



# Patterns of Fungal Community Assembly Across Two *Culex* Mosquito Species

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In the aquatic environment, mosquito larvae encounter bacteria and fungi that assemble into bacterial and fungal communities. The composition and impact of mosquito-associated bacterial community has been reported across larvae of various mosquito species. However, knowledge on the composition of mosquito-associated fungal communities and the drivers of their assembly remain largely unclear, particularly across mosquito species. In this study, we used high throughput sequencing of the fungal Internal transcribed spacer 2 (ITS2) metabarcode marker to identify fungal operational taxonomic units (OTUs) and amplicon sequence variants (ASVs) associated with field-collected *Culex restuans* and *Culex pipiens* larvae and their breeding water. Our analyses identified diverse fungal communities across larval breeding sites collected on a fine geographic scale. Our data show that the larval breeding site is the major determinant of fungal community assembly in these mosquito species. We also identified distinct fungal communities in guts and carcasses within each species. However, these tissue-specific patterns were less evident in *Cx. restuans* than in *Cx. pipiens* larvae. The broad ecological patterns of fungal community assembly in mosquito larvae did not vary between OTU and ASV analyses. Together, this study provides the first insight into the fungal community composition and diversity in field collected *Cx. restuans* and *Cx. pipiens* larvae using OTUs and ASVs. While these findings largely recapitulate our previous analyses in *Aedes albopictus* larvae, we report minor differences in tissue-specific fungal community assembly in *Cx. restuans* larvae. Our results suggest that while the fungal community assembly in mosquito larvae may be generalized across mosquito species, variation in larval feeding behavior may impact fungal community assembly in the guts of mosquito larvae.

**Keywords:** fungal community, *Culex pipiens*, *Culex restuans*, larvae, fungal diversity

## INTRODUCTION

Bacterial communities “i.e., microbiota” associated with mosquito larvae have been well-characterized across several mosquito species using culture-dependent and culture-independent methods (Rani et al., 2009; Chavshin et al., 2012; Gimonneau et al., 2014; Kim et al., 2015; Coon et al., 2016; Bascuñán et al., 2018; Galeano-Castañeda et al., 2019; Scolari et al., 2021). In addition, the impact of these communities on mosquito life history and vector competence have been

reported (Gaio et al., 2011; Dennison et al., 2014; Coon et al., 2016, 2017; Wang et al., 2018). Knowledge on mosquito-fungus interactions is largely confined to fungal entomopathogens of mosquito larvae and adults (Christophers, 1952; Jenkins, 1964; Scholte et al., 2004; Tawidian et al., 2019) or yeasts as food source for mosquito larvae and support larval development to adulthood (Asahina, 1964; Timmermann and Briegel, 1996; Steyn et al., 2016; Coon et al., 2017; Souza et al., 2019). However, mosquito-fungus encounters are not restricted to entomopathogenicity but can include symbiotic interactions that may further impact mosquito life history. More recently, studies have characterized the fungal communities of several species of field collected mosquito adults, including *Aedes aegypti*, *Aedes albopictus*, *Aedes japonicus*, *Aedes triseriatus*, *Anopheles coluzzii*, and *Culex quinquefasciatus* (Muturi et al., 2016; Krajacich et al., 2018; Thongsripong et al., 2018; Luis et al., 2019). In addition, recent studies report the fungal community composition and assembly in field collected *Ae. albopictus* and *Ae. aegypti* larvae (Tawidian et al., 2021; Zouache et al., 2022). However, it remains unclear whether similar patterns in fungal community assembly are observed in the larvae of other mosquito species.

Mosquito larval breeding sites harbor rich bacterial and fungal communities that vary across large and small geographic scales (Gimonneau et al., 2014; Coon et al., 2016; Bascuñán et al., 2018; Shelomi, 2019; Tawidian et al., 2021). The microbiota associated with field-collected mosquito larvae is largely influenced by the bacterial community in the aquatic habitat they reside in. Indeed, studies report a considerable overlap of bacterial taxa among mosquito larvae and their breeding environment (Gimonneau et al., 2014; Kim et al., 2015; Coon et al., 2016; Scolari et al., 2021). Similarly, fungal communities associated with field-collected *Ae. albopictus* and *Ae. aegypti* larvae reflect those in the larval breeding site (Tawidian et al., 2021; Zouache et al., 2022). In addition, tissue-specific fungal communities were observed in *Ae. albopictus* larval guts and carcasses, driven by mosquito feeding behavior and fungal mode of nutrition (ecological guild) (Tawidian et al., 2021). To date, no studies have characterized the fungal communities in larvae of any other mosquito species or the aquatic habitat they inhabit.

The overall goal of this study was to determine whether the fungal community assembly observed in the larvae of *Aedes* mosquitoes also hold true for *Culex restuans* and *Culex pipiens* mosquito larvae. In the United States, *Cx. restuans* and *Cx. pipiens* are mosquitoes of public health concern due to the transmission of the causative agent of West Nile Virus by the adult females (Andreadis et al., 2004; Andreadis, 2012; Rochlin et al., 2019). *Cx. restuans* and *Cx. pipiens* larvae exhibit a temporal pattern of prevalence in urban and suburban areas in east-central United States with *Cx. restuans* larvae being abundant in early summer, whereas *Cx. pipiens* larvae dominate in mid- to late summer (Jackson and Paulson, 2006; Gardner et al., 2013; Johnson et al., 2015). Larvae of both species occupy natural and human-made breeding sites (e.g., woodland pools, tires, catch basins, and storm drains) (Yee, 2008; Rochlin et al., 2019). In this study, we collected *Cx. restuans* and *Cx. pipiens* L4 larvae from various natural and human made mosquito breeding sites in the midwestern U.S. city of Manhattan in Kansas. To determine

the fungal community composition and diversity in breeding water and mosquito larval guts and carcasses, we amplified and sequenced the fungal internal transcribed spacer 2 (ITS2) metabarcode marker and analyzed the data using OTU and ASV-based approaches. Both approaches congruently identified the larval breeding habitat as the major driver of fungal community assembly in these mosquito species. In addition, we identified different fungal communities across mosquito tissues, likely driven by mosquito feeding behavior and fungal nutritional mode. However, the variation in fungal communities in the larval guts and carcasses was less evident in *Cx. restuans* larvae, potentially because of lesser larval feeding as reflected by the low OTU/ASV richness across *Cx. restuans*.

## MATERIALS AND METHODS

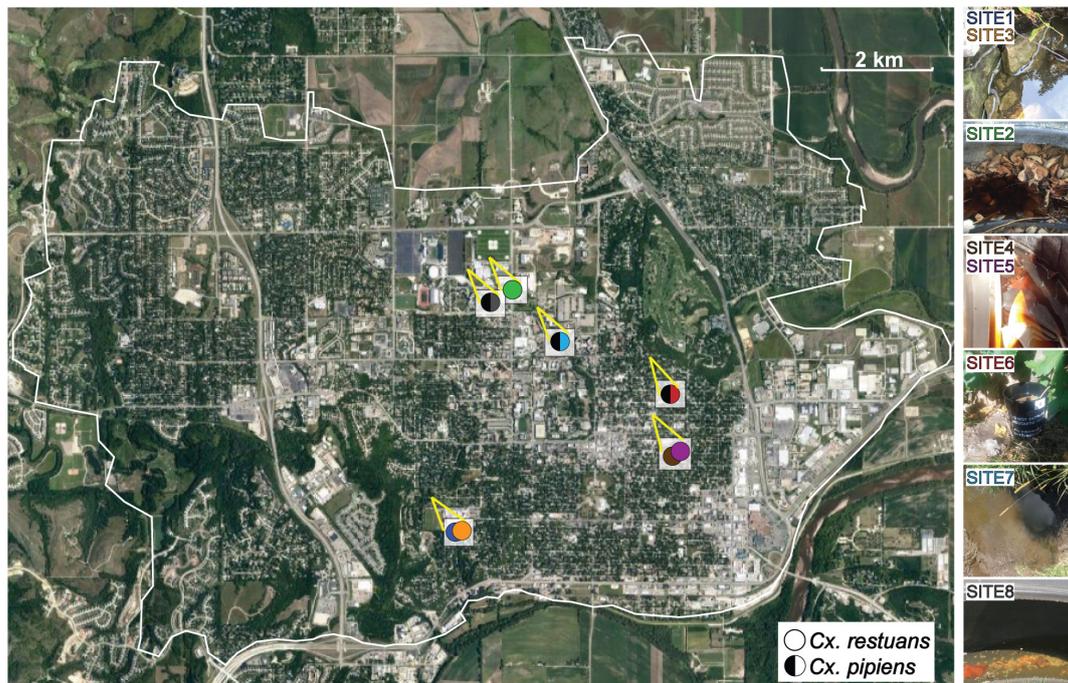
### Sampling

A total of ten *Cx. restuans* and *Cx. pipiens* L4 mosquito larvae and a single water sample (50 mL) were collected from each of eight larval breeding sites in Manhattan, KS. One breeding site (site 1) was sampled during 2017, whereas the remaining seven sites were sampled during 2018. Breeding sites 2, 4, 5, 6, and 8 were man-made containers consisting of tires, plastic containers, and a mosquito oviposition cup lined with seed germination paper (Anchor Paper Co., St Paul, MN, United States) (Figure 1). Sites 1, 3, and 7 were natural mosquito breeding sites consisting of small ponds (Figure 1). Site 1 was revisited a year after the first collection, and the revisited collection is referred to as site 3. Site 4 was revisited 2 months after the first collection and is referred to as site 5. Table 1 depicts sampling locations, dates, and mosquito species found at each larval breeding site. Mosquito larvae were collected in their breeding environments and transported to the laboratory in plastic containers (Bare Eco-Forward Rpet Deli Container).

### Sample Processing

In 2017, the larvae were incubated in their breeding water for 24 h at 27°C with 75% RH prior to gut dissection. In 2018, larvae were dissected immediately. Mosquito dissections were performed as described previously (Tawidian et al., 2021). In brief, larvae were washed six times with sterile Milli-Q water prior to gut dissection to eliminate fungal carryover from the breeding water and decapitated to remove loosely attached fungi from the breeding water on the mosquito mouth brushes. Sterile forceps and dissecting pins were used to dissect the larval guts, which included the gastric caeca, anterior midgut, posterior midgut, Malpighian tubules, and hindgut from the remainder of the body (carcass). Sterile net pieces were used as dissection controls to identify fungi introduced during the dissection process and to be subsequently removed from the community data. The larval breeding waters, dissected mosquito guts and carcasses, and controls were stored at -80°C until further processing.

The larval breeding water was filtered through 1-micron nuclepore membranes (Whatman®) immediately prior to nucleic acid extraction. From each of the collection sites, nucleic acids were extracted from ten individual guts and



**FIGURE 1** | Map depicting the collection sites of *Cx. restuans* and *Cx. pipiens* mosquito larvae and their breeding water in Manhattan, KS. Solid circles indicate *Cx. restuans* breeding sites while circles with half left black symbol indicate *Cx. pipiens* breeding sites. *Cx. restuans* breeding sites 1 and 4 were revisited in a May (site 3) and July (site 5) 2018, respectively.

**TABLE 1** | Locations, dates, and types of larval breeding sites across Manhattan, KS during 2017 and 2018.

Larval breeding site	Collection date	Latitude, longitude	Revisited sites	Mosquito species	Type of larval breeding site
1	09/24/2017	N 39° 18' 13.4", W 96° 59' 44.6"	Yes	<i>Cx. restuans</i>	Natural (Pond)
2	05/06/2018	N 39° 20' 15.4", W 96° 58' 78.4"	No	<i>Cx. restuans</i>	Man-made (Tire)
3	05/17/2018	N 39° 18' 13.4", W 96° 59' 44.6"	Yes	<i>Cx. restuans</i>	Natural (Pond)
4	05/28/2018	N 39° 19' 06.7", W 96° 57' 05.2"	Yes	<i>Cx. restuans</i>	Man-made (Rainwater bucket)
5	07/25/2018	N 39° 19' 06.7", W 96° 57' 05.2"	Yes	<i>Cx. restuans</i>	Man-made (Rainwater bucket)
6	03/08/2018	N 39° 19' 06.7", W 96° 57' 05.2"	No	<i>Cx. pipiens</i>	Man-made (Oviposition cup)
7	08/18/2018	N 39° 19' 74.7", W 96° 58' 28.3"	No	<i>Cx. pipiens</i>	Natural (Pond)
8	10/01/2018	N 39° 20' 19.4", W 96° 59' 01.6"	No	<i>Cx. pipiens</i>	Man-made (Tire)

ten corresponding carcasses as well as a water sample and dissection control using the DNeasy® PowerSoil® Kit (MoBio Laboratory, Carlsbad, CA, United States) following the manufacturer's instructions with minor modifications. The extracted DNA was quantitated and standardized to 2 ng/μL concentration using the NanoDrop™ 2000/2000c spectrophotometers (Thermo Scientific, Waltham, MA, United States).

## Total Nucleic Acid Extraction and Mosquito Identification

We identified mosquitoes by PCR amplification of the mosquito ITS2 region using the forward 5.8F (5'-TGTGAAGTGCAGGACACATG-3') and reverse 28R (5'-ATGCTTAAATTTAGGGGGTA-3') primers following the protocol described by Collins and Paskewitz (1996). PCR

cycling conditions were as follows: initial denaturation of 5 min at 94°C, 34 cycles of denaturing, annealing, and extension at 94°C for 30 s, 61°C for 30 s, 72°C for 30 s, respectively, followed by a final 72°C extension step for 5 min. The PCR included nuclease-free water as a PCR negative control. Amplicons were visualized by aliquoting 3 μL of each amplified product on 1% agarose gels in 1x TAE buffer at 100 V constant voltage for 30 min. Finally, the PCR products were purified on columns and sequenced using Sanger sequencing by Genewiz, Inc. (South Plainfield, NJ, United States).

## Fungal Internal Transcribed Spacer Library Construction and Sequencing

The total nucleic acids from breeding water and mosquito tissues were used for PCR amplification of the fungal ITS2 region in triplicate PCRs with the barcoded forward fITS7

(5'-GTGARTCATCGAATCTTTG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, as described in Tawidian et al. (2021). PCR cycling conditions were as follows: an initial denaturation of 30 s at 98°C, 35 cycles of denaturing, annealing, and extension at 98°C for 10 s, 54°C for 30 s, 72°C for 1 min, followed by a final 72°C extension step for 9 min. The PCR included nuclease-free water as a PCR negative control. For positive control, we included a mock fungal community created with DNA from ten fungal species belonging to the phyla Ascomycota (*Aspergillus niger*, *Chaetomium globosum*, *Penicillium notatum*, *Sordaria fimicola*, *Saccharomyces cerevisiae*), Basidiomycota (*Agaricus bisporus* and *Coprinus cinereus*), Chytridiomycota (*Phlyctochytrium acuminatum*), and Mucoromycota (*Phycomyces blakesleeanus* and *Rhizopus stolonifer*) to determine the sequencing depth, error rate, and amplification with the fungal primers. Aliquots of 5 µL of each amplified product were visualized on 1% agarose gels in 1X TAE buffer at 100 V constant voltage for 30 min. The remaining volume from triplicate PCRs were pooled and purified using Mag-Bind® RXNPure plus (Omega Bio-Tek; Norcross, GA, United States). A total of 200 ng of amplicons from each sample were pooled for Illumina MiSeq sequencing. Illumina MiSeq adaptors were ligated onto the amplicon library using a NEBNext® DNA MasterMix for Illumina Kit (KAPA Biosystems, Wilmington, MA, United States), and sequences were generated using a MiSeq instrument (2 × 300 cycles: Illumina, San Diego, CA, United States) at the Kansas State University Integrated Genomics Facility (Manhattan, KS, United States).

## Sequence Processing and Operational Taxonomic Unit Assignment

Fungal libraries were demultiplexed and the paired-end sequences processed through the mothur pipeline (v.1.38.1) (Schloss et al., 2009). As described in Tawidian et al. (2021), data denoising included filtering out ambiguous bases, mismatches to primers, and homopolymers longer than 10 bp. In addition, 110,151 chimeric sequences were removed using the VSEARCH algorithm (Rognes et al., 2016). The remaining 9,374,188 high-quality paired-end sequences were assigned to fungal taxa using naïve Bayesian classifier against the UNITE-curated International Nucleotide Sequence Database reference database (Wang et al., 2007; Abarenkov et al., 2010). Paired-end sequences assigned to Archaea, Plantae, and the protozoan phyla Cercozoa and Ciliophora were removed from the dataset through sequence processing in mothur. The filtered fungal sequences were pairwise aligned to generate a distance matrix that was clustered into fungal operational taxonomic units (OTUs) at a 97% similarity threshold using the average neighbor algorithm (UPGMA) using mothur (v.1.44.3) (Schloss et al., 2009). OTUs with less than ten sequence reads were considered as low abundance OTUs and removed from the dataset. In addition, OTUs assigned as unclassified fungi, unclassified Ascomycota, and unclassified Basidiomycota were manually cross-checked using NCBI's Basic Local Alignment Search Tool (BLAST).<sup>1</sup> BLAST revealed 296 non-fungal OTUs that were identified as

algae (62.8%), plants (24.7%), protozoa (7.77%), insects (3.7%), fish (0.67%), and bacteria (0.33%). The non-fungal OTUs were removed from the dataset prior to downstream data analysis. Further, OTUs detected in the negative PCR and dissection controls (279 OTUs accounting for 529,196 reads) were also removed from the dataset. This resulted in a fungal dataset consisting of 1,670 OTUs and 695,922 reads for *Cx. restuans* samples and 1,543 OTUs, and 698,411 reads for *Cx. pipiens* samples (**Supplementary Table 1**).

The non-rarefied OTU dataset was further filtered prior to downstream data analysis to exclude all paired mosquito gut and carcass samples with fewer than 1,034 sequence reads. This eliminated all mosquito samples from two *Cx. restuans* breeding sites (sites 4 and 5), one *Cx. pipiens* breeding site (site 6), a single gut and carcass pair from site 2, two pairs of gut and carcass samples from site 1 and 7, respectively, and three pairs of gut and carcass samples from site 3. In addition, two *Cx. restuans* breeding sites (sites 2 and 5) were co-occupied by three and two *Cx. pipiens* mosquito larvae, respectively. The identified *Cx. pipiens* larvae were removed from downstream analyses because of the low sample size. The final dataset used in downstream analyses included five breeding water samples (three from *Cx. restuans* breeding sites and two from *Cx. pipiens* breeding sites), 21 guts and 21 carcasses of *Cx. restuans* larvae, and 18 guts and 18 carcasses of *Cx. pipiens* larvae. In addition, we also rarefied the data to the sequencing depth equal to the lowest yielding sample (1,034 sequence reads). Analysis of the rarefied data were congruent to our analysis of the non-rarefied data (data not shown). However, subsampling resulted in loss of 92% of the sequence reads, including the loss of the rare taxa in the samples.

## Amplicon Sequence Variant Assignment

We used mothur (v.1.44.3) (Schloss et al., 2009) to compare the mosquito fungal community composition and assembly using OTUs and ASVs, while preserving the data denoising and cleanup performed in the OTU pipeline described above. For ASV assignment in mothur, pairwise aligned sequences were pre-clustered at a threshold of two nucleotide differences, prior to removing chimeric sequences through VSEARCH (Rognes et al., 2016). Upon removing chimeric sequences, ASVs were assigned to paired-end sequences using the UNITE-curated International Nucleotide Sequence Database reference database (Wang et al., 2007; Abarenkov et al., 2010). Similar to the OTU assignments, non-fungal ASVs and low abundance ASVs (accounting for less than ten reads) were removed from the dataset through the mothur pipeline. In addition, ASVs associated with the 296 non-fungal OTUs identified using BLAST were removed from the dataset. The final ASV dataset consisted of 3,303 ASVs, and 2,080,836 reads for *Cx. restuans* samples, and 2,548 ASVs, and 1,518,762 reads for *Cx. pipiens* samples (**Supplementary Table 2**). Similar to OTUs, ASVs were filtered to exclude all paired mosquito gut and carcass samples with fewer than 1,034 reads. The additional filtering steps eliminated the same mosquito samples and breeding sites as those listed with OTUs. Rarefaction analyses of the fungal ASVs were saturated for most samples (**Supplementary Figure 1B**) consistent with the high Good's coverage ( $0.998 \pm 0.003$  SD),

<sup>1</sup><https://blast.ncbi.nlm.nih.gov/Blast.cgi>

indicating that the sequencing depth was sufficient to capture the fungal diversity across most of the samples. Downstream ASV community analyses were performed as described for the OTUs. Results of the ASV community analyses are available in **Supplementary Data Sheet 1**.

## Community and Statistical Analyses

To assess the fungal diversity and richness in breeding water and mosquito guts and carcasses, alpha diversity indices, including observed richness ( $S_{Obs}$ ) and Shannon's diversity ( $H'$ ) were iteratively estimated (100 iterations) using *mothur* (v.1.44.3) (Schloss et al., 2009). The diversity indices were compared using non-parametric Wilcoxon signed-rank tests between paired mosquito guts and carcasses of each mosquito species separately. Breeding water samples were not included in the statistical analyses because of the low sample size after filtering. Wilcoxon signed-rank tests were performed using the GraphPad Prism version 8.4.3 for Windows (GraphPad Software, San Diego, CA, United States).

The Bray-Curtis dissimilarity matrices were computed for the fungal OTU data to compare and visualize the fungal community assemblages between the breeding water and mosquito guts and carcasses using non-metric multidimensional scaling (NMDS). To determine how much variation in the fungal community composition was inferred by larval breeding sites or mosquito tissue type, permutational multivariate analysis of variance (PERMANOVA) was performed. PERMANOVAs were performed using the *vegan* package in R.<sup>2</sup> In addition, Wilcoxon signed-rank tests on the Bray-Curtis distances were used to assess the similarity of the fungal community composition in the breeding water to each mosquito tissue type. To identify the fungal OTUs that drive the fungal community variation across mosquito tissue types, we used the indicator value index (IndVal) analysis (Dufrene and Legendre, 1997). In addition, the point-biserial correlation index (rpb) (Caceres and Legendre, 2009) was used to determine the tissue niche preference of the OTUs associated with the mosquito guts and carcasses. Both analyses were performed using the *indicspecies* package in R with 9,999 permutations (see text footnote 2). Finally, to determine the ecological guild of the identified OTUs, we assigned them to ecological guilds as saprophytes, plant pathogens, endophytes, animal pathogens, lichen parasites, fungal parasites, epiphytes, and animal endosymbionts using the FUNGuild annotation tool (Nguyen et al., 2016).

## RESULTS

### Temporal Prevalence of *Culex* Mosquitoes

We identified two *Culex* mosquito species collected from eight sites in Manhattan, KS, during 2017 and 2018 (**Figure 1**). *Cx. restuans* mosquito larvae were collected from five larval breeding sites, one in September 2017 and the remaining four during May-July 2018 (**Table 1**). While two *Cx. restuans* breeding

sites were co-occupied by *Cx. pipiens* mosquito larvae, the prevalence of *Cx. pipiens* larvae was low in the early season. However, during August-October 2018, *Cx. pipiens* mosquitoes were more widespread and collected from three larval breeding sites (**Table 1**). These results show that both mosquito species are widely distributed and may have a largely complementary temporal pattern of prevalence in Manhattan, KS.

### Fungal Community Composition in the Breeding Water and Mosquito Tissues

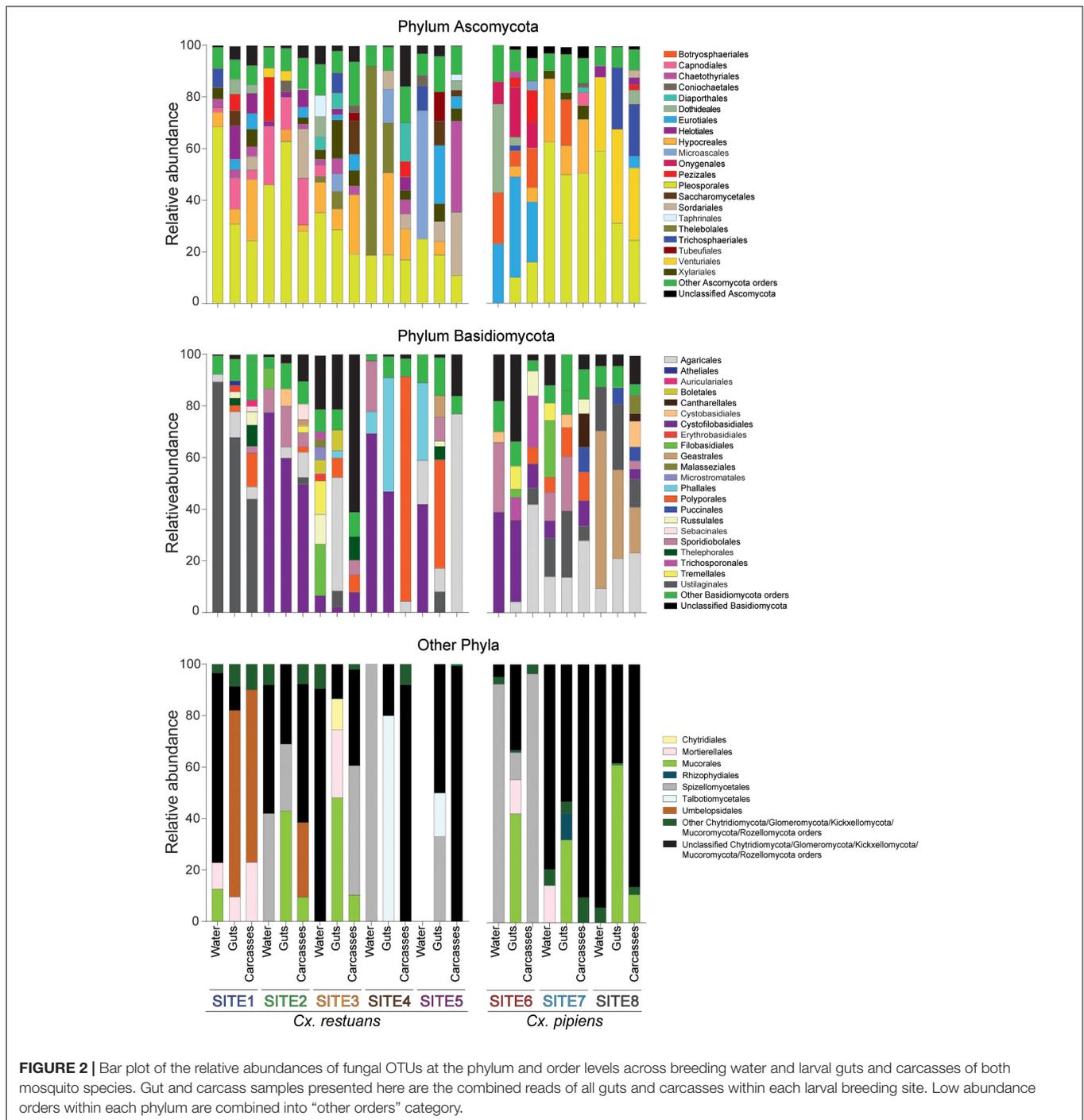
To determine the fungal community composition across larval breeding sites and mosquito tissues, we analyzed the fungal ITS2 metabarcoding markers of breeding water as well as mosquito gut and carcass samples. Our analyses revealed 1,670 OTUs associated with *Cx. restuans* samples and 1,543 OTUs with *Cx. pipiens* samples. Rarefaction analyses indicated that the fungal OTUs were saturated for most samples (**Supplementary Figure 1A**). In addition, the average Good's coverage across all samples was high ( $0.999 \pm 0.001$  SD). These results indicated that our sampling had sufficient sequencing depth and coverage to detect the fungal diversity across the samples.

To determine the fungal community composition across all samples, we plotted the relative abundances of the identified OTUs on the phylum and order levels (**Figure 2**). Twelve fungal phyla were identified accounting for 74.4% of total sequence reads. The phylum Ascomycota dominated with 45.8% of the sequences, followed by the phyla Basidiomycota (24.1%), Chytridiomycota (1.7%), Mucoromycota (1.7%), and Mortierellomycota (0.47%). A small proportion (0.63%) of the reads was assigned to seven rare phyla including Blastocladiomycota, Glomeromycota, Entorrhizomycota, Kickxellomycota, Monoblepharomycota, Rozellomycota, and Zoopagomycota (**Figure 2**). The remaining 25.6% of the reads were not classified beyond Fungi but were included in downstream analyses. In addition, the ascomycetous orders Pleosporales, Hypocreales, and Eurotiales were dominant and shared across most breeding water and mosquito samples (**Figure 2**). Within the phylum Basidiomycota, the order Ustilaginales was assigned the highest number of sequence reads (36.1%) followed by the order Agaricales (11.5%). As for the low abundance phyla, the majority of the remaining reads (90.5%) were assigned to the orders Mucorales and Mortierellales within the phylum Mucoromycota (**Figure 2**).

### Fungal Community Composition in *Culex* Mosquito Larvae Is Driven by the Aquatic Habitat

To identify whether the larval breeding site drives fungal community assembly in *Cx. restuans* and *Cx. pipiens* mosquito larvae, we analyzed the Bray-Curtis community dissimilarities for each mosquito species separately. We visualized the community data using non-metric multidimensional scaling (NMDS) (**Figure 3**). We used permutational multivariate analysis (PERMANOVA) to determine whether fungal communities were distinct across *Cx. restuans* and *Cx.*

<sup>2</sup><http://www.r-project.org/>

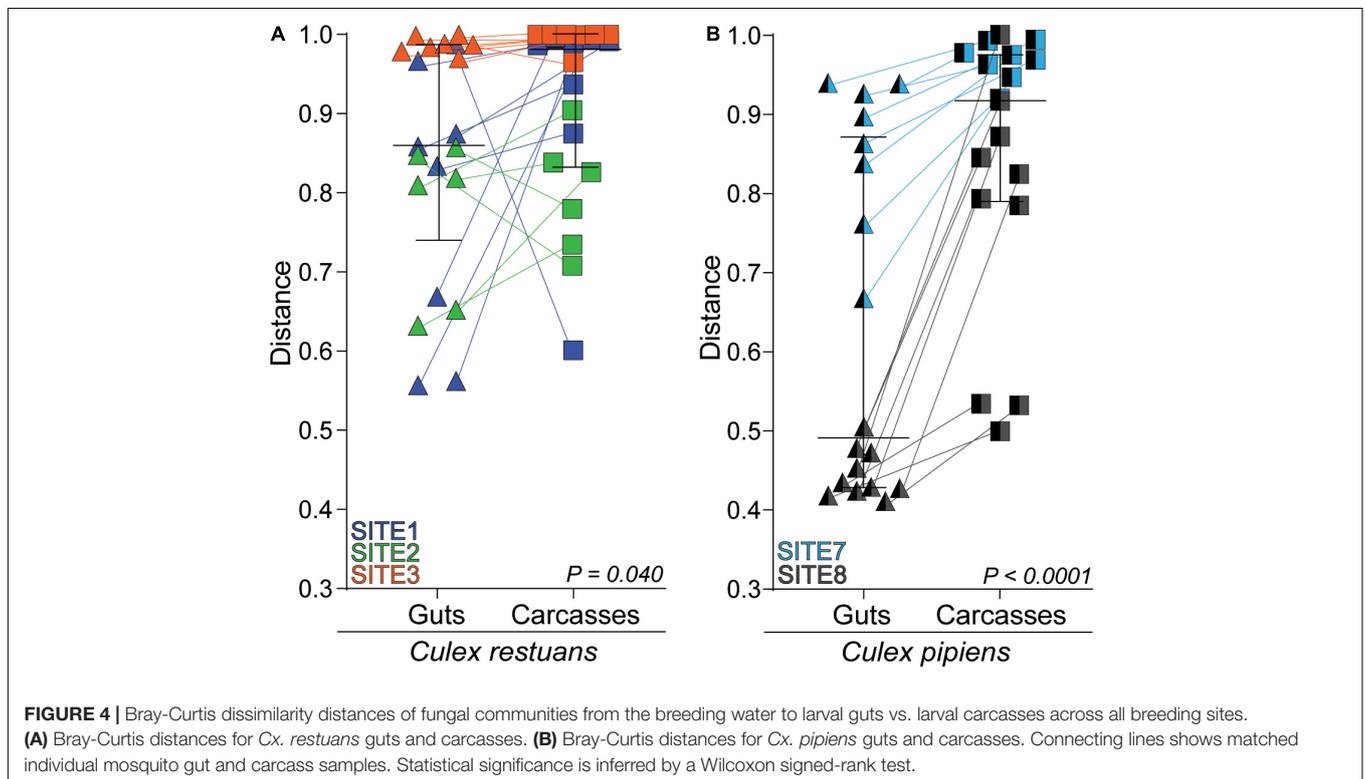
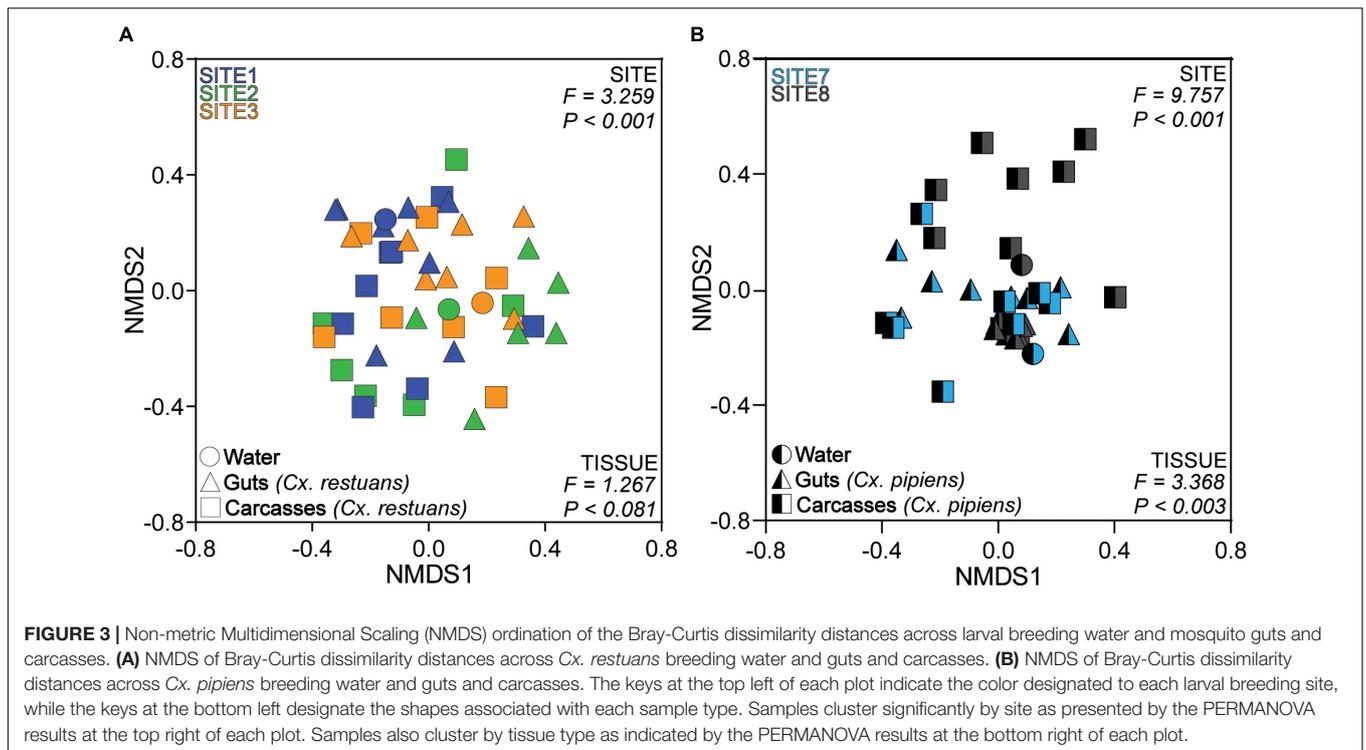


*pipiens* breeding sites. These analyses revealed distinct fungal communities across larval breeding sites. In addition, PERMANOVA analysis showed significant differences in mosquito-associated fungal communities across larval breeding sites, suggesting that the aquatic breeding environment is a major driver of fungal community assembly in the larvae of both mosquito species (Figures 3A,B). In addition, fungal communities differed in composition across guts and carcasses of *Cx. pipiens* mosquito larvae

with no evidence of tissue specificity in *Cx. restuans* larvae (Figures 3A,B).

## Fungal Diversity Varies Across Mosquito Tissue Types

To identify whether fungal communities varied across mosquito tissues within each breeding site, we visualized the Bray-Curtis distances from individual guts and carcasses using



NMDS (**Supplementary Figure 2A**) and compared them using PERMANOVAs. Our results revealed significant differences in fungal community composition among guts and carcasses for one of three *Cx. restuans* and two *Cx. pipiens* breeding sites. This was

also evident when we plotted the paired Bray-Curtis dissimilarity distances from the breeding water to guts and breeding water to carcasses for each mosquito species across all breeding sites (**Figure 4**). The results revealed that larval guts were more

similar to the breeding water than the paired carcasses, indicating that mosquito guts had fungal communities more similar to the breeding water than the carcasses did. While this pattern was evident for both mosquito species when analyzed across all breeding sites, within individual breeding sites, the fungal communities in *Cx. restuans* larval guts and carcasses did not significantly differ in their distance to the breeding water. This is likely because the distances between the guts and breeding water varied widely among sites in *Cx. restuans* larvae. This variability was not observed in the *Cx. pipiens* gut to breeding water distances (**Supplementary Figure 2B**).

In addition, our diversity analyses revealed variation in the fungal OTU richness ( $S_{Obs}$ ) and diversity (Shannon's  $H'$ ) diversity across mosquito guts and carcasses (**Figure 5**). In *Cx. restuans* larvae, OTU richness was significantly higher in guts than in carcasses (**Figure 5A**), whereas Shannon's diversity did not differ among mosquito tissues (**Figure 5B**). As for *Cx. pipiens* larvae, OTU richness as well as Shannon's diversity were significantly higher in guts than carcasses (**Figures 5C,D**). While these results indicate differences in fungal community composition and diversity across guts and carcasses of both mosquito species, the observed tissue specificity was more evident in *Cx. pipiens* than in *Cx. restuans* larvae.

## Tissue-Specific Indicator Fungi Reflect Mosquito Feeding Behavior and Fungal Niche Preference

To identify the OTUs that drive tissue-specific patterns in fungal community assembly in *Cx. restuans* and *Cx. pipiens* mosquito larvae, we used indicator taxon analyses using the indicator value index (IndVal) (**Figure 6** and **Supplementary Table 3**). We then used the FUNGuild annotation tool to assign the identified OTUs to ecological guilds. Three OTUs were identified as *Cx. restuans* gut indicators, whereas none were identified as indicators of carcasses (**Figure 6A** and **Supplementary Table 3A**). Two of three identified OTUs were assigned to unique ecological guilds (endophyte and fungal parasite). The third remaining OTU could not be assigned to an ecological guild. However, *Cx. pipiens* larval guts had higher number of indicators than *Cx. restuans* larvae. IndVal analysis identified 19 *Cx. pipiens* gut indicator OTUs (**Figure 6B** and **Supplementary Table 3A**), whereas carcasses were not assigned any indicators. The identified gut indicators OTUs were assigned to five ecological guilds [saprophyte (50%), plant pathogen (22.7%), endophyte (9.1%), animal pathogen (4.5%), and epiphyte (4.5%)] (**Figure 6B** and **Supplementary Table 3A**). The remaining 14.8% of indicator OTUs were not assigned to ecological guilds.

Finally, we used the point-biserial correlation index ( $r_{pb}$ ) to determine the role of fungal niche preference on fungal community assembly across mosquito tissue types (**Figure 6** and **Supplementary Table 3B**). Our results revealed three OTUs differentially abundant in *Cx. restuans* guts, all of which were also gut indicators (**Figure 6C** and **Supplementary Table 3B**). As for *Cx. pipiens* larvae, 14 OTUs were significantly associated with gut samples assigned to the ecological guilds [saprophyte (58.8%), plant pathogen (11.8%), endophyte (5.9%), animal pathogen

(5.9%), and epiphyte (1.2%)] (**Figure 6D** and **Supplementary Table 3D**). The remaining 11.8% of OTUs were not assigned to ecological guilds. The  $r_{pb}$  analyses did not identify any OTUs as significantly associated with the carcasses of either mosquito species.

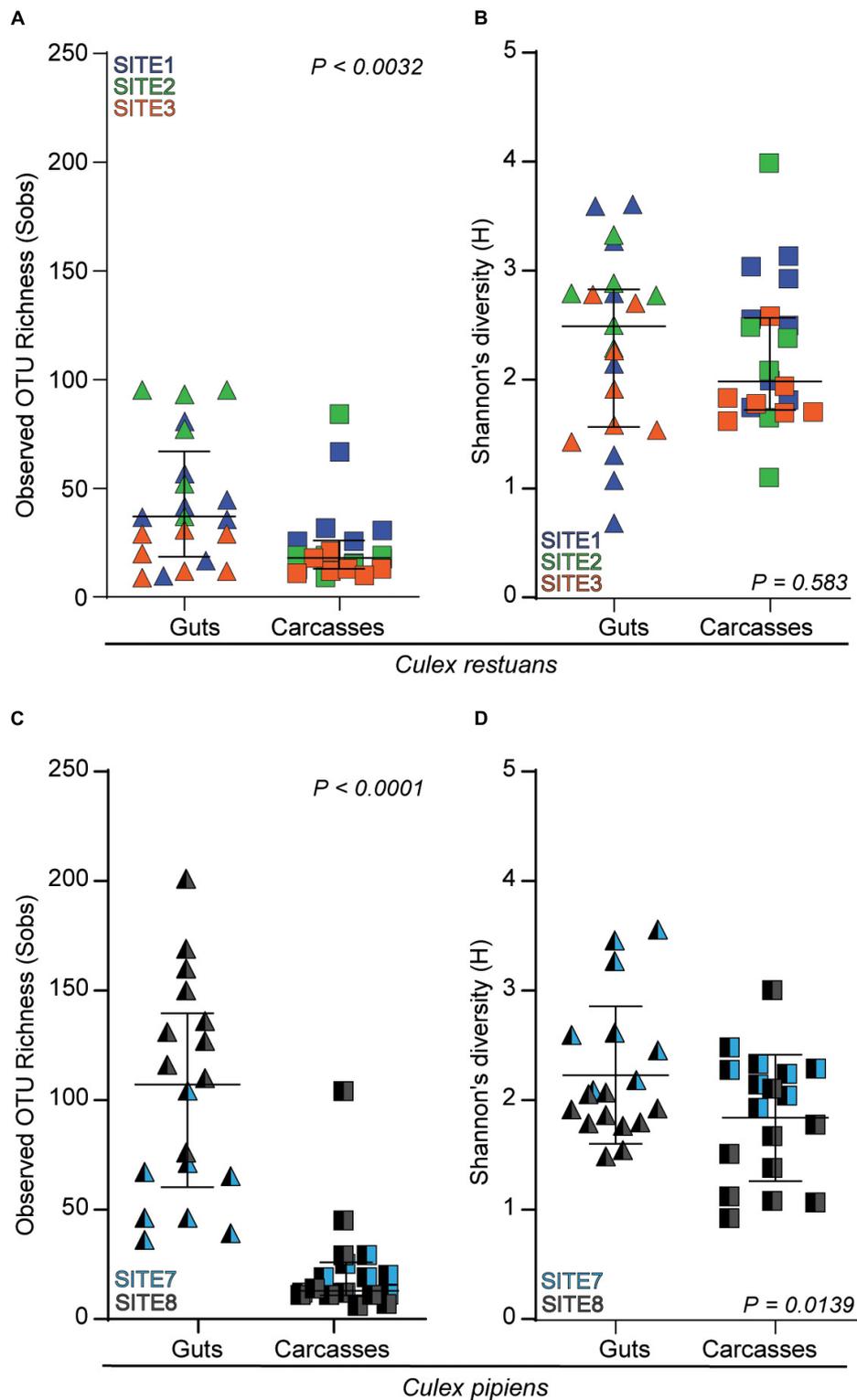
## DISCUSSION

This study provides fundamental insight into the composition and diversity of fungal communities associated with *Cx. restuans* and *Cx. pipiens* mosquito larvae. In addition, we determine whether the observed fungal community assembly in mosquito larvae were similar across mosquito species. Overall, our OTU and ASV data demonstrate that the breeding site was the major determinant of fungal community assembly in the larvae of both mosquito species. In addition, we observed tissue-specific fungal community assembly in larval guts and carcasses. However, variation in fungal diversity and composition across mosquito tissues was more evident in *Cx. pipiens* larvae compared to *Cx. restuans*.

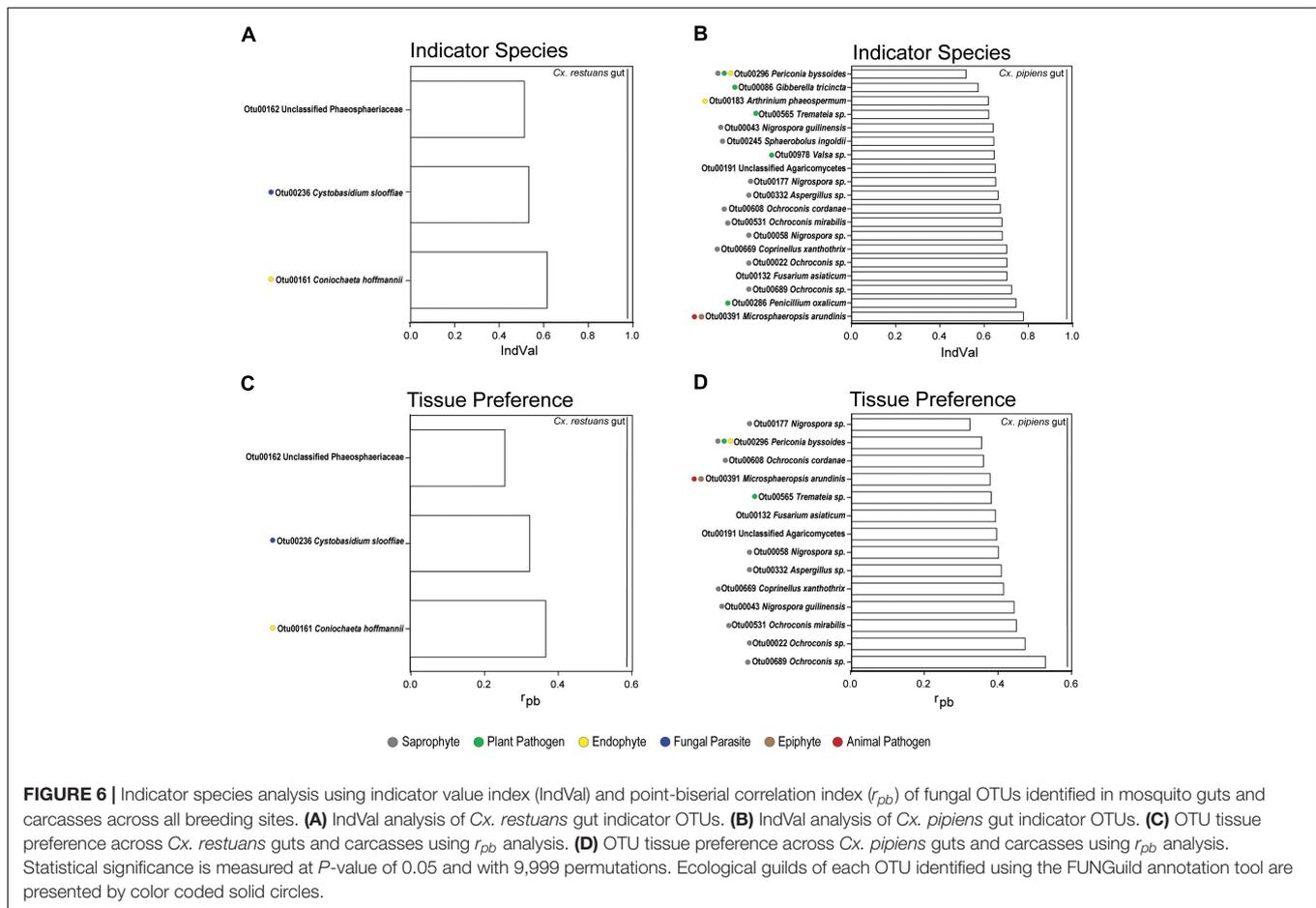
The fungal community associated with larval breeding water and mosquito guts and carcasses was dominated by the phyla Ascomycota and Basidiomycota, consistent with data reported in *Ae. albopictus* mosquito larvae collected in Manhattan, KS (Tawidian et al., 2021) and *Aedes* mosquito adults and larvae collected from the field (Muturi et al., 2016; Luis et al., 2019; Zouache et al., 2022). The observed phylum dominance was not surprising given the high prevalence of fungi in the phyla Ascomycota and Basidiomycota in habitats where mosquito larvae reside (Shelomi, 2019; Tawidian et al., 2021; Udjuh Obinna Godwin et al., 2021; Zouache et al., 2022). In addition, it can also be attributed to the amplification bias of the ITS2 primers (fITS7 and ITS4) toward fungi belonging to these phyla with low affinity for early-diverging fungal lineages, e.g., including taxa in the Chytridiomycota (Bellemain et al., 2010; Blaaidid et al., 2013; Bokulich and Mills, 2013).

The filamentous fungi and yeasts largely overlapped (42.6%) in *Cx. restuans* and *Cx. pipiens* mosquito larvae. The majority (96.4%) of fungal genera that overlapped across both *Culex* species were ascomycetes (68.7%) and basidiomycetes (27.7%). Similarly, an overlap of fungal genera (17.3%) was also observed between our *Culex* datasets and fungi detected by similar metabarcoding of larval *Ae. albopictus* tissues (Tawidian et al., 2021). This overlap (95.9%) was also largely attributable to ascomycetes and basidiomycetes. In addition, a considerable proportion of fungi (62.9% genus level and 15.1% species level) isolated from field collected mosquito larvae, including *Cx. pipiens* were also identified in our dataset (Badran and Aly, 1995; Pereira et al., 2009; Steyn et al., 2016). The observed overlap of ascomycetes and basidiomycetes across larvae of different mosquito species can be explained by the ubiquity and co-occurrence of these taxa in freshwater ecosystems (Manohar and Raghukumar, 2013; Panzer et al., 2015; Lepère et al., 2019; Shelomi, 2019; Tawidian et al., 2021).

The overall goal of this study was to determine whether the drivers of fungal community assembly in mosquito larvae



**FIGURE 5** | Alpha diversity indices as measured by observed OTU richness (Sobs) and Shannon's diversity (H) of larval guts and carcasses across collection sites. **(A)** OTU richness in *Cx. restuans* guts and carcasses. **(B)** Shannon's diversity in *Cx. restuans* guts and carcasses. **(C)** OTU richness in *Cx. pipiens* guts and carcasses. **(D)** Shannon's diversity in *Cx. pipiens* guts and carcasses. Statistical significance was determined using a Wilcoxon signed-rank test.



can be generalized across mosquito species. Our results show that fungal community assembly in *Culex* mosquito larvae is driven by the breeding site. This result corroborates our previous study assessing the fungal community assembly in *Ae. albopictus* larvae (Tawidian et al., 2021). Similarly, bacterial diversity in mosquito larvae also reflects that of breeding water, as reported for several species of field-collected mosquito larvae, including *Ae. japonicus*, *Ae. triseriatus*, *Ae. aegypti*, *Ae. albopictus*, *Anopheles albimanus*, *An. coluzzii*, *Anopheles darlingi*, *Anopheles nuneztovari*, *Culex tharsalis*, *Cx. quinquefasciatus*, and *Cx. restuans* (Duguma et al., 2013; Gimonneau et al., 2014; Kim et al., 2015; Coon et al., 2016; Bascuñán et al., 2018; Galeano-Castañeda et al., 2020; Scolari et al., 2021). To our knowledge, this is the first study that assesses the fungal community composition and diversity in field-collected *Cx. restuans* and *Cx. pipiens* mosquito larvae and their breeding water. We acknowledge that direct comparisons of fungal communities of *Cx. restuans* and *Cx. pipiens* larvae in our study are not possible, because the two species did not co-inhabit the larval breeding sites we sampled. However, we show that the fungal communities of both *Cx. restuans* and *Cx. pipiens* larvae cluster largely by site. Thus, these data provide support that the larval aquatic habitat is a major determinant of fungal community assembly in the larvae of both *Culex* species.

Our results further show variation in fungal community composition in the guts and carcasses of *Culex* mosquito larvae. While the fungal communities in the guts of *Culex* larvae were more similar to those in the breeding water than the carcasses, this pattern was more consistent in *Cx. pipiens* guts compared to *Cx. restuans*. The similarity of gut fungal community to that of the water can be attributed to the filter-feeding behavior of *Culex* larvae, which allows for the acquisition of fungi from the environment (Merritt et al., 1992). However, while similar, the gut fungal community of neither mosquito species was identical to that of the breeding water. This may be attributed to grazing behavior of *Culex* larvae on decaying plant detritus found in the aquatic habitat, in addition to the filter feeding behavior in the water column (Merritt et al., 1992; Yee et al., 2004; Winters and Yee, 2012). This notion is supported by the result of the *Cx. pipiens* indicator taxon analysis, in which the gut indicators were enriched with saprophytes, plant pathogens, and endophytes. These indicator taxa included obligate plant pathogens (family Ustilaginaceae or the rust diseases) and other plant pathogens, such as *Gibberella tricinata* (Desjardins, 2003; Geiser et al., 2014). These results corroborate earlier studies with *Ae. albopictus* larvae, in which the same taxa were indicators for the larval guts (Tawidian et al., 2021). Combined, these results suggest similar feeding behaviors of *Cx. pipiens* and *Ae. albopictus* larvae,

and support earlier observations by Yee et al. (2004). Our indicator taxon analyses further showed that *Cx. restuans* guts were less enriched with plant-associated fungi and had lower OTU richness than *Cx. pipiens* guts, irrespective of the greater sample size. This suggests that *Cx. restuans* larvae fed less on plant detritus than *Cx. pipiens* larvae do, resulting in the observed differences in the gut fungal communities. These results corroborate reports on *Cx. restuans* feeding behavior (Winters and Yee, 2012), suggesting that *Cx. restuans* larvae affect detrital decay less than *Ae. albopictus* larvae.

In comparison, carcasses of both mosquito species had lower fungal diversity and richness than guts and breeding water. In contrast to the *Ae. albopictus* dataset (Tawidian et al., 2021), indicator species analysis did not reveal entomopathogenic/entomotoxigenic fungi associated with the carcasses of either *Culex* species. This is perhaps not surprising, given the low abundance of known entomopathogens in larval breeding environments. However, analysis of the ASV dataset identified *Alternaria tenuissima* (ASV1048) as significantly enriched in *Cx. pipiens* carcasses. While there is no evidence of entomopathogenicity or entomotoxicity of *A. tenuissima* toward mosquito larvae, one study has reported the entomotoxicity of *A. tenuissima* extracts toward larvae of *Galleria mellonella* and adult *Acheta domesticus* (Salimova et al., 2021). Interestingly, a fungus within the genus, *Alternaria porri* was enriched in *Ae. albopictus* carcasses (Tawidian et al., 2021), and causes mortality in green apple aphids and lowers hatch rates of European corn borer eggs (Lynch and Lewis, 1978; Sarhan and Baji, 2009). *A. tenuissima* was also detected in the breeding water and guts of *Cx. pipiens* larvae, suggesting that this fungus is ingested and may disseminate from the gut into the larval body cavity. Future studies are necessary to determine the entomopathogenic and/or entomotoxigenic potential of *A. tenuissima* in mosquito larvae.

In conclusion, the present study provides the first comprehensive description of the fungal community composition and assembly in *Cx. restuans* and *Cx. pipiens* mosquito larvae. Our results show that the fungal community assembly remains generally similar between the two *Culex* species, and suggests that the observed variation is likely due to differences in larval feeding behavior. We show that the larval breeding site is the key driver of fungal community assembly in mosquito larvae. We further show that mosquito feeding behavior and fungal mode of nutrition contribute to differences in fungal community assembly across mosquito tissues. However, the observed tissue preference was more evident in *Cx. pipiens*

than in *Cx. restuans* larvae and broadly recapitulated our previous report focusing on *Ae. albopictus*. Future studies require greater sampling of breeding sites co-inhabited by *Cx. restuans* and *Cx. pipiens* larvae to better assess the impact of mosquito feeding behavior differences on fungal community assembly for these two species.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA798403.

## AUTHOR CONTRIBUTIONS

PT performed experiments and analyzed data. KM supervised the project. PT and KM drafted the manuscript and all authors contributed to the final version of the manuscript. All authors conceived the original idea, designed experiments, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.911085/full#supplementary-material>

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