Yeast Volatomes Differentially Affect Larval Feeding in an Insect Herbivore

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ABSTRACT Yeasts form mutualistic interactions with insects. Hallmarks of this interaction include provision of essential nutrients, while insects facilitate yeast dispersal and growth on plant substrates. A phylogenetically ancient chemical dialogue coordinates this interaction, where the vocabulary, the volatile chemicals that mediate the insect response, remains largely unknown. Here, we used gas chromatography-mass spectrometry, followed by hierarchical cluster and orthogonal partial least-squares discriminant analyses, to profile the volatomes of six Metschnikowia spp., Cryptococcus nemorosus, and brewer's yeast (Saccharomyces cerevisiae). The yeasts, which are all found in association with insects feeding on foliage or fruit, emit characteristic, species-specific volatile blends that reflect the phylogenetic context. Species specificity of these volatome profiles aligned with differential feeding of cotton leafworm (Spodoptera littoralis) larvae on these yeasts. Bioactivity correlates with yeast ecology; phylloplane species elicited a stronger response than fruit yeasts, and larval discrimination may provide a mechanism for establishment of insect-yeast associations. The yeast volatomes contained a suite of insect attractants known from plant and especially floral headspace, including (E)-terpineol, Z,E)-deca-2,4-dienoate (pear ester), (3Z)-myrcene, or (E)-farnesene. A wide overlap of yeast and plant volatiles, notably floral scents, further emphasizes the prominent role of yeasts in plant-microbe-insect relationships, including pollination. The knowledge of insect-yeast interactions can be readily brought to practical application, as live yeasts or yeast metabolites mediating insect attraction provide an ample toolbox for the development of sustainable insect management.

IMPORTANCE Yeasts interface insect herbivores with their food plants. Communication depends on volatile metabolites, and decoding this chemical dialogue is key to understanding the ecology of insect-yeast interactions. This study explores the volatomes of eight yeast species which have been isolated from foliage, from flowers or fruit, and from plant-feeding insects. These yeasts each release a rich bouquet of volatile metabolites, including a suite of known insect attractants from plant and floral scent. This overlap underlines the phylogenetic dimension of insect-yeast associations, which according to the fossil record long predate the appearance of flowering plants. Volatome composition is characteristic for each species, aligns with yeast taxonomy, and is further reflected by a differential behavioral response of cotton leafworm larvae, which naturally feed on foliage of a wide spectrum of broad-leaved plants. Larval discrimination may establish and maintain associations with yeasts and is also a substrate for designing sustainable insect management techniques.

KEYWORDS Yeast volatome, metabolomic profile, floral odorants, chemical signals, larval attraction, olfaction, Noctuidae, Lepidoptera
Microorganisms enable insect feeding and development on plants (1–4). Invisibly, they broadcast a rich bouquet of volatile metabolites to mediate communication with other microorganisms, plants and associated animals (5–7).

Plants also release volatile compounds in abundance, but there is mounting evidence that the production of volatiles by bacteria (8, 9), fungi (10–12), and yeasts (13–17) is equally prolific. The wide overlap in compounds released by plants (18) and microbes (19) further suggests that plant headspace includes volatiles that are produced by plant-associated epiphytic and endophytic microbes. A striking example is floral scent: bacteria and especially yeasts metabolize pollen, nectar, and other floral compounds and thus become a prominent source of volatiles (20–25).

Yeasts are widely associated with insects, since they require vectors for dispersal and outbreeding. In addition, larval feeding facilitates yeast growth on plant substrate. Yeasts provide, on the other hand, nutritional services to insects (26–32). This mutualistic interaction is facilitated by communication with volatile metabolites. Yeasts have apparently evolved the capacity to synthesize aroma compounds to attract insects (33–35), and insects, correspondingly, possess dedicated olfactory receptors tuned to yeast fermentation metabolites that signal suitable substrates for adult and larval feeding (36–39). Consequently, yeast volatiles, in addition to plant volatiles, play a part in host-plant and food finding in insect herbivores, including flies and moths (5, 40–44).

Investigations of the volatile metabolites of insect-associated yeasts serve a dual purpose. Plants and their insect herbivores are fundamental to many ecosystems. Olfactory recognition of food plants by insects is key to their interactions (45–47), and it is, accordingly, of fundamental interest to identify the microbial component of plant-insect communication. Furthermore, yeast metabolites or live yeasts facilitate insect management. Lack of environmentally benign, yet efficient control methods is an increasingly pressing issue in times of global change and increasing food insecurity (48–52). Yeast attraction of insects and their larvae for feeding (33, 44, 53–56) can be exploited for population control of herbivores (57, 58), as well as for improved crop pollination (59).

We sought to determine whether taxonomically related yeasts, isolated from different insects and habitats, differ with respect to their volatile metabolomes and whether cotton leafworm larvae behave differently toward them. We selected a Cryptococcus and several Metschnikowia yeasts, which have been isolated from insect larvae feeding on fruit or foliage, including the cotton leafworm, Spodoptera littoralis, a polyphagous noctuid moth (60). We investigated the volatomes of these yeasts by gas chromatography-mass spectrometry (GC-MS) and comparative multivariate discriminant analysis, affording unique volatile fingerprints. Differential larval attraction reveals the behavioral relevance of characteristic differences in yeast volatome composition.

**RESULTS**

**Yeast headspace analysis.** A phylogenetic tree of the yeasts investigated—Cryptococcus nemorosus, six Metschnikowia spp., and brewer’s yeast (Saccharomyces cerevisiae)—is shown in Fig. 1. These yeasts have all been found to occur in association with insects. The volatiles released during fermentation were investigated by GC-MS, and shown to contain a range of compounds, including largely methyl and ethyl esters but also terpenoids, straight and branched alcohols, aldehydes, ketones, acids, and five sulfur- and three nitrogen-containing volatiles (Fig. 2; see also Table S1 in the supplemental material). Of the 192 compounds found, 26 are not yet listed in databases of volatiles from yeasts, fungi, and bacteria, and 33 compounds are new for yeasts (17, 19).

Many of these yeast-produced compounds, including terpenoids such as linalool and farnesenes, and esters, such as pear ester, have also been found in plant headspace (18).

Figure 2 compares the volatomes of these eight yeasts. Variation across replicates is substantial, despite rigorous protocols used for yeast growing and headspace collection. However, species could still be separated according to headspace composition by...
a discriminant analysis (OPLS-DA M1, \( R^2_X(\text{cum}) = 0.768; R^2_Y(\text{cum}) = 0.918; Q^2(\text{cum}) = 0.756 \)) resulting in seven predictive components that explain 63% of the entire variation. Especially after grouping compounds into 14 groups by hierarchical cluster analysis (HCA) using the M1 loadings, Fig. 2 further visualizes that headspace composition is characteristic for each yeast.

Headspace composition further reflects taxonomic position. *M. andauensis*, *M. fructicola*, and *M. pulcherrima* share morphological and physiological characters, and the D1/D2 domain differs only with respect to a few nucleotides (61–63). The close relation between these three species (Fig. 1) is in line with their volatome composition in comparison to the more distantly related *Metschnikowia* species (Fig. 2 and 3).

Headspace composition also helped to clarify the taxonomic status of a yeast collected from apple, which had been tentatively and incorrectly determined as *Cryptococcus tephrensis*, according to morphological criteria. Visual inspection of its volatome fingerprint suggested this yeast to be closely related to *M. fructicola* (replicates 7 to 12 of *M. fructicola*; Fig. 2). This was then confirmed by sequencing the D1/D2 LSU rRNA gene, showing 99% similarity with the sequence obtained from *M. fructicola* (NCBI accession number KC411961; Fig. 1).

A three-dimensional score plot of the first three predictive components of M1 shows that *M. hawaiiensis* and *M. saccharicola* clearly separate according to the first three dimensions and that the other species are clustering with little overlap (Fig. 3). The other species diverged in the remaining dimensions and by HCA of the M1 scores (data not shown). Internal model robustness validation was performed by randomly excluding three observations for each yeast. In order to keep at least three observations, only one replicate was removed for *M. andauensis*, and *S. cerevisiae* was removed completely. Excluded observations were thereafter used as a prediction set in a new model made of the remaining observations [OPLS-DA M2, \( R^2_X(\text{cum}) = 0.686; R^2_Y(\text{cum}) = 0.879; Q^2(\text{cum}) = 0.596 \)]. A zero misclassification error (Fisher probability = \( 2.8 \times 10^{-19} \)) further corroborated the robust ability of OPLS-DA to distinguish between yeast headspace profiles.
FIG 2 Volatile compounds identified from headspace collections of fermenting yeasts by GC-MS (see also Table S1 in the supplemental material). Compounds were grouped according to hierarchical cluster analysis. Circles depict the relative abundance of compounds after normalization by abundance across species (rows) and observations (columns). Yeast species are listed according to the phylogenetic tree (Fig. 1). For abbreviations, see Fig. 1.
M. hawaiiensis, M. saccharicola, and M. lopburiensis. M. hawaiiensis has been isolated from morning glory flowers and is associated with drosophilid species (64), and M. saccharicola and M. lopburiensis have been found on sugarcane and rice leaves (65).

M. hawaiiensis and M. saccharicola were the most prolific producers of volatiles, several of which were not present in the other yeasts studied (Fig. 2). They separated clearly, according to OPLS-DA, from each other and the other yeasts (Fig. 3). The headspace of these two species has not yet been studied and contains a number of compounds which are new to databases of yeast volatiles (Table S1) (17, 19). They also contained several yet-unknown compounds, which were not found in commercial or our own libraries and which did not match commercially available standards.

Characteristic compounds for M. saccharicola were a range of putative sesquiterpenes and pear ester, a characteristic odorant of pear (66, 67), which is also a strong bisexual attractant for codling moth Cydia pomonella (68–70). Indole, a nitrous compound, was released by both M. lopburiensis and M. pulcherrima. Methanol, ethyl (E)-2-methylbut-2-enoate and ethyl furan-3-carboxylate are the primary class separators for M. lopburiensis. Methanol was also consistently found in M. saccharicola samples (Fig. 2).

The volatome of M. hawaiiensis was clearly separated from the other Metschnikowia spp. (Fig. 2 and 3), and methyl esters were key compounds in headspace class separation (Table S2). Perhaps coincidentally, two sulfur-containing compounds, methyl 3-methylthio-propanoate and 3-methylsulfanylpropan-1-ol, which are typical for M. hawaiiensis, are associated with the aroma of pineapple (71, 72).

M. andauensis, M. fructicola, and M. pulcherrima. Three very closely related species (Fig. 1) (63)—M. andauensis, M. fructicola, and M. pulcherrima—are morphologically and ecologically similar. They have all been found in larval frass of lepidopteran larvae (41, 62). Three compounds that differentiate M. fructicola from other yeasts were 2-phenyl ethanol, 3-ethoxy-propan-1-ol, and 3-methylbutan-1-ol. The top four discriminating compounds for M. pulcherrima were ethyl 3-methylbutanoate, ethyl propanoate, nonan-2-ol, and sulcatone, which showed a high correlation with butyl butanoate. Two methyl-branched short-chain carboxylic acids, heptan-4-ol and unknown 147, were highly characteristic for M. andauensis (Fig. 2; Table S2).

Cryptococcus nemorosus. Cryptococcus is polyphyletic, and several species, such as C. nemorosus, have been isolated from the plant phyllosphere and soil (73, 74).
Ethyl (E)-2-methylbut-2-enoate, aliphatic ketones, and aliphatic methyl-branched primary alcohols were the main volatiles that separated *C. nemorosus* from the other species. Strong correlations were observed for 6,10-dimethyl-5,9-undecadien-2-ol (fuscumol) and its respective ketone, 6,10-dimethyl-5,9-undecadien-2-one (geranyl acetone). Shared structures for classifying other species were also observed, namely, 2-pentylthiophene was shown to highly correlate with the class member *M. andauensis* (Table S2).

**Larval feeding on live yeasts.** We next investigated attraction and feeding of *S. littoralis* larvae in a choice test. Larval feeding was assayed in a petri dish with two drops of liquid medium, one with live yeast and the other without. Three yeasts—*M. andauensis* (*P* < 0.05), *M. pulcherrima* (*P* = 0.031), and *S. cerevisiae* (*P* = 0.002)—deterred feeding; more larvae fed on blank medium in their presence. Two species, *M. fructicola* (*P* = 0.06) and *M. saccharicola* (*P* = 0.096), had no significant effect, but *M. hawaiiensis* (*P* < 0.05), *M. lopburiensis* (*P* = 0.012), and *C. nemorosus* (*P* = 0.002) elicited larval attraction and feeding (Fig. 4A).

An orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to explore the correlation of yeast volatiles from different species with respect to behavioral activity. Three classes (shown in Fig. 4B) were used, which resulted in a model with two predictive and four orthogonal components [OPLS-DA M3, \( R^2_{Y(cum)} = 0.68; R^2_F(cum) = 0.947; Q^2_F(cum) = 0.812 \)]. Model M3 showed excellent classification performance (Fisher probability = 2.5 \( \times \) 10\(^{-19} \)). When plotting the two OPLS-DA predictive components, three groups separate clearly, showing that these yeasts can be distinguished with respect to their behavioral effect (Fig. 4B).

Among the eight yeast species, *M. andauensis* and *C. nemorosus* exhibited the strongest activity, resulting in larval avoidance and feeding, respectively (Fig. 4A). The volatile profiles of these yeasts show considerable overlap with the other species (Fig. 1), and we hypothesized that volatiles released by the other species could be used for sifting inactive volatile constituents and thus facilitate the search for bioactive candidate compounds. We constructed two models for this purpose. Volatiles of all species except *M. andauensis* were modeled against *C. nemorosus*, eliciting the highest rate of feeding which resulted in a model with two predictive and four orthogonal components [OPLS-DA M4, \( R^2_{Y(cum)} = 0.608; R^2_F(cum) = 0.984; Q^2_F(cum) = 0.933 \)]. *Metchnikowia andauensis*, which strongly deterred larvae from feeding, was likewise modeled against all other species except *C. nemorosus* [OPLS-DA M5, \( R^2_{Y(cum)} = 0.69; R^2_Y(cum) = 0.983; Q^2_Y(cum) = 0.572 \)]. Using M4 and M5, a shared and unique structure (SUS) plot was made to illustrate key compounds that are, compared to the other species, released in smaller or larger amounts by *M. andauensis* and *C. nemorosus*. The SUS-plot (Fig. 5) assigns two acids, several methyl branched esters, camphene, and the two unknown compounds
DISCUSSION

The yeasts studied here occur on plants and in connection with insect larvae feeding on these plants. A rich volatome found in all eight species may serve interactions within and between microbial taxa. The presence of many odor-active compounds also supports the idea that yeasts require animal vectors for dispersal and outbreeding. Unlike fungal spores, yeast spores are not adapted for wind-borne transmission. Needle-shaped ascospores, which are frequently found in the Metschnikowia clade, promote dispersal by flower-visiting flies, beetles, and bees. Yeasts, on the other hand, provide nutritional services to insect larvae and adults (26, 27, 30, 31, 33, 40, 75–78).

Larvae of cotton leafworm *S. littoralis* that naturally feed on foliage of a broad range of annual plants (79, 80) were attracted to volatiles of three yeasts (Fig. 4). Figure 2 illustrates that yeast headspace is at least as rich and complex as headspace of their food plants (81, 82) and that also many yeast compounds, including terpenoids, are shared by these plants. This raises the question of whether the larvae of insect herbivores become attracted to plant or to yeast odor for feeding or both. Especially for insect species found on a range of plant species, such as *S. littoralis*, volatiles from plant-associated yeasts may be sufficiently reliable signals, especially when these yeasts are part of the larval diet.

The question of to which extent yeast versus plant volatiles contribute to oviposition and larval feeding has been formally addressed in *Drosophila melanogaster*, where brewer’s yeast headspace alone elicits attraction and oviposition. *Drosophila* larvae complete their entire development on yeast growing on minimal medium, which supports the conclusion that the fruit merely serves as a substrate for yeast growth (40). In comparison, strict dissection of plant and microbial components is experimentally difficult in insects that require foliage for feeding. For example, the grape moth *Paralobesia viteana* was attracted to grape leaves after microbial colonies were washed.
A closer look at attractants identified from plant hosts produces a surprising insight: typical plant volatiles such as (Z)-3-hexenyl acetate, linalool, nonanal, or even (3E)-4,8-dimethyl-1,3,7-triene (DMNT), which play an important role in the attraction of P. viteana or the grape berry moth Lobesia botrana, are all produced by several yeasts (Fig. 2) (83, 84). Likewise, compounds from cotton headspace that elicit antennal or behavioral responses in cotton leafworm S. littoralis are also produced by yeasts (Fig. 2) (81, 85). Among these is again DMNT. Induced release of DMNT from plants following herbivore damage attracts natural enemies and deters some insect herbivores. In cotton leafworm, upwind flight to sex pheromone and cotton volatiles is suppressed by large amounts of DMNT due to its prominent effect on central olfactory circuits (47, 82, 86). DMNT is also a floral scent component across a wide range of plants (87) and an attractant of flies and moths (84, 88–90). Yeasts obviously contribute to DMNT release from flowers, since DMNT was found in all of the yeasts studied here (Fig. 2). In addition to DMNT, a wide range of volatiles cooccurs in yeasts and angiosperm flowers, for example, the typical Drosophila attractants acetoin, ethanol, ethyl acetate, 2-phenylethyl acetate, and 2-phenylethanol (Fig. 2) (91). 2-Phenylethanol, a typical yeast odorant (see, for example, reference 92), is also produced by green plants (see, for example, reference 93). In addition to an overlap of compounds produced by both plants and yeasts, fumigation of elderberry flowers with broad-spectrum antibiotics revealed that floral phyllospheric microbiota are unique producers of key floral terpenes (20). The yeasts investigated here all produce a range of terpenes (Fig. 2; Table S1).

Insect-yeast chemical communication evolved long before the emergence of flowering plants. Fungivory and herbivory on plants was initiated during the Early Devonian (~400 million years ago [Ma]) concurrent with the appearance of budding yeasts and prior to angiosperm pollination syndromes during the Cretaceous period (~100 Ma) (94–97). This lends support to the idea that a sensory bias for yeast-produced compounds, together with ubiquitous presence of yeasts in flowers, has contributed to the evolution of floral scent and insect-mediated pollination (23, 24, 91, 98, 99).

While the ecological and evolutionary consequences of chemical dialogue between plants, microbes and insects are unequivocal, it is yet largely unclear which of the many volatiles released by yeasts encode this interaction. A comprehensive analysis of yeast volatomes is a first and necessary step toward identifying the active compounds. Of the 192 volatiles (Fig. 2; Table S1), 33 are new for yeasts. Most of them were released by M. hawaiiensis, M. lopburiensis, and M. saccharicola, which are the most recently discovered species (64, 65). The database of yeast volatiles creates a basis for future studies, aimed at functional characterization of insect olfactory receptors and attraction bioassays, toward the identification of the behaviorally active compounds (100–102).

The overall species-specific volatome patterns showed variation between replicates, even though growth conditions were strictly controlled and sampling intervals were adjusted to cancel out growth-stage variations (Fig. 2). A general assumption in metabolomics is that identical genotypes produce the same steady-state metabolite concentrations under stringent conditions, whereas metabolic snapshots often show considerable biological variability. Metabolite-metabolite correlations derived from enzymatic reaction network activity may nonetheless be robust, despite considerable intrinsic, stochastic variation of metabolite concentrations obtained at momentary peeks into the state of an organism (103–105).

In spite of inherent variation among volatile samples, numerical headspace analysis by OPLS-DA, followed by HCA, revealed characteristic volatile fingerprints for each of the eight yeasts (Fig. 2 and 3) which align with the phylogenetic analysis based on sequences from the D1/D2 region (Fig. 1) and yeast taxonomy and ecology (Fig. 1) (63, 76). Moreover, we found that OPLS-DA exhibited a robust yeast-species assignment of
headspace samples and is therefore a useful tool for studying and classifying unknown species with regard to their volatiles (Fig. 3).

Volatile fingerprinting or chemotyping has previously been shown to differentiate between ectomycorrhizal, pathogenic, and saprophytic fungi, as a complement to genotyping (106, 107). This was confirmed by comparing *M. fructicola* and *M. andauensis*, which are taxonomically close and difficult to discriminate according to genotyping (Fig. 1) (108). They quantitatively and clearly separated according to headspace proportions, in addition to the production of methanol by *M. fructicola* (Fig. 2 and 3). Further support for the use of volatomes in species discrimination comes from an isolate from codling moth larvae, which had been misidentified as *C. tephrensis*. Comparison of headspace data with *M. fructicola* evidenced overlap of 101 compounds with significant coefficient values and nonzero confidence intervals for the whole data set. Subsequent DNA analysis identified this yeast as *M. fructicola* (Fig. 1).

The species-specific volatome differences are corroborated by a selective larval feeding response (Fig. 4), where cotton leafworm larvae, which are typical foliage feeders, prefer phyllosphere yeasts over the yeasts associated with fruit and frugivorous insects. Larvae avoided brewer’s yeast commonly found with *D. melanogaster* and *M. andauensis* and *M. pulcherrima*, which have been isolated from codling moth, *Cydia pomonella*, feeding in apple (41). It is yet unknown whether cotton leafworm forms associations with yeasts in natural habitats, but a consistent larval response to yeasts may establish and sustain such associations.

Identifying behaviorally active metabolites is key to understanding the ecology of insect-yeast interactions. Geranyl acetone, an aggregation pheromone component of *C. pomonella* larvae (109), is a distinctive compound for *C. nemorosus* (Fig. 2 and 5), which elicited the strongest larval feeding response (Fig. 4). Among the cotton leafworm olfactory receptors which have been functionally characterized, several are tuned to compounds produced by yeasts, and some even elicited larval attraction as single compounds, such as benzyl alcohol, benzaldehyde, or indole (110). For a more complete behavioral identification, it would probably be necessary to test compound blends, including candidate compounds from the headspace of *M. hawaiiensis*, *M. lopburiensis*, or *C. nemorosus* (Fig. 2, 4, and 5).

At the same time, our study reveals potential antifeedants. Camphene has indeed already been reported as a repellent in *S. littoralis* (111), and its acute larval toxicity has been shown in the sister species *S. litura* (112). In addition, *S. littoralis* females detect camphene, as well as 3-methylbutyl acetate (113), both of which are sign compounds for *M. andauensis* (Fig. 2, 4, and 5). Discriminant analysis points toward presence of attractive and antagonistic yeast volatiles and highlights compounds for future screening assays.

Cotton leafworm is polyphagous on a variety of crops, including vegetables in the Afrotropical and western Palearctic. Its sister species, *S. litura*, is found over Asia, Australasia, and Oceania, and the South-American species *S. frugiperda* has recently invaded Africa (52, 60, 79, 114). Global change and increasing food insecurity render insect control an ever more challenging and urgent task (51, 115–117). Detrimental environmental and health effects warrant the downregulation of conventional pesticides and accentuate the further development of biological insect control, comprising natural antagonists, insect pathogens, or semiochemicals.

Semiochemicals and pathogens are widely and successfully used as stand-alone techniques (118, 119), but semiochemicals could be combined with pathogens into lure-and-kill strategies (57, 58, 120). The current use of insect semiochemicals is based on controlled release formulations of synthetic chemicals, while yeasts could be used for live production of insect attractants (5, 121).

That yeasts would make suitable producers of insect attractants is supported by establishment of biofilms with strong survival ability, which enables postharvest control of fungal diseases in fruit (122–124). The combination of attractant yeasts for targeted ingestion of an insect baculovirus or a biological insecticide has been successful in laboratory and first field experiments against codling moth and spotted wing *Drosoph-
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MATERIALS AND METHODS

Yeast. Metschnikowia yeasts were purchased from the CBS-KNAW collection (Utrecht, Netherlands), except *M. fructicola*, which was isolated from apples (Alnarp, Sweden) infested with larvae of codling moth *Cydia pomonella* (Lepidoptera, Tortricidae), *Cryptococcus nemorosus* was isolated from cotton leafworm *Spodoptera littoralis* (Lepidoptera, Noctuidae), and European corn borer *Ostrinia nubilalis* (Lepidoptera, Crambidae) (62). *M. andauensis* and *M. pulcherima*, as well as occasionally *M. fructicola*, were found in apple and larval feces of caterpillars feeding on maize, corn earworm *Helicoverpa armigera* (Lepidoptera, Noctuidae), and European corn borer *Ostrinia nubilalis* (Lepidoptera, Crambidae) (62). *M. andauensis* and *M. pulcherima*, as well as occasionally *M. fructicola*, were found in apple and larval feces of caterpillars feeding on maize, corn earworm *Helicoverpa armigera* (Lepidoptera, Noctuidae), and European corn borer *Ostrinia nubilalis* (Lepidoptera, Crambidae) (62). *M. andauensis* and *M. pulcherima*, as well as occasionally *M. fructicola*, were found in apple and larval feces of caterpillars feeding on maize, corn earworm *Helicoverpa armigera* (Lepidoptera, Noctuidae), and European corn borer *Ostrinia nubilalis* (Lepidoptera, Crambidae) (62).

DNA isolation and yeast identification. Genomic DNA was isolated from overnight yeast cultures grown in liquid yeast extract-peptone-dextrose medium. Then, 2-ml portions of the overnight cell cultures were pelleted (13,000 rpm for 1 min) and washed with sterile double-distilled water (ddH₂O). The pellets were resuspended in 200 µl of lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris, 1 mM EDTA; pH 8), and 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1) mixture. Glass beads (200 µl) were then added to the tubes, and the samples were vortexed thoroughly for 3 min. To this mixture, 200 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) was added, followed by centrifugation at 13,000 rpm for 10 min. The upper aqueous phase was collected, 10 µl of RNase A (10 mg/ml) was added, and the mixtures were incubated at 37°C during 45 min. The DNA was precipitated using 300 µM sodium acetate and 3 volumes of cold absolute ethanol (99.9%), followed by centrifugation at 13,000 rpm for 10 min at 4°C. The DNA pellet was then washed in 70% cold ethanol (13,000 rpm for 10 min, 4°C), air dried, resuspended in 30 µl of TE buffer, and stored at –20°C until use.

The D1/D2 domain of the 26S rRNA gene was amplified with the universal primer pair NL-1 and NL-4 and the internal transcribed spacer (ITS) region with the primer combination ITS-1 and ITS-4 (132, 133). The PCR and product visualization on agarose gel were performed as previously described (91). PCR products were purified using ExoStar-IT (USB Corp.) according to the manufacturer’s protocol and sequenced using a BigDye v.1.1 terminator sequencing kit in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Fair Lawn, NJ).

All sequences were aligned in the program Bioedit 7.0.9.0 Sequence Alignment Editor (134). Each sequence was tested for identity and similarity against sequences deposited in the National Center for Biotechnology Information (NCBI) using Megablast search (accession numbers KF830191 and KC411961). Only similarities of >95% were considered. The phylogenetic tree of the yeast species investigated, based on nucleotide sequences of the D1/D2 domain of 26S ribosomal DNA (rDNA), was constructed using the neighbor-joining (NJ) method in MEGA 7.0 (135). The NJ method, based on evolutionary distance that minimize the total branch length during clustering of operational taxonomic units, is efficient and reliable for phylogenetic reconstructions (136). The evolutionary distances were calculated according to the Jukes-Cantor (JC) substitution model to minimize the bias due to nucleotide substitution during divergence. The JC substitution model considers the rate of substitution frequencies of all pairs of four nucleotides (A, T, G, and C) to be equal (137). The bootstrap values for the phylogenetic tree reconstruction were determined from 1,000 replications and are given next to the branch (Fig. 1).

Headspace collection and chemical analysis. Yeasts were grown in 100 ml of liquid minimal medium (138) in 250-ml culture flasks for 24 h in a shaking incubator (25°C, 260 rpm). Yeast headspace was collected by drawing charcoal-filtered air (0.125 liter/min) through a 1-liter gas wash bottle containing the yeast broth over a 35-mg SuperQ trap (80/100 mesh; Alltech, Deerfield, IL), which was held between plugs of glass wool in a 4-by-40-mm glass tube. Collections were done for ca. 24 h, at 20 to 22°C and 10 to 30 lux. The charcoal filter (50 g of activated charcoal) for incoming air and the SuperQ trap were connected with glass fittings to the wash bottle. All glassware was heated to 375°C for 10 h before use (139).

After volatile collections, the trap was extracted with 0.5 ml of redistilled hexane. Sample volumes were reduced to ca. 50 µl at an ambient temperature in Francke vials with an elongated tip (5 cm by 2 mm, inner diameter). Samples were stored in sealed glass capillary tubes at –19°C. The SuperQ trap was
wrapped in aluminum foil for protection from light. Before use, it was rinsed sequentially with 3 ml of methanol (redistilled >99.9% purity; Merck, Darmstadt, Germany) and hexane (redistilled, >99.9% purity; Labscan, Malmö, Sweden).

Yeast headspace collections were analyzed on a coupled gas chromatograph-mass spectrometer (GC-MS; 6890 GC and 5975 MS; Agilent Technologies, Palo Alto, CA), operated in the electron impact ionization mode at 70 eV. The GC was equipped with fused silica capillary columns (30 m by 0.25 mm; df = 0.25 μm), DB-Wax (J&W Scientific, Folsom, CA) or HP-5MS (Agilent Technologies). Helium was used as the mobile phase at an average linear flow rate of 35 cm/s. Two microliters of each sample were injected (splitless mode, 30 s, injector temperature 225°C). The GC oven temperature for both columns was programmed from 30°C (3-min hold) at 8°C/min to 225°C (5-min hold).

Data were exported in NetCDF file format and deconvoluted into compound spectra, elution profile, and peak area. We used MS-Omics software (Vedbaek, Denmark), the PARAFAC2 model (140), and the non-commercial package HDA (v0.910; P. Johansson, Umeå University, Umeå, Sweden), based on the H-MCR method (141). Both methods utilize covariation between samples to separate coeluting components and to pool mass spectra across samples, affording unambiguous spectra even at low signal-to-noise ratios. Approximately 70 and 30% of the peaks were separated by PARAFAC2 and H-MCR, respectively. However, neither deconvolution method produced satisfactory results for all compounds, which necessitated manual selection of the most feasible method in some cases.

Compounds were identified according to their retention times (Kovat's indices) and mass spectra, in comparison to the National Institute of Standards and Technology (NIST; v14) mass spectral library and authentic standards, on two columns. Extra care was taken to verify the identity of compounds showing high variation in abundance between yeast species. Compounds present in blank recordings of the sampling medium were subtracted.

Insects. A cotton leafworm Spodoptera littoralis (Lepidoptera, Noctuidae) laboratory colony was established using field-collected insects from Alexandria (Egypt) in 2010. This colony was interbred with wild insects from Egypt every year. Insects were raised on a semisynthetic agar-based diet (142) under the supervision of R. P. Ljunggren et al. Applied and Environmental Microbiology

ACKNOWLEDGMENTS

This study was supported by the Linnaeus environment Insect Chemical Ecology, Ethology, and Evolution (IC-E3; Formas, SLU); the Västernorrland County Administrative...
Board; the European Regional Development Fund of the European Union; and the Faculty of Landscape Architecture, Horticulture, and Crop Production Science (SLU, Alnarp, Sweden).

We gratefully acknowledge the Swedish Metabolomics Centre, Umeå, Sweden, for access to GC-MS evaluation software and helpful discussions. An anonymous reviewer and Cyrus Mahmoudi (comgraphix, Germany) provided constructive comments.

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