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Article

Presence of the yeast *Kodamaea ohmeri* associated with *Aethina tumida* (Coleoptera: Nitidulidae) collected in Africanized honey bee colonies from two apiaries of Yucatan, Mexico

Azucena Canto [a*]

Luis A. Medina-Medina [b]

Elisa Chan [a]

Rosalina Rodríguez [a]

[a] Centro de Investigación Científica de Yucatán AC. Unidad de Recursos Naturales, Calle 43, Num. 130, Colonia Chuburná de Hidalgo, Mérida, 97200, Yucatán, México.

[b] Universidad Autónoma de Yucatán, Facultad de Medicina Veterinaria y Zootecnia, Mérida, Yucatán, México.

* Corresponding author: azucanto@cicy.mx

Abstract:

Aethina tumida (Coleoptera: Nitidulidae), commonly known as the Small Hive Beetle (SHB), is becoming a significant pest in the beekeeping industry outside of its natural distribution range. In Mexico, recent reports indicate that the SHB is distributed throughout the Yucatan peninsula. The invasion of honey bee colonies by SHB it is mainly chemically mediated by volatiles produced by the yeast *Kodamaea ohmeri* which is regarded as a secondary symbiont of the SHB. It was analyzed the presence of this yeast in honey bee colonies of Yucatan based on the premise that symbionts are often conjointly distributed with their hosts, therefore the presence of *K. ohmeri* in hives will be closely associated with the presence of SHB. In managed Africanized honey bee (AHB) colonies, yeasts associated with adult beetles were isolated and identified and the results show that the SHB together with their associated yeast, *K. ohmeri*, have invaded AHB colonies in Yucatan.

It was also reported the presence of yeasts other than *K. ohmeri* associated with SHB that for the first time are recorded in a geographical region where they had not been recorded before.

Key words: *Aethina tumida, Apis mellifera*, Beetle-yeast association, Secondary symbiont, *Kodamaea ohmeri*, Small Hive Beetle, rDNA, Tropical beekeeping.

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Introduction

Aethina tumida Murray 1867 (Coleoptera: Nitidulidae), commonly known as the Small Hive Beetle (SHB), is an opportunistic scavenger which invades the nests of the honey bee *Apis mellifera*, becoming a significant pest in the beekeeping industry outside of its natural distribution range. SHB females proliferate within honey bee colonies, and their larvae consume the pollen, honey and bee brood present in the combs causing honey fermentation and collapse of the colony[1,2]. SHB was first reported in Mexico in 2007 in the state of Coahuila[3] and since then, it has been reported in other states, including Campeche, Michoacán, Jalisco, Quintana Roo, San Luis Potosí and Yucatan, which are the main honey producing states[4]. SHB was first reported in 2012 in apiaries located at northeast of the state of Yucatan[5], and recent reports indicate its presence throughout the Yucatan peninsula[6]. The invasion of the SHB adults into honey bee colonies is thought to be chemically mediated by volatiles that are produced by microbial fermentation of food reserves. One of the predisposing factors that allows SHB to become a pest in bee colonies is the association with the fermentative yeast *Kodamaea ohmeri*[7,8]. This yeast is a facultative or secondary symbiont of SHB and has been isolated from the digestive tracts of SHB adults, and their eggs and larvae[7,9-11] and is considered the primary factor responsible for fermenting the food stored in the colonies and producing the attractants sensed by other adult beetles[7,12,13].

In the interaction between symbionts and hosts, symbionts assist their hosts in colonizing new habitats and expanding to new geographical zones, mainly because they play a key role in the nutrition of host insects[14-17]. Insect-associated yeasts provide essential nutrients as sterols and produce allelochemicals to attract the insect dispersers for targeted dispersal to a new environment[17]. It is feasible to expect that *K. ohmeri* as symbiont of the SHB is being carried by the adults as they colonize new hives and apiaries. The purpose of this study was to analyze the

presence of yeasts, specifically *K. ohmeri*, associated with the SHB adults that invade managed Africanized honey bee (AHB) colonies in Yucatan. It is assumed that *K. ohmeri* is highly frequent in association with SHBs in this region, based on the premise that symbionts are often conjointly distributed with their hosts[15,16]. The results will help to elucidate the relationship between the SHB and its associated yeasts and to understand the beetle-yeasts impact on honey bee colonies, as well as to design adequate strategies to control this pest using SHB's symbiotic yeasts in neotropical environments.

Material and methods

The present study was conducted from February to July 2016 in AHB colonies from apiaries located in two different sites in the state of Yucatan, Mexico. One group of colonies was located in the experimental apiary of the Campus of Biological and Agricultural Sciences of the Autonomous University of Yucatan in the municipality of Merida (20° 51' 51.62" N; 89° 36' 45.35" W) and the other group was located in a private apiary of the municipality of Motul (21° 08′ 03″ N 89° 19′ 03″ W). The apiaries contained approximately 30 AHB colonies, from which a total of six colonies were randomly selected to sample. All combs, as well as the hive box, cover, bottom board, and artificial feeders (inside feeders) were removed from each colony and checked to detect and collect adult of the SHB.

Adult beetles were collected from the bottom board, brood combs and artificial feeders. In all these places was observed a great number of SHB individuals. Each beetle was collected using tweezers that were sterilized in 99 % alcohol to avoid contamination among beetles. Subsequently, each beetle was placed in a sterile bottle with a screw cap and was labeled according to the colony of origin, place inside the hive and number of specimen. Adult beetles were identified morphologically according to the standard methods for identification at the level of species[18]. In total, 27 live adult individuals (1 to 6 beetles per colony) were collected.

To obtain the yeasts, each individual beetle was placed inside the YPD (yeast extract-peptone-dextrose) agar plates and allowed to walk freely over the entire surface of agar plates during 40 min. There were not externally sterilize the beetles with 70 % alcohol and there were not rinsed with sterile water, with the purpose of keeping them alive and allowing the yeasts to be obtained by imitating the natural form of yeast dispersal inside the honey bee colonies. Each beetle was allowed to nibble the agar surface and move freely over the plate mimicking the way in which the beetles disseminate the yeast when they move through the honey bee combs. This method increases the probability of obtaining yeasts associated with the beetles' mouthparts and digestive system[15].

After the time of walk on the agar surface, each beetle was sacrificed and stored in 70 % alcohol. The agar plates were incubated at 25 °C and checked every 24 h to detect the growth of yeasts. It was monitored the growth of microbial colonies by observing each plate under a stereoscopic microscope at 50x magnification. To isolate and purify the yeasts from the agar plates, colonies of each morphotype were selected and were individually placed in Eppendorf tubes with 600 µL of sterile distilled water. The contents were re-suspended by shaking and seeded onto a new agar plate. The resulting plate was incubated at 25 °C for 5 d or until microbial growth was observed. This procedure was performed twice for each morphotype observed on the original agar plates.

All yeast morphotypes were stored in the Yucatan Scientific Research Center (CICY) yeast collection. Identification of species was performed through the symmetric sequencing of the D1/D2 domain (nucleotides 63-642 of *Saccharomyces cerevisiae*) of the large subunit (LSU) rRNA gene, following the standard DNA extraction protocols[19,20]. For DNA extraction, cells were grown for approximately 48 hours in YPD broth (3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 10 g of glucose per liter of distilled water) in a rotary shaker at 100 rpm at 27 ºC and harvested by centrifugation. The packed cells were immersed in liquid nitrogen for 10 min and then crushed in a mortar and placed in a sterile tube with 800 µL of buffer (50 mM Tris-HCl, 250 mM NaCl, 50 mM EDTA, 0.3 % sodium dodecyl sulfate). Twenty microliters of RNase were added to clean the samples, which were then heated in a thermoblock at 65 °C for 30 min and gently shaken every 10 min. They were cooled to 23 °C, and then 500 µL of chloroform was added to extract the DNA. The resulting volume was centrifuged at 1,300 rpm for 10 min. The supernatant was recovered, and 700 µL of isopropanol was added. Samples were gently mixed and centrifuged again for 5 min to precipitate DNA. The pellet was recovered and washed with 500 µL of 70 % ethanol and then centrifuged for 5 min. The supernatant was discarded, and the pellet was allowed to dry for 24 h at room temperature. Subsequently, the pellet was re-suspended in 70 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.4]). DNA samples were prepared for PCR using 5 µL (120-500 ng) of the diluted samples with 1 mL of TE buffer at 0.5X. To verify the extraction, 5 µL (120-500 ng) of DNA was used in a 1 % agarose gel electrophoresis using TBE (Tris-Borate-EDTA) 0.5X and a current of 100 V for 15 min. Subsequently, the DNA was quantified by spectrophotometry with a Nano Drop 2000 (ThermoFisher Scientific) and diluted to 20 ng/µL for PCR.

Amplification of the D1/D2 sequence was performed using the primers NL-1 (5´-GCATATCAATAAGCGGAGGAAAAG-3´) and NL-4 (5´-GGTCCGTGTTTCAAGACGG-3´)[20] and the following PCR protocol: 95 °C for 12 min followed by 40 cycles of denaturation at 94 °C for 15 sec annealing at 55 °C for 10 sec, extension at 72 °C for 20 sec, and a final extension of 5 min at 72 °C . The amplified DNA was prepared for sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions with the primers NL-1 and NL-4. The samples were individually injected for electrophoresis into an ABI 3730xl DNA Analyzer (Applied Biosystems). Sequences were aligned and assembled, and a

consensus sequence was obtained for each yeast isolate using Geneious Pro 8.1.7 bioinformatics software (Biomatters Ltd, Auckland, New Zealand). The GenBank nucleotide database was queried using the Basic Local Alignment Search Tool (BLAST)[21] to look for named yeast species with DNA sequences that matched the isolates of this study. All sequences yielded significant correlations with named yeast accessions in GenBank, with 98.8-100 % of sequence coverage and identity. The degree of divergence in the D1/D2 portion between the study sequences and concordant sequences found in the GenBank database did not exceed 1 %; therefore, they were considered co-specific sequences[22]. The sequences obtained in this study were deposited in GenBank under the accession numbers shown in Table 1.

Table 1: Yeast isolates resulted from SHB adults collected in colonies of Africanized honey bee *Apis mellifera* in apiaries of Yucatan, Mexico

Beetle specimen	AHB colony	Species	Site of collection	ND*	Strain designation CICY	GenBank accession number
1	1	*Kodamaea ohmeri*	Brood comb	0	CICYRN1044	MF431846
1	1	*Kodamaea ohmeri*	Brood comb	0	CICYRN1045	MF431847
1	1	*Meira argovae*	Brood comb	1	CICYRN1047	MF431848
2	2	*Kodamaea ohmeri*	Artificial feeder	0	CICYRN1048	MF431849
2	2	*Kodamaea ohmeri*	Artificial feeder	0	CICYRN1049	MF431850
2	2	*Citeromyces siamensis*	Artificial feeder	0	CICYRN1050	MF431851
2	2	*Kodamaea ohmeri*	Artificial feeder	0	CICYRN1051	MF431852
2	2	*Kodamaea ohmeri*	Artificial feeder	0	CICYRN1052	MF431853
2	2	*Citeromyces siamensis*	Artificial feeder	1	CICYRN1053	MF431854
3	2	*Kodamaea ohmeri*	Artificial feeder	0	CICYRN1054	MF431855
3	2	*Kodamaea ohmeri*	Artificial feeder	0	CICYRN1055	MF431856
3	2	*Citeromyces siamensis*	Artificial feeder	0	CICYRN1056	MF431857
3	2	*Kodamaea ohmeri*	Artificial feeder	0	CICYRN1058	MF431858
4	2	*Citeromyces siamensis*	Artificial feeder	0	CICYRN1059	MF431859
5	2	*Lachancea fermentati*	Artificial feeder	0	CICYRN1060	MF431860
6	3	*Kodamaea ohmeri*	Bottom board	0	CICYRN1062	MF431861
7	3	*Kodamaea ohmeri*	Bottom board	0	CICYRN1063	MF431862
7	3	*Kodamaea ohmeri*	Bottom board	0	CICYRN1064	MF431863
8	3	*Kodamaea ohmeri*	Bottom board	0	CICYRN1066	MF431864
9	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1069	MF431865
9	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1070	MF431866
10	4	*Kodamaea ohmeri*	Bottom board	1	CICYRN1072	MF431867
10	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1073	MF431868
11	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1091	MF431869
12	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1074	MF431870
13	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1075	MF431871

13	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1076	MF431872
14	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1089	MF431873
14	4	*Kodamaea ohmeri*	Bottom board	0	CICYRN1090	MF431874
15	5	*Kodamaea ohmeri*	Bottom board	0	CICYRN1077	MF431875
16	5	*Kodamaea ohmeri*	Bottom board	0	CICYRN1078	MF431876
17	6	*Kodamaea ohmeri*	Bottom board	0	CICYRN1081	MF431877
18	6	*Kodamaea ohmeri*	Bottom board	0	CICYRN1082	MF431878
19	6	*Kodamaea ohmeri*	Bottom board	0	CICYRN1084	MF431879
20	6	*Kodamaea ohmeri*	Bottom board	0	CICYRN1086	MF431880
20	6	*Kodamaea ohmeri*	Bottom board	0	CICYRN1087	MF431881
20	6	*Kodamaea ohmeri*	Bottom board	0	CICYRN1088	MF431882

* rDNA nucleotide differences between type strain from GenBank and conspecific isolate from this study.

Results

Adult beetles of *A. tumida* were found in all the AHB colonies examined and, individuals were found inhabiting mainly in the small cracks of the bottom board. The presence of yeasts was detected in the agar plates (each one depicting an individual beetle sample) after 5 d of incubation. Thirty-seven strains were obtained from 20 of the 27 collected beetles, of which four different yeast species were identified; three of them are reported for the first time associated to *A. tumida*. The most frequent yeast identified was *K. ohmeri* with 31 isolates, followed by *Citeromyces siamensis* with four isolates, *Lachancea fermentati* and *Meira argovae* with one isolate respectively (Table 1). Concerning the place where the individuals were collected inside the hive, SHB adults from the bottom board have associated only with *K. ohmeri*, while the beetles collected from the brood combs have associated with two, *K. ohmeri* and *M. argovae*. Beetles collected in the artificial feeders have associated with three species of yeasts, *K. ohmeri*, *C. siamensis* and *L. fermentati*.

Discussion

Of the four yeast species that were identified, only one can be considered as close-associated with SHB[11], *K. ohmeri*. This yeast was isolated from most of the beetles collected in the hive, and it is considered as secondary symbiont of *A. tumina*[7]. Results give evidence that symbionts are conjointly distributed with their hosts[15,16] and point out that the invasion of SHB in apiaries of this Mexican region have resulted in the expansion and distribution of *K. ohmeri* into new areas not been previously registered before.

In contrast, the other yeasts isolated from the SHBs cannot be defined as symbionts and probably were externally acquired by SHB adults during theirs movements through brood combs, feeders, and other structures inside the hive. *Citeromyces siamensis*, which belongs to Saccharomycetales order, is a fermentative yeast associated with high osmotic foods, such as salted squid and fermented soybeans[23]. In this study, it was isolated it from beetles that were feeding on sucrose syrup within the artificial feeders and probably this yeast was passively acquired by beetles feeding on the syrup. Unlike *C. siamensis*, another fermentative yeast, *L. fermentati* (Saccharomycetales) is associated with the gut of insects such as fruit flies and neuropters[24] and has been isolated from a variety of liquid substrates such as fruit juice and olive and tequila ferments[25]. In this study, *L. fermentati* was isolated from a beetle that was feeding on the artificial feeder, which is consider a circumstantial acquisition. *Meira argovae* is a basidiomycetous anamorphic yeast-like fungus belonging to class Ustilaginomycetes and has been reported to be associated with phytophagous mites[26] in bamboo shoots[27]. *Meira argovae* may have potential for controlling these mites in important crops because it secretes antagonistic substances[28]. *M. argovae* was isolated from a beetle collected in a bee brood comb of a colony, therefore, the acquisition of this yeast by the SHB adult may have been occurred when the beetle walked through brood combs.

Kod
amaea ohmeri (Saccharomycetales, family Metschnikowiaceae) was the most frequently isolated species in this study and it is also the only species that has been repeatedly isolated from fermented material found in *A. mellifera* colonies infested with SHB, as well as from within the body of beetles[2,7,8,29]. The importance of the relationship between SHBs and *K. ohmeri* yeast is not well understood, although the presence of *K. ohmeri* has been shown to increase the beetles' ability to invade and reproduce in *A. mellifera* colonies[7,30] because this yeast is responsible for producing volatile components in the food, which act as a strong attractant to other beetles[31].

The results showed that the SHBs together with their associated yeast, *K. ohmeri*, have invaded *A. mellifera* colonies in Yucatan, suggesting that the impact of the beetles on AHB colonies in this region may increase due to the presence of *K. ohmeri*. However, experimental studies are required to test the hypothesis that the presence of *K. ohmeri* isolated in this study increase the capacity of SHB to infest AHB apiaries. In Yucatan, AHB have similar behavior to their African ancestors, which entrap, encapsulate and confine SHB adults within the cracks and crevices of the hive and also remove the beetles' eggs and larvae, avoiding honeycomb fermentation, the production of chemicals and reducing the attraction of more beetles inside the colonies.

It has been proposed to use *K. ohmeri* to ferment pollen substitutes or the pollen itself, as bait for beetle traps to control SHB in the honey bee colonies[32]. In addition to the use of *K. ohmeri* in baits, experimental data using *C. siamensis*, *L. fermentati* and *M. argovae* are also needed to explore the role of these yeasts as attractants of beetles. Although *K. ohmeri* is not exclusive of SHBs as this yeast has been also isolated from nests of bumblebees such as *Bombus impatiens* and *Bombus pensylvanicus* that do not have SHBs in their colonies[12], it is plausible to assume that SHB are active disperser of *K. ohmeri* and other yeasts to new resources and hosts[8,16].

Conclusions and implications

The results pointed out that SHB is present in Africanized apiaries in the Yucatan area and that this colonization has also resulted in the presence of their facultative symbiont *K. ohmeri* and of other food- and invertebrate-associated yeasts, *C. siamensis*, *L. fermentati* and *M. argovae*, in substrates not previously recorded for these yeasts, and for the first time, in a region where they had not been observed.

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