N. pumilio*	1
10	November 2006	790	*N. antarctica*	1
11	November 2006	800	*N. antarctica*	2
12	November 2006	805	*N. antarctica/ N. dombeyi*	3
13	November 2006	805	*N. antarctica*	1
14	November 2006	790	*N. dombeyi*	1
15	December 2006	790	*N. dombeyi*	4
16	December 2006	1230	*N. pumilio*	1
17	January 2007	1187	*N. dombeyi*	2
18	December 2007	809	*N. antarctica*	2

[(a)]Please use this code number to locate site on the map in Figure 1. Note that site 1 was sampled in two different years, hence the letters *a* and *b* follow the site code number.

strains. Isolates were identified as *X. dendrorhous,* based on ITS sequence analysis of representative strains . In agreement with the MSP-PCR results, all sequences obtained here were 100 % identical to those Patagonian strains previously sequenced (for example DQ661028) (Fig. 3).

Other pigmented yeast species found in *C. hariotii* were *Rhodotorula mucilaginosa*, *Rhodotorula colostri*, *Cystofilobasidium infirmominiatum*, *Cystofilobasidium capitatum*, *Cystofilobasidium macerans*, and the yeast-like fungus *Aureobasidium pullulans*. *Cystofilobasidium* yeasts were the most frequently isolated pigmented species, and frequently occurred simultaneously with *X. dendrorhous* in the same sample of *C. hariotii.* Considering that *Cystofilobasidium* yeasts grow in orange colonies, produce amyloid compounds and may weakly ferment glucose, colonies of this yeast may potentially be misclassified as *X. dendrorhous*-like.

X. dendrorhous was found in 45% of the samples, corresponding to 10 sampling sites (Table 2). Samples of *C. hariotii* were taken from three *Nothofagus* species (*N. dombeyi*, *N. antarctica*, and *N. pumilio*), but *X. dendrorhous* was only recovered from *N. dombeyi* and *N. antarctica* (Table 2).

Ascostromata maturity influenced the *X. dendrorhous* recovery rate. Immature ascostromata were mostly negative for *X. dendrorhous*, while approximately 75 % of samples corresponding to mature and over-mature ascostromata were positive (Table 3).

DISCUSSION

DNA profiles obtained from MSP-PCR fingerprinting were identical to those of the strains previously found in this region, and thus differed from those obtained from other regions around the world (13, 15). Judging by the ITS sequences, Patagonian strains appear to be a genetically uniform and distinct population considering that the new isolates herein obtained shared identical ITS sequences to those of previous studies. As depicted in Figure 3, three main clades can be distinguished. Patagonian strains form a homogenous group and constitute a sister clade of the main clade of north hemisphere strains which are associated to exudates of Betulaceae trees from different geographical areas. The case of the type strain of *P. rhodozyma* CBS 5905^T isolated from Fagaceae is still not clear, as previously addressed (13). The third clade includes only two strains from *Cornaceae* trees in Japan and is the least represented and more distantly related group.

When considering the ecology of the north hemisphere strains of *X. dendrorhous* and the ephemeral nature of *Cyttaria* stromata, it becomes highly probable that the Patagonian population is actually associated to other tree-related substrates such as soil or leaves. However, very few cases of *X. dendrorhous* isolations from phylloplane have been reported so far (16, 24) and none from soil, which can be explained by a very low abundance maybe

Table 2. Site and sample information, and *X. dendrorhous* isolate distribution for different host tree species

Host tree species	number of sites positive for *X. dendrorhous*	number of samples	number of positive samples	% positive samples [(a)]	number of isolates
N. dombeyi	7	44	21	48	68
N. antarctica	3	10	4	40	4
N. pumilio	0	2	0	0	0

[(a)] Calculated as (number of positive samples /number of samples) x 100

Table 3. Sample and *X. dendrorhous* isolate distribution for different maturity stages of *C. hariotii* ascostromata

Maturity of ascostromata	number of samples	number of positive samples	% positive samples	number of isolates	% isolates corresponding to each maturity stage[(a)]	% samples with more than two isolates
Immature	13	3	23	3	6	0
Mature	34	25	74	42	82	21
Overmature	4	3	75	6	12	50

[(a)] Calculated over the total number of isolates for which maturity data was available (n = 51).

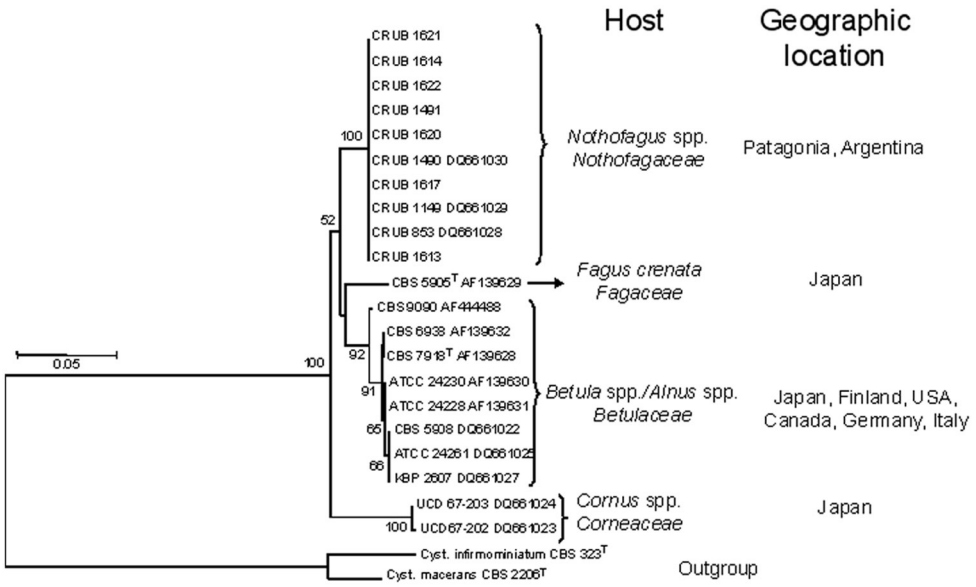

Figure 3. Phylogenetic relationships among *X. dendrorhous* strains inferred by ITS sequence analysis using the Neighbor-Joining method (18). Bootstrap analysis was performed with 1000 replicates (4). The evolutionary distances were computed using the Kimura 2-parameter method (10), and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (19). Sequences of CRUB strains lacking Genebank accession numbers were obtained in this study.

limited to a few spores. Due to the more favorable nutritional conditions of the stromata, opportunistic colonization by *X. dendrorhous* takes place when their fructification occurs in late spring. Similarly, *X. dendrorhous*-colonized exudates in the north hemisphere appear only in spring, providing this yeast the opportunity to propagate.

Based on the idea that the Patagonian strains are associated to the *Nothofagus* host, we previously proposed a host specificity model which suggested that the different yeast lineages (clades formed in Figure 3) colonize different tree species, and attribute genetic variation (intraspecific variation) to host specificity (13). The new information gathered in the present work supports this hypothesis, considering that the new isolates herein obtained from *N. antarctica* group together with those of the same genus. It remains to be studied if molecular markers with higher resolution could detect genetic subpopulations of Patagonian *X. dendrorhous* and if they are related to different *Nothofagus* species. This would help prove if the host specificity model can also be applied at host species level as well as at host family level, as already seen.

Several reports have shown the simultaneous presence of *X. dendrorhous* and *Cystofilobasidium* spp. (typically *C. infirmominiatum*, *C. macerans* and/or *C. capitatum*) in birch exudates (6, 7, 17, 21, 22). Apparently, when *X. dendrorhous* is found, *Cystofilobasidium* yeasts are also typically present. The opposite is not necessarily so, given that the natural distribution of *Cystofilobasidium* is broader than that of *X. dendrorhous*. Both genera belong to the monophyletic order *Cystofilobasidiales*, and thus share several physiological characteristics such as carotenoid production (3). Further studies are needed to understand the apparent niche overlap between *X. dendrorhous* and some *Cystofilobasidium* yeasts.

Despite the fact that about half of the total samples were positive for *X. dendrorhous*, positive samples yielded a low number of isolates, generally just one or two (only three samples yielded more than three isolates), which suggests that optimizing isolation procedures may be necessary to increase isolation recovery rates.

It is clear from recent research that habitats and distribution of *X. dendrorhous* are broader than originally suspected (13, 24). The present study, in particular, is the first report for *X. dendrorhous* growing on a *Nothofagus* species other than *N. dombeyi*, i.e. *N. antarctica*, and also shows that the occurrence of this yeast in *C. hariotii* is a general phenomenon in *Nothofagus* forests of NW Patagonia. We failed to find *X. dendrorhous* in *C. hariotii* from *N. pumilio,* likely due to the low sample size. Within Nahuel Huapi National Park, *N. pumilio* is usually restricted to altitudes above 1000 m.a.s.l. (2), and such altitudes were rarely covered in this study. However, in a previous work, we suggested that *X. dendrorhous* that had been isolated from lake water was originally from *C. hariotii* growing on *N. pumilio* surrounding the lake (15). Moreover, in a recent study dealing with the biodiversity of phylloplane fungi of *N. pumilio* at Nahuel Huapi National Park, a *X. dendrorhous* isolate was obtained (16). This result is in agreement with the proposal of *X. dendrorhous* as an epiphytic yeast (21, 24).

In a previous study we found, for a single location, that the proportion of *X. dendrorhous* isolates was higher

in mature ascostromata than in immature ascostromata (13); however, overmature ascostromata had never been studied. In the present study, more locations throughout Nahuel Huapi National Park were studied, and the great number of *X. dendrorhous* isolates recovered from mature ascostromata supported the previous observation. In addition, we observed that overmature ascostromata had a higher proportion of multiple (two or more) *X. dendrorhous* isolates than mature ascostromata. The increased isolate recovery rate for late maturity stages is probably related to the elevated sugar concentration in these stages (12). When mature, the fruiting bodies of *C. hariotii* have a sugar content of almost 10 % (mainly D-glucose, fructose, and sucrose) and contain polyols such as glycerol, D-mannitol, and D-arabinitol. Polyols are known to be key compounds for the development of the sexual cycle in *X. dendrorhous* (11).

It is clear that the nature and ecology of the sap-flows in the northern hemisphere and *Cyttaria* ascostromata in the southern hemisphere are quite different. However, both these substrates harbor *X. dendrorhous*. Thus, some careful analogies may be drawn between them. In both cases, *X. dendrorhous* is absent in the early stages of microbial colonization, and becomes more abundant in the later stages of colonization (6, 7, 21). However, in sap-flows, *X. dendrorhous* becomes one of the dominant species in the late phases of colonization (6, 7, 22), whereas in *C. hariotii*, *X. dendrorhous* does not outnumber sympatric ascomycetous fermenting yeasts frequently found in overmature ascostromata such as *Saccharomyces*, *Hanseniaspora*, *Pichia*, *Candida*, and *Zygosaccharomyces* (1, 20).

The second interesting analogy concerns the role of insects as vectors for *X. dendrorhous*. It has been suggested that insects are probably crucial as vectors for yeasts between sap-flows of different trees (21). The same is probably true for *X. dendrorhous* in *C. hariotii*. In the present work, it was not observed that *C. hariotii* ascostromata harboring insects/larvae had higher *X. dendrorhous* infection rates than those that did not.

In summary, *X. dendrorhous* was recovered in very different and distant locations throughout the sampling area, not only from *C. hariotii* infecting *N. dombeyi*, as previously documented, but also from *N. antarctica*. The Patagonian strains formed a genetically distinct population, supporting our previously suggested hypothesis that the association with different host species has determined genetically distinct *X. dendrorhous* populations worldwide. The abundance of this yeast increased with the maturity stages of the stromata and arguments were provided in favor of an epiphytic nature of this yeast when stromata (or exudates from the north hemisphere) are absent. Future studies will focus on the development of selective isolation media to increase the isolation recovery rate of *X. dendrorhous*, as well as on the study of molecular markers with better discriminatory power than those of the ITS region.

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