Methods

1. Specimens

Light traps were installed near peanut fields in Worth County (Georgia) and checked regularly. At each inspection, samples were collected in 70% ethanol and stored in a -20C freezer until specimens were sorted and burrower bugs identified with the aid of a stereo-microscope, and validated by molecular methods (see below). Burrower bugs were collected in light-traps during the months of April, May, June, August, September and October in 2020, and July, August, September and October 2021. DNA was extracted from these samples using the DNEasy Blood and Tissue kit (Qiagen) and stored in a -20C freezer.

1. Molecular validation of species identification

Species identification was validated for samples that were also submitted for RAD-seq sequencing. RAD-seq data was aligned against draft genomes that we sequenced for *P. billineatus* (XXaccessionnumberXX) and *D. lugubris* (XXaccessionnumberXX), using bowtie2 (Langmead & Salzberg, 2012). *D. lugubris* was first identified among the samples collected in 2021.

1. Diet Analysis

Gut content was profiled by PacBio sequencing based on the protocol published by Cooper et al. (2019). Briefly, DNA from all samples were amplified using primers targeting the chloroplast gene *trnF*: B49873-e: GGT TCA AGT CCC TCT ATC CC and A50272-F: ATT TGA ACT GGT GAC ACG AG (Taberlet et al. 1991). Different barcode combinations were added to these primers (forward and reverse), to allow multiplexing samples for sequencing (Suppl Table X).

In addition, ten negative control samples were included, to control for contamination in the reagents. All samples were sent for PacBio sequencing at the Washington State University Laboratory for Biotechnology and Bioanalysis.

1. *trnF* sequence analysis

3.1 Reference database

We built a custom database to define parameters to identify and classify our sequencing data. The entire RefSeq Plastid genomic database (NCBI, release 219) was downloaded, unzipped and concatenated into a single file. For the next steps we used Mothur (version 1.47; Schloss et al., 2009). We first performed an *in silico* PCR to amplify only the region targeted by our *trnF* primers B49873-e and A502720-F. Previously, we had found that most of our PacBio sequences were 400-600bp long (Cooper et al., 2019), but there were cases of sequences smaller than 100bp and greater than 1500bp. In fact, it is known that the gene *trnF* can greatly vary in size because of repeats and pseudogenes (e.g. Vijverberg et al. 1999; Yan et al., 2019 ). Based on this, we decided to take a conservative approach for quality-filtering our data, and excluded from our plastid database any sequence smaller than 100bp, or longer than 10K bp, which were likely non-target amplifications or plastids from unicellular organisms (e.g. algae). In addition, we also excluded all sequences with greater than 5 ambiguities. The median size of sequences passing these filters was 399bp, ranging from 101bp to 9119bp, for a total of 6,988 sequences. Next, we used custom scripts to add and format taxonomical rank information to the header section of each sequence, so that it can be read by the DADA2 classifier.

3.2 PacBio data

All data analyses were performed in R (version 4.2.1) and Rstudio (version 2023.06.1, Rstudio Team, 2023). PacBio data was quality-trimmed, assembled into ASV and classified using DADA2 (version 1.22.0; Callahan et al., 2016). Briefly, each sequencing batch (three total) started being processed separately as following: primer sequences were trimmed, and amplicons were removed if they presented unidentified nucleotides (“N”), and/or a quality score smaller than 3, and/or length smaller than 100bp. In addition, we set TruncQ to 2, and also excluded sequences with a value greater than 8 for maxEE, i.e. the maximum number of expected errors (EE), with EE defined as sum(10^(-Q/10)), where Q is the quality score. Next, sequences were dereplicated and we denoised sequences using DADA2 error estimation algorithm, with band size set to 32bp and the DADA2’s error model for PacBio data. All three batches of quality-trimmed, denoised PacBio sequences were merged, and chimeric sequences were identified and removed using the “removeBimeraDenovo” function in DADA2. Finally, all sequences were classified using our custom database as reference (trnF\_db11Feb22.fa), and the DADA2’s Bayesian naïve classifier algorithm.

After chimera removal and taxonomical assignment, data was transformed into a phyloseq object (McMurdie & Holmes, 2013) and decontaminated using the package “decontam” (Davis et al., 2017). We found that a number of likely contaminants were present in the control samples, such as sequences identified as *Citrus* and *Pinus*. After further investigation, we found that the water used to dilute the primer solutions was contaminated by these taxa, in addition to other plant and algae taxa. Although these are obvious contaminants, we cannot rule out the possibility that some of these contaminants may also be present as diet items, in particular taxa identified as native plants (e.g. *Carya* and *Pinus*). Therefore, we set up our filter to a moderately-high threshold of sensitivity (0.5) and removed all 15 ASV identified as contaminants from our samples. To further improve taxonomy, we also used the NCBI Blast tool against their nt database to classify the remaining ASVs without a genus ID (e-value = 1e-30, percent ID 90-100% for genus identification). Next, we removed any ASV with a classification other than Viridiplantae (Kingdom), or ASVs identified as mosses (Class Bryopsida). Therefore, only spermatophytes were left for downstream analysis.

The burrower bug genomes used to map RAD-seq data indicated that we had unintentionally sampled two species: one that aligned well to our reference *P. billineatus* draft genome (“high-mapper” species) and another one that presented poor alignment (“low-mapper” species). To determine which one was *P. bilineatus*, we PCR’ed the most recent batch of samples (Batch 3), which were sequenced for both RAD-seq and molecular gut content analyses. We targeted the COI region using primers BF3-BR2 (Elbrecht et al., 2019; Elbrecht et al., 2018; Elbrecht et al., 2017), amplifying a region 400bp in length. We compared these sequences to COI sequences generated for other, morphologically similar species found in the same area: *Dallasiellus lugubris*, *Cyrtomenus ciliatus*, and *Sehirus cinctus*, obtained from sequencing lab DNA extractions and from the NCBI nucleotide database. Using this method, we found that the low-mapper species is *Dallasiellus lugubris*, whereas the high-mapper samples were *P. billineatus* (Supplemental Figure). This method was used to identify species in samples belonging to batches 2 and 3, which were sequenced for both RAD-seq and diet analyses. DNA from batch 1 was only used for diet analysis.

For diversity analysis, we aggregated ASVs at the genus level. Alpha diversity (Shannon index) was calculated for each species and month of sampling. Principal Component Analysis (PCA) was used to evaluate beta-diversity using Euclidean distances to calculate a matrix of dissimilarity between samples. In order to account for different sampling depth, samples were rarefied to 100 sequences per sample, reducing the number of samples from 153 to 78 samples. Permutational multivariate analysis of variance (PERMANOVA) was used to test for batch effects, and variables to explain diet patterns observed within and between species. Sanger sequences and trees were assembled and analyzed using Geneious Prime® (version 2023.1.2). All other analyses were performed in R, using the following R packages: tidyverse (Wickham et al., 2019), ggplot2 (Wickham, 2016), RcolorBrewer (Neuwirth, 2022), ggside(Landis, 2022), vegan (Oksanen et al., 2022).

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