Multiplex Polymerase Chain Reaction Reveals Unique Trends in Pathogen and Parasitoid Infestations of Alfalfa Leafcutting Brood Cells

Justin Clements,1,5 Maggie Haylett,1 Brenda Nelson,1 Silas Shumate,1 Nicole Young,1 Benjamin Bradford,2,5 Doug Walsh,3 and Kurt Lamour4

1Department of Entomology, Plant Pathology, and Nematology, University of Idaho, Parma Research Station, ID 83660, USA, 2Department of Entomology, University of Wisconsin-Madison, Madison, WI 53706, USA, 3Department of Entomology, Washington State University, Prosser, WA 99350, USA, 4Department of Genome Science and Technology, University of Tennessee, Knoxville, TN 37996, USA, and 5Corresponding author, e-mail: justinclements@uidaho.edu

Subject Editor: Johanne Brunet

Received 15 February 2022; Editorial decision 6 June 2022.

Abstract

The alfalfa leafcutting bee Megachile rotundata Fabricius (HYMENOPTERA: Megachilidae) is an important pollinator for multiple agricultural seed commodities in the United States. M. rotundata is a solitary cavity nesting bee that forms brood nests where its larvae can develop. During the developmental stages of growth, brood can be preyed upon by multiple different fungal pathogens and insect predators and parasitoids, resulting in the loss of the developing larvae. Larval loss is a major concern for alfalfa (Medicago sativa L.) seed producers because they rely on pollination services provided by M. rotundata. Reduced pollination rates result in lower yields and increased production costs. In the present study, we examined the taxonomic composition of organisms found within M. rotundata brood cells using a multiplex PCR assay which was developed for the detection of bacterial, fungal, and invertebrate pests and pathogens of M. rotundata larvae. Known pests of M. rotundata were detected, including members of the fungal genus Ascosphaera, the causative agent of chalkbrood. The presence of multiple Ascosphaera species in a single brood cell was observed, with potential implications for chalkbrood disease management. The multiplex assay also identified DNA from more than 2,400 total species, including multiple predators and pathogenetic species not previously documented in association with M. rotundata brood cells.

Key words: Megachile rotundata, multiplex PCR, Ascosphaera

The alfalfa leafcutting bee Megachile rotundata Fabricius (HYMENOPTERA: Megachilidae) is an important pollinator for multiple agricultural commodities in the United States including alfalfa seed, canola, melons, and carrots (Pitts-Singer and Cane 2011, USDA ARS 2018). One of the primary agricultural commodities that uses M. rotundata for pollination is alfalfa (Medicago sativa L.) seed production, which provides the germplasm for alfalfa used for hay (Barnes 1980, Conrad and Klopfenstein 1988, Mueller 2008, Pitts-Singer and James 2017). Alfalfa seed production is concentrated in the Pacific Northwest and the Southwest United States. Notably, most of the alfalfa seed produced in California and Arizona is then planted in California and Arizona, while much of the seed produced in the Pacific Northwest is planted in the upper Midwest, where hay growers plant varieties requiring greater dormancy ratings. Alfalfa is the third most valuable field crop in the United States, and alfalfa hay production encompasses more than 17 million acres with a production value of more than 8.8 billion dollars annually in the United States (NAAIC 2017, USDA NASS 2018). Alfalfa forage production is only possible with the production of high-quality seed and an insect pollinator is used to generate a seed crop in alfalfa. For pollination, alfalfa seed producers rely on M. rotundata and, in limited areas, the ground dwelling alkali bee, Nomia melanderi, to fill this role (Barnes 1980, Bohart 1962, Cane 2008, Mueller 2008, Pitts-Singer and Cane 2011). The reliance on these bees has resulted in M. rotundata being one of the most heavily managed non-Apis pollinator species in the world (Pitts-Singer and Cane 2011).

M. rotundata is a solitary cavity nesting bee that constructs a nest for oviposition and larval development. Agriculturally managed M. rotundata will form nest and brood cells above ground in nesting boards placed within bee domiciles. Nest cells are generally composed of between 14 and 15 cut leaf pieces designed to encapsulate the developing brood. Individual female M. rotundata will provision the nest cells with pollen and nectar, lay a single egg on
the provisions, and seal the nest cell (Cane et al. 2011, Pitts-Singer and Cane 2011, MacIvor 2016). The brood will then undergo larval stage development and pupation into the adult stage (Pitts-Singer and Cane 2011). During this development the larvae will undergo five larval stages. After the fifth larval stage, the larvae will construct a cocoon within the nest cell. At this life stage the bee is known as a prepupa which then undergoes metamorphosis into the adult bee that emerges from the cell (Pitts-Singer and Cane 2011). This nest cell construction by a female bee requires multiple trips to flowering plants and makes alfalfa leafcutting bees a highly effective pollinator species (Pitts-Singer and James 2008, Pitts-Singer and Cane 2011). As with most pollinating species, including honey bees (Apis sp.), mason bees (Osmia bicornis), and blue orchard bees (Osmia lignaria), N. melanderi and M. rotundata are preyed upon by predatory and parasitic invertebrates and play host to numerous fungal pathogens (Tucher 1978, Gilliam et al. 1988, Rust and Torchio 1990, Pitts-Singer 2004, Gruber et al. 2011). The presence of invertebrate pests and fungal pathogens within M. rotundata brood cells is a major concern for alfalfa seed producers, as they can cause reduced pollination efficiency by resulting in brood loss. Common fungal pathogens include multiple Ascosphaera species which cause the disease known as chalkbrood. This disease only effects the developing brood (Pitts-Singer and Cane 2011). Ascosphaera are spore-cyst fungi within the family Ascosphaeraceae. Within the genus Ascosphaera, there are 28 different species (Wynns et al. 2012). While most, if not all, species of Ascosphaera are associated with bees or bee habitats, not all species are thought to be pathogenic, multiple species are proposed as saprophytes (A. atra, A. calicarpa, A. duiformis, A. fimicola, A. flavo, A. naganensis, A. pollenimica, A. subglobosa, A. tenax, A. verrucose), and the pathogenicity of some species is still unknown (Anderson et al. 1998, Vojvodic et al. 2012, Klinger 2015). However, recent studies have demonstrated that some of the species thought to be saprophytes have pathogenic capabilities, including A. atra and A. naganensis (Vojvodic et al. 2012, Krichilsky et al. 2021). Alfalfa seed growers are predominately concerned with the presence of A. aggregata within brood cells, as it is currently thought to be the major Ascosphaera species which results in M. rotundata brood loss (Goerzen 2002). However, multiple different Ascosphaera species have been detected in M. rotundata nest cells. For example, A. subglobosa, A. acerosa, A. asterophora, A. larvis, A. pollenimica, and A. proliperda have all been found in M. rotundata brood cells (Youssef et al. 1984, Goerzen 1991, Bissett et al. 1996, Wynns et al. 2012). Broods may also be attacked by other insects, including parasitic wasp species (such as Monodontomerus obscurus, Leucospis affinis, Pteromalus venustus, and Sapygus pumila), nest destroying beetles (including Tribolium audax, Tribolium brevicornis, and Tribodes ornatus), and cuckoo bees (Coelioxys and Stelis spp.), all of which feed on developing larvae and further decimate producers’ bee stocks (Eves et al. 1980). The presence of these pathogens and parasitoids can significantly impact the pollination of the alfalfa seed crop and subsequently decrease the crop yield by reducing pollinator populations and efficiency. Additionally, these pathogens and predators can reproduce within grower stocks, bee boards, and housing, and if not controlled, can result in a high abundance of dead bee larvae.

In order to protect the developing brood from different pathogens and parasitoids, growers often use a combination of disinfectants and lures and can also monitor cells for the presence of pathogens (Brindley 1976, Daves et al. 1979, Hill et al. 1984, James 2011). Detection of pathogens and parasitoids utilizes X-ray imaging as a diagnostic technique (Stephen and Undurraga 1976). However, the detection and classification of pathogens and predators within brood cells using X-ray imaging can be challenging, as it relies solely on visual assessment. With current detection practices limited to X-ray diagnostics and a few published polymerase chain reaction (PCR) amplifications for Ascosphaera species (James and Skinner 2005), we set out to examine the taxonomic makeup of M. rotundata brood cells through a multiplex deep sequencing PCR reaction. The multiplex was designed to amplify short sequence reads (150–200bp) to known and unknown pathogen, predators, and Ascosphaera species. If primers are designed correctly, multiplex technology can provide millions of short sequences which can be mapped back to reference sequences revealing the identity and composition of all species within a sample. As such, this technique can provide a glimpse into the complex biological niche of the M. rotundata brood cell.

In the current investigation, we examined the taxonomic makeup of M. rotundata brood cells to determine whether we could generate an Illumina-based DNA multiplex PCR assay that could confirm and potentially identify taxa associated with M. rotundata nest cells, including pathogens, predators, and parasitoids present in M. rotundata brood cells. To develop this assay, we classified the presence of different pathogens and predators within 4 populations of M. rotundata cells encompassing 4 grower populations from Idaho and Washington using X-ray imaging. We extracted total DNA from a set of 200 M. rotundata brood cells encompassing healthy brood cells, pathogen and predator infested brood cells, and brood cells with unknown infection status. Using an Illumina-based multiplex PCR assay we examined taxonomic makeup of the brood cells, including known and novel pathogens and predators. The analysis resulted in the identification of multiple species. Further, the multiplex analysis provided insight into which species of Ascosphaera are present in M. rotundata brood cells, including the detection of multiple Ascosphaera species within a single brood cell.

Materials and Methods

M. rotundata Sample Collection

M. rotundata samples were acquired from growers as part of the Parma Cocoon Diagnostic Laboratory service (Parma, Idaho). This service examines bee health for alfalfa seed growers through X-ray diagnostics. The Parma Cocoon Diagnostic Laboratory (PCDL) is an extension-oriented service that classifies the proportion of pathogen and parasitoid infected M. rotundata brood cells submitted by growers. PCDL uses X-ray imaging to visually classify brood cell fungal pathogens such as Ascosphaera aggregata and Ascosphaera larvis, insect parasitoids including imported chalcid wasps (Monodontomerus obscurus), cuckoo bees (Coelioxys and Stelis spp.), woodboring chalcid wasps (Leucospis affinis), long-tongued blister beetles (also known as sunflower beetles, Nemognatha lutea), Canadian chalcid wasps (Pteromalus venustus), and red-marked sapygids (Sapyga pumila), and predators and nest destroyers including American black flour beetles (T. audax), giant flour beetles (T. brevicornis), and checkered flower beetles (T. ornatus). Brood cells are shipped directly to the PCDL from commercial growers as a loose aggregate. Samples used within this experiment were acquired from multiple growers from the Pacific Northwest. Grower information cannot be disclosed as part of a confidentiality agreement. Upon arrival, samples were transferred to a sterile incubator held at 3.5°C. Samples were used in both X-ray and multiplex DNA analysis.

X-ray Analysis

Individual brood cells from each grower sample were lined up on a piece of contact film, all laboratory equipment and lab benches
were sterilized with 70% ethanol in-between populations, including using a Bunsen burner and ethanol to sterilize tweezers. In total, five 10-grm samples for each population were X-rayed using a Faxitron (Model 42855A) machine. Samples were X-rayed using Kodak Industrex-M radiographic film. Samples were processed at 20kVp for a minute exposure time. Samples were visually analyzed using defined in-house standards for fungal pathogens, invertebrate pests, dead larvae, pollen balls, and healthy larvae. After the brood cells were classified, they were removed from the contact film using sterilized tweezers and placed in sterile 1.5 ml microcentrifuge tubes (visual classification of cells can be found in Supplemental File S1).

**M. rotundata Brood Cell DNA Extraction**

DNA was extracted from individual cells using a modified CTAB extraction protocol. Individual brood cells were placed in 2 ml DNase/RNase-free homogenate tubes (Biospec, OK) with a single sterilized 6.4 mm diameter glass bead (Biospec, OK). Seven hundred and fifty µl of CTAB extraction buffer was added to each tube (OPS diagnostics, NJ). Samples were homogenized for 2 min in a Mini-beadator-16 (Biospec, OK). Tubes with homogenate were incubated at 60°C in a water bath for 30 min. Following the incubation period, samples were centrifuged for 10 min at 14,000 x g and the supernatant was transferred to new 1.5 ml tubes. Five µl of RNase solution A (20 mg/ml, Fisher Scientific, MA) was added and incubated at 37°C for 20 min. Three hundred µl of chloroform/isoamyl alcohol (24:1) was then added to each sample and vortexed for 5 s, then centrifuged for 1 min at 14,000 x g to separate the phases. The chloroform/isoamyl alcohol step was conducted twice. The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube. DNA was precipitated by adding 500 µl cold isopropanol. Samples were left for 12 h at −20°C. Samples were centrifuged at 14,000 x g for 10 min to pelletize DNA. Supernatant was decanted without disturbing the pellet and was subsequently washed with 1 ml of ice-cold 70% ethanol, and the samples were vortexed and centrifuged at 14,000 x g for 10 minutes. Ethanol was decanted and excess ethanol was removed from the pellet with a pipettor. Samples were air dried in a sterile PCR cabinet for 15 min. DNA was dissolved in 100 µl RNase/DNase-free H2O. DNA concentrations were determined using a Nanodrop 2000 (Thermo Fisher Scientific, MA). Samples were stored at −20°C until multiplex processing.

**M. rotundata Brood Cell Multiplex PCR and Data Analysis**

Total DNA from each sample was sent to Floodlight Genomics LLC (TN, USA). Floodlight Genomics used an optimized Hi-Plex approach to amplify targets in a single multiplex PCR reaction (Nguyen-Dumont et al. 2013). Primers were designed to amplify both known fungal pathogens (A. aggregata and A. larvis, as they are the two species of Ascosphaera screened by the Parma Diagnostic Laboratory), known invertebrate pests, and unknown bacteria (Supplemental File S2). The primers were also intentionally designed over large regions of genes used in phylogenetic investigations including cytochrome C oxidase, 18S ribosomal RNA, 28S ribosomal RNA, and bacterial V regions to be able to examine the overall taxonomic makeup of the M. rotundata brood cell. The sample-specific barcoded amplicons were sequenced on the Illumina HiSeq X platform according to the manufacturer’s directions. Floodlight Genomics delivered sample-specific short raw DNA sequence reads as FASTQ files. Annotation of the raw reads was performed with Geneious Bioinformatics Software (Auckland, New Zealand). Reads were grouped by both individual brood cell and as an aggregate of all brood cells. Raw reads were mapped to reference sequences (A. Aggregata, A. larvis, E. pilosula, L. affinis, M. obscurus, N. lutea, P. senustus, S. pumila, T. audax, T. brevicornis, T. ornatus, V. destructor) at 100% stringency for classification. Raw reads were also assembled (as an aggregate of all samples) into biological contigs using Geneious Bioinformatics Software (Auckland, New Zealand). Assembled contigs were uploaded into Blast2go (BioBam Bioinformatics, MA) for analysis. The National Center for Biotechnology Information (NCBI) nucleotide database was downloaded in May of 2021. The database was uploaded into Blast2go to generate a reference database. Contigs were blasted against the reference database with an E value cut of 10−10. Taxonomic identification of contigs was determined using R version 4.0.4 and the taxonomizr package (R-core team 2021). Phylogenetic trees were generated based on NCBI scientific name through phyloT v2 database 2021.1 (PhyloT 2021). Known insect parasites were classified through literature review.

**Ascosphaera DNA Extraction**

An additional set of M. rotundata sealed nest cells was classified as being infected with Ascosphaera (via X-ray diagnostics) and was placed within sterile 1.5 ml centrifuge tubes. The outside of the nest cells was sterilized prior to DNA extraction by washing with a sterilization solution designed to sterilize and remove contaminants from the outside of the M. rotundata nest cells. The sterilization solution comprised 5% 190 proof Ethanol, 1% Tween 20 (ThermoFisher Scientific, Waltham, MA), and 0.1% D-256 (Venco, St. Joseph, MO) and was generated in DNase/RNase-free water (ThermoFisher Scientific, Waltham, MA). Aliquots of 1 ml of sterilization solution were placed into 1.5 ml microcentrifuge tubes, and new aliquots were used for every nest cell. Nest cells were dipped in the sterilization solution aliquot using forceps for 30 s moving up and down within the solution. Cells were then washed with DNase/RNase-free water to remove any residues. Sterilized nest cells were then placed in sterile 2 ml DNase/RNase-free homogenate tubes (Biospec, OK) and allowed to completely dry before DNA extraction in a sterile PCR cabinet. Laboratory tweezers were sterilized between every nest cell with 70% ethanol and flame using a Bunsen burner. DNA was extracted from individual cells using a DNeasy Plant Pro Kit (Qiagen, Hilden, Germany). The DNeasy Plant Pro Kit requires limited steps compared to the CTAB method, resulting in less chance to contaminate the purified DNA. DNA concentration was determined using a Nanodrop 2000. Samples were stored at −20°C until PCR and multiplex processing.

**Ascosphaera Multiplex PCR**

Twenty IST1-5.8S-IST2 sequences from Anderson et al. 1998 were downloaded from NCBI, the IST2 region of the sequences was aligned using Geneious Prime tree builder global alignment. From the alignment we determined that the IST2 region had significant sequence differences that could be used to classify Ascosphaera species. Primers were designed to amplify the IST2 region of Ascosphaera (Supplemental File S2). Total DNA from 38 Ascosphaera samples were sent to Floodlight Genomics LLC. Floodlight Genomics used an optimized Hi-Plex approach to amplify targets in a single multiplex PCR reaction (Nguyen-Dumont et al. 2013). The sample-specific barcoded amplicons were sequenced on the Illumina HiSeq X platform according to the manufacturer’s directions. Floodlight Genomics delivered sample-specific short raw DNA sequence reads as FASTQ files. Annotation of the raw reads was performed with Geneious Bioinformatics Software (Auckland, New Zealand). To conduct the annotation, NCBI was mined for all reads corresponding
to the IST1-5.8S-IST2 region of Ascosphaera for use as references. This allowed us to examine multiple sequence reads from the same Ascosphaera species in case there were annotation errors in NCBI. Any unknown or unclassified Ascosphaera species from NCBI were removed from the analysis. Reads were treated as individual cells and mapped to reference sequences with a minimum overlap identity of 98% and only reads mapped to the single best match of the reference sequences were allowed. A threshold of 900 reads was used to determine the presence of Ascosphaera within a sample. The high threshold of 900 was used to eliminate the chance that a cell was not completely sterilized before extraction. U18362.1 sequence (A. apis) was identical to A. atra (GQ867794.1, Klinger et al. 2013), with the exception of two base pairs which were unannotated in U18362.1. Additionally, U18362.1 only had an 87.38% identity to the next closest A. apis IST1-5.8S-IST2 sequence (MH862580, Vu et al. 2018). As such, all reads mapped to U18362.1 were treated as A. atra.

Results

M. rotundata Brood Cell Multiplex PCR

In total, 21,974,525 short Illumina reads were acquired from Floodlight Genomics and mapped to a set of reference sequences to known pathogens and parasitoids of M. rotundata cells. In total, 607,769 reads were successfully mapped to a reference sequence (100% overlapping identity). Only A. aggregata, A. larvis, T. ornatus, and T. brevicornis were successfully identified within the samples using this method. A. larvis and A. aggregata were found within samples from each population analyzed. T. ornatus was classified in population 1 and T. brevicornis was classified in population 2. We noted that almost all samples had DNA which corresponded to both A. aggregata and A. larvis. This was most likely due to how samples were processed and shipped as aggregates, resulting in both fungal and invertebrate DNA contamination throughout the entire sample. While within regional samples it is possible to determine taxonomic classification, it was not possible within individual cells for this reason.

BLAST Assembly

To further explore the taxonomic makeup of the M. rotundata brood cells, we aggregated all reads into a single sample. In total, 51,056 contigs were assembled using Geneious Prime. The assembly was conducted using standard cutoffs and conditions provide by the software. The contigs were compared against the entire NCBI nucleotide database to investigate taxonomic composition of M. rotundata cells and explore new predators and pathogens of M. rotundata cells. This analysis was conducted as an aggregate of all brood cells to examine the general makeup of these complex biological systems. In total, there were 2,438 different species classified in the analysis (Supplemental File S3), including 13 archaeal, 1,716 bacterial, and 709 eukaryotic contigs which could be differentiated into biological classes (Supplemental File S4). The predominant orders of insects were Hymenoptera and Coleoptera (Fig. 1). Contigs within the class Insecta belonged to multiple different parasitic wasps, bees, and nest destroying beetles, and most of the classified parasitic taxa were associated with non-Apoidea parasitism insects. The detected insect parasites included the parasitic wasp taxa (including Chalcidoidea, Eupelminae, Melittobia spp., Mesopolobus spp., Pteromalus spp., Torymus spp., Pediothrus spp., Diaeretiella spp., Encarsia spp., Lysiphlebus spp., and Pavesia spp.), the cuckoo bee (Coelioxys pieliana), and predator and nest destroyers T. audax and T. brevicornis (Hobbs and Krunic 1971, Husband and Brown 1976, Eves et al. 1980, Gibson 1995, Habibpour et al 2002, Baur et al. 2007, Michener 2007, Stary et al. 2014, Kalyanasundaram and Kamala 2016, da Rocha-Filho et al. 2018, Suetsugu and Mita 2018, The Natural History Museum 2019, Rosanigo et al. 2020, Havelka et al. 2021, UC IPM 2021).

The BLAST results from the bacterial domain resulted in the identification of 21 phylums that could be broken into 46 different classes. The classes with the most species richness were Actinomycetia, Bacilli, Gammaproteobacteria, and Alphaproteobacteria, which made up more than 68% of the observed species. When we examined the bacteria species for known bee pathogens, we identified Spiroplasma apis, Melissosoccus plutonium, Serratia marcescens, and Lysinibacillus sphaericus, all of which have been associated with known diseases in bees (Funfhaus et al. 2018). We further investigated the division of fungal ascomycota found within M. rotundata brood cells. Members of the fungal division Ascomycota (Fig. 2) are known as sac fungi and include multiple known pathogens of bees within the class Eurotiomycetes. The contig assembly revealed multiple different known insect pathogens, including 20 Ascosphaera species. Multiple Ascosphaera species are known pathogens of bees and could be predating on and reproducing within the brood cells.

Ascosphaera Data Analysis

Deep sequencing of the IST2 region of Ascosphaera revealed the identification of the species of Ascosphaera associated with M. rotundata (Table 1). The amplification of the IST2 region resulted in the amplification of A. aggregata, A. atra, A. duoformis, A. naganensis, A. proliperda, A. solina, and A. subglobosa. A single Ascosphaera species (A. aggregata) was identified in 11 of the 27 successfully sequenced samples at a threshold of 900 sequencing reads or more while the presence of multiple Ascosphaera species was detected in the other 16 samples. Further, other Ascosphaera species were detected within the amplification, including A. larvis. However, these Ascosphaera were below the 900-sequencing read threshold for detection.

Discussion

The continued health and protection of M. rotundata is vital for the success of the alfalfa seed, alfalfa hay, and livestock industries. While M. rotundata larvae develop within their nest cell produced by the mother bee, they can be preyed upon by multiple different parasitoids, pathogens, and nest destroyers. Classification of the different pathogens, parasitoids, and predators has traditionally revolved around visual classifications and X-ray diagnostics. Within this investigation we set out to examine the taxonomic makeup of M. rotundata brood cells. Towards this end, we developed a multiplex PCR assay to examine the different Ascosphaera species within M. rotundata brood cells. We uncovered the presence of multiple Ascosphaera species within a single infected brood cell, suggesting that a developing brood could be co-infected by multiple Ascosphaera species.

We examined cells for the presence of known parasites, predators, nest destroyers, and fungal pathogens by first compiling genomic sequences from NCBI based on Eves et al. 1980, which provided current known parasitoids and pathogens of M. rotundata brood cells. From these sequences, we designed PCR primers and conducted a multiplex PCR reaction to confirm and classify known pathogens, parasites, predators, and nest destroyers of M. rotundata (Eves et al. 1980) within our samples to better understand the taxonomic
composition of *M. rotundata* brood cells. Approximately 600,000 reads were successfully mapped to compiled references sequences, and these reads revealed the presence of *A. aggregata*, *A. larvis*, *T. ornatus*, and *T. brevicornis* within our *M. rotundata* brood cell samples. After this mapping, there were over 21 million reads that remained unmapped. To explore unmapped reads and examine the taxonomic makeup of the *M. rotundata* cell, we chose to assemble the short reads into contigs and BLAST against the entire NCBI database.

The BLAST analysis revealed the diversity of the composition of *M. rotundata* cells, resulting in 2,438 different species classified. It is important to note that this investigation could not differentiate between environmental contaminants within the samples and matched contigs to the best, available NCBI sequence. Environmental DNA could have been picked up from the foraging mother *M. rotundata* from plant material as she provisioned her nest cells, including fecal material deposited by other insects or contamination from interactions with the grower. Further, a limited set of contigs were matched to DNA representing species not found in North America. Understanding the limitations of this investigation, we chose to examine likely fungal pathogens and insect predators and parasites. Within our BLAST investigation, we noted the presence of more than 20 *Ascosphaera* species. We further examined the presence of invertebrate pests and noted multiple species that have the potential to predate on insects, including bees. These included parasitic wasps, the cuckoo bee (*Coelioxys pieliana*) and predator/nest destroyers *T. audax* and *T. brevicornis*. However, with multiple species lacking sequence data in NCBI, it is hard to make a definitive identification. As such, our approach is useful for providing class and family identifications and to generate direction for future investigations.
including providing insight into future sequencing endeavors to produce more comprehensive data for taxonomic classification.

One interesting result from the BLAST analysis was the abundance of different *Ascosphaera* species found associated with *M. rotundata* cells. To better classify the identity of the *Ascosphaera* species in association with *M. rotundata*, we decided to sequence the ITS2 region of the IST1-5.8S-IST2 nucleic acid sequence of infected bee cadavers. The results suggest the presence of multiple different *Ascosphaera* species within the infected cadaver and the sanitized nest cell. While the presence does not directly indicate whether the *Ascosphaera* species is pathogenic, the association suggests a potential link to pathogenic traits. However, the pathogenicity of each species needs to be further explored. Assuming that the presence of *Ascosphaera* detected within the cells is associated with infectivity, our findings suggest that *M. rotundata* brood cells can be infected by multiple different *Ascosphaera* species, including being co-infected by multiple *Ascosphaera* species at the same time. Within the honeybee species *Apis mellifera*, co-infection by multiple *Ascosphaera* have been demonstrated, and co-infections with *A. atra* and *A. apis* resulted in higher mortality when compared to infection with *A. apis* alone, (Vojvodic et al. 2012). From our analysis, we noted that most infected brood were co-infected with multiple different *Ascosphaera* species including, *A. aggregata*, *A. atra*, *A. duoformis*, *A. naganensis*, *A. proliperda*, *A. solina*, and *A. subglobosa*. We also noted the presence of other *Ascosphaera* species, including *A. larvis*, within the samples. However, they were below the detection threshold cutoff, suggesting *A. larvis* within this investigation was not a predominate *Ascosphaera* species within the examined infected brood. Most samples were infected with *A. aggregata*, which is the major concern for alfalfa seed growers, but 5 of the infected brood samples had no *A. aggregata* detected within the samples (above the detection threshold) and were primarily infected with *A. atra*, *A. duoformis*, or *A. proliperda*. The findings suggest that other species of *Ascosphaera* (besides *A. aggregata*) may be contributing to chalkbrood within *M. rotundata*. Interestingly, Vojvodic et al. 2012 demonstrated that *Ascosphaera* species thought not to be pathogenic to bees (*A. atra*) was able to infect *A. mellifera* and cause mortality. We detected this *Ascosphaera* species in relatively high abundance within multiple cells. Laboratory investigations have demonstrated that *M. rotundata* brood can be co-infected with multiple *Ascosphaera* species. However, these studies also demonstrate that the most virulent species will out compete lesser-virulent *Ascosphaera* species (Klinger et al. 2015). In the present study, a similar trend was observed. We found that *A. aggregata*, a highly virulent species, was more abundant during co-infection compared to other species when they were present within the same sample. Our study was limited to a

![Fig. 2. Fungi division Ascomycota designation of contigs found within *M. rotundata* brood cells.](image)

* Unable to map Curvularia umbiliciformis, Neopetractis nodispora, Huriella flasksi, and Lilipila oculisporrella
Table 1. Multiplex amplification of *M. rotundata* cells infested with *Ascosphaera*

| Sample | Total reads | Total mapped reads | A. aggregata % of reads | A. atra % of reads | A. duoformis % of reads | A. naganensis % of reads | A. prolipera % of reads | A. solina % of reads | A. subglobosa % of reads |
|--------|-------------|-------------------|-------------------------|-------------------|------------------------|-------------------------|-------------------------|----------------------|-------------------------|
| Cell 94 | 77,919      | 47,013            | 46,709 99.35            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 104 | 282,506    | 176,881           | 176,881 99.46           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 129 | 211,313    | 115,963           | 98,814 85.21            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 130 | 326,567    | 191,318           | 189,771 99.19           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 132 | 81,211     | 44,158            | 35,733 80.92            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 136 | 192,195    | 112,013           | 111,130 99.21           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 138 | 257,281    | 153,167           | 152,078 99.29           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 197 | 73,888     | 44,158            | 35,733 80.92            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 266 | 163,785    | 8,335             | 3,196 38.26             | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 323 | 401,653    | 230,780           | 164,027 7.11            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 331 | 64,072     | 38,602            | 36,358 94.19            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 360 | 288,689    | 184,349           | 178,214 96.67           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 364 | 140,250    | 76,537            | 60,688 79.29            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 679 | 83,903     | 48,599            | 47,268 97.26            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 682 | 240,179    | 135,744           | 129,662 95.52           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 683 | 200,060    | 92,720            | 90,048 97.12            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 684 | 290,546    | 132,595           | 100,347 73.68           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 685 | 80,706     | 37,768            | 30,297 80.22            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 689 | 273,209    | 140,647           | 99.00                   | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 691 | 332,776    | 185,969           | 183,243 98.63           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 692 | 91,075     | 52,358            | 50,051 96.41            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 693 | 250,182    | 121,074           | 120,967 99.60           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 698 | 227,400    | 130,510           | 137,737 99.44           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 724 | 254,102    | 152,000           | 151,956 99.50           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 733 | 106,860    | 62,651            | 62,281 99.44            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 734 | 231,138    | 130,760           | 130,510 99.44           | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
| Cell 754 | 130,253    | 73,773            | 57,358 77.75            | 0 0               | 0 0                    | 0 0                     | 0 0                     | 0 0                  | 0 0                     |
subset of infected *M. rotundata* brood cells, and there is a possibility that other brood cells, particularly from different growing regions, could be infected with different *Ascosphaera* species which were not detected within this investigation. It is also possible that our alignments may be matching unclassified *Ascosphaera* species to annotated NCBI sequences. This suggests the need for more in-depth genomic data for this agriculturally relevant species. From our data, it is clear that multiple *Ascosphaera* species are associated with *M. rotundata* cells. More research should be conducted to determine how the interactions between different *Ascosphaera* species may be affecting their pathogenicity.

This investigation revealed the complex taxonomic diversity of *M. rotundata* brood cells, with more than 2,400 different known species of plants, fungi, bacteria, archaea, and insects comprising the brood cell. We further identified multiple different species that have the potential to predate on *M. rotundata* which should be examined in more detail, including species of *Ascosphaera* that may cause the disease phenotype known as charkbrook. Pollinators will continue to be a vital resource for alfalfa seed producers, and as such, the agricultural community and growers should be on the lookout for new and emerging pathogens and parasitoids of leafcutting bees.

**Acknowledgment**

This research was supported by funding from United States Department of Agriculture Alfalfa Pollinator Research Initiative (58-2080-0-009).

**Authors’ Contributions**

JC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing - original draft, Writing - review & editing. MH: Formal analysis, Methodology, Writing - review & editing. BN: Formal analysis, Methodology, Writing - review & editing. SS: Formal analysis, Methodology, Writing - review & editing. NY: Formal analysis, Methodology, Writing - review & editing. BB: Formal analysis, Methodology, Writing - review & editing. DW: Conceptualization, Methodology, Writing - review & editing. KL: Conceptualization, Methodology, Resources, Writing - review & editing.

**Supplementary Data**

Supplementary data are available at *Journal of Insect Science* online.

**Supporting information**

Supplemental File S1: *Megachile rotundata* bee cells classification used in multiplex analysis
Supplemental File S2: *Megachile rotundata* cells Multiplex, *Ascosphaera* PCR, and *Ascosphaera* cells Multiplex primers
Supplemental File S3: Taxonomic classification of contigs
Supplemental File S4: Taxonomic tree of class identification of *Megachile rotundata* bee cells

**References Cited**

Anderson, D. L., A. J. Gibbs, and N. L. Gibson. 1998. Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences. *Mycol. Res.* 102: 541–547.

Barnes, D. K. 1980. Alfalfa, pp. 177–187. In F. R. Water and H. H. Hadley (eds.), *Hybridization of crop plants*. American Society of Agronomy and Crop Science Society of America, Madison, WI.

Baur, H., F. J. Muller, G. A. Gibson, P. G. Mason, and U. Kuhlmann. 2007. A review of the species of Mesopodolobus (Chalcidoidea: Pteromalidae) associated with *Ceutorhynchus* (Coleoptera: Curculionidae) host-species of European origin. *Bull. Entomol. Res.* 97: 387–397.

Bissett, J., G. Duke, and M. Goettel. 1996. *Ascosphaera acerosa* sp. nov. isolated from the alfalfa leafcutting bee, with a key to the species of *Ascosphaera*. *Mycologia.* 88: 797–803.

Bohart, G. E. 1962. How to manage the alfalfa leaf-cutting bee (*Megachile rotundata* Fab.) for alfalfa pollination. *Utah Agric. Exp. Stat. Circ.* 144.

Brindley, W. A. 1976. Carbohydr control of chalcoid parasites from alfalfa leafcutting bees. *J. Econ. Entomol.* 69: 225–228.

Cane, J. H. 2008. A native ground-nesting bee (*Nomia melanderi*) sustainably managed to pollinate alfalfa across an intensively agricultural landscape. *Apidologie.* 39: 315–323.

Cane, J. H., D. R. Gardner, and P. A. Harrison. 2011. Nectar and pollen sugars constituting larval provisions of the alfalfa leaf-cutting bee (*Megachile rotundata*) (*Hymenoptera: Apiformes: Megachilidae*). *Apidologie.* 42: 401–408.

Conrad, H. R., and T. J. Klopfenstein. 1988. Role in livestock feeding—Greechop, silage, hay, and dehy, pp. 539–551. In A. A. Hanson, D. K. Barnes, and R. R. Hill Jr. (eds.), *Alfalfa and alfalfa improvement*, vol. 29. American Society of Agronomy and Crop Science Society of America, and Soil Sciences of America, Madison, WI.

Davis, H. G., J. D. Eves, and L. M. McDonough. 1979. Trap and synthetic lure for the checkered flower beetle, a serious predator of alfalfa leafcutting bees. *Environ. Entomol.* 8: 147–149.

Eves, J. D., D. F. Mayer, and C. A. Johansen. 1980. Parasites, predators, and nest destroyers of the alfalfa leafcutting bee, *Megachile rotundata*. *West. Reg. Exp. Est.* 32: 1–15.

Fünfhaus, A., J. Ebeling, and E. Genersch. 2018. Bacterial pathogens of bees. *Carr. Opin. Insect Sci.* 26: 89–96.

Gibson, G.A. 1995. *Parasitic wasps of the subfamily Eupelminae*. Associated Publishers, Florida, USA.

Gilliam, M., S. Taber, III, B. J. Lorenz, and D. B. Prest. 1988. Factors affecting development of charkbrook disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. *J. Invertebr. Pathol.* 52: 314–325.

Goerzen, D. W. 1991. Microflora associated with the alfalfa leafcutting bee, *Megachile rotundata* (Fab) (*Hymenoptera: Megachilidae*) in Saskatchewan, Canada. *Apidologie.* 22: 533–561.

Goerzen, D. W. 2002. Chalkbrook disease in alfalfa leafcutting bee populations. *Saskatoon* 527: S3 Extension Publ. No. 2002 – 02. Revised 2016.

Gruber, B., K. Eckel, J. Everaars, and C. F. Dormann. 2011. On managing the red mason bee (*Osmia bicornis*) in apple orchards. *Apidologie.* 42: 564–576.

Habbour, B., K. Kamali, and J. Meidani. 2002. Insects and mites associated with stored products and their arthropod parasites and predators in Khuzestan province (Iran). *IOBC WPRS Bull.* 25: 89–92.

Havelka, J., M. Kaluzhna, J. Danilov, and R. Rakauskaus. 2021. Pauses species (*Hymenoptera: Braconidae: Aphidiinae*) attacking *Euclanchis aphid* (Hemiptera: *Aphididae: Lachninae*) on coniferous plants in Lithuania: ecological and mitochondrial COI diversity. *Org. Divers. Evol.* 21: 561–573.

Hill, B. D., K. W. Richards, and G. B. Schaalje. 1984. Use of dichlorvos resin strips to reduce parasitism of alfalfa leafcutter bee (*Hymenoptera: Megachilidae*) cocoons during incubation. *J. Econ. Entomol.* 77: 1307–1312.

Hobbs, G. A., and M. D. Krunic. 1971. Comparative behavior of three chalcidoid (*Hymenoptera*) parasites of the alfalfa leafcutter bee, *Megachile rotundata*, in the laboratory. *Can. Entomol.* 103: 674–683.

Hubbard, R. W., and T. M. Brown. 1976. Insects associated with Michigan bumblebees (bombus spp.). *Gt. Lakes Entomol.* 9: 3.

James, R. R. 2011. Chalkbrood transmission in the alfalfa leafcutting bee: the impact of disinfecting bee cocoons in loose cell management systems. *Environ. Entomol.* 40: 782–787.

James, R. R., and J. S. Skinner. 2005. PCR diagnostic methods for *Ascosphaera* infections in bees. *J. Invertebr. Pathol.* 90: 98–103.
MacIvor, J. S., Krichilsky, E., M. Centrella, B. Eitzer, B. Danforth, K. Poveda, and H. Grab. 2021. Mixed infections reveal virulence differences between host-specific bee pathogens. *J. Invertebr. Pathol.* 129: 28–35.

Michener, C. D. 2004. *The bees of the world*, 2nd ed. Johns Hopkins University Press, Baltimore and London.

Muller, S. C. 2008. Producing Quality Alfalfa seed for the forage industry. In Proceedings, 2008 California Alfalfa & Forage Symposium and Western Seed Conference, San Diego, CA, 2–4.

Mueller, S. C., C. A. Garófalo, and G. A. Gibson. 2018. DNA barcoding to identify leaf preference of leafcutting bees. *R. Soc. Open Sci.* 5: 150623.

NCBI. 2021. Importance of Alfalfa. (https://www.ncbi.nlm.nih.gov/)

NAAIC. 2017. *The bees of the world*, 2nd ed. Johns Hopkins University Press, Baltimore and London.

Pitts-Singer, T. L. 2016. *The bees of the world*, 2nd ed. Johns Hopkins University Press, Baltimore and London.

PhyloT. 2021. A tree generator. (https://phylo.biobyte.de/)

Pitts-Singer, T. L., and R. R. James. 2008. Past and present management of alfalfa bees, pp. 105–123, in T. L. Pitts-Singer, and R. R. James (eds.), *Bee pollination in agricultural ecosystems*, vol. 9. Oxford University Press, New York, USA.

Pitts-Singer, T. L., and J. H. Cane. 2011. The alfalfa leafcutting bee, *Megachile rotundata*: the world’s most intensively managed solitary bee. *Annu. Rev. Entomol.* 56: 221–237.

R-Core Team. 2021. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

R Core Team. 2021. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rosanigo, M. P., H. J. Marrero, and J. P. Torretta. 2020. Limiting resources on the reproductive success of a cavity-nesting bee species in a grassland agroecosystem. *J. Apic. Res.* 59: 583–591.

Rust R.W, and P.F. Torchio. 1990. Induction and incidence of *Ascosphaera* infections in the blue orchard bee, *Osmia lignaria* propinqua (Hymenoptera: megachilidae), pp. 169–172, in VI International Symposium on Pollination 288.

Starý, P., N. G. Kavallieratos, A. Petrović, V. Žíček, E. Rakshani, S. Tomanović, Z. Tomanović, and J. Havelka. 2014. Interference of field evidence, morphology, and DNA analyses of three related Lysiphlebus aphid parasitoids (Hymenoptera: Braconidae: Aphidiinae). *J. Insect Sci.* 14: 1.

Stephen, W. P., and J. M. Undurraga. 1976. X-radiography, an analytical tool in population studies of the leafcutter bee *Megachile pacifica*. *J. Apic. Res.* 15: 81–87.

Suetugui, K., and T. Mita. 2018. *Pedioius metallicus* (Hymenoptera: Eulophidae): first record of a parasitoid wasp of the agromyzid fly *Naranagromyza tokunagai*, a serious pest of orchids. *J. Asia-Pac. Entomol.* 21: 1289–1291.

The Natural History Museum. 2019. Universal Chalcidoidea Database: Chalcididae. (www.nhm.ac.uk)

UC IPM. 2021. Pest management identification ‘Encarsia Formosa’. (http://ipm.ucanr.edu/natural-enemies/encarsia_formosa.html)

USDA ARS. United States Department of Agricultural Research Service. 2018. Alfalfa Leafcutting Bee (ALCB). (https://www.ars.usda.gov/pacific-west-area/logan-ut/pollinating-insect-biology-management-systematics-research/docs/alfalfa-leafcutting-bee-alcb/)

USDA NASS. United States Department of Agriculture National Agricultural Statistics Service. 2018. Crop production 2018 summary. (https://www.nass.usda.gov/Publications/Todays_Reports/reports/crop2018.pdf)

Vojvodic, S., J. J. Boomsma, J. Eilenberg, and A. B. Jensen. 2012. Virulence of mixed fungal infections in honey bee brood. *Front. Zool.* 1: 1–6.

Vu, D., M. Groenewald, M. De Vries, T. Gehrmann, B. Stielow, U. Eberhardt, A. Al-Hatmi, J. Z. Groenewald, G. Cardinale, J. Houbreken, et al. 2018. Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Stud. Mycol.* 91: 23–36.

Wynns, A. A., A. B. Jensen, J. Eilenberg, and R. James. 2012. *Ascosphaera subglobosa*, a new spore cyst fungus from North America associated with the solitary bee *Megachile rotundata*. *Mycologia.* 104: 108–114.

Youssef, N. N., C. F. Roush, and W. R. McManus. 1984. In vivo development and pathogenicity of *Ascosphaera proligerda* (Ascosphaeraceae) to the alfalfa leafcutting bee, *Megachile rotundata*. *J. Invertebr. Pathol.* 43: 11–20.