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# The beetle gut: a hyperdiverse source of novel yeasts

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## Abstract

We isolated over 650 yeasts over a three year period from the gut of a variety of beetles and characterized them on the basis of LSU rDNA sequences and morphological and metabolic traits. Of these, at least 200 were undescribed taxa, a number equivalent to almost 30% of all currently recognized yeast species. A Bayesian analysis of species discovery rates predicts further sampling of previously sampled habitats could easily produce another 100 species. The sampled habitat is, thereby, estimated to contain well over half as many more species as are currently known worldwide. The beetle gut yeasts occur in 45 independent lineages scattered across the yeast phylogenetic tree, often in clusters. The distribution suggests that the some of the yeasts diversified by a process of horizontal transmission in the habitats and subsequent specialization in association with insect hosts. Evidence of specialization comes from consistent associations over time and broad geographical ranges of certain yeast and beetle species. The discovery of high yeast diversity in a previously unexplored habitat is a first step toward investigating the basis of the interactions and their impact in relation to ecology and evolution.

## INTRODUCTION

Fungi have been suggested as determinants of plant diversity (van der Heijden *et al*. 1998), and plant diversification has been implicated as a major factor affecting the diversity of beetles and other insect groups (Farrell 1998). Direct relationships between fungi and insect evolution, however, remain under appreciated, although gut-inhabiting fungi are known to be essential to the nutrition of many insects (Martin 1987, Nardon & Grenier 1989, Vega & Dowd 2005). We isolated many yeasts associated with the digestive tract of insects by targeting beetles, most of which feed on basi-diomes, the spore-bearing structures of basidiomycetes (e.g. mushrooms, brackets) (Fig. 1), a strategy that allowed resampling of beetle taxa in 27 families. Over three years, but with intensive collecting for the equivalent of only 45 12 h days, we sampled beetles from the southeastern USA and Barro Colorado Island, Panama. Here we report the discovery of a large number of undescribed yeast species from the unexplored habitat of the insect gut and suggest that such associations promote fungal diversity and expansion of insects into nutrient-poor substrates.

## METHODS

### Beetle hosts and yeast isolation

Yeasts were isolated from beetles in the following taxa: *Anobiidae*, *Anthribidae*, *Biphyllidae*, *Carabidae*, *Cerambycidae*, *Chrysomelidae*, *Ciidae*, *Cucujidae*, *Curculionidae*, *Dermestidae*, *Derodontidae*, *Elateridae*, *Endomychidae*, *Erotylidae*, *Histeridae*, *Laemophloeidae*, *Latridiidae*, *Leiodidae*, *Melandryidae*, *Mordellidae*, *Mycetophagidae*, *Nitidulidae*, *Passalidae*, *Scarabaeidae*, *Staphylinidae*, *Tenebrionidae*, and *Trogossitidae*. Beetles were

surface disinfected with alcohol and rinsed in 0.7% saline solution before removal of the gut. The plating of the saline rinse solution after the alcohol was essential to provide a control to monitor the presence of surface microorganisms in each dissection. Yeasts were purified on acidified yeast-malt agar. More detailed methods, including collecting sites, DNA techniques, and phylogenetic analysis, have been published previously (Suh *et al*. 2003, Suh, Gibson & Blackwell 2004, Suh & Blackwell 2004, Suh, McHugh & Blackwell 2004). The complete SSU rDNA region also was sequenced for one isolate from each unique LSU rDNA genotype for parsimony analysis. Sequences have been deposited in Gen-Bank (AY227712–AY227727, AY227897–AY227899, AY242136–AY242352, AY309784–AY309919, AY-426946–AY426968, AY518520–AY518532, AY-520153–AY520423). Cultures are preserved in CBS and NRRL.

### Bayesian analysis

We applied a Bayesian analysis using various models that allowed for variable rates of discovery among yeast species. The best-supported model was a three rate-class variable frequency model (lnL=−316.14; 2 δlnL compared to the two rate-class variable frequency model is 20.87; 2 δlnL compared to the four rate-class variable frequency model is only −2.9). The three rate-class variable frequency model also is significantly better than the gamma model (2 δlnL= 20.87; note, although these two models are not strictly nested, a three-rate class approximation of the gamma model would be nested and would have a lower likelihood than our continuous approximation. Therefore, a chi-square approximation with three degrees of freedom is conservative in rejecting the gamma model in favour of the three-class model). A detailed description of the methods is provided by Pollock & Larkin (2004).

## RESULTS

About 650 yeasts isolated from beetle gut were characterized by sequencing 600 bp from the LSU rDNA D1/D2 loop and observing about 20 morphological and 80 physiological traits constituting the yeast standard description (Kurtzman & Fell 1998, Barnett, Payne & Yarrow 2000). Phylogenetic analysis of 196 new LSU rDNA D1/D2 sequences among previously described exemplar taxa showed that sequences from insect-associated yeasts often occurred in clusters that were distributed in at least 45 clades throughout the phylogenetic tree (Fig. 2). Some of the yeasts recovered were basidiomycetes (i.e. *Tremellales*), but the vast majority were ascomycete budding yeasts (*Saccharomycetes*), relatives of model yeasts such as *Saccharomyces cerevisiae*.

Among the 650 yeast isolates, we distinguished 290 unique D1/D2 loop genotypes. 39 (13%) of these were identical to previously sequenced yeasts, but 55 others (19%) differed by 1–5 bp from known yeasts; remarkably, 196 isolates (68%) differed by more than 5 bp from the closest previously known yeast genotype (Figs 2–3). In order to assess how many previously unknown species were represented, we used a species concept based on phylogenetic analysis of the combined SSU and LSU rDNA D1/D2 loop sequences to diagnose certain terminal groups as species (Suh, Gibson & Blackwell 2004), and these diagnoses corresponded well with other currently used species criteria (Kurtzman & Robnett 1998). The discovery of nearly 200 undescribed species from this one pilot study represents a substantial increase of more than 30% over all previously described yeast species in all habitats.

From the analysis of species discovery rates the mean estimate of the percentage of undescribed yeast species was 46.1% (range 33.7–53.8%), meaning that almost as many species remain undiscovered as have already been found. Results for the four rate-class variable frequency model (the second-best model) are very similar, and together these two models account for approximately 100% of the Akaike weights based on the Akaike information criterion (Akaike 1981). Thus, if only these localities were extensively resampled we estimate that the total

number of all known yeast species worldwide, including those already discovered by us, would increase by 50%.

## DISCUSSION

The number of all described species of all fungi is estimated to be 74 000–125 000, but this figure is probably an extreme understatement of actual numbers of extant fungi (Hawksworth 2001). Over the last decade, mycologists became interested in comparisons of the number of fungal species with the numbers of other groups of organisms, and worldwide estimates of 1.5 million species and higher have been proposed. Intense interest in biodiversity has led to the discovery of many new species of fungi in high-diversity habitats, including endophytes within above-ground plant parts and fungi from the rhizosphere (Arnold, Maynard & Gilbert 2001, Vandenkoornhuyse *et al*. 2002). Enumeration of animal-associated fungi, however, especially insects associates, has been addressed less often (Hawksworth 2001) with only a few exceptions (Lichtwardt, Cafaro & White 2001, Weir & Hammond 1997).

Our results indicate that insect gut habitats harbour an astonishing diversity of undescribed yeasts. There are no precise estimates for expected yeast numbers worldwide, but the rate of species discovery suggests that a plateau has not been reached and numbers will be extremely high, corresponding with our prediction that extensive resampling of our limited habitats collected would increase the number of known yeasts by 50%. In addition we consider the species criteria used to be conservative, since some yeasts with identical D1/D2 loop genotypes were resolved into subunits by ITS sequences and phenotypic traits; the additional taxonomic subunits sometimes corresponded with beetle host identity.

Additional support for the statistical prediction that large numbers of yeasts remain to be collected in our study areas comes from beetle diversity at those sites. In certain beetle groups, such as *Erotylidae* and *Tenebrionidae*, we suspect the total number of associated yeasts could approach the number of beetle species. For example we collected 40 species of erotylid beetles, only 11 of which had been reported to occur in Panama; these constitute fewer than 11% of the erotylid species previously reported from that country. Larger numbers of yeasts would be obtained if sampling were extended to other, more diverse habitats in the rest of the country, and even more if it were extended worldwide. Since 4500 erotylid species are currently recognized worldwide (probably an underestimate), and since our results indicate that each beetle species will on average be host for at least one new yeast species, at least 4500 new yeast species could be expected from this beetle group alone, 6–7 times the number of all currently recognized yeast species. The discovery of the great diversity of undescribed yeasts in association with beetles is stunning, especially when one extrapolates beyond the highly restricted taxonomic, temporal, ecological, and geographical scope of our study. Our findings raise physiological, ecological, and evolutionary questions, regarding the nature of the yeast-insect associations, the universality of associations worldwide, and the role that yeasts may play in habitat expansion and speciation of insects (Suh *et al*. 2003).

Although genome-level approaches (Rokas *et al*. 2003, Dietrich *et al*. 2004) may be needed to resolve certain relationships among yeast groups, our markers indicated that the insect gut yeasts occurred in clusters throughout the yeast phylogenetic tree. Taxa from one large ascomycete yeast clade, the *Candida tanzawaensis* clade (Suh, McHugh & Blackwell 2004), consisting of 164 isolates and representing 39 D1/D2 genotypes, were isolated from beetles in eleven families (Figs 2–3) and several other insect groups, including lepidopteran larvae. Only one species was known in this clade when our study began. Other examples of insect-associated clades are evident in the tree (Fig. 2), and the phylogenetic evidence and host relations suggest that divergence of yeasts may have occurred by occasional host switching to unrelated insects within a basidiome, followed by specialization with certain insects.

Host specialization was evident among some of the associated insects and yeasts in this study. For example, we isolated a *C. tanzawaensis* clade member, *C. choctaworum*, numerous times from the gut of *Neomida bicornis* (*Coleoptera: Tenebrionidae*) collected in basidiomes of *Fomitella supina* (*Basidiomycota: Polyporaceae*) at five different southern Louisiana localities in each of four different years. Another *C. tanzawaensis* clade yeast (*C. bolitotheri*) and its tenebrionid host (*Bolitotherus cornutus*) were associated at all collecting sites across an extremely broad range from Vermont to Louisiana, the entire north-south span of the eastern USA (Suh, McHugh & Blackwell 2004). A member of a different clade, *cfr Pichia stipitis*, was collected from the gut of a wood-ingesting passalid beetle (*Odontotaenius disjunctus*) from Pennsylvania, South Carolina, Georgia, and Louisiana (Suh *et al*. 2003).

Previous studies of gut fungus–insect associations (Martin 1987, Nardon & Grenier 1989, Vega & Dowd 2005) and the consistent, widespread, sometimes host specific associations reported here indicate a significant interaction between the organisms. Direct relationships between fungi and insect evolution, however, remain poorly studied, and the possibility that association with yeasts is an innovation that allowed expansion of insects into nutrient poor or intractable substrates needs to be considered further (Suh *et al*. 2003).

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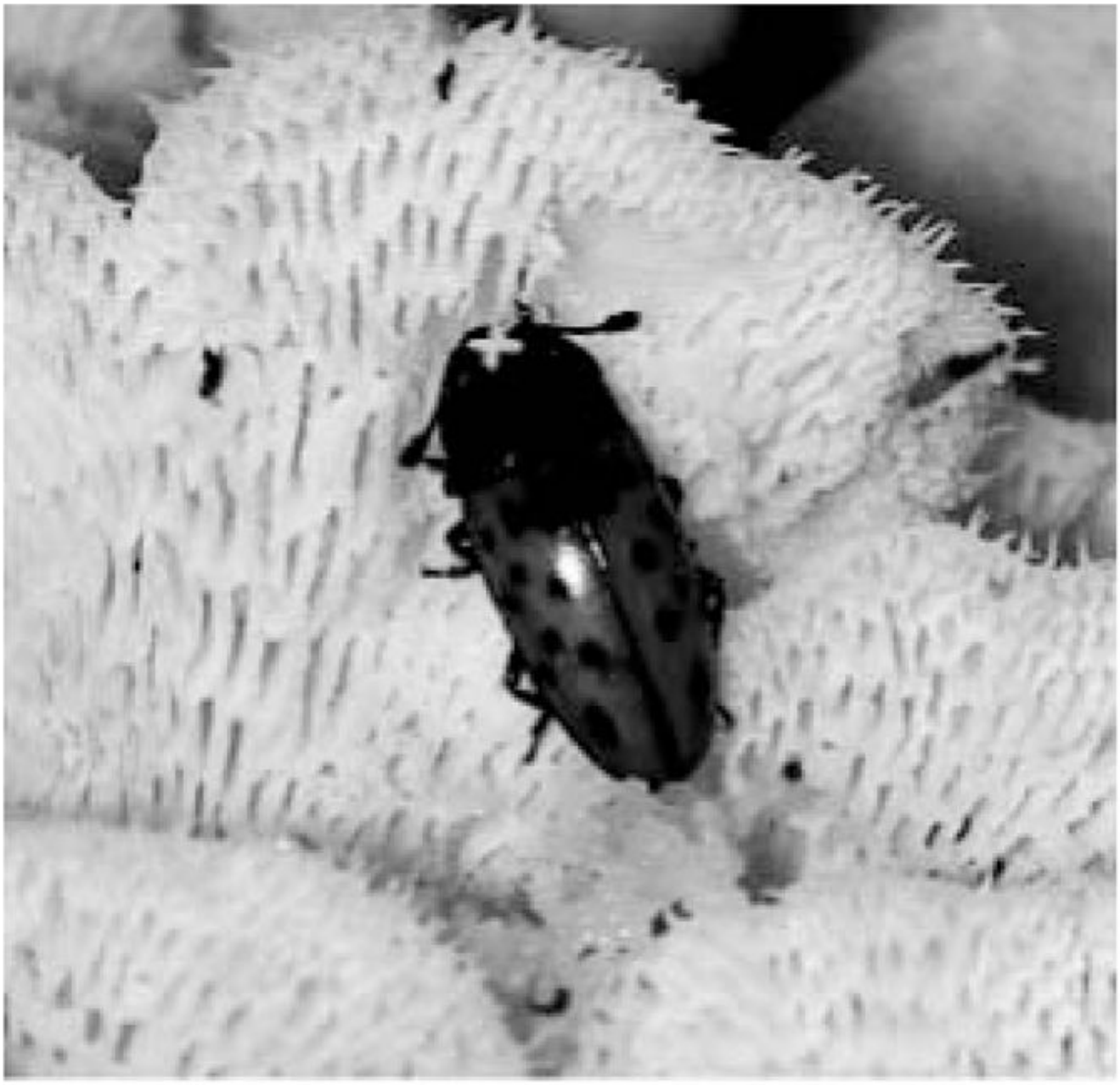

**Fig. 1.**
The 12 mm long beetle (*Erotylidae: Pselaphacus signatus*) spends most of its life on the basidiomata of *Polyporus tenuiculus* (*Polyporaceae*). Unseen here, yeasts in the gut of the beetle are an important component of a tripartite association. Photographed on Barro Colorado Island, Panama.

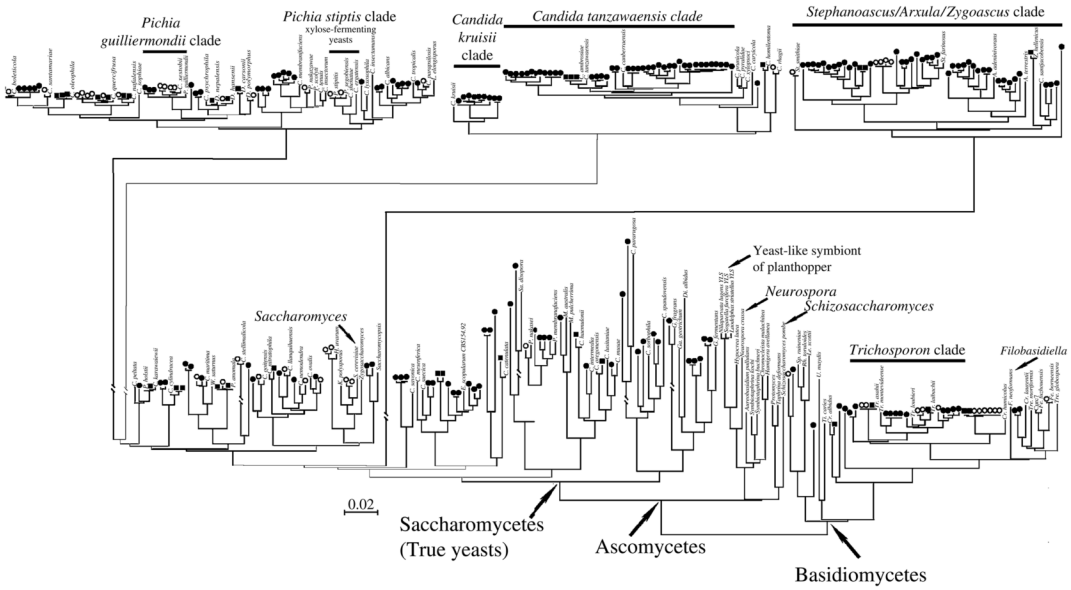


**Fig. 2.**

Bio Neighbour-Joining (BioNJ) tree using LSU rDNA and SSU rDNA sequences (see methods) based on most likely distance under Kimura's 2-parameter model estimating the phylogenetic position of nearly 400 taxa. Thicker branches were supported in more than 50% of 1000 bootstrap replicates. Comparisons based on LSU rDNA D1/D2 loop sequences, indicate many gut yeast genotypes were unknown previously and many new isolates varied greatly from known yeasts (filled circles=more that 5 bp different from previously known yeasts; open circles=1–5 bp different; solid squares=identical to known yeast). Reference taxa shown are closest relatives of the beetle gut yeasts. Taxon abbreviations are: A, *Arxula*; C, *Candida*; Cr, *Cryptococcus*; D, *Debaryomyces*; Di, *Dipodascus*; E, *Endomyces*; F, *Filobasidiella*; Fe, *Fellomyces*; G, *Geotrichum*; Ga, *Galactomyces*; H, *Hanseniaspora*; K, *Kluyveromyces*; L, *Lodderomyces*; Le, *Leucosporidium*; M, *Metschnikowia*; P, *Pichia*; Rh, *Rhodosporidium*; S, *Saccharomyces*; Sa, *Saturnispora*; Sp, *Sporidiobolus*; St, *Stephanoascus*; Ti, *Tilletia*; Tr, *Trichosporon*; Tre, *Tremella*; U, *Ustilago*; W, *Williopsis*; and Z, *Zygoascus*.

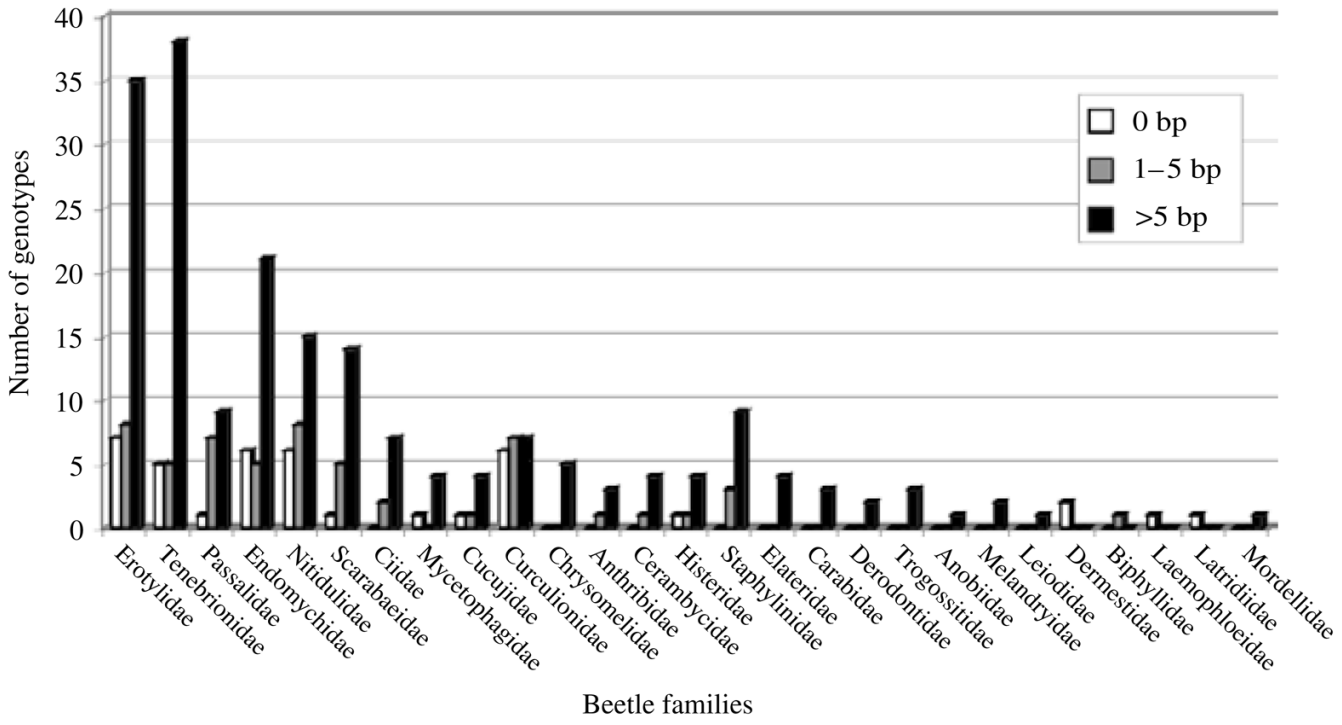


**Fig. 3.**

Yeast genotypes showing distribution among beetle taxa. Shaded bars indicate base pair differences in D1/D2 loop of LSU rDNA from closest known species. The isolates differing by more that 5 bp represent new taxa by any currently applied species concept. Yeast numbers are roughly proportional to number of beetles sampled in each family, and almost all beetles sampled contained gut yeasts.