Phylogenetic structure of the *Sporopachydermia cereana* species complex

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A large number of isolates previously referred to as members of the '*Sporopachydermia cereana* species complex' were examined by various DNA characterization methods, leading to the conclusion that the complex is in fact made up of 10 species, one of which contains three varieties. The sequences of the internal transcribed spacer (ITS) region and the D1/D2 divergent domains of the large subunit rDNA were determined for representatives of each taxon and specific primers based on differences in the ITS were designed for rapid identification of five of the taxa. Whereas the data provide additional elements for the calibration of the ITS as a criterion for species delineation, the emerging pattern is that the ITS region does not function as well as the D1/D2 domains as an evolutionary clock. Some taxa appear to be specific for the geographical regions where they were isolated, and the distribution of many taxa is mutually exclusive.

Keywords: *Sporopachydermia*, rDNA sequencing, specific primers, phylogeny, biogeography

INTRODUCTION

Lachance & Phaff (1998) accepted three species in the genus *Sporopachydermia*. They cautioned that one of these, *Sporopachydermia cereana*, is difficult to identify by conventional methods, as it is part of a complex of cryptic species. The expression '*Sporopachydermia cereana* species complex' (Lachance *et al.*, 1988; Starmer *et al.*, 1990) has been used to designate the large number of isolates from necrotic cactus tissue that share with *Sporopachydermia cereana* the ability to assimilate inositol and erythritol as carbon sources. Collectively, these represent the third most abundant component of the cactophilic yeast community. In this paper, we extend the use of that term to refer to any taxon that is difficult to differentiate from *Sporopachydermia cereana* by morphological and nutritional characters and we shall adopt the expression '*Sporopachydermia* clade' to designate the entire genus and related asexual species. Over a 25-year period, we have examined a large number of isolates at the levels of morphology, physiology, DNA base composition,

DNA–DNA reassociation, electrophoretic karyotyping and rDNA sequencing and have found that the complex is in fact an assemblage of several genetically distinct taxa. The phylogenetic structure of the clade has been clarified by rDNA sequencing. We now report these results.

METHODS

Micro-organisms. The strains used in this study (Table 1) were collected during various expeditions to study the ecology and biodiversity of yeasts associated with cacti and other sources by us or by other colleagues. Identification followed standard methods (Yarrow, 1998) as modified for cactophilic yeasts (Lachance *et al.*, 1988).

Assessment of sporulation, mating and inhibition reactions. Sporulation was assessed on McClary's acetate agar. In order to detect sporulation that arises after conjugation, cells were first mixed and incubated for 2 d on corn meal agar (Difco) and then transferred to acetate agar. The presence of conjugation figures or ascospores was assessed by light microscopy. Inhibition of strains by other strains in the *Sporopachydermia cereana* complex was assessed by inoculating potential inhibitor cultures as spots on lawns of potentially sensitive cultures and observing the formation of clearing zones. The medium was YM agar (Difco) buffered to pH 5.0 with 0.01 M sodium acetate.

DNA characterization. Extraction and purification of DNA, determination of mol% G+C content of DNA by ultra-

Abbreviations: CTAB, cetyltrimethylammonium bromide; ITS, internal transcribed spacer.

The GenBank accession numbers for the sequences reported in this paper are AF202900–AF202921.

Table 1. List of strains used in this study

DNA group-specific primer responses are given where available. Strains that induced (I) or were susceptible to (S) G₁ arrest, strains whose ITS or D1/D2 sequences were determined (Sequenced) and sporulating strains (Sp) are identified. Strains the sequences of which were used to design group-specific ITS primers are identified as Reference. ^w, Weak response.

Strain	Source	Primer	G ₁ arrest	Remarks
<i>S. cereana</i>				
D72-313 ^T	<i>Carnegiea gigantea</i> , Arizona, USA	IT3		Reference
D76-214C	<i>Stenocereus thurberi</i> , Baja California, Mexico	IT3		Sp
D76-406C	<i>Rathbunia</i> sp., Sonora, Mexico	IT3	S ^w	Sp
D81-413C	<i>Opuntia phaeacantha</i> , Arizona, USA			
D81-489B	<i>Opuntia ficus-indica</i> , Arizona, USA	IT3	I	
D81-676.2	<i>Opuntia lindheimeri</i> , Texas, USA			
W84-201.4	<i>Opuntia phaeacantha</i> , Arizona, USA	IT3	S	
W84-211.2	<i>Opuntia phaeacantha</i> , Arizona, USA	IT3		
W84-233	<i>Opuntia phaeacantha</i> , Arizona, USA	IT3		
W84-213.2	<i>Carnegiea gigantea</i> , Arizona, USA	IT3		
W84-238.2	<i>Opuntia phaeacantha</i> , Arizona, USA	IT3	I	
W85-212.4	<i>Opuntia phaeacantha</i> , Arizona, USA	IT3		
W85-814.2	<i>Opuntia phaeacantha</i> , Arizona, USA	IT3		Sp
W85-837.2	<i>Carnegiea gigantea</i> , Arizona, USA	IT3		
W87-2305.2	<i>Opuntia ficus-indica</i> , Island of Maui, Hawaii, USA	IT7 ^w	I	Sp, Sequenced
W87-2326.3	<i>Opuntia ficus-indica</i> , Island of Maui, Hawaii, USA	IT7 ^w	I, S ^w	
W87-2350.2	<i>Opuntia ficus-indica</i> , Island of Maui, Hawaii, USA	IT7 ^w	I	
W87-2411.2	<i>Opuntia ficus-indica</i> , Island of Maui, Hawaii, USA	IT7 ^w	S	
<i>'oaxacaensis'</i>				
D77-321B	Cactus, Oaxaca, Mexico	IT7		Homologous
S84-554.2	<i>Stenocereus pruinosus</i> , Tehuacan, Mexico	IT7		
S84-567.2	<i>Estontria chiotilla</i> , Tehuacan, Mexico	IT7		
S84-575.2	<i>Estontria chiotilla</i> , Tehuacan, Mexico	IT7		
S87-475.1	<i>Opuntia (Nopalea)</i> sp., Oaxaca, Mexico	IT7	S	Sp
S87-477.1	<i>Opuntia (Nopalea)</i> sp., Oaxaca, Mexico	IT7	I, S ^w	
S87-478.2	<i>Opuntia (Nopalea)</i> sp., Oaxaca, Mexico	IT7	S	
S87-479.2	<i>Stenocereus pruinosus</i> , Oaxaca, Mexico	IT7	S	
S87-487.2	<i>Opuntia (Nopalea)</i> sp., Oaxaca, Mexico	IT7	I	
S87-507.2	<i>Stenocereus pruinosus</i> , Chiapas, Mexico	IT7	S, I ^w	
S87-524.1	<i>Stenocereus pruinosus</i> , Chiapas, Mexico	IT7	S, I ^w	
<i>'stenocereana'</i>				
D82-449B	<i>Stenocereus hystrix</i> , Haiti	—		Sp, Sequenced
<i>'obscura'</i>				
D82-520A	<i>Opuntia moniliformis</i> , Dominican Republic	IT5		Reference
S82-496A	<i>Stenocereus hystrix</i> , Dominican Republic	IT5		
S84-553.3	<i>Stenocereus pruinosus</i> , Tehuacan, Mexico	IT5		
S84-566.3	<i>Estontria chiotilla</i> , Tehuacan, Mexico	IT5		
S86-298	<i>Cephalocereus lanuginosus</i> , Barquisimeto, Venezuela	IT5		Sequenced
S87-461.1	<i>Stenocereus</i> sp., Oaxaca, Mexico	IT5		Sequenced
S87-468.1	<i>Stenocereus</i> sp., Oaxaca, Mexico	IT5		
W91-476.2	<i>Opuntia quimilo</i> , Argentina	IT5		Sequenced
<i>'australis'</i>				
S86-297	<i>Cephalocereus lanuginosus</i> , Prudencio, Venezuela	IT6		
S86-303	<i>Acanthocereus</i> sp., Prudencio, Venezuela	IT6		
S86-314	<i>Opuntia wentiana</i> , Prudencio, Venezuela	IT6		
S86-358	<i>Cereus repandus</i> , Barquisimeto, Venezuela	IT6		
W91-460.3	<i>Opuntia tilcarensis</i> , Argentina	IT6 ^w	I	Sequenced
W91-478.1	<i>Opuntia quimilo</i> , Argentina	IT6	I, S ^w	Reference
W91-480.2	<i>Opuntia ficus-indica</i> , Argentina	IT6	I, S ^w	
W91-486.2	<i>Opuntia quimilo</i> , Argentina	IT6	I	

Table 1 (cont.)

Strain	Source	Primer	G ₁ arrest	Remarks
W91-487.1	<i>Opuntia quimilo</i> , Argentina	IT6		
C91-1243	<i>Pilosocereus arrabidae</i> , Brazil	IT6		
C91-1407	<i>Pilosocereus arrabidae</i> , Brazil		S	
'opuntiana'				
D77-33	<i>Opuntia stricta</i> , Australia	IT4 ^w	S ^w	Sequenced
D78-516D	<i>Opuntia stricta</i> , Australia			
D79-101A	<i>Opuntia stricta</i> , Australia			
D81-645.3	<i>Opuntia lindheimeri</i> , Texas, USA	IT4 ^w		
D82-424A	<i>Opuntia moniliformis</i> , Haiti	IT4 ^w	S	Sequenced
S82-450B	<i>Stenocereus hystris</i> , Haiti	IT4		
S82-459B	<i>Opuntia moniliformis</i> , Haiti	IT4		
D82-461A	<i>Opuntia moniliformis</i> , Haiti	IT4	I	Reference
S82-501B	<i>Stenocereus hystris</i> , Dominican Republic	IT4		
S82-504B	<i>Stenocereus hystris</i> , Dominican Republic	IT4		
S82-508B	<i>Stenocereus hystris</i> , Dominican Republic	IT4		
D82-571B	<i>Opuntia stricta</i> , Montserrat	IT4 ^w		
S82-572B	<i>Stenocereus hystris</i> , Dominican Republic	IT4		
S82-608C	<i>Cephalocereus royenii</i> , Virgin Islands	IT4		
D82-611C	<i>Cephalocereus royenii</i> , Montserrat			
S82-638C	<i>Melocactus intortus</i> , Virgin Islands	IT4		
D82-646C	<i>Melocactus intortus</i> , Virgin Islands			
S82-653B	<i>Cephalocereus royenii</i> , Virgin Islands			Sequenced
W83-1037.3	<i>Drosophila mulleri</i> , <i>Opuntia stricta</i> , Jamaica	IT4 ^w	I ^w	
W83-1042.4	<i>Drosophila stalker</i> , <i>Opuntia stricta</i> , Jamaica	IT4 ^w		
S86-242	<i>Neoraimondia</i> sp., Peru			Sequenced
G90-209.3	<i>Stenocereus</i> sp., Antigua	IT4 ^w		
G90-400.2	<i>Opuntia stricta</i> , Florida, USA	IT4 ^w	I ^w	Sp
W90-1004.2	<i>Opuntia stricta</i> , Ship Channel Cay, Bahamas	IT4 ^w		
W90-1029.2	<i>Opuntia stricta</i> , Hawksbill, Bahamas	IT4 ^w		
W90-1033.3	<i>Opuntia moniliformis</i> , Richard's Rock, Bahamas	IT4 ^w		
W90-1041.2	<i>Opuntia stricta</i> , Long Cay, Bahamas	IT4 ^w	I	
W90-1043.2	<i>Opuntia stricta</i> , Long Cay, Bahamas	IT4 ^w		
W90-1054.2	<i>Opuntia stricta</i> , Hall's Pond Cay, Bahamas	IT4 ^w	S	
W90-1115.1	<i>Opuntia stricta</i> , Conception Island S., Bahamas	IT4 ^w		
W90-1136.2	<i>Opuntia stricta</i> , Conception Island N., Bahamas	IT4 ^w		
W90-1156.1	<i>Opuntia stricta</i> , Little Torch Key, Florida, USA	IT4 ^w		
W93-333.2	<i>Opuntia stricta</i> , Alderlay Cay, Bahamas	IT4 ^w		
W93-357.5	<i>Opuntia stricta</i> , Conception Island N., Bahamas	IT4 ^w		
W95-917.3	<i>Opuntia stricta</i> , Australia	IT4 ^w		
'trichocereana'				
W91-449.2	<i>Trichocereus pasacana</i> , Argentina	—		Sp
W91-456.2	<i>Trichocereus pasacana</i> , Argentina	—	I	Sp, Sequenced
'centralis'				
S94-304.2	<i>Opuntia vulgaris</i> , Honduras	—		Sequenced
S94-310.1	<i>Opuntia guatemalensis</i> , Honduras	—		Sequenced
S94-313.1	<i>Opuntia guatemalensis</i> , Honduras	—		Sequenced
S87-428.4	<i>Opuntia (Nopalea)</i> sp., Chiapas, Mexico	—		Sequenced
'brasiliensis'				
C90-399	<i>Pilosocereus arrabidae</i> , Brazil	—		Sequenced
C90-1001	<i>Pilosocereus arrabidae</i> , Brazil	—		
C91-1764	<i>Pilosocereus arrabidae</i> , Brazil	—	S ^w	
'pachycereana'				
D76-228A	<i>Pachycereus pringlei</i> , Baja California, Mexico	—		Sequenced

Table 1 (cont.)

Strain	Source	Primer	G ₁ arrest	Remarks
‘<i>agaves</i>’				
W92-213.4	<i>Agave tequilana</i> , Jalisco, Mexico	—		Sequenced
W92-217.3	<i>Agave tequilana</i> , Jalisco, Mexico	—		
W94-257.2	<i>Agave tequilana</i> , Jalisco, Mexico	—		
W94-279.2	<i>Agave tequilana</i> , Jalisco, Mexico	—		
‘<i>paraquercuum</i>’				
W91-110.1	<i>Quercus rubra</i> , Ontario, Canada	—		Sequenced
<i>S. lactativora</i>				
CBS 6989 ^T	Hybrid culture	—		Sequenced
<i>S. quercuum</i>				
W80-118 ^T	<i>Quercus rubra</i> , Ontario, Canada	—		Sequenced

Table 2. List of oligonucleotides used as primers in this study

Calculated annealing temperatures (T_m) are given (°C). LSU, Large subunit; SSU, small subunit.

Primer	Sequence (5' to 3')	T_m (hybrid)	Remarks
NL1*	GCATATCAATAAGCGGAGGAAAAG	52.0	5' end of LSU D1, forward
NL4*	GGTCCGTGTTTCAAGACGG	52.1	3' end of LSU D2, reverse
IT1†	TAGGTGAACCTGCGGAAGGATCAT	55.4	3' end of SSU, forward
IT2*	CTTTTCCTCCGCTTATTGATATGC	52.0	5' end of LSU D1, reverse complement of NL1
IT3	GCAATCACTGGCTTAACAGATGT	51.6	ITS2, forward, strain D72-313 ^T (<i>S. cereana</i>)
IT4	CTGGCTGAAGAATATCCTCCAG	53.1	ITS1, reverse, strain D82-461A (' <i>opuntiana</i> ')
IT5	ACCCATCGCTGGCTTTTGGAGAT	53.4	ITS2, forward, strain D82-520A (' <i>obscura</i> ')
IT6	GCTAGAGAAATTGTCTCCAGCC	53.1	ITS1, reverse, strain 91-478.1 (' <i>australis</i> ')
IT7	GTGATCACTGGCTTATGAGATGATAGTCT	56.2	ITS2, forward, strain D77-321B (' <i>oaxacaensis</i> ')

* Primer sequence taken from O'Donnell (1993).

† Designed by alignment of the complete rDNA sequences of *Saccharomyces cerevisiae* (GenBank accession nos U53879 and Y13138) and *Schizosaccharomyces pombe* (GenBank accession no. Z19578).

centrifugation in CsCl and assessment of DNA reassociation by separation on hydroxyapatite were performed as described by Price *et al.* (1978), except that reference DNAs were labelled by radioiodination (Holzschu *et al.*, 1979).

Electrophoretic karyotyping. Cells were harvested from YM agar, washed in 0.05 M EDTA (pH 8) and adjusted to an optical density (660 nm) of 20. The cells were diluted 1:1 with 0.05 M EDTA and molten agarose (low-melting-point, Bio-Rad; final concentration 1% in 0.05 M EDTA) and poured into moulds. The agarose blocks were incubated overnight at 37 °C in buffered Pronase as recommended by McCluskey *et al.* (1990), washed several times in 0.05 M EDTA and stored in the cold (approx. 4 °C). Pulse-field electrophoresis was conducted in a Bio-Rad CHEF DRII apparatus at 84 V, 14 °C for 72 h, with the switching time increasing linearly from 120 to 900 s.

DNA sequencing and design of taxon-specific primers. The ITS region and the D1/D2 divergent domains of rDNAs were amplified by the PCR. Whole yeast cells were used as sources of templates. Cells growing on YM agar (Difco) were suspended in sterile water to a density of '1 + ' (Yarrow, 1998) and the suspension was added at a rate of 40% of the PCR volume. The amplification was conducted as recommended by the manufacturer of heat-activated *Taq* poly-

merase (AmpliTaq Gold, Perkin-Elmer) in a Perkin-Elmer System 2400 cycler. After 9 min at 95 °C, the reaction mixture was subjected to 35 cycles of 94 °C for 10 s, various annealing temperatures for 10 s and 72 °C for various extension times, as required. A final extension at 72 °C for 7 min was followed by cooling to 4 °C. The annealing temperatures were dictated by the calculated hybridization melting temperatures of the primers (DNAMAN version 4.0; Lynnon BioSoft). When the melting temperatures of various primers (Table 2) used together differed significantly, the annealing temperature was ramped from the highest to the lowest value at the rate of -0.2 °C per cycle and then remained constant for the remaining cycles. The extension time for separate amplification of D1/D2 or ITS regions was 20 s. Screening of strains for the presence or absence of group-specific sequences involved the use of three primers (Fell, 1993). For forward specific primers located in the ITS2 spacer, the reference primers were IT1 and NL4 and the extension time was 30 s, as the expected specific products were 0.7 kb long. For reverse specific primers located in the ITS1, the reference primers were IT1 and IT2 and the extension time was 5 s, to favour amplification of the 0.05 kb products. The primers were purchased from Procyon BioPharma.

For sequencing, the amplified DNA was concentrated and purified with MicroCon 100 concentrators (Amicon) or with Qiaquick PCR columns (Qiagen) and sequenced in an ABI sequencer at the John P. Robarts Research Institute (London, Ont.). The program CHROMAS 1.5 (Technelysium) was used to edit the sequence electrophoregrams. The program DNAMAN was used to align the sequences by CLUSTAL W (Thompson *et al.*, 1994), to design primers and to construct neighbour-joining trees.

RESULTS AND DISCUSSION

Provisional nomenclature

The designations used in the following discussion are based on all the information reported in this paper, with special emphasis on rDNA sequences or reaction with specific primers. They will be justified after reviewing the data. We shall ultimately propose the division of the species complex into ten species, one of which contains three varieties. The origin of the names is as follows. '*Sporopachydermia cereana sensu stricto*' applies to the species defined by Phaff *et al.* (1974) and Rodrigues de Miranda (1978) and further by Lachance & Phaff (1998). The epithet '*oaxacaensis*' applies to strains that originated from localities in three southern states of Mexico, Oaxaca being the most central. The

designation '*stenocereana*' applies to a single isolate from a Haitian columnar cactus. The epithet '*obscura*' applies to strains isolated sporadically from various host plants and geographical locations in the tropical Americas. Strains in the '*australis*' group were isolated in three South-American regions. The '*opuntiana*' group comprises isolates that originated primarily from Australia, the south-eastern United States and the Caribbean, mostly from prickly pears. Two isolates unique to the Argentinian cardón cactus are referred to as '*trichocereana*'. Four isolates from the Mexican state of Chiapas and two localities in Honduras were termed '*centralis*' (Central America). Representatives of certain Brazilian isolates (Rosa *et al.*, 1995) are designated '*brasiliensis*'. An isolate from the Mexican cardón cactus is referred to as '*pachycereana*'. Isolates from agave rots collected in the Mexican state of Jalisco (Lachance, 1995) are referred to as '*agaves*' and an isolate from a red oak exudate in Ontario is termed '*paraquercuum*'.

Growth characteristics

With minor exceptions, all known members of the *Sporopachydermia* clade assimilate D-xylose, ethanol, glycerol, *myo*-inositol and ethyl acetate as sole carbon

Table 3. Nucleotide base composition and DNA–DNA reassociation among selected strains of the *Sporopachydermia cereana* species complex

Results were obtained by the liquid-phase, hydroxyapatite method. Homologous reactions are shown in parentheses.

Strain	DNA G + C content (mol %)	Reference strain	
		D72-313 ^T	D82-461A
<i>S. cereana sensu stricto</i>			
D72-313 ^T	49.3	(100)	10
D76-214C	49.2	85	
D76-406C	48.5	73	
D81-413C	48.7	83	
D81-489B	49.6	82	
D81-676.2	49.4	80	
' <i>oaxacaensis</i> '			
D77-321B	48.6	32	
' <i>stenocereana</i> '			
D82-449B	50.4	39	
' <i>obscura</i> '			
D82-520A	45.1		8
' <i>opuntiana</i> '			
D77-33	42.6		78
D81-645.3	42.2		78
D82-424A	42.2	8	53
D82-461A	41.8		(100)
D82-571B	41.5		75
D82-611C	41.6		74
' <i>pachycereana</i> '			
D76-228A	43.5	7	7

sources and ethylamine and lysine as sole nitrogen sources. All grow at 30 °C or higher and are resistant to at least 10 µg cycloheximide ml⁻¹. Vitamins are required for growth, but amino acids are not. Members of the *Sporopachydermia cereana* species complex differ from the rest of the clade (*Sporopachydermia lactativora* and *Sporopachydermia quercuum*) by their ability to assimilate erythritol as sole carbon source.

Within the species complex, the assimilation of trehalose, cellobiose, salicin, L-sorbose, D-arabinose, D-ribose, 1-propanol, 2-propanol, 1-butanol, ribitol, xylitol, mannitol, glucitol, lactic acid, succinic acid, 2-ketogluconic acid and acetone as carbon sources is variable, as are utilization of cadaverine as sole nitrogen source and resistance to 0.5% tannin, 75 µg cetyltrimethylammonium bromide (CTAB) ml⁻¹, 500–1000 µg cycloheximide ml⁻¹ and 8 µg digitonin ml⁻¹. Weak utilization of L-arabinose may occur. Vegetative cells may be curved. Formation of ascospores is variable and occurs in single cultures grown on McClary's acetate agar. Sporulation was detected in several strains of *Sporopachydermia cereana* (all three varieties), a single strain of '*opuntiana*' and both strains of '*trichocereana*' (Table 1). Conjugation does not precede ascus formation, but in some cases cultures mixed in pairs undergo morphological distortions indicative of G₁ arrest. This can also be visualized on a Petri dish by plating a lawn of target cells and spot-inocula of inhibitory cells in a manner similar to that used for detection of killer toxins. Strains known to cause the inhibition or to be susceptible to it are indicated in Table 1. The inhibition was not specific, as most sensitive strains were inhibited by most inhibitor strains. The phenomenon appears to be independent of taxonomic position within the species complex. The optimum pH for these reactions is close to the optimum growth pH (about 5.5). We interpret this phenomenon as a remnant of a mating system that is no longer functional.

Taken together, the above properties do not clearly partition into groups of correlated responses on the basis of which the different taxa in the species complex can be defined. As will be shown below, even when the relatedness patterns among strains are known, the plasticity of many characters renders them nearly unusable for defining all taxa.

DNA base composition

The first indication that yeasts originally identified as *Sporopachydermia cereana* (or *Cryptococcus cereanus*) were not genetically homogeneous came from the determination of G + C contents in their nuclear DNA. Representative values are given in Table 3. Four classes emerged, namely 49–50 mol% for the type of the species and many similar strains, 41–42 mol% for a very large number of isolates that fit in the '*opuntiana*' group, 45 mol% for an isolate of the '*obscura*' group and 43.5 mol% for the single isolate designated as

'*pachycereana*'. The other two described species of *Sporopachydermia* (Lachance & Phaff, 1998) have values of 46 mol% (*Sporopachydermia lactativora*) and 38 mol% (*Sporopachydermia quercuum*).

DNA–DNA reassociation

DNA–DNA reassociation experiments (Table 3) confirmed the existence of four genetically distinct units and further indicated that the 49–50 mol% G + C strains in fact represent three separate populations, namely *Sporopachydermia cereana sensu stricto* plus strains designated as '*oaxacaensis*' and '*stenocereana*'. One '*opuntiana*' isolate (D82-424A) also appeared distinct from the rest, based on a 53% reassociation value.

Electrokaryotypes

Electrophoretic karyotypes for 60 strains are shown in Fig. 1. The patterns are characterized by the presence of four or more bands, many of which represent chromosomal DNAs that are larger than those of *Saccharomyces cerevisiae*. Although the patterns did not always allow each strain to be positioned within a group, they were useful in identifying replicate isolates or closely related strains and they allowed certain strains to be excluded as potential close relatives (e.g. the pattern for '*paraquercuum*' was substantially different from all others). Note, however, the important variation observed in some groups, for example '*obscura*'.

Sequence analyses

In view of the perplexing picture emerging from the above data, rDNA sequencing was applied to the resolution of the phylogenetic group structure in *Sporopachydermia*. One objective was to find indicator sequences and to use these to design group-specific primers that could be used to identify unknowns rapidly. Two important assumptions underlie this approach, namely (i) that strains that respond positively to a specific primer have essentially the same sequence as the reference strain and (ii) that the phylogeny of the rDNA reflects the history of the taxon (orthology).

In a preliminary test, D1/D2 divergent domains of the large subunit rDNA were examined in the type of *Sporopachydermia cereana* (sequence from Kurtzman & Robnett, 1998), members of the closely related '*oaxacaensis*' and '*stenocereana*' designations and the more distant strains D82-520A and D76-228A, representing '*obscura*' and '*pachycereana*' (at least one member of each group was eventually sequenced, Fig. 2). The inter-strain divergence values among the five sequences varied from 0.5 to 2.5%, but the variation was dispersed along the entire length of the D1/D2 domains, making the design of specific probes impractical. We therefore turned to the more variable

Fig. 1. Electrophoretic karyotypes of selected strains in the *Sporopachydermia cereana* species complex. See Methods for details. *Saccharomyces cerevisiae* (Bio-Rad standard) was used for comparison.

internal transcribed spacer (ITS) region. Strains D72-313^T, D77-321B, D82-449B, D82-520A, W91-478.1, D82-461A, W91-456.2, S87-428.4, D76-228A, CBS 6989^T (type strain of *Sporopachydermia lactativora*) and W80-118^T (type strain of *Sporopachydermia quercuum*) were sequenced first. Although the 5.8S gene was invariant in all strains, the minimum di-

Fig. 2. Phylogeny of the *Sporopachydermia* clade based on sequences of the D1/D2 region of the rDNA. The trees were constructed by neighbour-joining. Bootstrap values for 1000 iterations are shown as percentages. Bar, 1% DNA divergence.

Fig. 3. Phylogeny of the *Sporopachydermia* clade based on sequences of the ITS region of the rDNA. The trees were constructed by neighbour-joining. Bootstrap values for 1000 iterations are shown as percentages. Bar, 1% DNA divergence.

vergence between strains shown to be genetically distinct by DNA reassociation was 1.3% for the entire ITS cluster (ITS1–5.8S–ITS2), indicating that sufficient variation existed to discriminate between groups (Fig. 3). Primers IT3 to IT7 were designed and used sequentially, allowing the progressive assignment of most strains into groups without the need to sequence them all. Primers specific to the last eight taxa in Table 1 were not necessary, because these taxa could be identified either on the basis of other properties or by sequencing, being represented by only a few strains.

The ITS regions of additional strains (Fig. 3) were sequenced for the following reasons. In the '*opuntiana*' group, only the homologous reaction (strain D82-461A) gave a clear positive result by the three-primer method. Other members gave a weak signal for the specific primer pair as well as a weak but clear signal for the reference pair. The sequence of strain D77-33 was therefore determined and found to differ by 1.3% from that of the reference strain (D82-461A), with a two-base inversion in the priming site. In view of this result and of the 53% DNA-DNA reassociation value obtained between strains D82-461A and D82-424A (Table 3), the latter was also sequenced. Strains S82-653B and S86-242 (not included in Fig. 3) gave ambiguous reactions with primer IT4 and were sequenced. The pair-wise sequence divergence between the five sequenced strains in the '*opuntiana*' group varied from 0.9 to 2.0%, and variation existed in three adjacent bases located in the IT4 priming site. However, the phylogenetic analysis of the aligned ITS sequences showed that they belong to the same clade (the three most divergent representatives are included in Fig. 3).

On the basis of other data, the Hawaiian isolates (represented by strains W87-2305.2 and W87-2326.3) had been assigned to *Sporopachydermia cereana sensu stricto*. However, they gave negative results with primer IT3 and weak reactions with primer IT7 (specific to '*oaxacaensis*'). Sequencing (Fig. 3) confirmed that strain W87-2305.2 is indeed more closely related to strain D72-313^T (*Sporopachydermia cereana sensu stricto*, 1.3% divergence) than to strain D77-321B ('*oaxacaensis*', 3.1% divergence). The failure of primer IT3 to react with DNAs of strain W87-2305.2 and other Hawaiian isolates was caused by four substitutions near the 3' end of the primer sequence and the false-positive reaction with primer IT7 reflected the fact that this primer shared nine identical bases at the 3' end with the target site in strain W87-2305.2. Strains for which there was a clear positive signal for primer IT7 and a negative control signal were assigned to '*oaxacaensis*'.

Strain W91-460.3 reacted both with primer IT6 (specific to '*australis*') and with the control primers. Sequencing showed that the priming site of the strain differed from that of the reference strain (W91-478.1) by two substitutions and the overall ITS region differed by 2.9%, mostly due to a 15 base insertion in the ITS2 (which accounts for the longer branch for strain W91-460.3 in Fig. 3). However, the topology of the phylogram of the ITS region suggested that strain W91-460.3 belongs to the '*australis*' clade, and the D1/D2 domains of the two strains were identical (Fig. 2).

Three isolates from a Brazilian columnar cactus responded to none of the group-specific primers and shared distinct electrophoretic karyotypes and assimilation profiles. The ITS and D1/D2 sequences of the representative strain C90-399 differed by 5.0% and

1.1%, respectively, from those of any other strain sequenced (Fig. 3). Some recent isolates from agave had not been characterized by karyotyping or DNA reassociation, but were identified as *Sporopachydermia*-like on the basis of growth tests. Their DNAs did not react with any of the group-specific primers. These strains and a single isolate from red oak exudate ('*paraquercuum*') exhibited growth responses similar to those of '*brasiliensis*'. The pair-wise sequence differences between a representative from '*agaves*' and one from '*brasiliensis*' were 12.5% in the ITS and 2.7% in the D1/D2 regions, the highest divergence values obtained between these strains and any others.

Taxonomic interpretation

Faced with the task of assigning members of the *Sporopachydermia cereana* species complex to meaningful taxonomic units in the absence of clear heterothallic sexuality, we integrated the results obtained by various molecular approaches. A difference of 1.5% in DNA base composition determined by analytical ultracentrifugation has generally been interpreted as clear evidence for species delineation (Kurtzman, 1998). On that basis, we recognized the '*obscura*', '*opuntiana*' and '*pachycereana*' designations as representatives of distinct species, separate from *Sporopachydermia cereana sensu stricto*. Although a 1% difference in base composition left the position of the '*obscura*' group as distinct from *Sporopachydermia lactativora* in doubt, the matter was resolved by sequence analysis.

Numerous accounts have indicated that strains exhibiting 60% or more DNA reassociation can be regarded as conspecific (Kurtzman, 1998). Lower but non-trivial values, e.g. 25–60%, have generally but not always been associated with different varieties or even species, depending on other features relevant to the ability of yeasts to share effectively a common gene pool. The reassociation values of 32 and 39% obtained for the '*oaxacaensis*' and '*stenocereana*' designations (Table 3) are therefore problematic. The '*oaxacaensis*' group is indistinguishable from *Sporopachydermia cereana sensu stricto* by growth characteristics. Conversely, strain D82-449B ('*stenocereana*') differs in the assimilation of glucitol and is the only digitonin-resistant strain in the *Sporopachydermia* clade. On the basis of DNA reassociation, strain D82-424A would appear to represent a genetic variety of the '*opuntiana*' group, with a DNA reassociation value of 53% from the reference strain, but it is otherwise indistinguishable.

Kurtzman & Robnett (1998) published the D1/D2 sequences of all ascomycetous yeasts known at that time and new sequences are continuously being made available by various authors describing new species. This has allowed the formulation of certain generalizations on the significance of sequence divergence in species delineation, namely (i) that con-

specific strains seldom differ by more than three bases in their D1/D2 region and (ii) that strains differing by 1% (five or six bases) or more over this region are likely to be different species. A similar set of guidelines is not yet available for the ITS region, because a database of ITS sequences is only starting to accumulate. Five varieties of *Williopsis saturnus* examined by James *et al.* (1998) differed by only a few bases in the ITS regions. A similar situation exists among most physiological variants of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, but the two species are clearly distinct, differing by 2% or more in the ITS/5.8S region (Montrocher *et al.*, 1998). Species of *Zygosaccharomyces* and *Torulaspora* that are closely related on the basis of small subunit rDNA sequences (James *et al.*, 1996) exhibited various degrees of ITS divergence, ranging from almost none to nearly 40% in the ITS1 and 50% in the ITS2.

In the *Sporopachydermia* clade, D1/D2 and ITS/5.8S divergence values are loosely correlated ($r = 0.73$). Independent isolates that were nearly identical in their D1/D2 domains exhibited as much as 3% divergence in the ITS/5.8S region. The highest divergence values within the clade were approximately 14% for the ITS/5.8S region, corresponding to approximately 5% in the D1/D2 domains. By contrast, the divergence between *Sporopachydermia cereana* and *Saccharomyces cerevisiae* is 21% for D1/D2 and 27% for ITS/5.8S. A comparison between ITS/5.8S divergence and DNA–DNA reassociation data ($r = 0.97$) predicted that strains that give reassociation values of 100% are likely to be nearly identical in their ITS regions. It is interesting to note that *Sporopachydermia lactativora* and *Sporopachydermia quercuum* were found to be identical in a 168 base sequence located at the 3' end of the small (16–18S) rDNA subunit that has been used by some as a phylogenetic marker (Yamada *et al.*, 1992).

Considering the data as a whole, the most prudent interpretation in the present context is that *Sporopachydermia cereana* consists of three varieties, 'cereana', 'oaxacaensis' and 'stenocereana', and that all other designations represent distinct species. Strain D82-424A is provisionally considered an outlier within 'opuntiana'. Formal descriptions of these taxa by the authors involved in their respective discoveries will be forthcoming.

Biogeography and community structure

The distribution of the various members of the *Sporopachydermia cereana* complex appears to be based principally on geography and less on host-plant specificity (Table 1). Other members of the yeast community associated with cactus necroses are known to exhibit varying degrees of geographical or host partitioning (Starmer *et al.*, 1990). For example, *Pichia cactophila* belongs to a clade of eight cactophilic taxa (Kurtzman & Robnett, 1998), some of which are

distributed globally in all cactus species (e.g. *P. cactophila* itself), whereas others may be specific to a particular host plant and geographical region (e.g. *Pichia heedii* is restricted to the Sonoran region, mostly in cacti of the subtribe Pachycereinae). *Candida sonorensis* is isolated from cacti everywhere and is thought to be genetically homogeneous; the growth test responses are singularly invariant and they exhibit low DNA polymorphism when compared by arbitrary primer amplification (P. F. Ganter, personal communication). *Clavispora* comprises two species, one of which (*Clavispora lusitaniae*) is dominant in agave rots (Lachance, 1993) and regularly recovered from a broad range of other sources (Holzchu *et al.*, 1979), including cacti (Lachance *et al.*, 1986). The other (*Clavispora opuntiae*) occurs in cacti worldwide and the various genotypes within the species are distributed in certain geographical patterns (Lachance, 1990; Lachance *et al.*, 2000).

No members of the *Sporopachydermia cereana* species complex appear to be truly cosmopolitan. The findings that Hawaiian strains belong to the same genetic group as those in the Sonoran region and that the 'opuntiana' group occupies Australia and the Caribbean region are both consistent with the known history of anthropogenic cactus dispersal (Dodd, 1940; Fullaway, 1954). The elucidation of a species ('australis') the known range of which spans South America also makes sense, but the discovery of taxa that appear to be specific to more confined localities in Central America, Baja California, Haiti or Brazil is more puzzling, especially when juxtaposed with the recovery of 'obscura' strains in five distinct localities and four different host plant species (Table 1). Adding to this the existence of the species 'paraquercuum' in temperate oaks, the emerging suggestion is that the biodiversity of the species complex is much more complicated than anticipated and that further sampling of new habitats is likely to yield a large number of new taxa belonging to the group.

The broader distribution of 'obscura' raised the question of homogeneity within the species, since membership of the species was inferred from a specific primer with a relatively short recognition sequence. For this reason, the ITS regions of three additional strains, each from a different geographical origin, were sequenced (Table 1). The three sequences were identical and differed by a single substitution from that of strain D82-520A, the only representative of 'obscura' included in Fig. 3.

The interconnection between phylogeny and geography is also interesting from the standpoint of taxon overlap. Specifically, it appears that *Sporopachydermia cereana sensu stricto* and the species 'opuntiana', the two most abundant taxa, are separated geographically. The exception is the recovery of a rare strain (D81-676.2) of *Sporopachydermia cereana sensu stricto* in a Texan cactus sample (Table 1). Likewise, the three varieties of *Sporopachydermia cereana* appear mu-

Table 4. Selected characteristics of genetic groups within the *Sporopachydermia cereana* species complex

The genetic groups are identified as: 1, *Sporopachydermia cereana*; 2, '*oaxacaensis*'; 3, '*stenocereana*'; 4, '*obscura*'; 5, '*australis*'; 6, '*opuntiana*'; 7, '*trichocereana*'; 8, '*centralis*'; 9, '*brasiliensis*'; 10, '*pachycereana*'; 11, '*agaves*'; 12, '*paraquercuum*'. The tests are ranked in decreasing order of discriminatory power. Growth is classified as: —, no growth; s, slow; v, variable; w, weak; +, positive.

Growth test	1	2	3	4	5	6	7	8	9	10	11	12
Digitonin	—	—	+	—	—	—	—	—	—	—	—	—
Glucitol	+	+	—	w	+	+	+	+	—	—	—	—
Mannitol	+	+	+	—	+	+	+	+	—	—	—	—
Salicin	+	v	—	—	+	—	—	—	—	—	—	—
Cadaverine	+	+	+	+	+	+	+	+	+	—	+	+
Tannin	—	—	—	—	—	—	—	—	—	—	v	+
CTAB (75 mg l ⁻¹)	+	+	+	—	—	+	v	w	—	s	—	—
2-Propanol	v	w	s	w	w	v	w	w	s	w	s	s
Trehalose	+	+	—	—	+	v	—	—	s	—	+	+
Ribitol	+	+	w	v	s	v	+	+	w	w	v	—

tually exclusive in their distributions in the Sonoran region, southern Mexico and Haiti. In South America, the species '*australis*' appears to replace both '*cereana*' and '*opuntiana*' as the dominant species. Since there is no obvious association between yeast taxon and host-plant species, it would be difficult to implicate host plant chemistry as a determining factor. Perhaps this is a case where vicariance is a better explanation than dispersal for the observed distribution of taxa. Likewise, the growth inhibition of some strains by others (Table 1) does not follow geographical or even phylogenetic lines that could explain mutual exclusion of taxa. The possibility remains that members of the *Sporopachydermia cereana* species complex have alternate hosts that are region-specific and that the occurrence of these yeasts in cacti is accidental. We have no empirical evidence yet that this might be the case. When yeasts were collected from necrotic somatic tissue in the Caribbean in 1983 (Starmer *et al.*, 1987) and in Florida in 1985 (Starmer *et al.*, 1988), samples were also taken from decaying cactus fruit. These did not yield any cultures related to *Sporopachydermia*.

Identification

We have assessed carefully the usefulness of growth responses that exhibit variation within the *Sporopachydermia cereana* species complex. The most informative responses are listed in Table 4 in decreasing order of discriminatory power (between-group versus within-group variance). Most are insufficient to provide a clear definition of many of the taxa identified by DNA analyses. Identification will have to take into account the sources of isolation and may necessitate the use of group-specific primers or sequencing. In some cases, the maximum growth temperature allows a clear differentiation of taxa. For example, *Sporopachydermia cereana sensu stricto* generally grows at

45 °C or above, whereas '*opuntiana*' strains rarely grow above 42 °C. However, determination of maximum growth temperature is not entirely reproducible from one laboratory to another, due to variation in the equipment used to measure and control temperature, as well as in the culture conditions (e.g. broth versus agar, complex versus defined media). For this reason, these traits were not considered in the present analysis.

ACKNOWLEDGEMENTS

This work was funded by the Natural Science and Engineering Research Council of Canada (M.-A.L.) and the National Science Foundation, USA (H.J.P., W.T.S.). Some later collections were made possible through two cruises on the University of Miami research vessel *Calanus* (J.W. Fell). Permission to collect from agave plants on the estates of Tequila Herradura SA de CV, Amatitán, Mexico, is gratefully acknowledged. We thank D. Cornejo, P.F. Ganter, R. Metsger, F. Peris and C.A. Rosa for the gift of strains or collection assistance, I. Goodman for laboratory assistance and P. Campbell and H. Schneider for sequence determinations.

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