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Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts

SUNG-OUI SUH,* CHRISTOPHER J. MARSHALL,† JOSEPH V. McHUGH‡ and MEREDITH BLACKWELL*

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803 USA, †Department of Entomology, Smithsonian Institution, National Museum of Natural Science, Washington, D.C., USA, ‡Department of Entomology, University of Georgia, Athens, GA 30602 USA

Abstract

During a survey of insect gut micro-organisms, we consistently isolated *Pichia stipitis*-like yeasts (Fungi: Ascomycota, Saccharomycetes) from the wood-ingesting beetles, *Odontotaenius disjunctus* and *Verres sternbergianus* (Coleoptera: Passalidae). The yeasts were isolated from passalid beetles over a wide area, including the eastern and midwestern USA and Panama. Phylogenetic analyses of the nuclear encoded small and large subunit rRNA gene (rDNA) sequences distinguished a well-supported clade consisting of the passalid yeasts and *Pichia stipitis*, *P. segobiensis*, *Candida shehatae* and *C. ergatensis*. Members of this clade have the ability to ferment and assimilate xylose or to hydrolyse xylan, major components of the polysaccharide, hemicellulose. Sexual reproduction was present in the passalid isolates but was rare among the gut yeasts of other beetles to which they were compared. Minor genetic and phenotypic variation among some of the passalid yeasts was detected using markers from the internal transcribed spacer region of the rDNA repeat unit, morphology, and *in vitro* metabolic tests. The consistent association of xylose-fermenting yeasts of almost identical genotypes with passalid beetles across a broad geographical distribution, suggests a significant symbiotic association.

Keywords: *Enteroramus dimorphus*, evolution, LSU rDNA, SSU rDNA, symbiosis, wood decomposition

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Introduction

Numerous associations between invertebrate animals and endosymbiotic micro-organisms have been described over the past century (Buchner 1965; Nardon & Grenier 1989). Invertebrates rely on microbes for various metabolic functions, including synthesis of amino acids, vitamins, lipids, sterols and pheromones, degradation of nutritional substrates, and detoxification of inhibitory compounds. A continuum of insect–fungus symbiotic associations exists in terrestrial ecosystems. These range from casual interactions in a shared habitat to strict obligate endosymbioses. Martin (1987) emphasized the contributions of microbial enzymes to the survival of various insects: anobiid beetles are able to live in cigarette packs because the tobacco is detoxified by yeast-like fungi (Dowd 1989, 1991), siricid wood wasps acquire fungal enzymes to degrade woody plant parts (Gilbertson 1984), and termites use cultivated fungi or gut symbionts to break down their cellulosic foodstuffs. Microbes profoundly affect the abilities of insects to utilize intractable nutritional resources and occupy habitats that otherwise would be unavailable to them.

The present work grew from a need to obtain yeasts for comparison with the gut-inhabiting yeasts of fungus-feeding beetles. We investigated passalid beetles (Coleoptera: Passalidae) for gut yeasts because: (i) passalids inhabit the same woody substrates used by certain basidiomycete fungi and the beetles that eat those fungi; (ii) fungi assist insects and other animal hosts by degrading the complex polysaccharides (cellulose and hemicelluloses) and phenylpropane polymers (lignin) that comprise the secondary cell walls of woody plants; (iii) passalid beetles are subsocial, they live in colonies of related individuals with overlapping generations, which could promote associations with symbionts; and (iv) yeast-like fungi of undetermined

Correspondence: Meredith Blackwell. Fax: 01 225578 2597; E-mail: mblackwell@lsu.edu

taxonomic placement had been reported previously from passalids (Lichtwardt *et al.* 1999).

Odontotaenius disjunctus (Illiger) is broadly distributed in eastern North America from Ontario and Quebec southwards to Lake Okeechobee, Florida (Schuster 1994). The beetles may grow to a length of 3.5 cm; they develop rapidly by maturing from egg to adult in several months. They have subsocial behaviour with adults tunnelling into moist rotten logs and stumps that have been decayed for several years by white rot fungi in temperate regions (Gray 1946; Schuster & Schuster 1997) or within 3–4 months in the tropics (Rodriquez & Zorrilla 1986). Adult beetles tear and masticate the ingested wood to a fine grain. First-instar larvae presumably require wood that is first processed by the adults (Pearse *et al.* 1936; Gray 1946; Schuster & Schuster 1985), and both adults and larvae feed on the masticated wood plastered on the walls of the tunnels by the adults.

Several earlier studies reported gut micro-organisms to be absent (Pearse *et al.* 1936; Gray 1946; Schuster & Schuster 1985), but because both adults and larvae ingest faeces, predigestion of the faecal pellets by microbial action has been postulated to occur and compared to an 'external rumen.' Details of the process by which the wood ingested by *O. disjunctus* is degraded are not clear, but it is unlikely that Trichomycetes (Fungi: Zygomycota) and an unclassified fungus noted as regular inhabitants of the gut (Heymons & Heymons 1934; Lichtwardt *et al.* 1999) are involved. Analysis of the chemical composition of passalid faecal pellets from the field and under laboratory conditions showed that available nitrogen increased with time (Rodriquez 1985; Rodriquez & Zorrilla 1986). Behavioural observations of many species of passalids indicated that *O. disjunctus* is characteristic of the family as a whole (Schuster & Schuster 1997).

Here we report the consistent isolation of closely related yeasts (Fungi: Ascomycota, Saccharomycetes) from *O. disjunctus* in the eastern USA and Kansas and from a second passalid species with a similar life history, *Verres sternbergianus*, from Panama. Phylogenetic analysis groups the undescribed yeasts in a well-supported clade with four other yeast taxa, including *Pichia stipitis*. The ability of *P. stipitis* and a few other yeasts to ferment xylose has led to intense interest in their use in biotechnology to produce ethanol from wastes with high xylose content (van Dijken *et al.* 1986; Jeffries & Kurtzman 1994; Biely & Kremnicky 1998; Winkelhausen & Kuzmanova 1998; Jeffries & Jin 2000; Ward & Singh 2002).

Methods and materials

Host beetles

Yeasts were isolated from the gut and external surface of specimens of *Odontotaenius disjunctus* (Passalidae: Proculini) collected from rotten wood in Pennsylvania and several different localities in Georgia, South Carolina and Louisiana, and from *Verres sternbergianus* (Passalidae: Proculini) from Barro Colorado Island, Panama (Table 1). Vouchers were deposited at the Georgia Museum of Natural History, Collection of Arthropods.

Yeast isolation and culture

Beetles collected from decaying logs were frozen until dead and submerged in 95% ethanol for 1–2 min to disinfect their surfaces. The alcohol wash was followed by a 0.7% saline rinse; the rinse liquid was plated on acidified YM agar (Difco YM broth, 2% plain agar, adjusted to pH 3.5 with HCl) as a negative control. Forceps, dissecting needles and minute insect pins were used to dissect the beetles on sterile microscope slides under a dissecting microscope. The beetle gut was removed aseptically, cut into pieces and transferred to tubes containing 0.7% saline. Gut segments were crushed in the saline solution with a pipette tip and streaked with a loop onto the surface of acidified YM agar plates. Plates were incubated at 25 °C, and after 3 days single colonies were streaked for purification. This procedure was replicated one or more times. Cultures were maintained on YM agar. A culture established from the original type material of *Enteroramus dimorphus* (KS-42-W2) from the gut of *O. disjunctus* collected in Kansas (Lichtwardt *et al.* 1999), was compared with the other yeasts isolated in this study. Morphological observations and metabolic tests comprising the yeast 'standard description', were performed on the passalid yeasts listed in Table 1, according to established methods (Kurtzman & Fell 1998; Barnett *et al.* 2000). The cultures established in this study have been deposited in the Agricultural Research Service (ARS) Culture Collection (NRRL Y-27547–Y-27555).

DNA methods

Yeast cells were harvested from agar cultures, and the nucleic acids were extracted and purified following the procedures of Lee & Taylor (1990). The primer sets NS1-NS8, LS1-LR5 and ITS5-ITS4 were used for amplifying SSU and LSU rRNA genes (rDNA), and 5.8S rDNA and internal transcribed spacer (ITS) sequences (White *et al.* 1990; Hausner *et al.* 1993), respectively, using the polymerase chain reaction (PCR). PCR products were purified using a DNA purification kit (Bio-Rad Laboratories). The purified double-stranded PCR products were used as templates for sequencing with an ABI PRISM™ BigDye Terminator Cycle sequencing kit, version 2 (PE Applied Biosystems). The complete sequences of SSU rDNA, 5.8S rDNA including ITS and the D1/D2 region of the LSU rDNA were sequenced with the primers NS1, NS2, 18H, NS8, ITS1, ITS4, LS1 and LR3 using an ABI

Table 1 Yeasts isolated from passalid beetles and GenBank DNA accession numbers

DNA group*	Yeast isolate no.	Host and other information	GenBank rDNA accession no. LSU	SSU	ITS/5.8S
PASS1	BG 01-5-4-2-1	*Odontotaenius disjunctus*; Passalidae Burke Co., Shell Bluff, GA (March 22, 2001) (NRRL Y-27547)	AY227721	AY227898	AY227901
	GA 012-1-1	*Odontotaenius disjunctus*; Passalidae Clarke Co., Athens, GA (July 9, 2001) (NRRL Y-27548)	AY227720	AY227897	AY227900
	BG 02-7-16-1	*Odontotaenius disjunctus*; Passalidae Orangeburg Co., SC (August 13, 1999) Yeast isolate provided by C. E. Beard (NRRL Y-27549)	AY227723	—	AY227903
	BG 02-2-11-6-5	*Odontotaenius disjunctus*; Passalidae East Baton Rouge Parish, Baton Rouge, LA (February 11, 2002) (NRRL Y-27550)	AY227724	—	AY227904
	BG 02-4-1-3-1	*Odontotaenius disjunctus*; Passalidae East Baton Rouge Parish, Baton Rouge, LA (April 3, 2002) (NRRL Y-27551)	AY227725	—	AY227905
	BG 03-3-25-1-3	*Odontotaenius disjunctus*; Passalidae Oxford, PA, in red oak log (20 March 2003) (NRRL Y-27554)	AY325109	—	AY325111
	BG 03-3-25-1-5†	*Odontotaenius disjunctus*; Passalidae Oxford, PA, in red oak log (20 March 2003) (NRRL Y-27555)	AY325110	—	AY325112
	BG 02-7-14-003-1-1	*Verres sternbergianus*; Passalidae Barro Colorado Island, Panama (July 14, 2002) (NRRL Y-27552)	AY227726	—	AY227906
	BG 02-7-14-003-2-1	*Verres sternbergianus*; Passalidae Barro Colorado Island, Panama (July 14, 2002) (NRRL Y-27553)	AY227727	—	AY227907
PASS5	KS-42-W2	*Odontotaenius disjunctus*; Passalidae Douglas Co. KS (September 19, 1997) (NRRL Y-27535)	AY227722	AY227899	AY227902

*DNA group based on the sequence of D1/D2 region in LSU rDNA.
†Metabolic tests not performed.

PRISM 377 Automated DNA sequencer (PE Applied Biosystems).

GenBank accession numbers for DNA sequenced from passalid beetles in this study are listed in Table 1. In addition to the new yeast sequences in Table 1, several other yeasts were sequenced in this study [*Candida ambrosiae* (NRRL YB-1316), *Candida tanzawaensis* (NRRL Y-17324), *Candida ernobii* (acquired as *Candida karawaiewii*) (ATCC 22994), *Candida xestobii* (ATCC 24001), *Symbiotaphrina buchneri* (CBS 420.63), and *Symbiotaphrina kochii* (CBS 250.77)], and their GenBank accession numbers are listed in the reference data below. LSU rDNA sequences were used to distinguish yeast genotypes (Kurtzman & Robnett 1998); in this study PASS1 and PASS5 are named using the first four letters of the beetle family and a unique number (Table 1).

Data analysis

DNA sequences were aligned with sequences obtained from GenBank using the multialignment program CLUSTAL x (Thompson *et al.* 1997). The newly sequenced yeasts were analysed with LSU and SSU rDNA sequences of other yeasts and fungi obtained from GenBank. GenBank accession numbers of SSU and LSU rDNA sequences, respectively, were as follows: *Arxula adeninivorans* (AB018123; U40094), *Aureobasidium pullulans* (M55639; AF050239), *Brettanomyces naardenensis* (X85110; U76200), *Candida albicans* (M60302; AF156536), *Candida ambrosiae* (AY227712; AY013716), *Candida ergatensis* (AB013524; U45746), *Candida insectamans* (AB013518; U45791), *Candida insectorum* (AB013565; U45753), *Candida intermedia* (AB013571; U44809), *Candida ernobii* (acquired as *Candida karawaiewii*) (AY227714; U94921),

Candida kruisii (AB013543; U45718), *Candida lyxosophila* (AB013522; U76204), *Candida odintsovae* (AB054570; U70182), *Candida parapsilosis* (AB013588; AF485969), *Candida shehatae* var. *insectosa* (AB013584; U45773), *Candida rhagii* (AB018172; U45729), *Candida tenuis* (AB013516; U45774), *Candida tanzawaensis* (AY227713; U44811), *Candida xestobii* (AY227715; U45707), *Chromocleista malachitea* (D88323; AB000621), *Dipodascus albidus* (X69840; U40081), *Galactomyces geotrichum* (X69842; U40118), *Hamigera avellanea* (D14406; AF454075), *Hanseniaspora uvarum* (X69844; U84229), *Hypocrea lutea* (D14407; U00739), *Kluyveromyces polysporus* (X83825; U68548), *Lodderomyces elongisporus* (X78600; U45763), *Neurospora crassa* (X04971; U40124), *Pachysolen tannophilus* (AF132030; U76346), *Pichia anomala* (AB054562; AF330115), *Pichia guilliermondii* (AB013587; AF374616), *Pichia segobiensis* (AB054288; U45742), *Pichia stipitis* (AB054280; U45741), *Protomyces inouyei* (D11377; U84344), *Saccharomyces cerevisiae* (Z75578; J01355), *Saccharomycopsis capsularis* (X69847; U40082), *Stephanoascus farinosus* (AB000660; U40132), *Symbiotaphrina buchneri* (AY227716 AY227718), *Symbiotaphrina kochii* (AY227717; AY227719), *Taphrina deformans* (U00971; AF492038), *Williopsis saturnus* var. *mrakii* (Y11318; U94929), yeast-like symbiont of *Laodelphax striatellus* (AF267232; AF267235), yeast-like symbiont of *Sogatella furcifera* (AF267234; AF267237), yeast-like symbiont of *Nilaparvata lugens* (AF267233; AF267236), *Zygoascus hellenicus* (AF294751; U40125), *Zygosaccharomyces rouxii* (X90758; U72163). The alignments were optimized visually, and ambiguous regions were excluded from the analyses. *Protomyces inouyei* and *Taphrina deformans*, determined to be basal ascomycetes in previous phylogenetic studies, were designated as outgroup taxa.

Maximum parsimony analyses were performed using PAUP 4.0b10 (Swofford 2002). Heuristic tree searches were executed using the tree bisection–reconnection branch swapping algorithm with random sequence analysis. Bootstrap values of the most parsimonious tree were obtained from 1000 replications. Base-pair differences in a gene were counted using BLAST 2 sequences (Tatusova & Madden 1999) or from a manually aligned sequence database.

Results

Yeast cultures

PASS1 yeasts (Table 1) were isolated from the gut and external surface of about 22 adult passalid beetles (*Odontotaenius disjunctus* and *Verres sternbergianus*) and purified on YM agar. More than 100 colony-forming units were obtained from the gut of every beetle, except for a single anomalous individual from which no yeast was isolated. Although other yeast species were not cultured from the passalids, we cannot rule out the presence of yeasts that would not grow under our culture conditions or that were present in low population numbers. The PASS1 and PASS5 cultures were used as a source of genomic DNA for sequencing. Approximately 1730 base pairs (bp) of SSU rDNA representing most of the gene; about 600 bp of the LSU rDNA gene, including the variable D1/D2 region; and about 600 bp of the ITS and 5.8S region were obtained from PCR products of the passalid yeasts.

Relationships of the yeasts

Phylogenetic analysis using a combined LSU and SSU rDNA sequence database of the eight passalid isolates and a diverse group of other yeasts chosen from among all described species of yeasts, resulted in a single most parsimonious tree (Fig. 1). The passalid isolates formed a well-supported clade with two closely related taxa (*Pichia stipitis* and *P. segobiensis*), hereafter called the *P. stipitis* clade. *Candida shehatae* was a sister taxon to the *P. stipitis* clade, and *C. ergatensis* was sister to the *P. stipitis* and *C. shehatae* clade and the basal member of the lineage (bold typeface, Fig. 1). The other relationships obtained were consistent with results from previous studies (Kurtzman & Robnett 1998).

Genetic and metabolic similarity among passalid yeast isolates

Two passalid sequences were distinguished by comparison of LSU rDNA (PASS1, PASS5, Table 2), following a convention used in the study of yeasts (Kurtzman & Robnett 1995, 1998). Other PASS isolates from Panama exist, but these are from passalid beetles with a different habit, and they will be discussed in a future study. The PASS1 and PASS5 LSU rDNA sequences varied by only 1 bp from each other, as did their SSU rDNA sequences. ITS sequences revealed greater variation (Table 2). The PASS1 ITS sequences from the USA (Pennsylvania BG 03-3-25-1-3, BG 03-3-25-1-5; Georgia BG 01-5-4-2-1, GA 012-1-1; South Carolina BG 02-7-16-1; Louisiana BG 02-2-11-6-5, BG 02-4-1-3-1) were identical, but varied by 1 bp from the Panama PASS1 ITS sequences from *Verres sternbergianus* (BG 02-7-14-003-1-1, BG 02-7-14-003-2-1). The PASS5 (Kansas KS-42-W2) ITS sequence varied by 1 bp from the USA PASS1 isolates and by 2 bp from the Panamanian PASS1 isolate. *Pichia stipitis* and *P. segobiensis* were more variable, differing from the passalid isolates by 6–7 bp and 16–18 bp, respectively (Table 2). BLAST searches did not reveal other similar sequences deposited in GenBank or collected by us.

Besides the DNA sequences, approximately 20 morphological and 80 physiological traits were compared *in vitro* for the PASS1 isolates to other clade members. The PASS1 and PASS5 isolates varied in relatively minor details when compared to the reference cultures of *P. stipitis* and

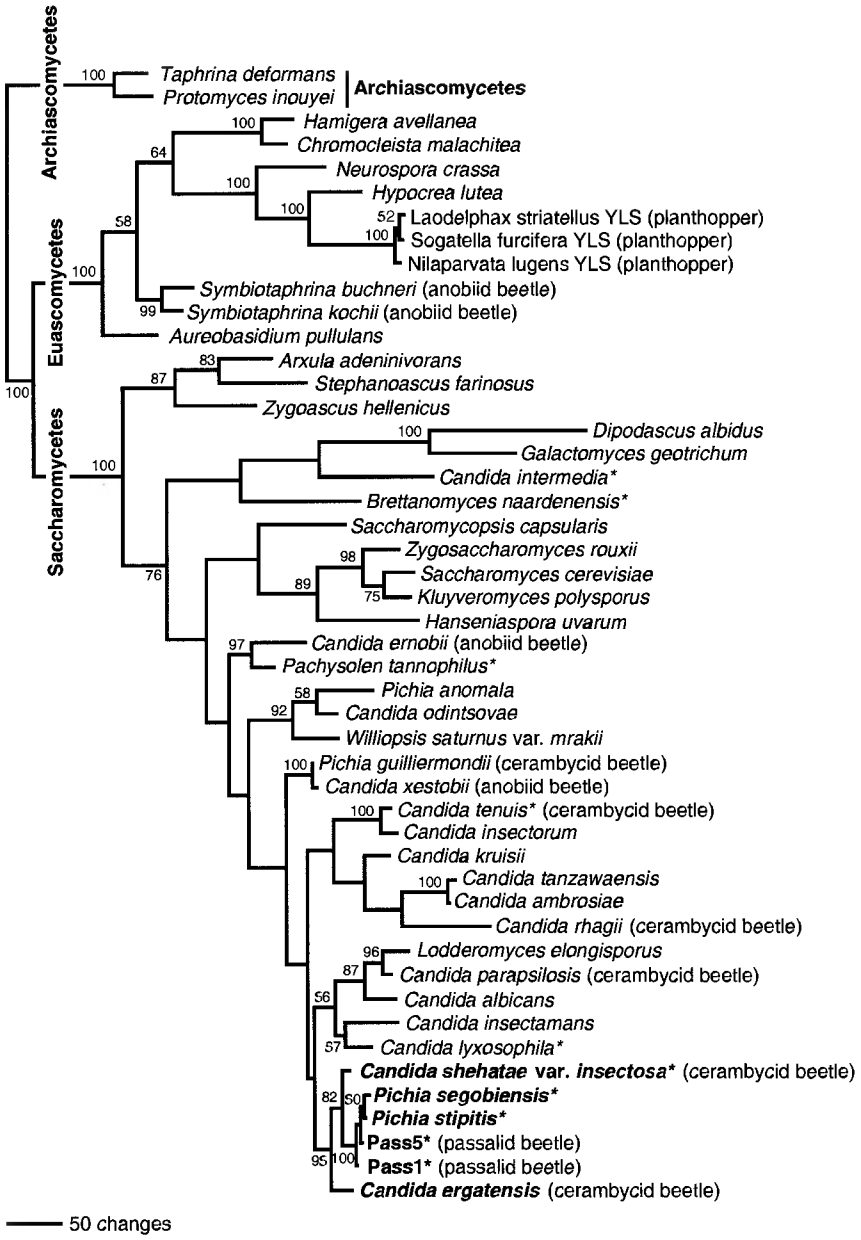

Fig. 1 Single most parsimonious tree obtained from combined LSU and SSU rDNA sequence data. The xylose-fermenting clade, including yeasts isolated from passalid beetles, appears in bold typeface, and subphyla of ascomycetes are indicated on branches. Yeasts that ferment xylose are indicated by an asterisk (*); note that five species outside of the *Pichia stipitis* clade possess the rare trait. Tree length = 3589; consistency index = 0.4266; homoplasy index = 0.5734; retention index = 0.6450; rescaled consistency index = 0.2751. Numbers on tree branches indicate the percentages of bootstrap samplings derived from 1000 samples that supported the internal branches by 50% or higher.

P. segobiensis discussed in the literature (Kurtzman & Fell 1998; Barnett *et al.* 2000), such as ability or rate of fermentation and assimilation of several carbon compounds and growth at certain temperatures (Table 3). Most members of the clade, including PASS1 and PASS5 isolates, fermented and assimilated xylose (Kurtzman 1990; Jeffries & Kurtzman 1994), albeit often delayed until about 10 days after inoculation. The exception was the basal member, *C. ergatensis*, as reported in the literature (Kurtzman & Fell 1998; Barnett *et al.* 2000). However, this species, along with *P. stipitis*, has been reported to hydrolyse xylan, a component of hemicellulose composed of D-xylose residues (Biely & Kremnicky 1998). In addition most of the clade members synthesize a wide range of vitamins (myo-inositol, pantothenate, thiamine, pyrodoxine, niacine), but only PASS1 isolates produced small amounts of biotin *in vitro*. Again, *C. ergatensis* was the outlier producing fewer vitamins, requiring both biotin and thiamine for growth (Kurtzman & Fell 1998; Barnett *et al.* 2000).

Two morphological differences were detected among clade members. The symbiotic filamentous growth that marked the PASS5 isolate *in situ* was absent in culture, and the PASS1 isolates varied in production of pseudohyphae or hyphae in culture. The other members of the clade all form pseudohyphae or true hyphae in culture. Sexual reproduction occurred in the passalid isolates, *P. stipitis* and

Table 2 Comparison of LSU rDNA (577 bp) (bold figures) and ITS/5.8S rDNA (567 bp) bp differences in rRNA gene of yeasts from passalid beetles and closest relatives

Locality	Yeast isolates		1	2	3	4	5	6	7	8	9	10	11	12
PASS1														
USA (GA)	BG 01-5-4-2-1	1	—	0	0	0	0	0	0	1	1	1	6	16
	GA 012-1-1	2	**0**	—	0	0	0	0	0	1	1	1	6	16
USA (SC)	BG 02-7-16-1	3	**0**	**0**	—	0	0	0	0	1	1	1	6	16
USA (LA)	BG 02-2-11-6-5	4	**0**	**0**	**0**	—	0	0	0	1	1	1	6	16
	BG 02-4-1-3-1	5	**0**	**0**	**0**	**0**	—	0	0	1	1	1	6	16
USA (PA)	BG 03-3-25-1-3	6	**0**	**0**	**0**	**0**	**0**	—	0	1	1	1	6	16
	BG 03-3-25-1-5	7	**0**	**0**	**0**	**0**	**0**	**0**	—	1	1	1	6	16
Panama (BCI)	BG 02-7-14-003-1-1	8	**0**	**0**	**0**	**0**	**0**	**0**	**0**	—	0	2	7	17
	BG 02-7-14-003-2-1	9	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	—	2	7	17
PASS5														
USA (KS)	KS-42-W2	10	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	—	7	17
France	*Pichia stipitis* (JCM 10742)	11	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	—	18
Spain	*Pichia segobiensis* (JCM 10740)	12	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**2**	—

PASS1 and PASS5 designations are based on unique LSU rDNA sequences.

Table 3 Variable traits* of PASS1, PASS5, *Pichia stipitis* and *P. segobiensis* among about 100 traits tested

Variable trait	PASS1†	PASS5	*Pichia stipitis*‡	*Pichia segobiensis*‡
Morphology				
Pseudohyphae§	+/–	–	+	+
Fermentation				
Maltose	D	D	+/D	–
Cellobiose	–	–	D/–	–
Starch	–	–	D/–	–
Carbon assimilation				
L-Sorbose	–	–	D/–	D
L-Arabinose	+/D	D	+/–	–
D-Arabinose	+/D	+	+/D	D/–
L-Rhamnose	+/–	–	+	–
α-Methyl-D-glucoside	+	+	+	D
Maltose	+	+	+/D	–
Lactose	–	+	+/–	–
Soluble starch	+/D	D	+	–
Erythritol	+/D	–	+	–
L-Arabinitol	+/D/W	–	D/–	–
D-Gluconate	+/D	+	D/–	–
DL-Lactate	D/W	D	D/–	–
Propane 1, 2 diol	+/D/W	W	D/–	–
Nitrogen assimilation				
D-Glucosamine	W	–	–	–
Vitamin requirements				
W/O PABA	+/W	+	+	+
W/O Biotin	W/–	W	–	–
W/O Biotin, Thiamin	W/–	–	–	–
Growth temperature				
at 35 °C	+	–	+/–	+
at 40 °C	–	–	–	+

*Variable reactions: +, positive reaction; –, negative reaction; D, delayed positive reaction; W, weak positive reaction.
†Data obtained from eight PASS1 isolates listed in Table 1.
‡Data from Barnett *et al.* (2000).
§Observation on corn meal agar after 7 da (Kurtzman & Fell 1998).

P. segobiensis, all of which usually produced two hat-shaped ascospores per ascus. *Candida shehatae* and *C. ergatensis* are asexual yeasts.

Discussion

Symbiotic passalid yeasts

Yeasts sometimes have been considered conspecific if they share identical or similar sequences for about 600 bp in the D1/D2 loop region of the LSU rDNA (Kurtzman & Robnett 1998), and, using this conservative measure, PASS1, PASS5 and *Pichia stipitis* might be considered conspecific by some yeast systematists. The chance determination that the PASS5 isolate (KS-42-W2), previously described as *Enteroramus dimorphus* (Lichtwardt *et al.* 1999), is a yeast shows the utility of a dense DNA database for identification of yeasts if not for routine species delimitation. The morphology and physiology of the passalid isolates and *P. stipitis* also are similar, and consistent with small differences sometimes observed among isolates of a single taxon. The PASS1 and PASS5 isolates have not been distinguished as species distinct from *P. stipitis*, because without greater geographical sampling and additional genomic markers, we do not know if the differences detected among the isolates (Tables 2 and 3) are significant. There is, however, the possibility that certain yeast populations have become isolated in association with the passalids.

Interestingly, the capacity of fungi to reproduce sexually is usually correlated with their degree of symbiosis and method of transmission. Sexual reproduction by the passalid-gut yeast isolates implies that their biology is not strictly tied to that of their coleopteran hosts and that they are not simply clonally propagated by vertical transmission. Thus, if xylose-fermenting yeasts help to improve the fitness of the beetles, it may be necessary for the beetles to repeatedly co-opt these yeasts from the environment. Of course, given the subsocial behaviour of passalid beetles with requisite intergenerational transfer of gut fauna, associations lasting more than a generation are likely. Furthermore, as has happened with termite endosymbionts, association with yeasts may have played a fundamental role in the evolution of subsocial behaviour, a characteristic of all Passalidae.

Wood-decay and the importance of microbes

This is the first specific report of a widespread association of passalid beetles with a known endosymbiotic organism. Earlier researchers suggested microbes were involved in passalid digestion, but such micro-organisms were not identified in these studies (Pearse *et al.* 1936; Gray 1946; Schuster & Schuster 1985). The isolation of other yeasts belonging to the *P. stipitis* clade from different wood-ingesting beetles and their habitats, indicates that Passalidae are not the only beetles to make use of yeasts having the rare ability to ferment and assimilate xylose (Fig. 1) (Martin 1987; Nardon & Grenier 1989). In the yeast pathway xylose is converted to xylitol, and then, xylulose; conversion of xylulose is by the pentose phosphate pathway to fructose-6-phosphate to provide a substrate for oxidation or fermentation (Jeffries & Jin 2000; Jackson & Nicolson 2002). Xylose is usually not found as a soluble sugar in nature, unlike sucrose and fructose, so the insect gut offers a place in which hemicellulose can be broken down for assimilation (Jeffries & Jin 2000).

Some wood decay may be required before the invasion of adult passalids. Early in the wood decay process, bacteria, some of which fix nitrogen, and fungi, including yeasts and stain fungi, invade fallen timber. For example stain fungi help to remove extractives such as phenolics that can inhibit fungal enzymes. Later in the succession, wood-decaying basidiomycetes contribute their decay enzymes to the process. In the relatively early stages of the white rot process (Alexopoulos *et al.* 1996), lignin–cellulose bonds are cleaved to expose unprotected hemicellulose that is vulnerable to fungal enzymes (Eriksson *et al.* 1990; Blanchette 1991).

Our findings do not preclude the possibility that gut micro-organisms in addition to the PASS1 and PASS5 yeasts may be involved in the digestion of ingested wood in passalids; for example we have not attempted to isolate bacteria, nor are we certain that other eukaryotes are absent. Several trichomycetes are known to partition themselves within the gut of passalids and they could also be involved (Lichtwardt *et al.* 1999, 2001).

Beetle invasions into woody substrates may have been facilitated by their association with yeasts. The association of passalids from distant localities with yeasts of such high genetic similarity suggests a significant commensal or symbiotic relationship.

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