Symbiotic yeasts from the mycangium, larval gut and woody substrate of an African stag beetle *Xiphodontus antilope* (Coleoptera: Lucanidae)

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Abstract

Female stag beetles (Lucanidae) possess internal mycangia to maintain microbial cultures. Yeasts from these mycangia may help with larval nutrition in nutrient poor woody substrates, but only a few Lucanidae taxa have been studied and all reports originate from Europe and Asia. We identify the first mycangial yeasts of a South African endemic Lucanidae beetle, *Xiphodontus antilope*, using nuclear ribosomal RNA and ITS DNA sequence data. In addition we identified yeasts from the larval gut, fecal matter, frass and woody substrate surrounding larvae and pupae. The mycangium of *X. antilope* was confined to females and is structurally similar to all other Lucanidae. Unlike most Lucanidae that seemingly associate with single species of yeast, or whose mycangia contain yeast monocultures, three yeast species were commonly isolated from *X. antilope*. *Scheffersomyces coipomoensis* was the most numerically dominant species on most substrates and in most individuals, but a second, undescribed, *Scheffersomyces* species was present in high numbers. A third species, also undescribed and unrelated to *Scheffersomyces*, was recovered from all mycangia but could not be detected in the

larval gut, fecal matter, frass or woody substrates. We confirm a close association of *Scheffersomyces* yeasts with Lucanidae globally, but other taxa may also be involved. We show that the predominant mycangial yeasts also form the predominant yeasts within the larval gut and the woody substrates around the larvae and pupae. This combined external and internal colonization by the same yeasts may provide enhanced opportunities for nutrient acquisition, but this needs validation in future studies.

Keywords: cellobiose, gut flora, lignicolous, mycangium, saproxylic, symbiosis

Introduction

Wood comprises primarily of polymers such as cellulose, hemicellulose, and lignin that are nutritionally inaccessible to insects without the help of wood-digesting microbes such as bacteria, protozoa and fungi (Haack and Slansky 1987). Insects that rely on nutrients derived from wood therefore often form close associations with wood-decay microorganisms (Beaver 1989; Kostovcik et al. 2015). When there is mutual benefit to the insects (e.g. nutrition) and the microbes (e.g. dispersal), the evolutionary development of specialized propagule-carrying structures on or within the insect bodies can result. These structures, or mycangia, vary from simple pits in the exoskeleton, to complex sac-like structures with associated glands within insect bodies, as in bark and ambrosia beetles (Six 2003), lizard beetles (Toki et al. 2012), Lymexylidae beetles and wood wasps (Francke-Grosmann 1967), and *Euops* (Attelabidae) (Grebennikov and Leschen 2010). When complex mycangia are only associated with females, at least some of the microbes within these structures are likely important for larval development.

The larvae of most stag beetle species (Coleoptera: Lucanidae) develop within dead wood. Adult females of all species examined to date possess an internal sac-like mycangium, attached to the intersegmental membrane between tergites eight and nine, near the abdominal tip (Fremlin and Tanahashi 2015; Tanahashi and Hawes 2016). These structures contain symbiotic bacteria and yeasts (Kuranouchi et al. 2006; Tanahashi et al. 2010; Hawes 2013; Miyashita et al. 2015; Tanahashi et al. 2017). Lucanidae genera evaluated for the presence of mycangia to date include *Aegus*, *Aesalus*, *Ceruchus*, *Dorcus*, *Prosopocoilus*, *Lucanus*, *Neolucanus*, *Prismognathus*, *Figulus*, *Nicagus*, *Nigidius*, *Platycerus* and *Sinodendron* (Tanahashi et al. 2010; Hawes 2013; Tanahashi and Hawes 2016, Tanahashi et al. 2017). However, this only represents a small fraction of the total number of extant genera, geographically restricted to Europe and Asia. It remains to be demonstrated that mycangia are universal to the family as hypothesized (Fremlin and Tanahashi 2015).

Yeasts isolated from Lucanidae mycangia are commonly members of the genus *Scheffersomyces* but other taxa are often also involved (Tanahashi et al. 2010; Tanahashi and Hawes 2016; Tanahashi et al. 2017). For example, *Scheffersomyces* yeasts have been detected from numerous genera, including *Ceruchus*, *Sinodendron*, *Dorcus*, *Lucanus*, *Platycerus*, *Prismognathus* and *Prosopocoilus* (Tanahashi et al. 2010; Tanahashi and Hawes 2016; Tanahashi et al. 2017 and unpublished data referred to in this paper). The mycangia of members of the subfamily Lucaninae often contain monocultures of *Scheffersomyces*, and different Lucaninae species often associate with different species or genetically different strains of these yeasts (Tanahashi et al. 2010; Tanahashi and Hawes 2016; Tanahashi et al. 2017). However, the mycangium of *Sinodendron cylindricum* contains two dominant yeasts, a *Sugiyamaella* sp. and another undescribed yeast (Tanahashi and Hawes 2016). This apparent flexibility in associations with multiple yeast taxa currently seems restricted to this species, but more data are required to corroborate this (Tanahashi and Hawes 2016).

Members of *Scheffersomyces* and *Sugiyamaella* dominate yeast communities within the larval gut of *S. cylindricum* in Europe, the only species for which larval gut symbionts have been quantified (Tanahashi and Hawes 2016). The absence of *Scheffersomyces* from the mycangium of this species is peculiar as *Scheffersomyces* is commonly associated with numerous Lucanidae species, but may be due to either the limited sample size in a previous assessment (only two individuals assessed, in one of which the mycangium was devoid of any culturable yeasts) (Tanahashi and Hawes 2016), or due to associations of the larvae with yeasts from the surrounding woody environment after hatching. More comparative studies between yeast associates within mycangia and larvae are needed, as it appears that all yeast taxa studied to date can metabolize xylose in wood, which may aid larval nutrition (Tanahashi and Hawes 2016; Tanahashi et al. 2017).

It is likely that the mycangial yeasts are deposited in wood from mycangia during egg laying, as the adult gut contains little to no yeasts, or only yeast species that are not associated with larvae and mycangia (Tanahashi and Hawes 2016). Deposited yeasts may be ingested by the hatching larvae either from the egg shell or the surrounding wood (Tanahashi et al. 2010). Once inside the gut these may help with nutrition, but, although untested, living cells may also pass through the gut in the fecal matter and colonize the wood surrounding the larval galleries. Here these yeasts may aid in external breakdown of woody components (Tanahashi et al. 2018). Interestingly, Lucanidae larvae often mix undigested wood (frass) with fecal matter inside tunnels and later ingest this mixture (Araya 2006; Kamata 2014, cited in Tanahashi et al. 2018), a process that can enhance nutrient uptake (Tanahashi et al. 2018). Symbiotic yeasts in the midgut are retained during larval shedding, but these are lost at pupation (Fremlin and Tanahashi 2015; Tanahashi and Hawes 2016). When pupating, the yeasts likely occupy walls of the pupal chamber until eclosion (Fremlin and Tanahashi 2015). Yeasts enter the newly-eclosed female mycangia from the pupal chamber walls through an active process during specialized post-eclosion behavior (Fremlin and Tanahashi 2015). Although likely, it remains

to be tested whether mycangial yeasts pass unharmed through the digestive tract of the larvae and whether these also colonize the wood surrounding the tunnels of the larvae, the accumulating frass within tunnels and the walls of the pupal chamber.

In the present study we set out to determine whether the South African endemic *Xiphodontus antilope* possesses a female-specific mycangium as other members of the family assessed in the Northern Hemisphere. We characterize the yeast communities from the mycangia and larval gut and compare the taxa isolated to those known from other Lucanidae. In addition, we characterize the yeasts in larval fecal matter, the frass within larval tunnels, and the woody substrate surrounding the tunnels of larvae and in the pupal chamber. We test the hypotheses that all Lucanidae females possess mycangia (Tanahashi and Hawes 2016) and that these are predominantly colonized by yeast monocultures (Tanahashi et al. 2010; Tanahashi and Hawes 2016; Tanahashi et al. 2017). The identity of the yeasts from *X. antilope* will elucidate host-microbe specificity in the Lucanidae. Identifying yeast communities in *X. antilope* larval fecal matter, frass and the wood surrounding the tunnels will add support for the hypothesis of an "external gut system" maintained by Lucanidae larvae as proposed by Tanahashi et al. (2018).

Materials and methods

Beetle, frass and wood collection

A chance encounter in November 2018 (summer) led to the discovery of a log colonized by the infrequently encountered *Xiphodontus antilope* (Lucanidae) in an Afromontane forest patch near the town of Knysna, Western Cape Province, South Africa (34°01'39.9"S 23°14'27.1"E). The genus *Xiphodontus* is endemic to the Afrotropical Region and contains three species (Bartolozzi and Werner 2004; Bartolozzi 2005). *Xiphodontus antilope* is confined to Afromontane forest patches in South Africa (Bartolozzi and Werner 2004; Bartolozzi 2005) and nothing is known regarding its breeding habits. Recently however, Bartolozzi et al. (2019)

collected both larvae and adult *X. endroedyi* from the base of dead and senescent Proteaceae trees and shrubs in scrubland vegetation (Fynbos) in the Eastern Cape Province of South Africa, where they were boring into the decaying wood. In the present study, the 230 x 47 cm log was identified as a fallen branch of a Candlewood tree (*Pterocelastrus tricuspidatus*) and was at a fairly advanced state of brown rot decay. We collected all Lucanidae larvae and adults of *X. antilope* (Fig 1) encountered from about half of the length of the log, leaving the rest undisturbed for normal larval development. Larvae of instars 1 – 3, adult males, and adult females were found intermixed, with all individuals fairly evenly distributed throughout the woody substrate. Adults were found with fully hardened integuments within their pupal chambers at the end of larval galleries. Larvae were surrounded by light-brown colored frass inside the galleries, suggesting that they may be engaged in coprophagy like other members of the family (Araya 2006; Kamata 2014, cited in Tanahashi et al. 2018). These galleries often crossed between different individuals and in some areas of advanced activity no more traces of galleries could be seen in the mass of frass and woody debris.

Adults (7 males and 12 females) were placed individually into 45 ml vials containing moistened filter paper. Larvae were placed into 45 ml vials containing 20 ml substrate that surrounded these in the larval tunnels (woody debris and frass from the immediate surrounds) in pairs of the same instar [2 vials containing 1st instar larvae, 7 vials containing 2nd instar larvae (one of these with only a single individual), and 1 vial containing 3rd instar larvae]. In addition, frass that surrounded 8 larvae in tunnels (2nd instar larvae) were aseptically collected and placed in sterile 2.5 ml Eppendorf tubes. Similarly, pieces of brittle wood (ca. 10 x 5 mm) were aseptically removed from the gallery surface, from the end of the advancing galleries of the same 8 larvae, and pupal chambers of 4 adult males and 4 adult females, and placed in separate 2.5 ml Eppendorf tubes. All vials were kept at ambient temperature (18 – 22˚C) for 3 – 5 days until processing in the laboratory.

Larval identification

In the laboratory, one adult male, one adult female, and two 1st instar individuals were selected for molecular confirmation of larval identity. These individuals were euthanized by placing in a -80˚C freezer overnight before DNA extraction. A single leg of each adult, and all legs of each larva were removed and placed in separate Eppendorf vials containing 300 µl extraction buffer (DNeasy Blood and Tissue Kit, Qiagen, Germany), 70 µg PCR grade Proteinase K (Roche Applied Science, Mannheim, Germany) and 5 glass beads. Tubes were incubated at 35˚C for 12 hours after which the contents were homogenized in a Tissue Lyser (Qiagen Retsch, Walpole, MA, USA) and genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocols. The mitochondrial 16S DNA molecular marker was amplified for each individual using the primers, reaction mixtures and protocols outlined in Kim and Farrell (2015) and sequenced at CAF (Central Analytical Facility, Stellenbosch University, South Africa). Sequences were compared to sequences stored on GenBank, (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) using the Basic Local Alignment Search Tool (BLAST) to confirm identity as belonging to members of the Lucanidae and were aligned with each other in Bioedit V.7.2.5 (Hall 1999) to confirm conspecific status of larvae and adults.

Isolation of yeasts from larvae and adults

Five adult males, ten adult females and seven 2nd instar larvae (one from each tube) were surface sterilized by washing in 70% ethanol for 60 s, followed by 30 s in 3% sodium hypochlorite and washed in sterile water for 60 s. Adults were euthanized by removing their heads using a scalpel and hereafter dissected in sterile phosphate-buffered saline (PBS, pH 7.4). Adults were dissected to expose the hind gut and other organs at the tip of the abdomen to verify the presence, placement and structure of a mycangium. If present, the mycangium was separated from the rest of the organs and integument under sterile conditions and placed in individual 2.5 ml Eppendorf tubes containing 200 µl PBS. Surface-sterilized larvae were placed individually within sterile petri-dishes and left in the dark at ambient room temperature for three hours. Any

fecal matter produced within the petri dishes were transferred to individual 2.5 ml Eppendorf tubes containing 200 µl sterile PBS using sterile earbuds. For transfer of fecal matter to the tubes, the tips of the earbuds soaked with fecal matter were cut off, placed within the tubes, and the tubes vigorously shaken using a vortex mixer for 1 minute. Hereafter the larvae were surface-sterilized again and the midgut, hindgut and cecum-like sacs from each were removed, washed in sterile PBS, and placed in individual 2.5 ml Eppendorf tubes containing 200 µl sterile PBS. Contents in tubes containing insect tissues were homogenized using a small handheld plastic pestle. Hereafter, these primary solutions were used to generate a dilution series for each individual sample by adding 20 µl to new tubes containing 180 µl PBS for 4 successive dilutions, vigorously mixing the homogenate between steps. 100 µl per tube were used to inoculate two 9-cm diameter potato dextrose agar (PDA) plates (20 µl homogenate per plate) amended with Streptomycin Sulphate Salt (0.04 g/L) to suppress bacterial growth, and spread using a glass spreader. Plates were incubated at 20˚C for 6 d in the dark.

Isolation of yeasts from wood and frass

The pieces of wood from larval galleries and pupal chambers were surface sterilized following the same procedure as for the beetles. Hereafter, a 3 x 3 x 3 mm piece was removed from each of the eight samples and placed in individual 2.5 ml Eppendorf tubes containing 200 µl sterile PBS. Frass samples were not surface sterilized. Instead, for each sample ca. 3 x 3 x 3 mm (volume) of frass was placed in 2.5ml Eppendorf tubes containing 200 µl sterile PBS. Contents in tubes were homogenized using a small hand held plastic pestle. These primary solutions were used to generate dilution series and plated onto PDA as described above.

Quantification and molecular identification of yeasts

For quantification of yeasts, plates containing between 30 – 300 colonies were selected and the yeast colonies were grouped according to their morphological traits. The number of colonies of each morphotype (colony forming units = CFU) were counted on each selected plate, two plates

per sample, where after the CFU's of each yeast morphotype were averaged between the two plates per sample. One individual of each yeast morphotype per individual sample was then randomly selected and purified on new PDA plates for molecular characterization. These counts were used to calculate the number of CFU's present in the original primary solutions. Colonies of morphotypes for which less than three colonies were observed on selected plates were ignored in the present study.

Molecular identification of yeasts

A single colony per purified culture of each reference yeast strain was harvested and placed in Eppendorf tubes with 500 µl TES buffer (100 mM Tris–HCl, pH 8.0; 10 mM EDTA; 2% (w/v) SDS), 70 µg PCR grade Proteinase K (Roche Applied Science, Mannheim, Germany) and three glass beads. Cells were disrupted using a Tissue Lyser (Qiagen Retsch, Walpole, MA, USA) and genomic DNA was extracted following Möller et al. (1992). Approximately 2,270 bp of the nuclear ribosomal RNA region (18S rRNA, ITS1, 5.8S rRNA, ITS2, and 26S rRNA) were amplified using primer pairs NS1-NS4, NS3-NS6, NS5-NS8 and NS7-NL4 (White et al. 1990). Reaction mixes (25 µl) contained 2.5 µl of 2.5 mM $MgCl_2$, 0.5 µl of 10 µM of each primer, 12 µl 2× KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA), 7.5 µl ddH2O and 2 µl template DNA. PCR reaction conditions were set at denaturation at 95˚C for 2 min, followed by 35 cycles (denaturation at 95˚C for 30 s, annealing at 50 – 55˚C (depending on primer pair) for 30 s, and elongation at 72˚C for 60 s) and termination with a final elongation step at 72˚C for 8 min. All PCR products were sequenced in both directions at CAF (Central Analytical Facility, Stellenbosch University, South Africa) using the PCR primers.

Base calling for sequences was verified using Chromas V.2.6.6 (Technelysium Pty Ltd, Tewantin, Australia) and consensus sequences were generated and aligned using BioEdit V.7.2.5 (Hall 1999). All generated sequences were deposited in GenBank. Hereafter sequences were subjected to BLAST searches (Altschul et al. 1997) on the NCBI website

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) for provisional identifications. As we generated more lengthy sequence reads than most yeast sequences on GenBank, default BLAST searches often retained suboptimal matches as top matches. We therefore regrouped top matches according to percentage similarity for these preliminary identifications. From these we were able to identify closest matching taxa to our sequences.

For phylogenetic analyses we used the data sets compiled by Tanahashi and Hawes (2016), Tanahashi et al. (2017), Urbina and Blackwell (2012) and Liu et al. (2018) as base and added appropriate sequences available from GenBank for analyses. The data sets of Tanahashi and Hawes (2016) and Tanahashi et al. (2017) included numerous lengthy sequences (of the same regions determined in the present study) of yeasts isolated from Lucanidae from the northern hemisphere and various outgroups. Sequences were also obtained from studies on closely related taxa from Liu et al. (2018). Sequences were aligned using BioEdit V.7.2.5 (Hall 1999). For phylogenetic analyses, both Bayesian Inference (BI) and Maximum Likelihood (ML) approaches were used, implemented on the CIPRES Science Gateway v3.3 platform (Miller et al. 2010, http://www.phylo.org). Analyses for BI were conducted using MrBayes v.3.2 (Ronquist et al. 2012) using the nst = mixed command to average over nucleotide substitution models and including a gamma factor for among-site rate variation. Two independent Markov Chain Monte Carlo (MCMC) chains were run for 10,000,000 generations, sampling every 2000 generations and the first 25% of values were discarded as burnin. For ML analyses we used the BlackBox version of RAxML v8.2.12 (Stamatakis 2014). The GTR model of nucleotide substitution with a gamma correction was used to search for the best ML phylogeny. Branch node confidence was calculated using the bootstopping criterion (Pattengale et al. 2010) to ensure adequate bootstrap replicates.

Results

Beetle identification

Based on morphological characters of mature male beetles, the taxon collected in the present study conforms to the description of *Xiphodontus antilope* as highlighted by Bartolozzi (2005). Using the protocol of Kim and Farrell (2015) we generated mitochondrial 16S DNA sequences of 1001 bp length for the two adult beetles and the two larvae in this study (GenBank numbers: MT269940−MT269943). All four sequences were identical and we therefore conclude that the adults and larvae evaluated in the present study is indeed *X. antilope* and that we have identified the larvae of this species here for the first time as developing within rotten logs in Afromontane forests of South Africa.

Yeasts from insects

Dissection of adults revealed the presence of a sac-like mycangium in all females that were absent in all males dissected. Similar to other species in the family, the mycangium of *X. antilope* has a trapezoid shape in ventral view and attached to the intersegmental membrane between tergites eight and nine near the abdominal tip (Tanahashi et al. 2010; Fremlin and Tanahashi 2015; Tanahashi and Hawes 2016).

Only three morphotypes of yeasts grew on the PDA plates containing homogenate from the 10 female mycangia. Except for two mycangia devoid of culturable yeasts, all mycangia contained these same three morphotypes. The first morphotype formed shiny white circular colonies with elevated centers and an entire edge. It was the predominant morphotype in all mycangia (Table 1). Morphotype 2 was morphologically similar to morphotype 1 except that colonies were only a third in size. This morphotype was usually the second most predominant morphotype from female mycangia (Table 1). However, colonies of morphotype 3 were also present in high numbers in some female mycangia (Table 1). Morphologically morphotype 3 differed substantially from the other two in that colonies were waxy in colour and had wavy margins. It was similar in size to colonies of morphotype 2. From each of the 8 females that housed yeasts

in their mycangia we randomly selected one colony of each morphotype as representatives for purification and molecular characterization (n=24 isolates from mycangia in total).

We identified two morphotypes of yeasts from the gut of the 7 2nd instar larvae assessed. Colonies of these two morphotypes were indistinguishable from morphotype 1 and 2 from female mycangia. Both morphotypes were present within larval gut homogenates of 4 larvae with three larvae only containing yeasts conforming to morphotype 1. Numbers of each morphotype varied considerably between different individuals (Table 1) and either one could be predominant (data not shown). From each of the 7 larvae we randomly selected one colony of each morphotype as representatives for purification and molecular characterization (n=12 isolates from larval gut in total).

Only 4 of the surface-sterilized larvae produced fecal matter. From these, we observed the same two morphotypes of yeasts that were isolated from the larval gut. Yeast numbers followed those observed from the larval gut in that when the one morphotype was dominant within the gut of a larva, the same morphotype was dominant within the fecal matter originating from that particular larva. Morphotype 2 was not observed on plates of fecal matter originating from the two larvae for which this morphotype was also absent in the gut. From the fecal matter of each of the 4 larvae we randomly selected one colony of each morphotype as representatives for purification and molecular characterization (n=6 isolates from fecal matter in total).

Yeasts from wood and frass samples

Two predominant yeast morphotypes were isolated from the 8 frass, 8 wood samples in larval tunnels and 8 samples from pupal chambers. All samples produced colonies of at least one of these. Again these were indistinguishable from Morphotype 1 and 2 from female mycangia and from the larval gut. Numbers of the two morphotypes varied considerably between different individual frass and wood samples (Table 1), but generally when one was predominant in a

frass sample, it was also predominant in the wood surrounding the tunnel from where the frass was collected (data not shown). In a few cases only a single morphotype was identified (data not shown). Samples from walls of pupal chambers of 1 male and 3 females were dominated by colonies of morphotype 1. Samples from walls of pupal chambers of 3 male and 1 female were dominated by yeasts that conform to morphotype 2. From two of the samples taken from the walls of the pupal chamber of males, only yeasts of morphotype 2 were isolated. From these samples we selected 27 isolates for purification and molecular characterization.

Molecular identification of yeasts

Molecular characterization confirmed our preliminary classification of predominant yeasts into only three morphotypes. Sequences of individuals of each morphotype were identical in all cases. For morphotype 1 we were able to successfully amplify 2262 bp of the nuclear ribosomal RNA marker (18S rRNA, ITS1, 5.8S rRNA, ITS2, and 26S rRNA). This region for morphotype 2 was slightly longer at 2267 bp. For morphotype 3, this marker yielded a considerably shorter consensus sequence of only 1925 bp. BLAST searches on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for provisional identification of yeast morphotype 1 retrieved closest matches based on total score as *Scheffersomyces coipomoensis* (JCM 8916: GenBank LC120359.1; 100% identity) and *Scheffersomyces lignosus* (JCM 9837: GenBank LC120360.1; 96% identity). Based on identities, morphotype 1 was closest to *Scheffersomyces coipomoensis* (JCM 8916: GenBank LC120359.1; 100% identity). BLAST searches for provisional identification of yeast morphotype 2 retrieved closest matches based on total score as *Scheffersomyces coipomoensis* (JCM 8916: GenBank LC120359.1; 98% identity) and *Scheffersomyces lignosus* (JCM 9837: GenBank LC120360.1; 96% identity). However, based on identities, morphotype 2 was closer to *Scheffersomyces ergatensis* (ATCC 22589: GenBank NG_064960.1; 99% identity). Identity of morphotype 3 was less clear as BLAST searches

retrieved a closest matches based on total score as *Candida hispaniensis* (likely to be transferred to the genus *Yarrowia;* Liu et al. 2018) (GenBank LS992273.1; 87% identity). The Blast Tree View function in GenBank corroborated the uniqueness of this sequence as it did not group closely with any known taxa.

All BI runs converged, with combined estimated sample sizes > 5400. BI and ML analyses produced very similar trees, and confirmed the placement of morphotype 1 and 2 in the genus *Scheffersomyces* with strong support (Fig. 2). These two taxa were resolved as *S. coipomoensis* (no base pair differences with sequences from the type) and a (poorly supported) lineage sister to a clade consisting of *S. amazonensis*, *S. lignosus*, *S. queiroziae* and *S. coipomonensis* respectively (Fig. 2), hereafter referred to as *Schefferomyces* sp. morphotype 2. The phylogenetic placement and identity of morphotype 3 was less clear. This taxon resolved closest to the genus *Yarrowia* with strong support, but was well separated from all species currently recognized in this clade for which data were available (Fig. 2) – we subsequently refer to this species as mycangial isolate morphotype 3.

Discussion

In this study, we confirmed the presence of a female-specific mycangium in *Xiphodontus antilope*, a rare Lucanidae beetle restricted to South Africa. The shape and placement of this mycangium was similar to all other Lucanidae species evaluated to date (Tanahashi et al. 2010; Fremlin and Tanahashi 2015; Tanahashi and Hawes 2016). From the mycangia we isolated three dominant yeast species, namely *Scheffersomyces coipomensis, S*. sp. morphotype 2 and an undescribed taxon with closest known affinity to *Yarrowia* – mycangial isolate morphotype 3. *Xiphodontus antilope* represents the first African member of the globally-distributed Lucanidae to be evaluated for mycangial yeast associates and we provide independent evidence that these structures may be universal for the family (Fremlin and Tanahashi 2015; Tanahashi

and Hawes 2016). We also provide evidence that members of the subfamily Lucaninae may be universally closely associated with *Scheffersomyces* (Tanahashi et al. 2010, Hawes 2013, Fremlin and Tanahashi 2015, Tanahashi et al. 2017), a genus that contains numerous taxa closely associated with wood-feeding insects (Suh et al. 2003; Urbina and Blackwell 2012; Urbina et al. 2013). The two *Scheffersomyces* species were also common in the larval gut and fecal matter, frass, on gallery walls and in the pupal chambers, pointing towards the existence of both an internal and an "external gut system" maintained by Lucanidae larvae in woody substrates (Tanahashi et al. 2018).

In contrast to all other Lucaninae species studied to date, the mycangium of *X. antilope* contained multiple species of yeasts instead of monocultures (Tanahashi et al. 2010, Hawes 2013, Fremlin and Tanahashi 2013, Tanahashi et al. 2017). This is similar to the situation for *Sinodendron cylindricum* (Tanahashi and Hawes 2016) from the subfamily Syndesinae (Kim and Farrell 2015). In that study it was speculated that association with multiple yeasts may reflect less specialized insect-yeast associations possibly similar to early evolutionary stages of these symbioses (Tanahashi and Hawes 2016). Our results may contradict this view as *X. antilope* from the subfamily Lucaninae is also associated with multiple yeast species and we isolated the same three species of yeasts from the mycangia of multiple individuals, albeit from only a single decaying stump at a single locality. Therefore, at least some consistency was found in these associations. It is possible that associations with multiple yeast species may be more of ecological significance than a reflection of less specialized associations due to early evolutionary stages, as there may be several advantages of having multiple yeast associates. For example, different yeast taxa will have different wood metabolizing properties, optimal growth temperatures, moisture requirements and antimicrobial properties. Increasing the numbers of possible associates may therefore increase chances of the successful use of a larger diversity of woody substrate types in habitats that present a large diversity of tree species such as found in Afromontane forests. Alternatively, the transition from multiple yeast mycangial associates to

yeast monocultures is more phylogenetically constrained to only some members of the Lucaninae, with others (such as *X. antilope*) retaining a putatively ancestral generalist association.

Yeast numbers from female mycangia varied considerably between individuals. Also, two adult mycangia did not yield any culturable yeasts. It is unknown whether this variability is the result of differences in maturity of the adults or the unsuccessful transfer of yeasts to mycangia upon female eclosion, but all adults used in assessments here were already fully sclerotized, presumably after the process of acquiring mycangial yeasts (Fremlin and Tanahashi 2015). Offspring from females such as these may therefore need to acquire yeasts from the woody substrate after hatching. The acquisition of beneficial yeasts for larvae such as these may be enhanced when numerous overlapping generations of the same species inhabit the same substrate as was found for this species. If not the case and the eggs are laid on substrates devoid of conspecifics, these larvae may associate with new yeast strains, which may offer additional explanation for the presence of multiple yeast species within the mycangia and gut of *X. antilope.*

Yeast numbers were particularly high in the larval gut, however, only the two dominant *Scheffersomyces* were isolated in the 7 larvae studied. It has been speculated that *Scheffersomyces* yeasts from Lucanidae mycangia likely aid larval nutrition by utilizing inaccessible polymers such as xylose and cellobiose (Breznak and Brune 1994, Geib et al. 2009). Both *S. coipomensis* and *S.* sp. morphotype 2 group within the cellobiose-fermenting *Scheffersomyces* clade (Urbina and Blackwell 2012) and may well optimize nutritional gain from nutritionally poor woody resources ingested by the larvae (Haack and Slansky 1987). These same two yeasts were also dominant in fecal matter and frass (mixture of fecal matter and woody particles) and these can therefore pass through the larval gut unharmed. These yeasts will likely continue metabolizing woody materials in the frass and, as shown here, also on the wood surrounding the galleries. It is therefore likely that the yeasts also aid in external

breakdown of these woody components for later ingestion by the larvae (Tanahashi et al. 2018). This may also explain why Lucanidae larvae generally engage in coprophagy (Araya 2006; Kamata 2014, cited in Tanahashi et al. 2018). However, it still needs to be experimentally demonstrated that yeasts associated with Lucanidae mycangia do indeed aid beetle nutrition, survival or fecundity.

The same yeasts found within the larval gut, fecal matter and on woody substrates were also found colonizing the walls of the pupal chambers. Therefore, after eclosion the yeasts that colonized the pupal chambers are readily available to be picked up by the mycangia of the newly-eclosed female as described by Fremlin and Tanahashi (2015). However, one caveat identified in the present study is the apparent absence of one of the mycangium-dominating yeast species from the larval gut, fecal matter, frass, wood substrates and pupal chamber samples. Mycangial isolate morphotype 3, a taxon most closely allied to *Yarrowia*, was found in almost all mycangia (except the two individuals that yielded no culturable yeasts) and was often quite abundant. This species grew at the same rate on media as *S.* sp. morphotype 2, which excludes its apparent absence from other structures and woody substrates due to competitive exclusion by other yeasts on media, as reason for non-detection. We conclude that this species must therefore be present in yeast assemblages either in extremely low numbers within larvae and substrate, or that these proliferated only for a short time when adults freshly eclosed and acquired the mycangial yeasts from the pupal chamber walls. The relevance of this yeast for the development and life cycle of *X. antilope* needs further investigation. A similar system may also exist in the case of *S. cylindricum*, where an undescribed yeast species seemingly closely allied to *Candida tunisiensis* was isolated from the mycangium, but not larval gut (Tanahashi and Hawes 2016). Whether this taxon is commonly associated with the mycangium of *S. cylindricum* remains to be determined, as only two female individuals and one larva were available for study (Tanahashi and Hawes 2016).

To conclude, we identified common yeast associates of an African Lucanidae species for the first time. All of these are first reports as yeast associates of Lucanidae and two likely represent undescribed species. Yeast associates may therefore vary considerably between different Lucanidae species and, as was the case here, also between different individuals of the same species. Most of these yeasts likely aid beetle nutrition and can use common compounds in wood that are usually inaccessible to insects (Haack and Slansky 1987). These same capabilities are often of industrial use (e.g. Dussán et al. 2019) and the taxa from Lucanidae should be tested for such applications in future studies. *Scheffersomyces* yeasts have now been reported as associates of ca. 15 Lucanidae species in East Asia (Tanahashi et al. 2010; Liu et al. 2018; Tanahashi et al. 2017), Europe (Hawes 2013; Tanahashi and Hawes 2016) and in Africa (this study). However, the family contains more than 1200 species world-wide (Kimm and Farrell 2015 and references therein). Unravelling these associations and characterizing the specific types of interactions will aid our meager understanding of insect-fungus interactions in terms of evolutionary history and biological relevance. In addition, bacterial, archaea, and protist symbionts may play a large role in Lucanidae nutrition (Kuranouchi et al. 2006; Tanahashi et al. 2009; Tanahashi and Kubota 2013) and, although ignored in the present study, were often seen in samples from mycangia here and elsewhere. Also, our characterization of the yeast communities here were only based on culture dependent methods. Studies have shown that these techniques are often not representative of true communities due to biases in growth on prepared media and often biotrophic nature of arthropod-associated microbes (e.g. Rani et al. 2009). Although not without biases, culture independent methods should be used in future studies to elucidate the full complement of fungi and other microbes involved in this multi-taxon symbiosis.

Compliance with Ethical Standards

Welfare of animals: No ethical approval required, but animals were euthanized using methods that cause minimum discomfort.

Conflict of Interest: The authors declare that they have no conflict of interest.

Author contribution: All authors contributed to the study conception and design. Material preparation and data collection was performed by Francois Roets and analysis was performed by Kenneth Oberlander. The first draft of the manuscript was written by Francois Roets and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figure 1: Male (top left), female (top right) and 2nd instar larva (bottom) of *Xiphodontus antilope* collected from a rotting log of *Pterocelastrus tricuspidatus* from Knysna forest in South Africa.

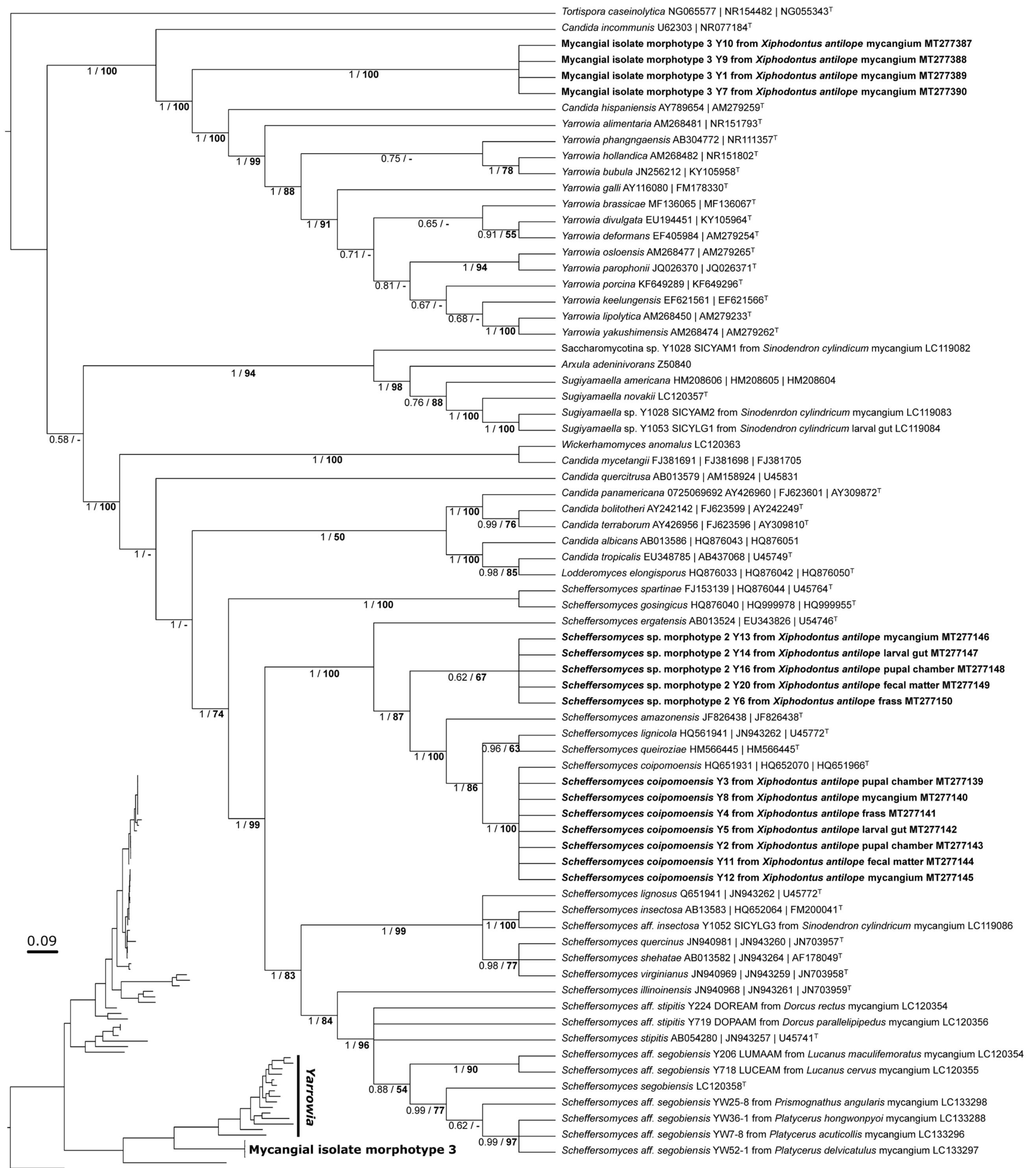

Figure 2: Bayesian Inference consensus tree based nuclear ribosomal RNA and ITS DNA sequence data for yeasts isolated in the current study (bold) and other closely related taxa. Isolate numbers in brackets and bold indicate reference numbers for isolates housed at the Department of Conservation Ecology and Entomology, Stellenbosch University, South Africa.

Values above nodes to the left indicate posterior probabilities obtained through Bayesian inference, and those on the right indicate bootstrap values obtained from maximum likelihood analyses. GenBank numbers of sequences used for analyses are indicated after taxon labels. [T] = sequences originating from type strains. Left insert: Consensus phylogram indicating branch lengths in substitutions per site. Note the substantial branch length separating mycangial isolate morphotype 3 from members of the *Yarrowia* clade.

Table 1: Numbers of colony forming units (CFU) of yeast morphotypes isolated from *Xiphodontus antelope* female mycangia, larval gut (2nd instar), larval frass in galleries in wood, wood samples from larval gallery walls and samples from pupal chamber walls.

Origin	n	Colony forming units (CFU)					
		Morphotype 1 (*S. coipomoensis*)		Morphotype 2 (*Scheffersomyces* sp.)		Morphotype 3	
		Range	Average (SD)	Range	Average (SD)	Range	Average (SD)
Mycangium	10	$0 – 2.7 \times 10^3$	7.4×10^2 (9.2×10^2)	$0 – 1.0 \times 10^3$	3.7×10^2 (3.8×10^2)	$0 – 1.0 \times 10^3$	2.9×10^2 (3.5×10^2)
Larval gut	7	$1.3 \times 10^3 – 4.2 \times 10^5$	2.0×10^5 (1.5×10^5)	$0 – 4.7 \times 10^5$	1.9×10^5 (1.9×10^5)	n.a.	n.a.
Fecal mater	4	$3.8 \times 10^2 – 4.7 \times 10^3$	2.1×10^3 (1.6×10^3)	$0 – 2.4 \times 10^3$	1.2×10^3 (1.2×10^3)	n.a.	n.a.
Larval frass	8	$0 – 2.8 \times 10^5$	6.1×10^4 (8.9×10^4)	$0 – 3.9 \times 10^5$	1.0×10^5 (1.2×10^5)	n.a.	n.a.
Gallery wall	8	$0 – 1.1 \times 10^3$	1.9×10^2 (3.6×10^2)	$0 – 1.1 \times 10^3$	2.9×10^2 (3.8×10^2)	n.a.	n.a.
Pupal chamber ♂	4	$0 – 8.7 \times 10^2$	2.4×10^2 (3.7×10^2)	$2.3 \times 10^2 – 5.4 \times 10^3$	4.6×10^2 (4.3×10^2)	n.a.	n.a.
Pupal chamber ♀	4	$3.2 \times 10^3 – 1.4 \times 10^4$	1.3×10^3 (9.0×10^2)	$2.3 \times 10^2 – 1.7 \times 10^4$	1.1×10^3 (1.3×10^3)	n.a.	n.a.