Biological Journal of the Linnean Society, 2009, **98**, 325–342. With 3 figures

Gut-associated yeast in bark beetles of the genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae)

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Received 11 December 2008; accepted for publication 21 April 2009

Scolytine bark beetles are the most destructive pests of conifers; they sometimes aggregate in such large numbers that they actually kill their hosts. They maintain close relationships with yeasts and fungi, in particular those that are assumed to aid in digestive, detoxification processes and pheromone production. In this study, 403 yeast strains were isolated from the guts, ovaries, eggs and frass of nine bark beetle species in the genus *Dendroctonus* Erichson. The beetles were collected from 10 conifer species at 34 locations in Mexico, Guatemala and the USA. Yeast identification was based on partial DNA sequences from 18S rDNA, 26S rDNA and internal transcribed spacer (ITS1), as well as morphological and physiological characteristics. A combined phylogenetic analysis delimited 11 clades with sequences similar to *Candida arabinofermentans*, *C. ernobii*, *C. membranifaciens* (including *C. lessepsii*, *Pichia mexicana* and *P. scolyti*), *C. oregonensis*, *C. piceae*, *Kuraishia capsulata* (including *K. capsulata* and *K.* cf. *molischiana*), *Pichia americana*, *P. canadensis*, *P. glucozyma*, *P. guilliermondii* and an undescribed species of *Candida*. Nucleotide divergences between the major clades were at least 5% while, with the exception of 30 isolates, yeasts within clades differed from named reference species at fewer than 1% of the nucleotide sites. There do not appear to be obligate relationships between particular yeasts and specific anatomical partitions, nor between particular yeasts and bark beetle species. Some yeasts do appear to be preferentially associated with bark beetles feeding on different conifer genera and therefore host plant defences may limit yeast community diversity in *Dendroctonus*. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **98**, 325–342.

ADDITIONAL KEYWORDS: 26S rDNA – *Candida* – 18S rDNA – internal transcribed spacer 1 (ITS1) – *Kuraishia* – *Pichia*.

INTRODUCTION

Diverse associations between fungi and insects are well established (Ishikawa, 2003). There are many reports of ascomycetous and basidiomycetous fungi that are intimately associated with phytophagous Coleoptera, Homoptera, Hemiptera, Isoptera and Lepidoptera (Nardon & Grenier, 1989; Schafer *et al.*, 1996; Blackwell & Jones, 1997; Suh *et al.*, 2003; Zhang, Suh, Blackwell, 2003). In particular, it is very well known that bark beetles of the weevil subfamily Scolytinae increase their host-colonizing potential by means of symbiotic relationships with fungi, which are carried within specialized structures called the mycangia, or on the body surface (Paine, Raffa & Harrington, 1997; Six, 2003). Although there is some information on the potential roles of yeast in certain biological processes of insects (Vega & Dowd, 2005),

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these associations are not well understood. Bark beetle-associated yeasts are not an exception; little has been documented about their taxonomy, distribution, abundance or functional roles (Whitney, 1982; Six, 2003). Experimental evidence suggests that certain yeasts found in the alimentary canals of bark beetles may be involved in digestive and detoxification processes, as well as the production of pheromones, which are essential for the beetles'chemical communication (Borden 1982; Paine *et al.*, 1997).

The function of bark beetle-associated yeasts cannot be examined unless their diversity and habitats are known. Previous studies of bark beetle-associated yeasts have been hampered by two serious limitations. The first one involves the method of isolation. Organisms were isolated from macerates of entire beetles or entire alimentary canals (Shifrine & Phaff, 1956; Moore, 1972; Leufvén, Bergström & Falsen, 1984); this approach made it impossible to link individual yeasts with a particular anatomical region of the insect. The second limitation involves the methods of taxonomic identification; traditionally based on the morphology of vegetative and sexual stages, in conjunction with physiological tests (Shifrine & Phaff, 1956; Lu, Allen & Bollen, 1957; Callaham & Shifrine, 1960; Moore, 1972; Bridges, Marler & McSparrin, 1984; Leufvén *et al.*, 1984). Variable physiological responses and phenotypic plasticity hampered accurate taxonomic identification in earlier studies. DNA sequences from ribosomal genes and internal transcribed spacer regions are now widely used for the rapid identification of yeasts (Kurtzman, 1994; Kurtzman & Robnett, 1998; Brookman *et al.*, 2000; Lim *et al.*, 2005). The gene 18S rDNA is informative at the higher taxonomic levels, the 1/2 domains of 26S rDNA at genus and species levels, while the internal transcribed spacer (ITS) is divergent in yeasts at the species level (Berbee & Taylor, 1999).

The aim of this study was to assess the diversity of yeasts isolated from specific regions of the alimentary canal of bark beetles; these systems include well-defined microhabitats that might be inhabited by specific mycota. For comparative purposes, yeasts were also isolated from frass, ovaries and eggs. While 26S rDNA is often used to distinguish closely related species and assess relationships among species, using concatonated sequences of multiple genes may offer a more promising approach (Hanage, Fraser & Spratt, 2006). Therefore, partial sequences of 18S rDNA, the 1/2 domains of 26S rDNA and ITS1 were analysed in a combined phylogenetic analysis to identify yeasts. We use the term clade to indicate a group of isolates with similar genotypes at species level, as defined by rDNA. We hypothesize that the clades thus identified represent biological entities having real existence; these clades might or might not be delimited by the physiological tests traditionally used in yeast taxonomy.

MATERIAL AND METHODS

Sample collection

Bark beetles ($N = 450$) were collected from eight species of *Pinus* (pine) L., *Picea engelmannii* (spruce) Parry ex Engelm. and *Pseudotsuga menziesii* var. *glauca* (Douglas-fir) (Mirb.) Franco, at 34 geographic locations in Mexico, Guatemala and Oregon (USA) (Table 1). The beetles, which represent nine species of *Dendroctonus* Erichson, colonize different host trees and have different distributional ranges. The host plants all belong to the conifer family Pinaceae. Insects, frass and eggs were obtained during the first stages of colonization, using fine forceps, from trees and insect galleries. Live insects were transported to the laboratory in sterile vials containing fragments of bark or moist tissue paper. Frass and eggs were placed separately in sterile Eppendorf tubes. Taxonomic identification was based on external morphological characteristics and the shapes of the seminal rods in males (Wood, 1982). Heads and pronota were deposited as vouchers in the Departamento de Zoología insect collection at Escuela Nacional de Ciencias Biológicas, Mexico (ENCB-IPN, Mexico).

Yeast isolation

Resin was removed from live insects with cotton swabs and 96% ethanol. To disinfect the body surfaces, beetles were twice submerged and shaken gently for 2 min in phosphate buffer solution (PBS) with a mixture of penicillin (100 U/mL), streptomycin (0.1 mg/mL) and amphotericin B (0.25 mg/mL). Specimens were dissected under sterile conditions using forceps, dissecting needles and minutin insect pins. The elytra, wings and tergites were removed to expose the abdomen, prior to aseptic extraction of the gut and ovaries. The anterior midgut, posterior midgut and hindgut were morphologically identified (Díaz *et al.*, 1998, 2003; Díaz, Cisneros & Zúñiga, 2000) and sectioned with a razor blade. Gut regions and ovaries were indiviudally crushed in 200 μL of RPMI 1640 medium (Moore, Gerner & Franklin, 1967) (Gibco, Grand Island, NY, USA), supplemented with yeastolate (3.33 g/L) (Invitrogen Carlsbad, CA, USA), lactoalbumin hydrolysate (3.33 g/L) (Invitrogen), 600 μL vitamin premix (Sigma, Mexico), 10% fetal calf serum (Sigma), 6 mM L-glutamine (Invitrogen) and non-essential amino acids. This medium was used because preliminary assays showed that it favoured both slow-growing and fast-growing yeasts. Samples were placed in microplate wells and

Table 1. Geographical locations and *Dendroctonus* species included in this study

Geographical location	Code	Latitude/longitude	Species	Host plant	No. of insects
1. Parque Nacional El Ajusco, México, D.F.	Aj	19° 13′ 00″ N	*D. mexicanus*	*Pinus teocote* (Pteo)	10
		99° 14′ 00″ W	*D. valens*		8
2. Centro Interdisciplinario de Ciencias de la Salud, IPN, Estado de México, México	C	19° 04′ 00″ N	*D. mexicanus*	*Pinus montezumae* (Pmont)	10
		98° 58′ 00″ W	*D. valens*		8
3. Zoquiapan, Estado de México, México	Zoq	19° 20′ 15″ N	*D. valens*	*Pinus hartwegii* (Phart)	8
		98° 42′ 15″ W			
4. Predio 1, Sierra Fría, Aguascalientes, México	A1	22° 06′ 43″ N	*D. mexicanus*	*Pinus leiophylla* (Pleio)	10
		102° 29′ 14″ W	*D. valens*		8
5. Predio 2, Sierra Fría, Aguascalientes, México	A2	22° 10′ 37″ N	*D. mexicanus*	*Pinus leiophylla*	10
		102° 36′ 36″ W	*D. valens*		8
6. Predio 3, Sierra Fría, Aguascalientes, México	A3	22° 10′ 11″ N	*D. mexicanus*	*Pinus leiophylla*	10
		102° 38′ 45″ W			
7. Opopeo, Sta. Clara del Cobre, Michoacán, México	M1	19° 29′ 19″ N	*D. mexicanus*	*Pinus leiophylla*	10
		101° 35′ 28″ W	*D. valens*		8
8. Pascuala, Michoacán, México	M2	19° 26′ 45″ N	*D. valens*	*Pinus leiophylla*	7
		102° 09′ 58″ W			
9. Lagunas de Montebello, Chiapas, México	Ch1	16° 06′ 49″ N	*D. frontalis*	*Pinus oocarpa* (Pooc)	10
		91° 43′ 33″ W	*D. mexicanus*		10
			D. valens		8
10. Carpinteros, Metzquititlán, Hidalgo, México	H	20° 34′ 00″ N	*D. approximatus*	*Pinus teocote*	8
		98° 33′ 00″ W	*D. mexicanus*		9
			D. valens		7
11. San Joaquín, Querétaro, México	Q1,2	20° 53′ 58″ N	*D. mexicanus*	*Pinus pseudostrobus* (Ppseu)	10
		99° 34′ 34″ W	*D. valens*		8
12. Ciénega de la Vaca, Durango, México	D1	24° 05′ 20″ N	*D. adjunctus*	*Pinus arizonica* (Pariz)	8
		105° 31′ 00″ W	*D. approximatus*	*Pseudotsuga menziesii var. glauca* (Psmen)	8
			D. pseudotsugae		8
13. La Taunita, Ejido La Manga, San Dimas, Durango, México	D2	24° 25′ 49″ N	*D. rhizophagus*	*Pinus arizonica*	8
		105° 59′ 38″ W			
14. La Joya del Cerro, Ejido La Manga, San Dimas, Durango, México	D2	24° 22′ 08″ N	*D. pseudotsugae*	*Pseudotsuga menziesii var. glauca*	9
		105° 58′ 15″ W			
15. Puentecillos, San Dimas, Durango, México	D3	24° 21′ 10″ N	*D. pseudotsugae*	*Pseudotsuga menziesii var glauca*	10
		105° 54′ 39″ W			
16. Predio Núñez, San Dimas, Durango, México	D4	24° 22′ 10″ N	*D. pseudotsugae*	*Pseudotsuga menziesii var glauca*	9
		105° 54′ 39″ O			
17. Cuatro Vientos, Madera, Chihuahua, México	Chih1	29° 12′ 05″ N	*D. rhizophagus*	*Pinus arizonica*	8
		108° 26′ 52″ W			

Table 1. *Continued*

Geographical location	Code	Latitude/longitude	Species	Host plant	No. of insects
18. Camino Cuerno Verde, El Largo, Madera, Chihuahua, México	Chih2	29° 36′ 25″ N 108° 17′ 59″ W	*D. rhizophagus*	*Pinus arizonica*	8
19. Puerto Blanco, El Nopal, Gpe. y Calvo, Chihuahua, México	Chih	26° 05′ 31″ N 107° 02′ 11″ W	*D. pseudotsugae*	*Pseudotsuga menziesii var. glauca*	8
20. Predio La Laja, Bocoyna, Chihuahua, México	Chih3	27° 55′ 53″ N 107° 35′ 52″ W	*D. rhizophagus*	*Pinus arizonica*	9
21. San Juan, Madera, Chihuahua, México	Chih4	29° 47′ 06″ N 108° 18′ 42″ W	*D. rhizophagus*	*Pinus arizonica*	9
22. Las Antenas, Nevado de Colima, Jalisco, México	J	19° 35′ 18″ N 103° 37′ 00″ W	*D. adjunctus*	*Pinus hartwegii*	8
23. Rancho Los Angeles, Cañón de Jade, Arteaga, Coahuila, México	Co	25° 20′ 02″ N 100° 33′ 51″ W	*D. pseudotsugae*	*Pseudotsuga menziesii var. glauca*	8
24. Puerto La Cruz, San Antonio de las Alazanas, Arteaga, Coahuila, México	Co	25° 13′ 49″ N 100° 24′ 56″ W	*D. adjunctus* *D. approximatus* *D. valens*	*Pinus teocote*	8 8 8
25. El Terrero, San Pablo Etla, Oaxaca, México	Ox1	17° 10′ 29″ N 96° 41′ 27″ W	*D. adjunctus*	*Pinus hartwegii*	9
26. Mayavisia, Sta. Catarina Albarradas, Díaz Ordaz, Oaxaca, México	Ox2	17° 05′ 29″ N 96° 17′ 29″ W	*D. approximatus* *D. mexicanus*	*Pinus hartwegii*	8 10
27. Sta. María Albarradas, Díaz Ordaz, Oaxaca, México	Ox3	16° 58′ 43″ N 96° 11′ 52″ W	*D. approximatus*	*Pinus hartwegii*	9
28. Parque Arqueológico Iximché, Tecpán, Depto. de Chimaltenango, Guatemala	Gua1	14° 44′ 09″ N 90° 59′ 04″ W	*D. approximatus* *D. valens*	*Pinus hartwegii*	8 10
29. Morán, Reserva de la Biosfera Sierra de las Minas, Depto. Zacapa, Guatemala	Gua2	15° 09′ 47″ N 89° 29′ 40″ W	*D. valens*	*Pinus hartwegii*	9
30. Spring Creek, La Grande, Oregon, USA	GO	45° 19′ 00″ N 118° 19′ 00″ W	*D. brevicomis* *D. valens*	*Pinus ponderosa* (Ppon)	10 10
31. Tollgate Road, Umatilla Nacional Forest, Walloma-Walloma Range Distric, La Grande, Oregon, USA	GoTo	45° 41′ 41″ N 118° 02′ 39″ W	*D. rufipennis*	*Picea engelmannii* (Piengel)	10
32. Rock Springs, La Grande Range District, Walloma-Whitman National Forest, La Grande Oregon, USA	GoRo	45° 19′ 11″ N 118° 34′ 15″ W	*D. rufipennis*	*Picea engelmannii*	10
33. Mt. Emily Road No. 31, Wallowa-Whitman National Forest, La Grande Range District, La Grande, Oregon, USA	GoEmi	45° 29′ 40″ N 118° 07′ 45″ W	*D. pseudotsugae*	*Pseudotsuga menziesii var. glauca*	10
34. Balmer Creek Reservation, La Grande, Oregon, USA	GoBa	44° 58′ 48″ N 117° 33′ 43″ W	*D. pseudotsugae*	*Pseudotsuga menziesii var. glauca*	10

incubated for 2 days at 28 °C (previously determined to be the optimal growth temperature).

To isolate yeasts, 20 µL of RPMI culture were streaked on Sabouraud dextrose agar (SDA) plates, which were incubated at 28 °C for 2 days. This procedure was replicated twice. All yeast colonies were characterized using phenotypic traits (texture, colour, surface, elevation and margin). Four to six colonies with different morphologies were selected from each plate and streaked for purification in SDA. Finally, single colonies were cultured in Sabouraud dextrose broth at 28 °C for 2 days. Frass and eggs were inoculated directly in Sabouraud broth and incubated under the same conditions. A total of 650 yeasts was isolated and 403 of these were selected for complete biochemical characterization and sequencing of the three genes. Axenic cultures of each strain were stored at –70 °C in 50% glycerol and voucher cultures were deposited in the strain collection at the Departamento de Microbiología, ENCB-IPN, Mexico.

DNA extraction and amplification

Total genomic DNA was extracted from each yeast isolate following the protocol of Lehmann, Lin & Lasker (1992), except that cell lysis was achieved by heating at 65 °C for 45 min. PCR amplifications were performed in a thermocycler (Geneamp PCR System 9700) on 25-µL reactions containing 20 ng of DNA template, 0.4 µM of each primer, 2 mM magnesium chloride ($MgCl_2$), 200 µM of each deoxynucleotide triphosphate (dNTP) and 1.2 U *Taq* DNA polymerase (Invitrogen). An initial 5-min denaturation step at 94 °C was followed by 35 cycles: 94 °C for 1 min, 67 °C for 1 min and 72 °C for 1 min. The fragment of 1/2 26S rDNA was obtained using the conserved primers (rRNA1, rRNA2) of Fell (1993). The 18S rDNA target segment was obtained using the primers 18-1F (5′-GCCAGCAGCCGCGGTAATTCCAGC-3′) and 18-2R (5′-GCGACCATACTCCCCCCAGAACCC-3′), designed for this study. Amplification conditions were similar for both fragments, except the annealing temperature was 65 °C for 18S rDNA. The ITS1 fragment was amplified using the primers (ITS1, ITS2) of Lin *et al.* (1995). The initial denaturation step, 94 °C for 5 min, was followed by 35 cycles: 94 °C for 5 min, 50 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were visualized on 1% agarose gels stained with 0.5 µg/mL ethidium bromide solution and compared with a 100-bp DNA ladder (Gibco, BRL, Gaithersburg, MD, USA). PCR products were purified with the GFX PCR DNA Kit (Amersham Biosciences, UK), then sequenced using Big Dye Terminator v3.1 and an ABI Prism Model 3100 automated sequencer (Applied Biosystems, Japan). Both strands were sequenced.

Phylogenetic reconstruction

Sequences of each of the three DNA regions were aligned independently using CLUSTAL X v1.7 (Thompson *et al.*, 1997), manually edited to achieve positional homology, then concatonated and included in a combined phylogenetic analysis. When the gene regions were concatenated, each isolate had a unique haplotype and therefore all isolates are represented as terminals. The approximate fragment lengths were 660 bp (26S rDNA), 767 bp (18S rDNA) and 409 bp (ITS1). The 18S and 26S rDNA sequences had few insertions or deletions; however, indels were frequent in the ITS1 region. Because indels may reflect single evolutionary events, regardless of size (Giribet & Wheeler, 1999), they were replaced in each gene by a coded binary matrix based on presence/absence (Simmons & Ochoterena, 2000). Both matrices (with and without gaps) were analysed. Resulting topologies were used to identify the clades, but not to establish phylogenetic relationships within individual clades or between clades. Clades are named for reference sequences from GenBank, which were included in the phylogenetic reconstructions. *Schizosaccharomyces pombe* Linder was used as outgroup in all analyses (Berbee & Taylor, 1999).

To estimate the degree of incongruence between the three data partitions, Templeton's non-parametric test was performed (Templeton, 1983). Individual topologies were built for each marker because (1) different genes sampled from the same isolate may not have identical phylogenetic histories (Rosenberg & Nordborg, 2002), (2) concatenated sequences may provide erroneous support for incorrect species trees (Degnan & Rosenberg, 2006) and (3) the sequences of individual regions differ in size and therefore some regions may be more informative than others.

Maximum parsimony analyses were performed using PAUP* v4.0b10 (Swofford, 1998). Heuristic tree searches used tree bisection–reconnection (TBR) and a branch-swapping algorithm with 100 random stepwise additions, with 100 trees saved for each pseudoreplicate. To find the most parsimonious trees, a successive approximation analysis was performed on the a posteriori weighted data set, based on the rescaled consistency index derived from trees obtained by unweighted analysis (Farris, 1969). Analysis of this weighting scheme used the same heuristic search conditions as that for unweighted data. Branch support was obtained with 500 bootstrap replicates. Consensus trees were built for the total evidence analyses and for individual molecular markers.

The average numbers of nucleotide differences were calculated, within and between clades, for the 26S rDNA sequences (Nei, 1987). The delimitation of

yeast clades at particular nodes was based on the average number of pairwise nucleotide differences between clade members and the named reference sequences. Sequences obtained in this study were deposited in GenBank (Table 2).

Ecological associations

The sources of the isolates included within the clades shown in Figure 1 were reviewed to determine if particular yeasts were consistently isolated from specific anatomical isolation sites, beetle species, host trees or geographic localities. A series of multiple correspondence analyses (Legendre & Legendre, 1998) were also performed, using STATISTICA v.7.0, to further explore preferential associations between yeast clade and substrate or locality. We first analysed isolation site, host beetle and locality together and then analysed variables separately (eliminating outliers and reanalysing particular data partitions as described in RESULTS).

Physiological characterization

The 403 yeast isolates included in the phylogenetic analyses were further characterized using 40 assimilation and fermentation tests, as well as morphological features, according to Yarrow (1998). To explore the presence of metabolic similarities among isolates, a cluster analysis with the unweighted pair-group method with arithmetic mean (UPGMA) with NTSYS-PC v.2.02j (Rohlf, 1998) was carried out using all physiological test responses as attributes. Pairwise similarities among isolates were calculated by single matching index and the reliability of the corresponding dendrogram was evaluated by means of the cophenetic correlation coefficient using a Mantel test (Legendre & Legendre, 1998).

RESULTS

Phylogenetic reconstruction

The combined sequence data set comprised 1836 bp, of which 1004 were parsimony-informative characters. Eleven clades were delimited in the total evidence analysis (Fig. 1, clades A–K). Topologies with and without gaps (data not shown) were similar and recovered the same clades. Sequences included in these clades were similar to sequences of *Candida arabinofermentans* Kurtzman & Dien (clade E), *C. ernobii* (Lodder & Kreger-van Rij) S. A. Meyer & Yarrow (clade B), *C. membranifaciens* (Lodder & Kreger-van Rij) Wickerham & K. A. Burton (clade A) including *Pichia scolyti* (Phaff & Yoneyama) Kreger-van Rij (subclade 1), *C. lessepsii* Suh, Nguyen & Blackwell (subclade 2) and *P. mexicana* Miranda, Holzschu, Phaff & Starmer (subclade 3), *C. oregonensis* Phaff & do Carmo-Sousa (clade K), *C. piceae* Kurtzman (clade F), *Candida* sp. (clade C), *Pichia americana* (Wickerham) Kurtzman (clade H), *P. canadensis* (Wickerham) Kurtzman (clade I), *P. glucozyma* (Wickerham) Kurtzman (clade G), *P. guilliermondii* Wickerham (clade J) and *Kuraishia capsulata* (Wickerham) Y. Yamada, Maeda & Mikata (clade D) (Fig. 1). The number of isolates within each clade varied considerably (Table 2). The three most frequently isolated yeasts, *P. americana* (clade H, $N = 90$), *Candida* sp. (clade C, $N = 65$) and *C. ernobii* (clade B, $N = 53$), accounted for over half of the isolates. At the other end of the spectrum were *Pichia glucozyma* (clade G) and *P. canadensis* (clade I), represented by three and one isolates, respectively (Fig. 1).

Templeton's test showed that the three data sets (18S rDNA, 26S rDNA and ITS1) were incongruent ($Ts = 123$, $P = 0.02$). The clades shown in Figure 1 were recovered in the individual analyses of 26S rDNA and ITS1, but only clades *C. oregonensis*, *P. americana* and *P. guilliermondii* were consistent with the 18S rDNA topology (data not shown). The total evidence and ITS1 analyses delimited three subclades within the *C. membranifaciens* clade (A): *C. lessepsii* (2), *P. mexicana* (3) and *P. scolyti* (1). In addition, a subclade of the *K. capsulata* clade, defined as *K.* cf. *molischiana* Dlauchy, Péter, Tornai-Lehoczki & Kurtzman (D**), was identified only with the ITS1 (Table 3). The 26S nucleotide diversity between clades (including subclades) was always greater than 3.8% (Table 4). The nucleotide diversity between an isolate and its reference species was usually < 1%; however, 30 isolates differed by > 1% (Fig. 1, Table 2).

Ecological associations

Most yeast clades shown in Figure 1 included isolates from various anatomical isolation sites, beetle species and geographic localities. For example, even the rather small *Candida arabinofermentans* clade (E) included yeasts isolated from both the guts and frass of *Dendroctonus adjunctus* Blandford, *D. approximatus* Dietz and *D. pseudotsugae* Hopkins, collected in three mountain systems (at Chihuahua, Coahuila, Durango and Jalisco). Two yeasts were isolated exclusively from a single beetle species: *Pichia scolyti* (Fig. 1, clade 1) from *Dendroctonus pseudotsugae* and *K.* cf. *molischiana* (clade D**) from *D. rufipennis* (Kirby). All yeasts shown in Figure 1 were isolated from multiple gut regions, sometimes from the same individual (data not shown), but some yeasts were not recovered from frass. Eggs and ovaries yielded fewer yeast cultures and associated yeasts therefore appear to be less diverse.

Table 2. GenBank accession numbers, total number yeast isolates from each clade, nucleotide diversity between isolated strains of different species according to 1/2 of 26S rDNA and closest matches from BLAST searches

Clades GenBank accession numbers	Total number of isolates	Nucleotide diversity (D1/D2 of 26S rDNA) < 1%	> 1%	Closest match in BLAST
C. arabinofermentans (E) *EF041859-EF041874 †EF090741-EF090757	16	15	1	*C. arabinofermentans* (AF017248)
C. ernobii (B) *EF041893-EF041949 †EF090838-EF090890, EF090798	53	53	0	*C. ernobii* (U70241)
C. membranifaciens (A)				
C. lessepsii (A2) *EF041987-EF042008, EF042046-EF042050 †EF090778-EF090801, EF093441-EF093444	19	17	2	*C. lessepsii* (AY640214)
P. mexicana (A3) *EF042031-EF042045 †EF093426-EF093440	19	19	0	*P. mexicana* (U45797)
P. scolyti (A1) *EF042009-EF042030 †EF093445-EF093466	20	18	2	*P. scolyti* (U45788)
C. oregonensis (K) *EF041875-EF041892, AY212911-AY212912 †EF090758-EF090777 AY249517-AY249518	17	13	4	*C. oregonensis* (U44815)
C. piceae (F) *EF041950-EF041983, AY212917 †EF090802-EF090837, AY249523	33	27	6	*C. piceae* (AF153672)
Candida sp. (C) *EF016026-EF016081, AY212913-AY212916, AY212907 †EF093189-EF093258, AY249519-AY249522, AY249513	65	65	0	*Candida* sp. (AY242329)
P. americana (H) *EF015940-EF016025, EF042087-EF042088, AY212914 †EF093300-EF093389 AY249520	90	88	2	*P. americana* (U73575)
P. canadensis (I) *EF016107 †EF093299	1	1	0	*P. canadensis* (U75415)
P. glucozyma (G) *EF041984-EF041986 †EF0932976-EF093298	3	1	2	*P. glucozyma* (U75520)
P. guilliermondii (J) *EF015908-EF015939, AY212908-AY212910 †EF093390-EF093425, AY249514-AY249516	32	32	0	*P. guilliermondii* (AB105436)
K. capsulata (D)	26	15	11	*K. capsulata* (U70178)
K. cf. *molischiana* (D‡) *EF042051-EF042086 †EF093259-EF093295	9	9	0	*K.* cf. *molischiana* (DQ026030)

*GenBank accession numbers of 1/2 of 26S rDNA from yeast isolates in this study.
†GenBank accession numbers of 18S rDNA and internal transcribed spacer 1 (ITS1) region from yeast isolates in this study.
‡Subclade defined only ITS1.

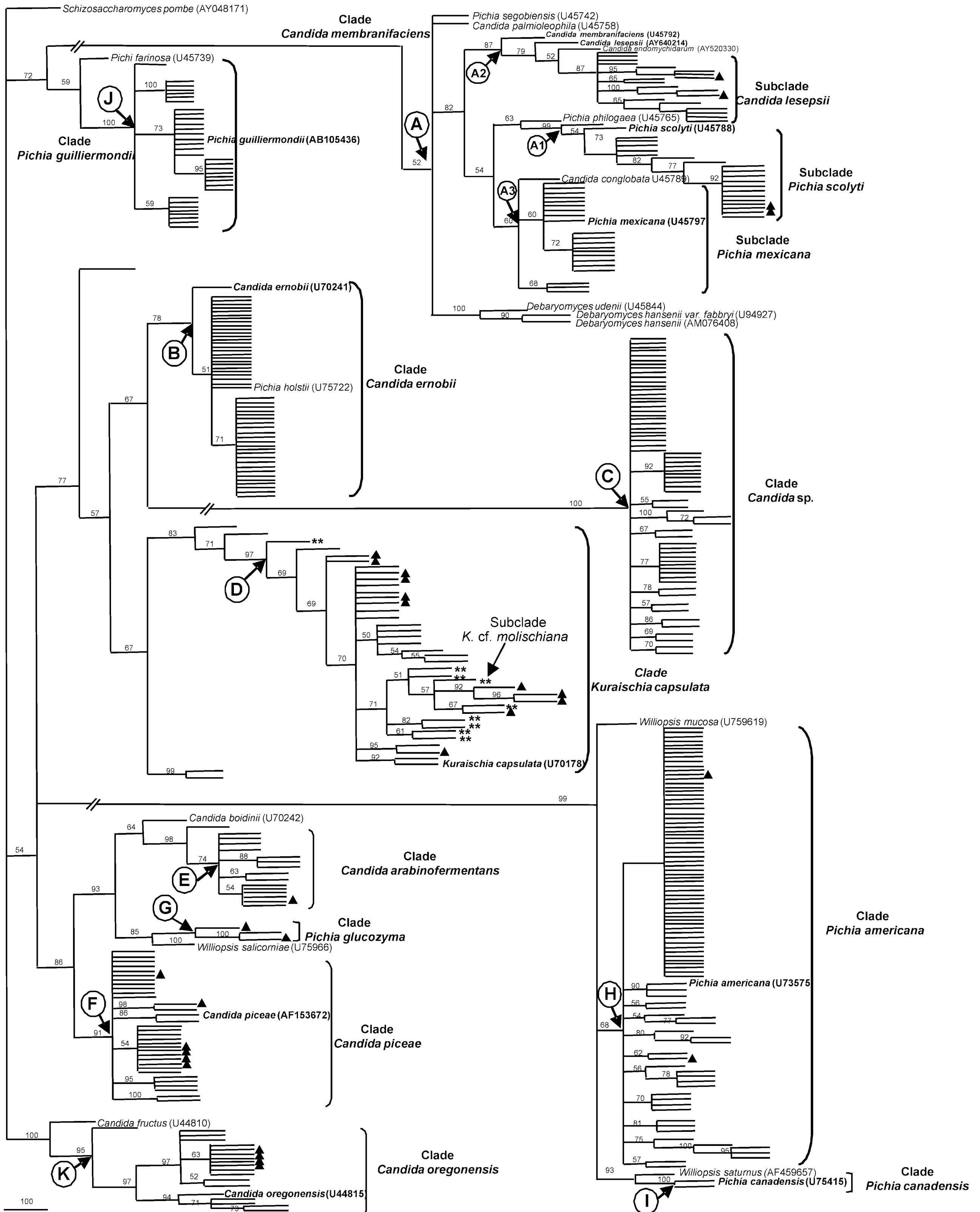
Schizosaccharomyces pombe (AY048171)
Clade
Candida membranifaciens
Pichia segobiensis (U45742)
Candida palmioleophila (U45758)
Candida membranifaciens (U45792)
Candida lesepsii (AY640214)
Candida endomychidarum (AY520330)
Subclade
Candida lesepsii
Pichi farinosa (U45739)
Clade
Pichia guilliermondii
Pichia guilliermondii (AB105436)
Pichia philogaea (U45765)
Pichia scolyti (U45788)
Subclade
Pichia scolyti
Candida conglobata U45789)
Pichia mexicana (U45797)
Subclade
Pichia mexicana
Debaryomyces udenii (U45844)
Debaryomyces hansenii var. fabbryi (U94927)
Debaryomyces hansenii (AM076408)
Candida ernobii (U70241)
Pichia holstii (U75722)
Clade
Candida ernobii
Clade
Candida sp.
Subclade
K. cf. molischiana
Clade
Kuraischia capsulata
Kuraischia capsulata (U70178)
Williopsis mucosa (U759619)
Candida boidinii (U70242)
Clade
Candida arabinofermentans
Clade
Pichia glucozyma
Williopsis salicorniae (U75966)
Clade
Pichia americana
Pichia americana (U73575
Candida piceae (AF153672)
Clade
Candida piceae
Candida fructus (U44810)
Clade
Candida oregonensis
Candida oregonensis (U44815)
Williopsis saturnus (AF459657)
Pichia canadensis (U75415)
Clade
Pichia canadensis

Figure 1. Majority-rule consensus tree of the 100 most parsimonious trees among bark beetle-associated yeasts from this study and from selected yeasts from GenBank based on total evidence analysis give DNA regions [tree length 2063.63 steps; consistency index (CI) = 0.4336; retention index (RI) = 0.9117; rescaled consistency index (RC) = 0.3953]. Branch lengths are proportional to the number of nucleotide differences; support values for internal nodes are bootstrap values. The arrows indicate the clades. E, *C. arabinofermentans*; B, *C. ernobii*; A, *C. membranifaciens* (1, *P. scolyti*; 2, *C. lessepsii*; 3, *P. mexicana*); K, *C. oregonensis*; F, *C. piceae*; C, *Candida* sp.; H, *P. americana*; I, *P. canadensis*; J, *P. guilliermondii*; G, *P. glucozyma*; D, *K. capsulata* (D**, subclade *K.* cf. *molischiana* only with ITS1). *Schizosaccharomyces pombe* was used as outgroup in the analysis (▲). Isolated with nucleotide diversity > 1%.

◄

Table 3. Clade defined according to the total evidence analysis (TEA), 1/2 domains of 26S rDNA, 18S rDNA and internal transcribed spacer 1 (ITS1) topologies

Clades	TEA	D1/D2 domains of 26S rDNA	ITS1	18S rDNA
C. arabinofermentans (E)	D	D	D	ND
C. ernobii (B)	D	D	D	ND
C. membranifaciens (A)	D	D	D	ND
C. lessepsii (A2)	*		*	
P. mexicana (A3)	*		*	ND
P. scolyti (A1)	*		*	
C. oregonensis (K)	D	D	D	D
C. piceae (F)	D	D	D	ND
Candida sp. (C)	D	D	D	ND
P. americana (H)	D	D	D	D
P. canadensis (I)	D	D	D	D
P. glucozyma (G)	D	D	D	ND
P. guilliermondii (J)	D	D	D	D
K. capsulata (D)	D	D	D	ND
K. cf. *molischiana* (D†)			D	

*Subclades defined only with TEA and ITS1.
†Subclade defined only with ITS1.
D, well-defined clade; ND, no-defined clade.

When yeast clades were ordinated with anatomical isolation site, host beetle and locality, the two principal axes explained only 29% of the total variation. Subsequent analyses (yeast clade and anatomical isolation site, yeast clade and beetle host, yeast clade and tree genus) were more informative. In the correspondence analysis of yeast clade and anatomical isolation site, the two principal axes explained 72.97% of the variation (Fig. 2A). We thought the results might be biased by the relatively small sample sizes of yeasts from eggs, ovaries and, to a lesser degree, frass. When reproductive structures were deleted and the analysis repeated, the axes accounted for 88.2% of the variability and, when all outliers (posterior midgut, frass and eggs) were deleted, the two axes accounted for 89.9% of the variation. In the analysis of yeast clade and beetle species, the two axes explained 63.08% of the variation (Fig. 2B). When yeast clades were ordinated with tree species, the two axes explained only 39.36% of the variability (Fig. 2C), but, when tree species were coded by genus, they explained 70.66% of the variability (data not shown). When localities were analysed as separate variables, the two axes lacked explanatory value (they accounted for 38.36 of the variability), but when localities were coded by mountain system, the two axes explained 77.68% of the variation (data not shown).

Physiological characterization

Yeast isolates within clades often had highly variable responses to the physiological tests (see SUPPORTING INFORMATION). Most isolates assimilated inulin, cellobiose and D-xylose, as well as salicin; results in most other physiological tests varied among isolates. The cluster analysis among isolates recovers two large groups (Fig. 3): yeast clades (A + B + C +

Table 4. Percentage of nucleotide divergences among clades estimated according to Nei (1987)

	Cara (E)	Cern (B)	Cles (A2)	Pmex (A3)	Psco (A1)	Core (K)	Cpic (F)	Csp (C)	Pame (H)	Pcan (I)	Pglu (G)	Pgui (J)	Kcap (D)
Cern	13.6												
Cles	16.5	12.6											
Pmex	13.8	9.3	52.7										
Psco	14.8	10.7	5.6	6									
Core	27.9	25.6	25.9	26.3	26.7								
Cpic	7.8	13	18.1	15.4	15.8	26.3							
Csp	12.2	5	16	13.4	14.3	26.2	13.7						
Pame	11.6	13.4	16.9	14	15	23.7	14.2	13					
Pcan	19.5	19.8	21.7	17.9	19.6	29.4	21.2	19.2	11.6				
Pglu	10.4	15.3	20.3	17.5	17.8	28.2	7	16.8	16.2	24.4			
Pgui	19	14.9	16	11.9	12.8	25.6	18.5	17	17	22.8	21.6		
Kcap	15.7	14.3	16.8	15	15.6	24	14.5	16.1	11.8	19	17.5	20.6	
Kmol (D**)	17.8	15.9	18.5	16.2	17	26.8	15.5	18	14.2	21.6	17.9	23.3	3.8

Clade E, *Candida arabinofermentans*; clade B, *C. ernobii*; clade 2, *C. lessepsii*; clade 3, *P. mexicana*; clade 1, *P. scolyti*; clade K, *C. oregonensis*; clade F, *C. piceae*; clade C, *Candida* sp.; clade H, *Pichia americana*; clade I, *P. canadiensis*; clade G, *P. glucozyma*; clade J, *P. guilliermondii*; clade D, *Kuraishia capsulata*; subclade D**, K. cf. *molischia*.

D + I + J) and (E + F + H + K). Within the first group, the main division was between J and the remaining yeasts, with the three subclades of A jointed forming a metabolic cluster, the clade D + subclade D** + I jointly forming a cluster and B + C + A (in part) forming a third cluster. In the second group, H + F (in part) made up one cluster, while E + F (in part) + G formed a second cluster.

DISCUSSION

Gut yeast diversity

DNA sequence data (26S rDNA) have revealed that insect guts harbour an astonishing diversity of yeasts (Lim *et al.*, 2005; Suh, Nguyen & Blackwell, 2005a; Suh *et al.*, 2005b). In this study, the total evidence analysis showed that the yeasts isolated from the guts, ovaries, eggs and frass of *Dendroctonus* belong only to the genera *Candida* Berkhout, *Pichia* (E. C. Hansen) Kurtzman and *Kuraishia* Y. Yamada, K. Maeda & Mikata. The use of 26S rDNA in rapid species identification, with sequences that differ by approximately 1% hypothesized to represent different species, is not without pitfalls: isolates representing independent lineages sometimes have identical 1/2 loop genotypes (Kurtzman & Robnett, 1998). This lack of resolution has sometimes led to the a posteriori use of ITS1 sequences or phenotypic traits to resolve taxonomic status (e.g. Lin *et al.*, 1995). In this study, we decided to link together three molecular regions for phylogenetic analysis and recognize the isolates belonging to the resulting clades as putative species. The 11 main clades recovered in the total evidence analysis had moderately strong support (bootstrap values > 70%) and between-clade nucleotide divergences ranged from 5 to 29.4% (Fig. 1; Table 4). Topologies derived from individual gene partitions were consistent with total evidence tree, but support was less robust and not all clades were recovered (data not shown).

The subclades within *C. membranifaciens* (*C. lessepsii*, *P. mexicana*, *P. scolyti*) and *Kuraishia capsulata* (*K.* cf. *molischiana*) were well defined only in the total evidence and ITS1 analyses. These closely related subclades were recognized in our subsequent analyses because they have been identified in previous phylogenetic analyses and described using other biological attributes (Gábor *et al.*, 2005; Suh *et al.*, 2005a). In this study, average nucleotide divergences between subclades were much lower than most interclade divergences: 3.8% between *Kuraishia capsulata* and *K.* cf. *molischiana* and 5.3–6% among the three subclades embedded within *C. membranifaciens* (Table 4). In the cluster analysis, subclade isolates formed joint metabolic clusters (Fig. 3). Further

Figure 2. Correspondence analyses: ordination of clades with respect to axes I and II from presence/absence of isolates according to anatomical isolation site (A), *Dendroconus* species (B) and host plants (C). Axes I and II explain for: A, 72.97% ($P = 0.003$); B, 63.08% ($P = 0.0001$); C, 39.36% ($P = 0.001$) of the total variation. Abbreviations of clades (◆):E, *Candida arabinofermentans*; B, *C. ernobii*; 2, *C. lessepsii*; 3, *P. mexicana*; 1, *P. scolyti*; K, *C. oregonensis*; F, *C. piceae*; C, *Candida* sp.; H, *P. americana*; I, *P. canadensis*; J, *P. guilliermondii*; G, *P. glucozyma*; D, *K. capsulate*; D**, *K.* cf. *molischiana*. P, pine; Ps, Douglas-fir; Pi, spruce. Abbreviations of host plant, see acronyms in Table 1.

studies are needed for a full taxonomic description of the isolates within the *Candida* sp. clade, as well as the 30 isolates that differed from their reference sequences by > 1%.

Although such studies may well increase our estimates of *Dendroctonus* gut yeast species richness, we believe that, by sampling individual anatomical regions from nine beetle species ($N = 450$) feeding on 10 conifer species, collected at 34 localities, we have assembled a fairly comprehensive data set. The diversity of culturable gut-associated yeasts in *Dendroctonus* appears to be unusually low compared with other insects. Suh *et al.* (2005b) analysed 650 yeast isolates from the digestive tracts of beetles in 27 families (including many fungus feeders) and recognized at least 45 yeast clades. In an initial survey of gut yeasts from Neotropical wood-boring beetles, Berkov *et al.* (2007) sampled 18 beetles at a single locality and 34 isolates yielded six gut yeasts, with an additional two yeasts from body exteriors. Mankowsky & Morrell (2004) reported 18 species in 11 genera from the intrabuccal pocket, exoskeleton and frass of wood-dwelling carpenter ants (*Camponotus vicinus* Mayr) collected at two locations. Conifer-associated bark beetles seem to yield relatively low diversity even when body surfaces are sampled. A recent study of fungal communities on the surfaces of *Dendrocnotus ponderosae* Hopkins and *Ips pini* (Say) incorporated both cultural and molecular methods and 730 isolates and 250 clones yielded only 14 yeast species in five genera (Lim *et al.*, 2005). Even including culture-free analysis, Lim *et al.* (2005) documented an even higher

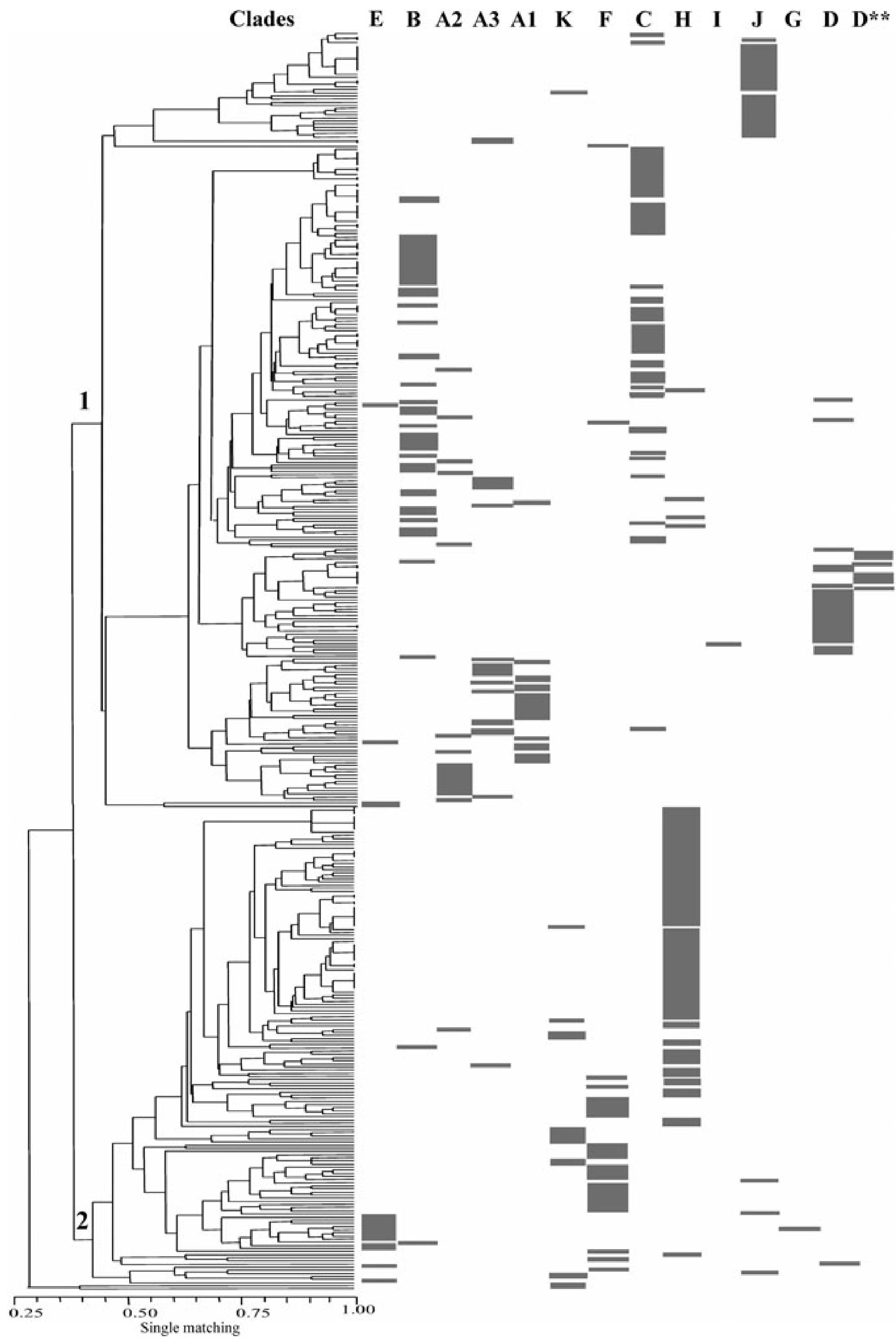
Clades
E B A2 A3 A1 K F C H I J G D D**
1
2
0.25 0.50 0.75 1.00
Single matching

Figure 3. Dendrogram obtained by unweighted pair-group method with arithmetic mean (UPGMA) for the 403 yeast strains with 40 physiological tests (see SUPPORTING INFORMATION). The positions into dendrogram of isolates included into clades obtained by phylogenetic reconstruction are shown at right. Abbreviations of clades: E, *C. arabinofermentans*; B, *C. ernobii*; 2, *C. lessepsii*; 3, *P. mexicana*; 1, *P. scolyti*; K, *C. oregonensis*; F, *C. piceae*; C, *Candida* sp.; H, *P. americana*; I, *P. canadensis*; J, *P. guilliermondii*; G, *P. glucozyma*; D, *K. capsulate*; D**, *K.* cf. *molischiana*.

ratio of isolate to yeast species than we found in our study. Limited bacterial communities also appear to be present in *Dendroctonus frontalis* Zimmermann (Vasanthakumar *et al.*, 2006) and *D. micans* (Kugelann) (Yilmaz *et al.*, 2006).

Ecological associations

In *Dendroctonus* (and many other insects-associated gut yeasts), little is known about yeast transmission, localization or fate within the digestive tract. If yeasts are primarily vertically transmitted, obligate symbionts, they would need to evade digestion within the gut. We would expect consistent associations between particular yeasts and their insect hosts and low diversity in yeasts isolated from conspecific insects. This appears to be the case in anobiid (deathwatch) beetles and some cerambycids; intracellular yeasts are housed in specialized mycetocytes (Douglas, 1989) where they are presumably protected from digestive enzymes. The yeasts are maternally transmitted during oviposition and ingested when first intar larvae eat the eggshell (Douglas, 1989). Recent studies of several Neotropical wood-boring cerambycids found no support for strict vertical transmission, but larvae inhabiting the same branch sometimes gave rise to the same yeast (Berkov *et al.*, 2007). If yeasts are primarily horizontally transmitted and part of a facultative, multispecies symbiosis, we would expect greater variability among yeasts isolated from conspecific insects. Yeast fate would depend on the outcome of interactions within the insect gut: those with the insect itself, with the feeding substrate and among microbes ingested with the feeding substrate. This might explain the observations of Suh *et al.* (2003) that fungus-feeding beetles, as a group, are associated with high yeast diversity (including many undescribed species), but low diversity of other gut organisms. In contrast, overall gut yeast diversity appears to be low in wood-boring passalid beetles [*Odontotaenius disjunctus* (Illiger)], but the total gut community is more diverse and distinct from that of the fungus-feeding beetles.

There is, thus far, no experimental evidence to explain why the gut yeast diversity in *Dendroctonus* appears to be so low. With the exception of yeast clades 1 and D**, all yeasts were isolated from multiple beetle species. Certain yeasts do appear to be preferentially associated with particular beetles: when beetle species and yeast clades from all localities were pooled in the correspondence analysis, the two principal axes explained 63.08% of the variation. *Dendroctonus pseudotsugae,* which feeds on Douglas-fir, was the only bark beetle that yielded *Pichia scolyti* (1) and appears to be preferentially associated with *C. ernobii* (B), *K. capsulata* (D) and *C. arabinofermentans* (E). Spruce-feeding *D. rufipennis* was the only beetle associated with *K.* cf. *molischiana* and it also gave rise to the infrequently isolated yeasts *P. glucozyma* (G) and *P. canadensis* (I). There is, however, little evidence of obligate association (see SUPPORTING INFORMATION). The number of yeast clades isolated per beetle species ranged from 1 to 11 (with a mean ± SE of 6.2 ± 1.18) and yeast species were isolated from multiple hosts (mean of 4.7 ± 0.5). Because of the lack of specific associations between beetle species and yeast, this study does not suggest strict vertical transmission via eggs. Maternal transmission cannot be ruled out as a contributing factor, however, because some yeasts were isolated from ovaries and eggs. Female reproductive structures were not always available and thus sample sizes were therefore lower, but they were extensively sampled when present. Almost all yeasts represented by at least 10 isolates were isolated from both the gut and either ovaries or eggs (see SUPPORTING INFORMATION), but the reproductive structures consistently yielded fewer yeast cultures than gut partitions.

Most yeasts were isolated from all three gut partitions, but not always in the same abundance (see SUPPORTING INFORMATION). *Pichia guilliermondii* and *C. ernobii* were isolated most frequently from the posterior midgut and this may explain its presence as an outlier in Figure 2A. The three most frequently isolated yeasts (*Pichia americana*, *Candida* sp. and *C. ernobii*) were abundant in all gut partitions and frass (the first two were also regularly isolated from ovaries). Overall, only half of the yeasts found in the gut were recovered in the frass (the lower diversity in both frass and eggs probably explain why they appear as outliers in Fig. 2A). The presence of culturable yeast in freshly deposited beetle frass suggests that at least some yeasts (species and individuals) survive gut passage and are dispersed by the beetle. Yeasts that were not recovered in the frass may not

have survived or they have been represented by few individual cells and difficult to isolate.

There may be different selection pressures present operating on yeast communities in different gut partitions and, although the strength of selection probably differs across beetle development stages, a critical period occurs during host tree colonization. At this point, both insects and yeasts must withstand the toxic effects of diverse host plant metabolites as they tunnel into the bark. Conifers have diverse defences within the phloem, including parenchyma cells specialized for the synthesis and storage of polyphenolic compounds (Franceschi *et al.*, 2005). These cells, often in concentric rings that protect the cambium, frequently store starch or lipids, but the phenolic compounds are thought to deter feeding and inhibit fungal growth. In addition, members of the family Pinaceae have radial ducts lined by epithethial cells that secrete abundant, constitutive terpenoid resin into the lumens (Franceschi *et al.*, 2005). The resin accumulates under pressure and, when released, it can first entrap or deter invading organisms and subsequently seal off the wound. In the most aggressive bark beetles, aggregation pheromones are required to attract insects in sufficient numbers to overwhelm the host. Volatile monoterpenes from resin in the phloem ducts are likely to regulate host plant location and, in some cases, are the source of the aggregation pheromones that enable bark beetles to subdue a tree (Seybold *et al.*, 2006). The metabolism of many monoterpenes takes place in the anterior midgut where pheromone synthesis takes place (Díaz *et al.*, 2000; Hall *et al.*, 2002; Nardi *et al.*, 2002). Monoterpene derivatives, which are more soluble but apparently more toxic, are stored in the posterior midgut and hindgut (Seybold *et al.*, 2006). In this study, yeasts were sometimes isolated from different gut regions from the same individual beetle and preliminary data suggest that these isolates sometimes differ in their ability to metabolize monoterpenes (G. Zúñiga, unpubl. data). Interactions with monoterpenes or their derivatives could therefore affect the abundance of particular yeasts in different gut regions.

In the correspondence analysis of yeast clades and host tree species, the two principle axes had low explanatory value (39.36% of the variability, Fig. 2C), but there were three clusters associated with host tree genera. The first cluster included pines (sampled primarily in Mexico and Guatemala, but also in Oregon), the second, Douglas-fir (sampled in both Mexico and Oregon) and the third, spruce (sampled in Oregon). When tree species were coded by genus rather than species and reanalysed, the two axes explained 70.66% of the variation (data not shown). Species of *Dendroctonus* tend to feed on subsets of potential hosts within particular conifer genera (Kelley & Farrell, 1998) and most pine feeders form a single clade, while spruce-, Douglas-fir- and larch-feeding species form a second clade. This suggests that host plant chemistry may regulate both beetle host association and the species richness of the gut yeasts implicated in monoterpene metabolism.

We were intrigued by the results of the analysis of yeast clade and locality; explanatory value increased when localities were classed into mountain systems. In Mexico, both the host plants and bark beetles have complex distributions: their ranges are extensive but discontinuous, on mountain systems that differ in temperature, rainfall and relative humidity. Because this study was designed to investigate associations between yeasts and anatomical isolation sites, rather than geographic locality, we cannot currently suggest a rationale: it would be necessary to design studies to test explicit biogeographical hypotheses.

Physiological characterization

The cluster analysis shown in Figure 3 graphically demonstrates the physiological variability in yeast isolates that, according to sequence data, represent members of the same species. In many cases, there is a rough concordance between yeast clade and metabolic cluster, but there are almost always outliers and the index of similarity shows that there can be considerable variability even among isolates included in a cluster. In this study, bark beetles feeding on different plant genera appear to be associated with distinct yeast communities and, although most yeasts were isolated from all gut partitions, there were differences in abundance. This raises the possibility that different yeasts are making different metabolic contributions to their hosts. Spruce- and Douglas-fir-feeding beetles most frequently yielded yeasts in the first large metabolic cluster (Fig. 3), while yeasts in the second metabolic cluster were frequently isolated from pine feeders. It is also interesting to note that the widely distributed yeast *P. guilliermondii* (clade J), in this study isolated in greatest abundance from the posterior midgut, makes up most of an independent cluster within the first large metabolic group.

Potential roles of yeast

Insect guts are clearly favourable sites for microbial colonization. Microorganisms must be localized in specific gut regions before their precise function can be studied; this requires detailed anatomical characterization. Although yeasts associated with *Dendroctonus* have been studied for decades, the anatomy and structural organization of the beetle guts have only recently been documented (Díaz *et al.*, 1998, 2000,

2003). In previous studies, yeasts were isolated from macerated larvae or adult beetles (Shifrine & Phaff, 1956; Lu *et al.*, 1957; Moore, 1972; Bridges *et al.*, 1984; Leufvén *et al.*, 1984; Leufvén, Bergström & Falsen, 1988). Detailed descriptions of gut organization in *Dendroctonus* permitted the isolation of yeasts from specific anatomical gut partitions and reduced the risk of contamination from other body sites. Frass was collected directly from galleries created by early colonists, thereby reducing the likelihood of contamination by co-occuring organisms, and this was not the case in other studies (Bridges *et al.*, 1984).

Few works have systematically studied the roles of yeast communities in bark beetle guts (Brand *et al.*, 1977; Leufvén *et al.*, 1984; Delalibera, Handelsman & Raffa, 2005) and almost nothing is known about the compartmentalization of the digestive process in *Dendroctonus*. While *Dendroctonus* species spend most of their life cycle on cortex, they are phloemphagous. Phloem has a relatively low nutritional content (Slansky & Scriber, 1985) and microorganisms may provide bark beetles with nitrogenous compounds including essential amino acids, vitamins, fatty acids or sterols (Ayres *et al.*, 2000; Six, 2003). All yeasts isolated from *Dendroctonus* assimilated inulin, cellobiose and D-xylose; therefore, our findings suggest that the yeasts' capacity for enzymatic degradation gives them the potential to contribute nutrients to the insect host. Future studies may document the presence of yeast-specific enzymes that hydrolyse or convert carbohydrates to simpler sugars that can be assimilated by insects, as has been widely documented in other insect groups (Koch, 1960; Schafer *et al.*, 1996; Nasir & Noda, 2003; Suh *et al.*, 2003; Vega & Dowd, 2005).

Alternatively, the assimilation of salicin (a toxic phenol glycoside) supports a potential role of yeast in detoxification. Phenol metabolism might enable beetles to withstand defence compounds stored in the relatively nutritious polyphenolic parenchyma cells, which are the most abundant living cells in conifer secondary phloem (Franceschi *et al.*, 2005). The phenolic compounds in conifers have been poorly studied relative to terpenoids, which give conifers their characteristic aromas, and are implicated in host location, host specificity and pheromone synthesis. Previous studies have shown that some yeasts, such as *Candida* (*Hansenula*) *capsulata* (Wickerham) Kurtzman and *C. nitratophila* (Shifrine & Phaff) Meyer & Yarrow associated with *Ips typographus* (L.) (Leufvén *et al.*, 1984, 1988; Hunt & Borden, 1989), *Pichia pini* (Holst) Phaff associated with *D. ponderosae* (Hunt & Borden, 1989) and *C.* (*Hansenula*) *holstii* (Wickerham) Kurtzman, *P. bovis* van Uden & do Carmo-Sousa and *P. pini* isolated from *D. frontalis* (Brand *et al.*, 1977), are able to oxidize the α-pinene of tree resin into both *cis*- and *trans*-verbenol and eventually to verbenone. *Cis*- and *trans*-verbenol are used as aggregation pheromones, while verbenone is an anti-aggregation pheromone. Experimental data (in progress) show that the gut yeasts identified in this study are also able to transform α-pinene, a monoterpene that is toxic for bark beetles, into *cis*-verbenol and *trans*-verbenol.

Phloem-feeding is considered an ancestral condition in the scolytids (Farrell *et al.*, 2001). All conifers sequester polyphenols, but only conifers in the (relatively recent) family Pinaceae produce copious constitutive resin and only Pinaceae are attacked by extremely aggressive bark beetles (Franceschi *et al.*, 2005). *Dendroctonus* is associated exclusively with members of the Pinaceae and its ancestral host appears to be pine (Kelley & Farrell, 1998). Although resin terpenoids may provide the compounds implicated in aggregation, all bark beetles feeding on conifers would benefit from a strategy to overcome the phenolic compounds defending the most nutritious cells in the phloem.

Interactions between insects and gut microbes are generally considered mutualisms. Gut yeasts clearly have the potential to make diverse contributions to fitness in *Dendrocnotus* and insects are assumed to provide gut microbes with a favourable environment for growth, ready access to macronutrients and dispersal. In this study, some beetles failed to yield yeasts: for instance, the 10 specimens of *D. frontalis* collected from *Pinus oocarpa* Schiede ex Schltdl. yielded a single yeast isolate. This, plus the lack of obligate associations between yeast clades and particular species of *Dendroctonus* – or specific anatomical isolation sites – suggests that yeasts are facultative symbionts of *Dendroctonus*. Specific roles have not yet been elucidated. Because at least some yeasts were isolated from both frass and eggs, there is at least the potential for either vertical or horizontal transmission. The preferential association between certain yeasts and bark beetles feeding on different host genera (pine, Douglas-fir or spruce) suggests that these conifers might harbour a limited subset of yeasts, which are ingested as beetles tunnel through the bark to construct galleries or during larval feeding. This would explain why all yeasts identified in this study have been previously isolated from gallery walls, pupal chambers, xylem tissues and exoskeletons of other bark beetles (Shifrine & Phaff, 1956; Callaham & Shifrine, 1960; Moore, 1972; Brand *et al.*, 1977; Bridges *et al.*, 1984; Leufvén *et al.*, 1984; Lim *et al.*, 2005), whole insects (Leufvén *et al.*, 1984; Lim *et al.*, 2005), as well as frass collected from *Tsuga heterophylla* (Raf.) Sarg and – an angiosperm – *Ulmus americana* L. (Kurtzman, 1994).

This study provided a broad overview of the association between gut yeasts, anatomical isolation site, bark beetle species and host plant. Further studies will focus on a single species of *Dendroctonus* that can be reared in the laboratory. This will enable us to investigate yeast acquisition and localization, the impact of yeasts on insect nutrition and fitness and complex ecological interactions between yeasts and other gut symbionts, in a controlled setting. This interdisciplinary approach will ultimately help us better understand the evolution of interactions between *Dendroctonus* and its gut microbiota, which appear to support the beetles' success in their lethal assaults.

ACKNOWLEDGEMENTS

We thank Jaime Villa, Jane L. Hayes, Gene Paul, Enrico Ruíz, Antonio Olivo, Francisco Bonilla and Jorge E. Macías-Samano for assistance in collecting insects. We also thank Sung Oui Suh, Ken Raffa, John Rinehart, Jorge E. Macías-Samano, Josep Guarro, Christine Johnson, Robert Anderson and two anonymous reviewers for comments and valuable suggestions regarding the manuscript. This work was financed partially by CONACyT (27585N), CONACyT (40955-Q) and CONAFOR (2002-COI-6020). FNR was a CONACyT fellow (129086); EG was a SPI-IPN fellow; ZG and NL were members of the PIFI-IPN programme. AB was supported by The National Science Foundation RIG 0542276.

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SUPPORTING INFORMATION

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

Table S1. Biochemical tests of yeast isolates from each clade.

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