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Identification of Yeasts from the Gut of *Cotinis nitida* using ITS and LSU rRNA D1/D2 Gene Sequences

by

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Undergraduate honors thesis under the direction of

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the Upper Division Honors Program.

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& Agricultural and Mechanical College
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Abstract

The insect digestive tract is host to many organisms including yeasts that may provide benefit the host. I isolated microbes from the plant-ingesting *Cotinis nitida* (Coleoptera: Scaraebiidae) in order to determine if they were present and to attempt to determine if the microbes are beneficial to the insects. Yeast associates were isolated from the gut of the beetles collected in Oklahoma and Louisiana. These yeasts were treated with a lysis buffer and heated to lyse the cell wall and obtain the yeast DNA. PCR followed by DNA purification prepared the DNA samples for sequencing. About 600 bp of the D1/D2 region of the LSU and ITS rRNA gene regions were compared with previously described yeasts using BLAST searches and PAUP* analyses. Many branches of the most parsimonious tree were unresolved or had low bootstrap values. Closely related yeasts, however, were evident, and the newly isolated yeasts included 12 strains related to previously described members of three clades: *C. tropicalis*, *C. corydali*, and *Trichosporon* spp. Only about 50% of the insects examined contained culturable yeasts. It is interesting that *C. tropicalis* has been implicated as an attractant in other insects. *Candida corydali* was present in *Cotinis* samples taken from both locations, which suggests a close association, the basis of which is not known. Previously, *C. corydali* was collected from neuropterans in the Baton Rouge region.

Introduction

We live in a world that has a greater biodiversity than previously realized, and the complex interactions among the organisms are just being recognized in many cases. Methods used in the characterization of the organisms and their interactions have changed dramatically since Darwin's voyage aboard the H.M.S. Beagle. Microscopes are used routinely to include microbes, and molecular methods are used to distinguish and discover unculturable taxa. Many new analytical techniques (microarrays) can be used to help to determine function among interacting organisms.

1,500 species are categorized as ascomycete yeasts in the Kingdom Fungi[1]. The yeasts make up about 1% of living organisms described [1]. It is hypothesized that a large number of yeast taxa have not yet been discovered. Although the number of described yeasts is relatively small, their presence is common in our environment where they interact and form relationships with many other types of organisms. Eukaryote-yeast interactions include pathogenic associations such as endocarditis in humans caused by *Candida albicans* and saprobic fermentation of the pentose sugar, xylose, in the gut of beetles[2]. Beetles living in diverse habitats comprise a large group of hosts for yeasts, and the guts of many species of beetles

have been examined for the presence of new yeast species. Although some yeasts are involved in the digestive physiology of certain hosts, there are many other interactions between yeasts and insects, including production of pheromones.

Fungi

Roughly a billion years ago, a lineage split to produce animals and their sister group, fungi [3]. Studies of the major diversifications of fungi support the divergence of 5-7 phyla. The two largest phyla within the Kindgom Fungi, Ascomycota and Basidiomycota, diverged most recently and comprise approximately 98% of all fungi described [3]. Of the two phyla, Ascomycota is the larger, containing close to 64% of described fungi. Ascomycetes use a variety of nutritional modes in nature, including mutualism (e.g., lichens composed of green algal and blue green bacterial associates), saprobes feeding on dead or decaying organic matter, and parasites that cause animal mycoses and plant diseases.

The Ascomycota contains the subphylum Saccharomycotina that includes one of the most famous industrial yeasts used in baking and brewing, *Saccharomyces cerevisiae* [3]. In addition to the industrial species, many other yeasts that make up the Saccharomycetes live in natural communities. The group is characterized by the presence of naked asci that are not protected by fruiting bodies. Following meiosis, ascospores either form haploid colonies or mate with sister ascospores to reestablish the diploid state [3]. Yeast genomes are small (12-

15 mbp) and contain few to no introns, thus they are exceptional models for eukaryotic exploration and experimentation.

Insect-associated yeasts are not confined to a monophyletic group but are dispersed throughout the ascomycete yeast clade [4]. Although some of these fungi are model organisms heavily used in molecular biology, few researchers study their biodiversity and evolutionary relationships. The lack of insight into the world of insect-yeast associations makes these aspects of their study compelling. Although a few yeasts were widely known to occupy the gut of insects, only recently was it recognized that a number of species are present in diverse groups of insects [2]. Isolation of microbes from the gut contents of beetles and other arthropods has provided organisms for investigating insect-fungal relationships. High beetle diversity in associations with insects allows for examination of the many parallel interactions that might be considered as natural experiments to provide greater insight into yeast-arthropod symbiotic regulation. The beetle selected for this study is the green June bug, *Cotinis nitida*.

Cotinis nitida

Cotinis nitida, the green June bug, is a common agricultural pest of fruit crops, ranging from New York south to the Gulf states and as far West as Missouri. Its name is derived from the Latin root "nitid" which refers to its distinct shiny green appearance [5]. *C. nitida* females lay their eggs in soil with high organic content.

The larvae hatch from the eggs after prolonged rainfall and develop in tiny burrows in the earth until their second spring when the larvae pupate. *C. nitida* adults often emerge in late summer and can be seen in large groups flying near wooded areas and suburban environments that have lawns or turf [5]. The larvae and adults have completely different diets. The larvae feed on plant roots, giving them the stigma "pest" by industrial crop growers, and the adults feed on ripening or fermenting fruits and on leaves of shrubs. While these beetles are usually seen alone, feasting on commercial fruits, they actually are often associated with other beetles. The introduced Japanese beetle *Popillia japonica* facilitates the feeding habits and aggregation of *C. nitida* populations by puncturing fruit that the green June bugs were previously unable to eat [6]. *C. nitida* have blunt, spatulate mandibles that are physically unable to puncture the taunt skin of fruit such as grapes and peaches. *P. japonica* pierces the skin of the fruit with its sharp, pointed mandibles and contaminates the fruits with yeasts that induce fermentation to produce kairomones (specialized pheromones) that attract *C. nitida* [7]. The flesh of the pierced fruit emits more fermentation compounds and interacts with the air to attract more green June bugs than intact fruit. The Japanese beetle is also part of a pheromone-based scavenging system. Crabapple leaves damaged by Japanese beetles or other insects such as fall webworms produce aliphatic compounds, phenylpropanoid-derived compounds, and terpenoids [8]. The pheromones in turn attract more Japanese beetles and might facilitate Japanese beetle aggregation and mating [9]. Studies by Réginière, Rabb, and Stinner have shown that attractants for Japanese beetles actually attract larger numbers of green June bugs than Japanese beetles [9].

Also, in field trapping experiments green June bugs and Japanese beetles are attracted to similar pheromones [10]. This suggests that the green June bug and Japanese beetle share an interdependent relationship and are a part of an ecological cycle, and such an association is of special interest because the Japanese beetle is an introduced species and *C. nitida* is native.

Symbiosis

A brief explanation of symbiosis provides a better understanding of a possible relationship between yeasts and their hosts. Symbiosis refers to the shared ecological relationship between two or more organisms, and depending on the benefits or harm that each organism experiences, the relationship is characterized as mutualistic in which both organisms benefit; commensalistic in which one organism benefits and the other receives no benefit or harm; or parasitic in which one organism benefits and the other is harmed. The exact nature of these relationships is variable and exists in countless environments ranging from the luminescent bacteria present in the lure of the deep sea anglerfish to the fluke, *Fasciola hepatica*, that feeds on the liver of sheep. Microbial symbiosis is essential in the guts of humans for proper digestion and absorption of nutrients. The importance of functioning symbiotic relationships has been studied in the human gastrointestinal tract, and the proper function of the gastrointestinal tract and much of the human body relies on its association with its resident microbes [14]. These microorganisms inhabit almost all environmentally exposed surfaces of the body.

The gastrointestinal tract alone harbors over 10^{14} microorganisms from over 1,000 species [14]. *Bacteroides fragilis* is a prominent human gastrointestinal symbiont that inhibits the colonization of the potentially pathogenic bacterium *Helicobacter hepaticus* through the production of polysaccharide A. If defective polysaccharide or no polysaccharide A is present, *H. hepaticus* may colonize and produce inflammatory cytokines in colonic tissues and lead to disease (e.g. ulcers). Also, polysaccharide A is a requirement for interleukin-10-producing $CD4^{+}$T cells of the immune system that protects against inflammatory disease[14]. That molecules regulated by certain bacterial microorganisms in the human gastrointestinal tract can mediate the balance between health and disease suggests the importance of preservation of such microorganisms and the potential for therapeutic treatment.

More knowledge of insect gut organisms could provide insight into the interactions among gut microbes and vertebrates. In addition, yeasts and other microbes in the gut of wood-ingesting beetles have fermentative enzymes, and they have the potential to produce biofuels from plentiful inexpensive resources [15]. Other yeasts such as *Yarrowia lipolytica* offer alternatives in bioremediation by degrading hydrocarbons to decontaminate radioactively polluted sites [16].

That certain yeasts reside in the gut of beetles and are conserved across considerable geographical distances suggests that the conserved yeasts are involved in a close association with beetles, such as *C. nitida*. My research seeks to find possible conserved beetle-gut yeasts, determine the characteristics of yeasts

residing in the beetle guts, and find the possible benefit of these yeasts to the beetles in their native environments.

DNA Barcodes

Systematists have been able to devise more effective methods for the rapid identification of new taxa as well as the cataloguing of previously described species. This ability has allowed for genetic classification systems as a faster, more accurate alternative to testing the physiological or morphological characteristics of the microorganisms. Because the genetic properties of microorganisms are marked by high variability between similar organisms, the genomes of these microbes serve to distinguish taxa species. One method used in species identification is DNA barcoding. DNA barcodes use a specific, variable region of a genome for comparison between species. The part of the genome used for identification reveals conserved base-pairs between similar species and differing base-pairs within differing species. The comparison of the variability of these base pairs at specific loci within the genome also gives insight into evolutionary relationships, as more highly conserved regions of base-pairs between species are more evolutionarily conserved than the hypervariable regions [17].

While some barcoding methods employ the use of Cytochrome C oxidase 1 (CO1), a 648 base-pair segment of mitochondrial CO 1 [17], the internal transcribed spacer (ITS) and ribosomal large subunit (LSU) of the D1/D2 region of the rRNA

gene are better regions to use for most fungi. The LSU and ITS regions used here are 400-600 base pairs based on the model yeast *Saccharomyces cerevisiae*. Identification employing the CO1 gene has been most extensively used in the animal kingdom and has had a species-level resolution of more than 95% of taxa from studies focusing on animal lineages [17]. The effectiveness of the CO1 gene in differentiation of animal species has led to the assumption that the gene would be effective in distinguishing taxa universally, but the use of multiple assays, such as the combination of LSU and ITS, presents a more comprehensive region for analysis in fungi, and universal primers are available. CO1 barcoding of fungi requires many specially designed probes that do not prime CO1 for all fungi. Debate about effective barcoding regions, however, soon will be moot with the rapid improvement and reduced cost of whole genome sequencing.

Polymerase Chain Reaction

DNA sequences from the fungal genome can be obtained from yeast cultures using the polymerase chain reaction (PCR) for DNA amplification. The reaction involves two sets of priming RNA base-pair sequences created specifically for the gene region to be amplified, autoclaved distilled H_2O, and a stock solution of nucleotides that combined with sample DNA will constitute the amplified DNA. Taq polymerase and its buffer are also required in order to polymerize the newly forming DNA. In order for the reaction to move towards completion, high temperature must be applied to denature DNA by breaking the hydrogen bonds that

hold the nucleotide bases together in the double helix form. Once the bonds are broken, the temperature is reduced to a state of incubation so that primers attach to the individual DNA strands and taq polymerase initiates the replication of the DNA strands using the stock nucleotides. After the time allotted for DNA replication elapses, the temperature is raised again, and the process repeats approximately 30 more times to produce a substantial amount of replicated sample DNA. The process of DNA replication is very precise. If the temperature doesn't fall below a certain value during the incubation period, the reaction may not proceed forward, and likewise, if the denaturing temperature is too low, a sufficient amount of DNA will not be replicated. Thus, the annealing and temperatures of various primers must be specifically determined for each DNA reaction [18]

The PCR product is most often verified using electrophoresis. In this process, the PCR DNA products are combined with a dye and loaded into wells of a gel (e.g., 0.8% agarose 0.5xTB) that has been placed in an electrophoresis chamber filled with a buffer solution. The migration of the samples is induced by an electric current that acts on the negatively charged phosphate groups of the DNA samples. The samples follow current that flows from the negative to the positive pole of the chamber. After the samples have traveled a sufficient distance on the gel, seen by the migration of the loading dye, the presence of the actual PCR product is verified under UV light. The distance traveled by the sample in the gel is proportional to its density measured in sedimentation coefficients, Svedberg units, and compared to a ladder, a set of proteins of known density that are used to map electrophoresis products [18].

DNA Purification and Identification

Phylogenic analysis of the combined ITS and LSU rDNA D1/D2 region sequences can be used to indentify taxa based on he short DNA segments and to detect previously unknown species [2]. Following amplification by PCR, DNA segments must be purified in preparation for identification. An alkaline phosphatase such as shrimp alkaline phosphatase (SAP) and exonuclease 1 are included in a second PCR of the DNA. The SAP helps to purify the DNA by dephosphorylating the remaining nucleotide bases, and with the enzyme exonuclease I, the residual primers and single strands of DNA are degraded [19]. To prepare the samples for DNA sequencing a second PCR reaction is run to incorporate BigDye™ or a similar identification dye and its buffer to bind with the DNA samples. Washing the samples with sodium acetate and ethanol removes any extra accompanying materials so that they may be processed by DNA sequencers.

Sequences of newly isolated yeasts can be compared quickly with the LSU and ITS rDNA fungal sequences obtained from GenBank or some other public database. Programs such as PAUP* 4.0b10 facilitate the identification process by analysis of aligned sequences between multiple species or by comparison with single sequences in BLAST searches. Sequences differing by more than 5 base pairs from the closest known genotype generally have been considered to constitute a

new species based on comparisons with species based on sexual reproduction criteria [15], but additional study is required before this step is taken. The sequences also can be used to determine a phylogeny, usually based on parsimony criteria, to further evaluate evolutionary relationships.

A necessary step in novel species characterization is the determination of physiological characteristics. Assimilation tests show these characteristics of yeasts by inoculation of the yeast to varying carbon and nitrogen sources. Yeast cells are transferred from standard media plates to media tubes of varying carbon sources and are allowed to incubate. The yeasts are measured by and culture turbidity and by product production at certain time intervals to determine rate and ability of the yeasts to assimilate and ferment the carbon source. Knowledge of the yeast physiological characteristics derived from these tests helps to resolve the nature of the relationships between the yeast and its host [1].

Materials and Methods

Living specimens of *Cotinis nitida* were collected in flight from Midwest City, Oklahoma on August 21, 2008 and from the Louisiana State University campus, Baton Rouge, Louisiana on August 22, 2008. Extraction techniques were taken from Suh, Zhang, Nguyen, Gross, and Blackwell [18]. Seven beetles from Louisiana and fourteen from Oklahoma were placed in individual Petri dishes that were parafilmed and moved to 5˚C for 5 minutes. The beetles were removed from the freezer and surface disinfected with chilled 95% EtOH wash. The beetles were transferred to a second empty Petri dish and washed with a 0.7% saline solution. The saline wash was streaked onto YM agar plates (1000ml distilled water, 3g yeast extract, 3g malt extract, 5g Bacto Peptone, 10g glucose, 2% agar) as a control for possible exterior microbial contaminants. The gut of the beetle was extracted with forceps and transferred to a 0.5ml Eppendorf tube containing 50ul of 0.7% saline solution. The gut was crushed using a pipette tip and plated on YM agar. All plates were incubated at room temperature for 72 hours. Plates showing growth provided inoculation for isolation on AYM agar (YM agar, 0.7ml HCL). The freshly plated cultures were then incubated at room temperature for 72 hours. Colonies on AYM plates were determined to be yeasts or other microbial organisms based on morphology using a wet mount slides. Yeast colonies were frozen at -80˚C after growing the yeasts in YM broth tubes, and transferring them to cryotubes with addition of 1ml of 60% glycerol.

Plates containing yeasts were used for PCR [18]. Yeast cells were transferred to 1.5ml Eppendorf tubes containing lysis buffer (Tris-HCl, EDTA, 3% SDS) and glass beads. The tubes were vortexed and placed in a hot bath (~65˚C) for 35 minutes. Samples were then centrifuged for 10 minutes and the supernatant was collected. 3ul of 2-mercaptoethanol and 50ul of lysis buffer were added to each supernatant sample followed by addition of 300ul of phenol-chloroform. The samples were vortexed for 30 seconds and the upper layer was transferred to a fresh tube. 300ul of phenol-chloroform was added a second time and the samples were vortexed. The resulting upper layer was added to 500ul of isopropanol and 20ul of 3M sodium acetate. The tubes were inverted to mix the solution and then centrifuged for 1 minute. The supernatant was discarded and 700ul of 70%EtOH was added to the tubes which then rested for 5 minutes. The top layer was removed by pipetting, and 700ul of 100% EtOH was added to each tube and spun down using the centrifuge. The top layer was decanted and discarded, and the tubes were inverted for 15 minutes on a paper towel to dry the DNA pellet formed in the bottom of the tube. 25ul of autoclaved water was added to each tube to re-suspend the DNA. This DNA was used to perform the PCR protocols [Fig. 1; 20] for the LSU and ITS regions of the yeast genome. Protocols were taken from Suh, McHugh, and Blackwell [20].

			Vi	Vf
MASTER MIX	Ci	Cf	1	13.00
H2O			11	146.25
Buffer	5	1	5	65.00
Mg2SO4 25 mM	25	4	4	52.00
DNTp 10 uM	10	0.4	1	13.00
LS1	100	3	0.8	9.75
LR3	100	3	0.8	9.75
TAQ 5U/ul	5	1	0.3	3.25
DNA 10 ng/ul			2	-
Final volumen			25	299.00
Volumen without H2O			14	23.00
Program	Tem	Time (min)		
Pre-denaturation	95	4		
Denaturation	95	1	Cycles	
Anneling	55	0.5	35.00	
Extension	72	1		
Final extension	72	10		
Stop	4	-		

			Vi	Vf
MASTER MIX	Ci	Cf	1	13.00
H2O			11	146.25
Buffer	5	1	5	65.00
Mg2SO4 25 mM	25	4	4	52.00
DNTp 10 uM	10	0.4	1	13.00
ITS1	100	3	0.8	9.75
ITS4	100	3	0.8	9.75
TAQ 5U/ul	5	1	0.3	3.25
DNA 10 ng/ul			2	-
Final volumen			25	299.00
Volumen without H2O			14	23.00
Program	Tem	Time (min)		
Pre-denaturation	95	5		
Denaturation	95	1	Cycles	
Anneling	57	0.5	35.00	
Extension	72	1		
Final extension	72	10		
Stop	4	-		

Fig. 1 Examples of PCR protocol for amplification of LSU and ITS regions of yeast genome [23]

Bacterial PCR was also attempted with the non-yeast samples using the same concentrations and reaction temperatures but 1492R and 27F primers [21]. Yeast PCR products were verified using gel electrophoresis. The 3ul of the products were combined with 3ul of loading dye and were loaded into the wells of a 0.8% agarose 0.5x TBE gel and electrophoresed at 100V in a 0.5x TBE solution for 30 minutes. The products were viewed under UV light to verify the presence of DNA products.

The samples were purified by combining 20ul of PCR product with 0.5ul exonuclease 1, 0.5ul SAP, and 1.0ul water. The mixtures were placed in the PCR thermocycler at 37˚C for 45 minutes, 80˚C for 15 minutes, and held at 10˚C.

To prepare the samples for sequencing, the BigDye protocol was used [18]. Each sample was combined with 0.5ul of BigDye Terminator, 1ul of 5uM primer, and

1ul of BigDye buffer. Reactions were run using the LR3 primer for the LSU region and ITS4 for the ITS region. The amount of each sample to be combined with the 2.5ul of BigDye mixture was determined relative to the intensity of the bands shown through electrophoresis. The combined DNA and BigDye solutions were brought up to 5ul through addition of autoclaved PCR water. The solutions were centrifuged for 10s and then sequenced using a PCR. The reaction involved 25 cycles of 96˚C for 10s, 50˚C for 5s, and 60˚C for 4 minutes.

The samples were further purified using ethanol precipitation [18]. The samples were moved to 0.5 ml tubes, and 2ul of 3M sodium acetate and 50ul of 100% EtOH were added. The tubes were vortexed then incubated at room temperature for 10 minutes. The tubes were centrifuged for 20 minutes followed by removal of the EtOH by pipette, taking care not to disturb the bottom of the tube. The procedure was repeated with 250ul of 70% EtOH. The remaining EtOH in the bottom of the tubes was evaporated off. Rehydration of the samples with 15ul of Hi-Di allowed for the samples to be sent to the LSU DNA sequencing facility for final base-pair sequencing.

Resulting base-pair sequences were put through BLAST analysis to evaluate closely related species. DNA sequences were aligned using the multi-alignment program PAUP* 4.0b10 [24].

Results

Yeasts were cultured from 12 of 21 of the individual guts. Only one yeast species was present in each of the 12 guts. Most similar taxa were generally determined by BLAST searches. BLAST analysis of the LSU and ITS rRNA indicated that both RNA regions gave consistent results in determining *C. corydali* as the closest match. The *Trichosporon* and *C. tropicalis* isolates, however, gave somewhat different results for the LSU and ITS RNA regions.

Within the LSU of rRNA, samples OK4 and OK6B differed by five base-pairs or more from comparison strains, *C. corydali* (ATCC MYA-4357) and *Trichosporon moniliforme* (C107X-Y32). The ITS showed greater variability with LA2, LA5, LA6, OK2, OK4, OK5A, and OK10 differing by more than 5 base-pairs between each strain. LSU gap numbers were overall lower than those of the ITS.

PCR of the yeast-associated bacteria using the 1492R and 27F primers failed to amplify the DNA.

PAUP* analyses verified that the yeasts occured in two Saccharomycetales clades (*Candida corydalis* and *C. tropicalis*) and one basidiomycete clade containing *Trichosporon* sp. [Fig. 2]. *Trichosporon* species and *C. tropicalis* were present in the gut of Louisiana beetles only. *C. corydali* was isolated from both Louisiana and Oklahoma beetles.

A heuristic search was executed with the tree bisection-reconnection branch-swapping algorithm and random sequence analysis in PAUP* [Fig. 2]. Most isolates fell into one of two clades, *Candida corydali* and *C. tropicalis*, that appear as sister clades with the sampling used, although the support is not high (78%). The *Candida corydalis* clade also contained 2 subclades with subclade A containing two Louisiana samples similar to *C. corydali*, and subclade B containing six similar samples, five from OK and one from LA. The *C. tropicalis* clade contained three subclades with subcade A consisting of *C. tropicalis*, subclade B consisting of one LA isolate and subclade C consisting of two LA isolates. The third clade, the *Trichosporon* clade, consists of five closely related *Trichosporon* strains and *Trichosporon* sp., a more distantly related clade member from OK. Isolates from *C. corydali* subclade B and *Trichosporon* sp. may be undescribed species.

Table 1. Base-pair analysis of isolated species obtained through BLAST

Sample ID	Closest Known Species	Host Source	LSU rRNA Sequences					ITS rRNA Sequences				
			# of BP	Identities[a]	Gaps[b]	BP differences[c]	Strain Designation[d]	# of BP	Identities	Gaps	BP differences	Strain Designation
LA1A	*Candida corydali*	Baton Rouge, LA	584	583	1	0	ATCC MYA-4357	487	485	2	0	ATCC MYC-4357
LA1B	*Candida corydali*	Baton Rouge, LA	584	581	2	1	ATCC MYA-4357	457	456	1	0	ATCC MYC-4357
LA2	*Candida tropicalis*	Baton Rouge, LA	471	471	0	0	*C. tropicalis* strain LYSC-3	434	419	5	10	*C. tropicalis* strain HK68D
LA3A	*Candida corydali*	Baton Rouge, LA	545	545	1	1	ATCC MYA-4357	470	470	0	0	ATCC MYC-4357
LA5	*Candida tropicalis*	Baton Rouge, LA	498	497	0	1	*C. tropicalis* strain LYSC-3	446	418	10	18	*C. tropicalis* strain HK68D
LA6	*Candida tropicalis*	Baton Rouge, LA	498	497	0	1	*C. tropicalis* strain LYSC-3	456	436	8	12	*C. tropicalis* strain HK68D
OK2	*Candida corydali*	Midwest City, OK	351	346	4	1	ATCC MYA-4357	488	475	3	10	ATCC MYC-4357
OK4	*Candida corydali*	Midwest City, OK	497	492	0	5	ATCC MYA-4357	473	462	2	9	ATCC MYC-4357
OK5A	*Candida corydali*	Midwest City, OK	496	494	1	1	ATCC MYA-4357	490	476	3	11	ATCC MYC-4357
OK6B	*Trichosporon moniliforme*	Midwest City, OK	457	446	4	7	C107X-Y32	485	484	0	1	ATCC 46490
OK10	*Candida corydali*	Midwest City, OK	451	451	4	2	ATCC MYA-4357	487	473	4	10	ATCC MYC-4357
OK11	*Candida corydali*	Midwest City, OK	550	548	1	1	ATCC MYA-4357	464	436	6	2	ATCC MYC-4357

[a]Number of base-pair matches of isolated species with closest known species
[b]Unannalyzed base-pairs in sequence
[c]Mismatches in sequence, gaps excluded
[d]Notation conserved from BLAST database

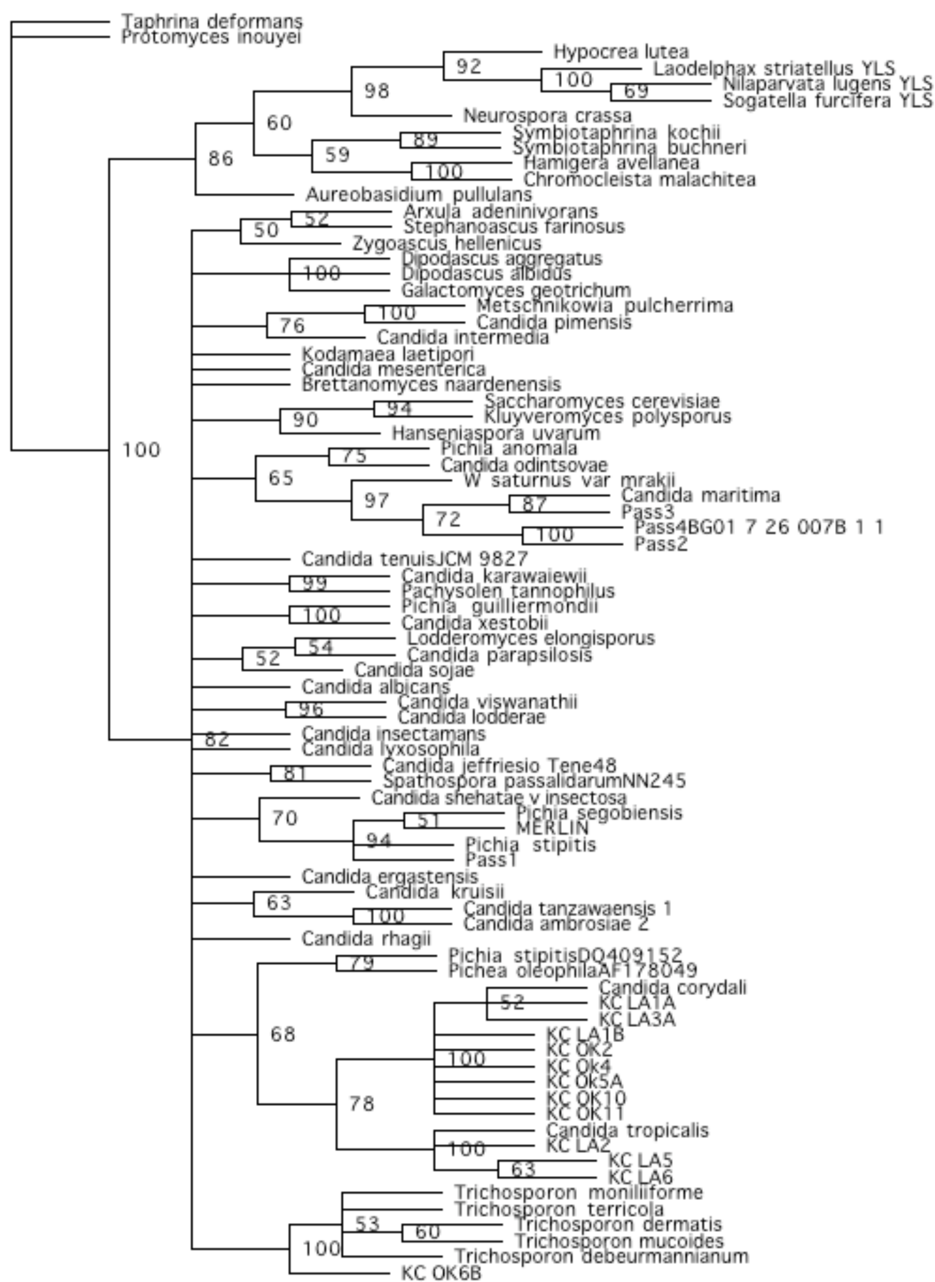

Fig. 2 Phylogenetic tree used for identification of KC, LA, and OK isolates by comparing approximately 600 base-pairs from the LSU rDNA region. Although isolates are positioned near certain *Candida* and *Trichosporon* species, many branches are poorly supported (<90%) by bootstrap analysis. The DNA regions used in the analysis defined a number of clades of closely related yeasts, but the relationships among the clades is not resolved.

Discussion

Diversity of culturable gut yeasts from *Cotinis nitida* is somewhat low compared to some other beetles previously studied in the lab. This may be tied to the feeding behavior of the beetles. As adults, *C. nitida* appears to eat fruit and probably obtain nutrients primarily from the simple sugars within the fruit, a substrate that the beetles probably can digest with their native enzymes. Since only about half the beetles sampled contained gut yeasts, the yeasts do not seem to be essential to the beetle nutrition.

During the development of *C. nitida*, the gut flora undergoes a change. Shortly after the last molt and development of the adult, the beetle houses a fermentative flora that in time with a change of diet turns to an obligately respiratory microbiota [11]. One of the yeasts known to reside in the green June bug gut is *Candida tropicalis*. This yeast has also been found in figs infected by the fly *Zapronius indianus* [12]. Because both insects have similar diets and sometimes are hosts of *C. tropicalis*, this yeast could aid in the digestion and processing of nutrients when it is present. Alternatively, the aggregation of green June bugs was induced by using fruit cultures containing *Candida tropicalis* and *Candida krusei*, which were known to be endemic gut flora, than with damaged fruit cultures [13]. This relationship could be indicative of a conserved mutualistic relationship between *C. tropicalis* and fruit-eating insects in which the yeasts produce pheromones.

I recovered only three yeast taxa from the gut of the beetles. It is important to point out that in cases when both cultivation of gut organisms plus non-culture methods are compared, the number of unculturable microbes usually greatly exceeds that of culturable species. My findings may have underestimated the number of gut microbes. Therefore, it is important to realize that other organisms may be present that are involved in interactions with the beetles and the microbial community. For example, although some bacteria were isolated and cultured, these were not found in large numbers using the methods of yeast isolation, and this group of organisms were neglected in the study.

C. tropicalis previously was reported to be a common resident of the gut flora of *C. nitida* in which it was involved in producing aggregation pheromones [13]. The sex of the beetles and sex ratio were not determined in my study, but if the sex ration were near 1:1, the skewed presence of *C. tropicalis* in approximately only half the beetles might be explained. The discovery of the other yeasts, *C. corydali*, was unexpected. Previously this species was known only from associations with neuropteran insects in Louisiana [22].

It is not clear if new species were discovered in the study. Yeast systematists [1] have used a 1% difference (>5bp) in LSU rRNA (the yeast "barcoding" region) sequence as a rough estimate of distance between species. Using this criterion, OK4 and OK6B have an LSU base-pair difference great enough to qualify for further study to determine if new species are involved. It was interesting that among the three regions of the rRNA repeat examined ITS was not always the most variable region,

nor was SSU always the most conserved. The ITS regions of LA2, LA5, LA6, OK2, OK4, OK5A, and OK10 were marked with high variability, and LA2, LA5, and LA6 contained 5 or more gaps in their sequences. The large number of gaps could account for the low bootstrap value of the LA5, LA6 branch in Fig. 2.

The potential discovery unique species in this study will require additional evaluation of physiological and DNA characteristics to determine if they are in fact distinctive.

Conclusion

Three different yeast taxa were found in the *C. nitida* gut: *C. tropicalis, C. corydali,* and a *Trichosporon* species. The diversity of culturable gut yeasts from the *Cotinis nitida* beetles were low in comparison to some other beetles studied for yeast-insect associations [22, 23, 24]. This might be tied to feeding behavior. Because only half of the beetles sampled contained gut yeasts, the yeasts are probably not essential to nutrition. It is interesting to note that if the sex ratio for the beetles were 1:1, the yeast-associations seen in this study could be tied to the sex of the beetle.

Because *Candida tropicalis* is associated with some fruit-eating insects [12] and more favorably induces the feeding of *Cotinis nitida* when added to fruit cultures [13], *C. tropicalis* could induce its uptake by the insect host. Potential vitamins and digestive enzymes produced by the yeast could be favorable to the insect host.

The Oklahoma samples OK4 and OK6B have a base pair difference greater than 5 in the LSU rRNA gene and could be novel. Further evaluation of physiological and DNA characteristics are needed in order to properly classify these yeasts.

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