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ORIGINAL ARTICLE

Similarities and spatial variations of bacterial and fungal communities in field rice planthopper (Hemiptera: Delphacidae) populations

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Abstract

Rice planthoppers are notorious plant sap-feeding pests that cause serious damage. While several microbes in rice planthoppers have been broadly characterized, the abundance and diversity of bacteria and fungi in field planthoppers are largely unknown. This study investigated the bacterial and fungal community compositions of Chinese wild rice planthoppers *Laodelphax striatellus* and *Sogatella furcifera* using parallel 16S rRNA gene amplicon and internal transcribed space region sequencing. The bacteria varied significantly

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between the species and were partitioned significantly by sexes, tissues and host environments in each species. The majority of bacteria were affiliated with the genera *Wolbachia*, *Cardinium*, *Rickettsia* and *Pantoea*. The abundance of *Wolbachia* was negatively correlated with that of *Cardinium* in both planthopper species. Compared with bacteria, the abundance and diversity of fungi did not differ between sexes but both were enriched in the gut. The bacterial community as a whole showed no significant correlation with the fungal community. The majority of fungi were related to *Sarocladium*, *Alternaria*, *Malassezia*, *Aspergillus* and *Curvularia.* A phylogenetic analysis revealed that these fungi were closely related to botanic symbionts or pathogens. Our results provide novel insights into the bacteria and fungi of rice planthoppers.

Key words fungal diversity; *Laodelphax striatellus*; microbiota; *Sogatella furcifera*; planthoppers

Introduction

Microbial symbionts are prevalent in diverse insects (Baumann, 2005; Douglas, 2015; Pietri *et al.*, 2016). Insects that feed on plant sap need these symbionts in particular because plant sap usually lacks some essential nutrients (like amino acids and vitamin Bs) that can only be supplemented by microbial symbionts (Baumann, 2005). Many plant sap-feeding insects harbor primary symbionts localized in specialized tissues (usually named the bacteriome/mycobiome) that are strictly vertically transmitted, such as *Buchnera* and *Portiera* in aphids and whiteflies, respectively (Baumann, 2005). Additionally, plant sap-feeding insects harbor secondary symbionts that do not exist in all individual insects and may occupy multiple tissues in one individual (Oliver *et al.*, 2010). Microbial symbionts influence multiple aspects of insect biology, such as contributing essential amino acids to the host

(Douglas, 2017), manipulating host reproduction (Duron *et al.*, 2008), and protecting the host against biotic and abiotic stresses (Brownlie & Johnson, 2009; Six & Wingfield, 2011). The diversity of the microbiota is affected by multiple factors, including the host's genetic background, physiological condition, sex and age (Bing *et al.*, 2013; Broderick *et al.*, 2014). Environmental conditions, such as temperature, humidity and diet, also influence the microbial composition (Wernegreen, 2012; Bing *et al.*, 2018; Lax *et al.*, 2019).

The small brown planthopper, *Laodelphax striatellus* (Fallén), and the white-backed planthopper, *Sogatella furcifera* (Horváth), are two notorious insect species that cause severe damage to rice (*Oryza sativa*). They are phylogenetically close and are members of Delphacidae (Hemiptera) (Song & Liang, 2013). They both feed exclusively on plant sap and oviposit in rice tissues. Additionally, *L. striatellus* and *S. furcifera* migrate long distances, which seriously increases the area that they damage (Otuka, 2013; Chen *et al.*, 2019) and their ability to transmit plant viruses. However, they transmit different viral spectra. The former transmits viruses such as rice stripe virus, rice black-streaked dwarf virus, and barley yellow striate mosaic virus (Falk & Tsai, 1998; Li *et al.*, 2011; Cao *et al.*, 2018), while the latter transmits viruses such as southern rice black-streaked dwarf virus (Xu *et al.*, 2014). These planthopper-transmitted viruses cause great economic losses.

Like most plant sap-feeding Hemipteran insects, rice planthoppers are associated with diverse symbionts, including intracellular bacterial symbionts and yeast-like symbionts (YLS) (Noda, 1974; Chen *et al.*, 1981; Tang *et al.*, 2010; Zhang *et al.*, 2010; Bing *et al.*, 2019). Rice planthoppers are used as powerful insect models for research on host–microbe interactions (Huo *et al.*, 2018). The reproductive manipulators *Wolbachia* and *Cardinium* are prevalent and alter the sex ratios of hosts *L. striatellus* and *S. furcifera* through cytoplasmic incompatibility (CI) (Noda, 1984; Kittayapong *et al.*, 2003; Nakamura *et al.*, 2012; Zhang *et al.*, 2013). The symbiont *Spiroplasma* colonizes and kills some *L. striatellus* infected male

individuals (Sanada-Morimura *et al.*, 2013). In addition, recent high-throughput analyses have detected other bacteria, like *Serratia*, *Pantoea* and *Chryseobacterium*, in *L. striatellus* populations (Li *et al.*, 2017). A library analysis of the 16S rRNA gene showed that *S. furcifera* harbors mostly *Wolbachia*, followed by *Cardinium* and then *Pantoea agglomerans* (Kang, 2014). Being prevalent in the fat bodies of all rice planthoppers, YLS are considered primary symbionts of rice planthoppers (Noda, 1974). Although several other species of YLS have been detected in rice planthoppers, most research has been limited to studying only *Nilaparvata lugens* (Dong *et al.*, 2011; Pang *et al.*, 2012; Hou *et al.*, 2013). To our knowledge, the fungal microbial communities in *L. striatellus* and *S. furcifera* have never been explored extensively. Previous studies mainly yielded snapshots of the rice planthoppers' microbiome, because they were limited to specific microbial species or to relatively few sequences from only laboratory-reared planthoppers. The extent of the variation in microbiota within field populations should be explored on a large scale.

To extend our understanding of the rice planthoppers' microbiome, we performed the thorough culture-independent high-throughput sequencing of the 16S rRNA gene for bacteria and internal transcribed spacer (ITS) region for fungi and conducted a systematic investigation of the microbial communities associated with field *L. striatellus* and *S. furcifera* populations that live in distinct environments. We analyzed variations in the microbial communities of various tissues of different insects collected from multiple locations on rice farms. The correlations between bacteria and fungi in the samples were calculated to provide insights into microbe–microbe interactions. We specifically aimed to answer the following questions: (i) Do the phylogenetically closely related insects *L. striatellus* and *S. furcifera* harbor similar microbiota? (ii) Do their microbiota vary among sexes, tissues and host environments? These systematic and comparative analyses establish

a comprehensive understanding of the rice planthoppers' microbiome and yield insights into microbial symbiont interactions in plant sap-feeding insects.

Materials and methods

Sample collection

Rice planthoppers were collected on eight rice farms scattered throughout Jiangsu Province, China in 2018 (Fig. 1A, Table S1). The environments surrounding these farms were distinct. For example, the LYG planthopper population was collected from rice plants that grew on alkali soil near the sea, while the SQ population was collected from rice plants irrigated with pure lake water. After collection, planthoppers were kept with the rice leaves on which they were caught. Later, the planthoppers were sorted by morphology and then surface sterilized in 75% ethanol three times and washed with sterile distilled water before dissection. The guts and reproductive tissues (ovary or testes) were dissected under aseptic conditions using forceps sterilized in 1× phosphate-buffered saline. Dissections were performed within 24 h of collection. Three individual planthoppers or the tissues of five planthopper individuals were pooled as one sample. The tissues were stored in 1.5-mL sterile tubes in liquid nitrogen before DNA extraction. A total of 175 samples were collected for the microbiota analysis.

DNA extraction and PCR screening

The total DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Diagnostic PCR was also conducted with the DreamTaq Green PCR master mix (Thermo Fisher Scientific, Carlsbad, CA, USA)

to verify the classifications of planthopper species using specific mitochondrial cytochrome oxidase subunit I primers. The primer sequences are listed in Table S2.

Amplicon sequencing

The bacterial and fungal communities of both *L. striatellus* and *S. furcifera* were assessed by the high-throughput sequencing of the 16S rRNA gene and the internal transcribed spacer (ITS) region, respectively (Fig. 1B). The V3–V4 region of the bacterial 16S rRNA gene was amplified using the primer set 515f/806r, and the fungal ITS region was amplified using the ITS1/ITS2 primers as previously described (White *et al.*, 1990; Klindworth *et al.*, 2013). We performed two-step PCR to generate amplicon libraries, as recommended by Illumina (Illumina, 2013). Briefly, PCR amplifications were performed using the DreamTaq Green PCR master mix (Thermo Fisher Scientific) in an ABI Veriti 96 Thermal Cycler (Applied Biosystems, Waltham, MA, USA). The PCR products were cleaned using Hieff NGS DNA-selection Beads (YEASEN, Shanghai, China), according to the manufacturer's instructions. The Illumina sequencing adapters, including sample-specific barcodes, were added to the purified PCR products during a second PCR reaction using the TruePrep Index Kit V3 for Illumina (Vazyme, Nanjing, China). Final PCR products were purified with Hieff NGS DNA-selection Beads (YEASEN), and equalized and normalized using the dsDNA HS assay kit for Qubit (YEASEN). Samples were quantified and pooled in equimolar ratios using a Qubit 4 Fluorometer (Invitrogen, Waltham, MA, USA). Sequencing was performed on the Illumina MiSeq platform at the Plant Protection College, Nanjing Agriculture University using the MiSeq Reagent Kit v3 (2× 300 bp) (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

For fungal amplicon sequencing, the ITS universal primers not only amplified the fungal ITS region but also other eukaryotic fragments. PCR of the ITS regions from planthopper samples yielded two bands: one of ~400 bp and one of ~1200 bp. Preliminary Sanger sequencing results showed that the smaller amplicons were mainly from fungi and the larger ones were from insects (Fig. S1). To enrich fungal sequences in the library, we excised the smaller bands from the gels and purified the PCR products to isolate the fungal fragment using an Axygen Gel Extraction Kit (Axygen, Corning, NY, USA). Then, the purified products were used as template in a second PCR reaction, and the following procedures were as described above (Fig. 1B).

Microbial community analysis

A total of 14 270 455 paired-end reads of 16S rRNA V3–V4 amplicon sequences and 6 444 946 reads of ITS amplicon sequences were generated to survey the bacterial and fungal communities, respectively. The sequenced paired-end Illumina reads were first analyzed using AfterQC (version 0.9.6) for quality control (Chen *et al.*, 2017). We used DADA2 (Callahan *et al.*, 2016) implemented in QIIME2 (version 2018.8.0) (Bolyen *et al.*, 2019) to construct an operational taxonomic unit (OTU) table for OTU representative sequences. On the basis of the quality control results, 10 bp at the 5′- and 3′-ends of the sequence data were removed. Finally, 21 311 ± 21 370 reads (mean ± SD) and 2728 ± 5459 reads representing bacterial and fungal sequences, respectively, were obtained for the microbial community analysis. The microbial communities were then analyzed using QIIME2 (version 2018.8.0) (Bolyen *et al.*, 2019). We used the GreenGenes 13.08 database with a 99% identity (DeSantis *et al.*, 2006) and the feature-classifier plugin with the classify-sklearn method (Bokulich *et al.*, 2018) to assign bacterial taxonomies to representative OTU sequences. The UNITE

database (version 7 released on 01.12. 2017) (Nilsson *et al.*, 2018), which is composed of fungal singleton sequences identified by taxonomic experts, was used to assign fungal OTUs. To exclude OTUs other than fungi, such as those of insects or plants, we used BLAST to query the representative OTU sequences against the NCBI NR database and excluded OTUs annotated to the ITS of rice planthoppers or plants (*Oryza* sp.) (Fig. 1B). Information on fungi was obtained from the application FUNGuild (Nguyen *et al.*, 2016).

To avoid potential sequencing artifacts, we set the sampling depth to 1500 reads for bacteria and to 500 reads for fungi and removed samples with low sequence reads for the diversity analysis (Fig. S2). After quality control and rarefaction cutoffs, 167 bacterial samples, containing 122 bacterial OTUs, and 118 fungal samples, containing 748 fungal OTUs, were left for subsequent analyses. The samples harbored an average of 10.86 ± 0.32 bacterial OTUs (mean ± SE), but only 4 OTUs made up 90% of the total sequences. An average of 15.03 ± 0.47 fungal OTUs were identified from all the samples, and 31 OTUs accounted for 80% of the total sequences. Alpha-diversity analyses were performed using observed OTUs for microbial richness and Shannon indexes for microbial species diversity. The diversity index values were compared with Kruskal–Wallis test results after Benjamini–Hochberg correction. Beta-diversity comparisons were performed using the Unweighted-UniFrac methods for presence/absence metrics and the Weighted-UniFrac methods for relative abundance metrics. The distance matrices were further analyzed using an adonis (also named as Permutational Multivariate Analysis of Variance, PERMANOVA) analysis (Anderson, 2001) with 999 permutations to compare the differences in microbial communities among species, sexes, tissues and host environments (locations). To better represent variations within each factor, we analyzed the testing factor and consolidated the other factors. Alpha- and beta-diversity analyses were conducted using the R packages "vegan" (Oksanen *et al.*, 2007) and "phyloseq" (McMurdie & Holmes, 2013) on R 3.5.3 (R

Core Team, 2018). Diversity visualization was realized using the R package "ggplot2" (Wickham, 2016).

Molecular phylogenetic analysis

To further clarify the taxonomic positions of the abundant microbial OTUs, we inferred their phylogeny using closely related sequences from NCBI. The 16S rRNA gene and ITS sequences were aligned using MUSCLE (Edgar, 2004) and were manually trimmed using MEGA 7 (Kumar *et al.*, 2016). The maximum-likelihood phylogenetic tree was constructed using IQ-TREE 1.6.5 (Nguyen *et al.*, 2015) with the best-fitting nucleotide substitution model (option'-m AUTO'). Node support was calculated using 1000 ultrafast bootstraps. The tree was visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Accession numbers

The raw Illumina sequencing data for the 16S rRNA and ITS sequences have been deposited in the GenBank database under BioProject numbers PRJNA555522 and PRJNA555662, respectively.

Results

Comparison of bacterial communities

We first analyzed the bacterial microbes associated with wild *L. striatellus* and *S. furcifera*. Alpha-diversity analyses showed that bacterial species richness (observed OTUs)

and phylogenetic diversity (Shannon) were both significantly different between the two species of rice planthoppers (Fig. 2, Table S1). Bacteria were more abundant and more diverse in *L. striatellus* than in *S. furcifera* ($\chi^2 = 67.39$ and $P < 0.0001$ for observed OTUs; $\chi^2 = 13.61$ and $P = 0.0002$ for Shannon index, Kruskal–Wallis test; Fig. 2). A principle components analysis of beta-diversity based on Unweighted and Weighted UniFrac analyses showed that the bacterial community structures significantly differed qualitatively and quantitatively between the two planthopper species ($R^2 = 0.016$ and $P = 0.001$ for Unweighted; $R^2 = 0.031$ and $P = 0.001$ for Weighted, adonis; Fig. 2, Table 1), indicating that the bacterial community varied among rice planthoppers.

In *L. striatellus*, the males harbor a more diverse bacterial community than the females, while the bacterial abundance levels in both genders are similar (Table 1, Fig. S3). No significant differences in bacterial abundance and diversity were detected across locations. Although different tissues harbored similar amounts of bacteria, the gut had a more diverse bacterial community (Table 1, Fig. S4). The Unweighted UniFrac analysis showed that the bacterial communities did not significantly differ among sexes, tissues or host environments (Table 1). However, when the relative abundance was included (Weighted UniFrac analysis), the bacterial community structures among samples taken at different locations became significantly different ($P = 0.004$, adonis; Table 1).

In *S. furcifera*, both the bacterial abundance and diversity in males were greater than in females (Table 1, Fig. S3). In addition, the beta-diversity analysis showed that the bacterial community structure was significantly different between female and male samples ($P < 0.05$ for Weighted and Unweighted UniFrac analysis, adonis; Table 1). At different locations, *S. furcifera* had similar bacterial diversity levels but different abundance levels (Table 1). The bacterial community varied qualitatively in samples from different locations ($P = 0.007$, adonis, Unweighted UniFrac analysis; Table 1). No significant differences in bacterial

abundance or diversity levels were detected across *S. furcifera* tissues (Fig. S4). However, the Weighted UniFrac analysis showed significant quantitative differences in the bacterial community across tissues (Table 1).

Relative abundance of bacteria at the genus level

The taxonomic analysis revealed that the bacterial communities in the two planthopper species were mainly dominated by *Wolbachia*, which accounted for >80% of the bacterial reads (Fig. 3A, Table S3), followed by *Cardinium*, *Pantoea* and *Rickettsia.* Bacteria from these four genera accounted for 90% of the identified reads in both species (92.9% for *L. striatellus* and 90.9% for *S. furcifera*). In addition, *Wolbachia* and *Pantoea* were more abundant in *L. striatellus* than in *S. furcifera* (*L. striatellus* vs *S. furcifera* = 87.21% ± 0.21% vs 82.58% ± 1% for *Wolbachia* and 1.67% ± 0.07% vs 0.12% ± 0.06% for *Pantoea*; mean ± SEM).

In *L. striatellus*, the relative abundance levels of *Wolbachia* were significantly different among locations (χ^2 = 14.13 and P = 0.015, Kruskal–Wallis test). Although the relative abundances of other bacteria were not significantly different among sexes, tissues and host environments, the Spearman correlation analysis showed the relative abundance of *Wolbachia* was significantly negatively correlated with *Cardinium*, *Pantoea* and *Rickettsia* in *L. striatellus* (Fig. 3B). However, *Cardinium* was positively correlated with *Rickettsia* (Fig. 3B).

In *S. furcifera*, the proportion of *Wolbachia* was higher in the females than in the males (female = 86% ± 1.18% vs male = 80% ± 1.47%, P = 0.007, Kruskal–Wallis test). On the contrary, *Rickettsia* was more abundant in the males than in the females (female = 0.87% ± 0.34% vs male = 1.7% ± 0.14%, P < 0.001, Kruskal–Wallis test). In addition, the

proportions of *Rickettsia* and *Cardinium* were significantly different among tissues in *S. furcifera*. *Rickettsia* was more abundant in testes and gut (χ^2 = 26.07, P < 0.001, Kruskal–Wallis test) compared with *Cardinium* (χ^2 = 23.37, P < 0.001, Kruskal–Wallis test). As in *L. striatellus*, the relative abundance of *Wolbachia* was negatively correlated with *Cardinium* in *S. furcifera* (r = −0.27, P < 0.001, Spearman). In addition, *Wolbachia* were negatively correlated with *Chryseobacterium* (r = −0.69, P = 0.018, Spearman). However, the *Cardinium* abundance in *S. furcifera* was negatively correlated with *Rickettsia* in *S. furcifera* (r = −0.28, P = 0.014, Spearman) (Fig. 3C), which contrasted results in *L. striatellus*.

Phylogenetic analysis of bacteria from planthoppers

Because the 16S rRNA genes generated by MiSeq sequencing were short, we manually built phylogenetic trees containing related sequences. *Wolbachia* in *L. striatellus* and *S. furcifera* belonged to supergroup B (Fig. S5), which is consistent with previous phylogenetic analyses using a set of five multilocus sequence typing markers (Zhang *et al.*, 2013). *Cardinium* from rice planthoppers grouped together with *Cardinium* from other Delphacidae members, such as *Euides speciosa* and *Dicranotropis hamata* (Fig. S6), indicating that *Cardinium* may have a relatively long evolutionary history of living within Delphacidae planthoppers. *Rickettsia* were phylogenetically grouped into two groups (Fig. S7). However, because the sequences used for the phylogenetic analysis were short, we could not identify which rice planthoppers *Rickettsia* spp. inhabit. Furthermore, the phylogenetic tree showed that *Pantoea* was *Pantoea agglomerans* (Fig. S8), a pathogen causing plant disease and opportunistic mammalian infections (Cruz *et al.*, 2007).

Comparison of fungal communities

Compared with those of bacteria, the fungal compositions, as reflected by ITS sequences, were much more complex in *L. striatellus* and *S. furcifera* (Fig. S3). Both the numbers of observed OTUs and Shannon indexes were much greater for fungi than bacteria. Alpha-diversity analyses of fungal ITS reads showed that *L. striatellus* samples had higher levels of fungal richness (observed OTUs) than *S. furcifera* samples (Fig. 4A, Table S1). However, their phylogenetic diversities (Shannon indexes) were similar (Fig. 4B). The UniFrac (Unweighted and Weighted) metrics revealed that fungal community structures among rice planthopper species were significantly different in both quality and quantity (Fig. 4C, 4D). However, although the fungal populations in *L. striatellus* and *S. furcifera* samples were significantly different from each other, the combined first two principle coordinates of the Unweighted UniFrac analysis explained only 9% of the data variation, with each explaining < 6% of the variation in the composition of fungi, as assessed by the adonis test (Fig. 4C), suggesting the complex natures of fungal communities in rice planthoppers.

The alpha- and beta-diversity analyses of fungi showed similar patterns in *L. striatellus* and *S. furcifera* samples (Table 1). There was no significant difference in the fungal communities between female and male planthoppers in either *L. striatellus* or *S. furcifera* (Fig. S3, Table 1). Planthoppers from within populations collected at different locations also contained similar OTU numbers and diversity levels of fungi (Table 1). However, the fungal community composition differed significantly among the different locations (R^2 = 0.087 and P = 0.001 for *L. striatellus*; R^2 = 0.15 and P = 0.001 for *S. furcifera*, adonis, Unweighted UniFrac analysis; Table 1). The fungal abundance and diversity levels were significantly different among the various tissues in both species (Table 1). In addition, the structure of fungal community was also significantly qualitatively

different among *L. striatellus* tissues (R^2 = 0.065 and P = 0.002 for *L. striatellus*; R^2 = 0.091 and P = 0.001 for *S. furcifera*, adonis, Unweighted UniFrac analysis; Table 1). In *L. striatellus*, the guts tended to harbor greater numbers and more diverse fungi compared with other tissues, while in *S. furcifera*, this was true in the testes and guts (Fig. S4).

Relative abundance of fungi at the genus level

No genus accounted for more than 20% of the identified fungi. Phylogenetic-based assignments of fungal sequences showed that the five most abundant fungal genera were *Sarocladium*, *Alternaria*, *Malassezia*, *Aspergillus* and *Curvularia* (unidentified reads were excluded), which collectively accounted for 41.9% of the fungal composition (Fig. 5, Table S4), while the top four bacterial genera accounted for a much higher percentage of the total bacterial composition. Among them, the proportions of *Sarocladium* and *Aspergillus* were significantly greater in *L. striatellus* than in *S. furcifera* (*L. striatellus* vs *S. furcifera* = 12.72% ± 1.54% vs 2.33% ± 0.67% for *Sarocladium* and 1.53% ± 0.59% vs 0.43% ± 0.19% for *Aspergillus*, mean ± SEM), while *Curvularia* was more abundant in *S. furcifera* than in *L. striatellus* (*L. striatellus* vs *S. furcifera* = 0.5% ± 0.17% vs 2.81% ± 0.9%).

The taxonomic analysis revealed that *Sarocladium* accounted for 20.33% of the taxonomically assigned fungal reads on average in *L. striatellus* and was the most abundant fungal genera (Fig. 5A). After *Sarocladium*, *Alternaria* accounted for 14.78% and *Malassezia* accounted for 6.22%. The proportions of *Sarocladium* and *Malassezia* were significantly in *L. striatellus* samples from different locations (P = 0.004 for *Sarocladium*; P < 0.001 for *Malassezia*, Kruskal–Wallis test) and different tissues (P < 0.001 for *Sarocladium*; P = 0.008 for *Malassezia*, Kruskal–Wallis test). The correlation analysis showed that *Alternaria* was

negatively correlated with *Malassezia*, *Sarocladium* and *Aspergillus* in *L. striatellus* (Fig. 5B), while *Malassezia* and *Sarocladium* were positively correlated (Fig. 5B).

In *S. furcifera*, the most prevalent fungal genera were *Alternaria* (12.75%), *Malassezia* (11.82%) and *Sarocladium* (5.69%) (Fig. 5). The proportions of *Alternaria* and *Malassezia* in *S. furcifera* were significantly different among various tissues ($\chi^2 = 9.39$ and $P = 0.024$ for *Alternaria*; $\chi^2 = 21$ and $P < 0.001$ for *Malassezia*, Kruskal–Wallis test). The relative abundance of *Sarocladium* was higher in males than in females ($\chi^2 = 6.44$, $P = 0.011$, Kruskal–Wallis test) and varied among *S. furcifera* from different locations ($\chi^2 = 14.28$, $P = 0.014$, Kruskal–Wallis test). A correlation analysis showed that *Alternaria* was negatively correlated with *Malassezia* (Fig. 5C), while *Aspergillus*, *Malassezia* and *Sarocladium* were all positively correlated in *S. furcifera* (Fig. 5C).

Phylogenetic analysis of fungi from planthoppers

A phylogenetic analysis of fungal ITS region sequences showed that *Sarocladium* OTUs identified in *L. striatellus* and *S. furcifera* mainly belonged to *Sa. implicatum* (Fig. S9), a fungal symbiont of a *Brachiaria* grass (Poaceae) (Kago *et al.*, 2016). In addition, *L. striatellus* and *S. furcifera* harbored other fungi associated with Poaceae plants, including *Sa. zeae* and *Sa. oryzae* (Fig. S9). The former is a maize symbiont, and the latter is a rice pathogen (Bridge *et al.*, 1989). The majority of detected *Malassezia* OTUs from *L. striatellus* and *S. furcifera*, were *M. globose* and *M. restricta* (Fig. S10). *Malassezia* has also been identified in house dust mites (Klimov *et al.*, 2019). The phylogenetic tree was not able to accurately assign every *Alternaria* and *Aspergillus* fungal ITS sequence to a species (Figs. S11, S12). FUNGuild predictions have shown that *Alternaria* contains animal pathogens, endophytes, plant pathogens and wood saprotrophs (Nguyen *et al.*, 2016). From *L. striatellus*

and *S. furcifera*, we detected a diverse range of fungal OTUs that live with plants, including *Al. destruens* (Cook *et al.*, 2009).

Co-occurrence of bacteria and fungi

Because some bacteria and fungi were sequenced from the same samples, we determined the correlations between bacteria and fungi within rice planthoppers. We found neither the number of observed OTUs nor the diversity of bacteria within the community were significantly correlated to those of fungi in *L. striatellus* or *S. furcifera* (Fig. 6), suggesting that bacteria and fungi may have different ecological niches in rice planthoppers. However, in *L. striatellus*, the abundance of *Wolbachia* was slightly positively correlated with that of *Aspergillus* (r = 0.23, P = 0.048, Spearman) (Fig. S13A). In *S. furcifera*, the relative abundance of *Rickettsia* was positively correlated with that of both *Malassezia* and *Sarocladium* (r = 0.56 and $P < 0.001$ for *Malassezia*; r = 0.36 and P = 0.027 for *Sarocladium*, Spearman) (Fig. S13B). These results imply that some bacterial symbionts may have mutualistic interactions with fungi in host planthoppers.

Discussion

The results presented here further our understanding of the diversity of microbes (including bacteria and fungi) in rice planthoppers and plant sap-feeding insects in general. On the basis of our collective sequencing evidence, we concluded that microbiota in wild *L. striatellus* and *S. furcifera* are distinctly different from each other. The relative abundance levels of bacteria and fungi were not correlated in *L. striatellus* and *S. furcifera*, revealing the independence of the bacterial and fungal communities. Although *L. striatellus* harbors a more abundant and

diverse bacterial community than *S. furcifera*, the communities in both species of rice planthoppers were dominated by the intracellular bacterial symbiont *Wolbachia.* The fungal communities were much more diverse than the bacterial communities. We further demonstrated that some bacteria and fungi may be positively or negatively correlated in planthoppers. Thus, our findings are indicative of the complex interactions between microbes of different plant sap-feeding insects and suggest the importance of evaluating microbiota diversity in insects that share an ecological niche.

Although *L. striatellus* and *S. furcifera* were sampled from the same rice plant, their bacterial and fungal communities varied significantly, indicating that host phylogeny is involved in shaping the overall microbial community. This is consistent with research in Lepidopteran mulberry-feeding insects (Chen *et al.*, 2018). The large dagger moth *Acronicta major* and the mulberry pyralid *Diaphania pyloalis* both feed on mulberry leaves but have distinct abundance and diversity levels of microbiota (Chen *et al.*, 2018). Studies of the bacterial and fungal communities of *Drosophila* species showed that the effects of host diet are much greater than those of host species (Chandler *et al.*, 2011; Chandler *et al.*, 2012). However, a recent field assay revealed that some *Drosophila* species have gut-specialized bacteria (Martinson *et al.*, 2017). The drastic differences in microbiota between rice planthopper species may result from various physiological conditions in different insects.

Bacteria were significantly more abundant in the male rice planthoppers compared with female hosts, which was in agreement with previous findings in ticks (Van Treuren *et al.*, 2015). The bacterial community structures were similar between *L. striatellus* sexes, but varied significantly between *S. furcifera* sexes, suggesting genotype, compared with sex, has a greater influence on determining the bacterial microbiota (Kovacs *et al.*, 2011; Van Treuren *et al.*, 2015). The bacterial microbiota of different sexes were similar in the spotted wing *Drosophila suzukii*, in which the microbiota are determined by diet (Bing *et al.*, 2018).

Female planthoppers usually harbor more intracellular bacteria because they are larger than males. There were no significant differences in the diversity level or community structure between sexes of fungal microbes, suggesting that bacteria and fungi are relatively loosely correlated in rice planthoppers.

The detection of symbionts in reproductive tissues (especially ovary) indicates their maternal transmission. Indeed, symbionts like *Wolbachia* and *Cardinium* are vertically transmitted among rice planthoppers (Bing *et al.*, 2019). The bacterial abundance was similar among different tissues in *L. striatellus* and *S. furcifera*, while, the fungal abundance was significantly different. The gut harbored more fungi than the remaining tissues, suggesting that planthoppers obtained fungi from feeding. Many fungal ITS reads were phylogenetically assigned to plant endosymbionts (such as *S. implicatum* and *A. destruens*), implying the horizontal transmission of plant symbionts through insects. However, whether rice planthoppers obtain plant fungal endosymbionts through feeding and how fungi affect host biology have not been experimentally confirmed.

The bacterial and fungal abundance and diversity levels within rice planthopper populations of different locations were similar. However, the bacterial and fungal community structures in *L. striatellus* and *S. furcifera* were significantly different among locations. *L. striatellus* is more resistant to cold temperature compared with *S. furcifera* and overwintering in the collection region (Jiangsu Province, China). *S. furcifera* migrates, probably traveling from a limited region in southern China (Chen *et al.*, 2019). The bacterial microbiota of sap-feeding insects, such as *Ixodes* ticks and *Bemisia tabaci* MEAM1 and MED cryptic species (Jing *et al.*, 2014; Van Treuren *et al.*, 2015), vary significantly among different geographical locations. Thus, environment may influence the microbiota of plant sap-feeding insects.

The bacterial communities in field populations of *L. striatellus* and *S. furcifera* were dominated by *Wolbachia*, which accounted for more than 80% of the bacterial reads,

followed by *Cardinium*, *Rickettsia* and *Pantoea*. Previous surveys using diagnostic species-specific primers showed that *L. striatellus* and *S. furcifera* are heavily infected by *Wolbachia*, which was phylogenetically assigned to supergroup B (Zhang *et al.*, 2013). Our results confirmed the prevalence of *Wolbachia* and elucidated its absolute dominance in field rice planthoppers. *Wolbachia* induces CI in both species (Noda *et al.*, 2001; Ju *et al.*, 2017). The somatic *Wolbachia* distribution in insects may be associated with more functions, including, but not limited to, manipulating host reproduction (Pietri *et al.*, 2016). *Wolbachia* infections increase the fecundity of *L. striatellus* by provisioning biotin and riboflavin (Ju *et al.*, 2019). As the most dominant symbiont in *L. striatellus* and *S. furcifera*, the effects of *Wolbachia* on host biology should be studied more thoroughly.

Diagnostic PCR showed that *S. furcifera* is commonly co-infected with *Wolbachia* and *Cardinium* (Nakamura *et al.*, 2012; Zhang *et al.*, 2012a). However, *Cardinium* has been rarely detected in *L. striatellus* (Zhang *et al.*, 2013). In this study, *Cardinium* was identified in both *L. striatellus* and *S. furcifera*. The variation in the *Cardinium* infection rate between our sequencing and a previous survey may have resulted from our pooling of 3–5 individuals in one sample instead of individually screening insects. The *Cardinium* found in *L. striatellus* and *S. furcifera* were closely related to those found in other Delphacidae members, indicating that *Cardinium* has an ancient association with planthoppers. The successful amplification of *Cardinium* from ovary, testis, midgut and the remaining carcass of *S. furcifera* suggests that *Cardinium* distributes throughout individuals and may be mobile (Zeng *et al.*, 2018). *Cardinium* appears to have no effect on the fecundity of females, but hastens the nymphal development (Zhang *et al.*, 2012b). Importantly, *Cardinium* induces CI and is the leading factor of CI in *S. furcifera* when co-infected with *Wolbachia* (Nakamura *et al.*, 2012; Zhang *et al.*, 2012a; Bing *et al.*, 2019). Intracellular symbionts influence the rest of the microbiome

(Fromont *et al.*, 2019). Our data showed that *Cardinium* had a negative correlation with *Wolbachia*, but details of the interactions between these two symbionts are unclear.

Another intracellular bacteria, *Rickettsia*, accounted for more than 1% of reads in both *L. striatellus* and *S. furcifera* samples. *Rickettsia* is a human pathogen that causes spotted fever. Although *Rickettsia* is important in many arthropods (Perlman *et al.*, 2006), the existence of *Rickettsia* in rice planthoppers was previously ignored. A phylogenetic analysis showed that *Rickettsia* OTUs from planthoppers belonged to two clades. However, based on partial 16S rRNA sequences, we cannot determine the exact *Rickettsia* species. The biology of *Rickettsia* needs more exploration in rice planthoppers.

Pantoea species are associated with rice pests, like the rice water weevil *Lissorhoptrus oryzophilus, N. lugens*, *L. striatellus* and *S. furcifera* (Kang, 2014; Zhang *et al.*, 2016; Li *et al.*, 2017; Zhang *et al.*, 2018). The *Pantoea* sequence obtained in this study was closely related to that from *L. oryzophilus* and was phylogenetically assigned to *P. agglomerans*, a plant pathogen causing human disease (Cruz *et al.*, 2007), which implies the horizontal transmission of *Pantoea* among rice pests. Without experimental validation, we do not know if *Pantoea* vectored by rice pests causes any rice disease. However, because *Pantoea* can be isolated and cultured *in vitro*, this can be easily confirmed in the near future.

Both *L. striatellus* and *S. furcifera* feed exclusively on plant sap, which is nutritionally depauperate in the essential amino acids and other nutrients (Douglas, 2006). Thereby, plant sap-feeding insects harbor symbionts that supplement their dietary deficiencies. Multiple Sternorrhyncha phloem-feeding insects, like *B. tabaci* and *Acyrthosiphon pisum*, harbor very few bacterial taxa and all harbor primary symbionts (Jing *et al.*, 2014). Species in the Auchenorrhyncha (including the cicadas, leafhoppers and spittlebugs) broadly specialize on using phloem and xylem for food and harbor the primary symbiont *Sulcia*, which may have allied with the Auchenorrhyncha since the origin of the suborder (Moran *et al*., 2005). The

Hawaiian planthopper *Oliarus filicicola* also harbors *Sulcia* as a primary symbiont (Bennett & Mao, 2018). However, *Sulcia* reads were neither observed in our dataset nor in other high-throughput sequencing analyses (Li *et al.*, 2017; Zhang *et al.*, 2018; Zhang *et al.*, 2019; Zhang *et al.*, 2020), suggesting that *Sulcia* does not exist in rice planthoppers.

Primary symbionts are obligatory and essential. When the primary symbionts do not function well, other symbionts often become obligatory and jointly support the growth and survival of host insects (such as *Sulcia* and *Ophiocordyceps* fungi in cicadas) (Matsuura *et al.*, 2018). Rice planthoppers harbor YLSs, which provide essential amino acids and sterols to planthoppers (Yukuhiro *et al.*, 2014). A phylogenetic analysis based on 18S and 26S rRNA genes placed the rice planthopper YLSs into the filamentous ascomycetes *Cordyceps* (Euascomycetes: Hypocreales: Clavicipitaceae), which are parasitic mainly in insects and other arthropods (Suh *et al.*, 2001). There were very few *Cordyceps* YLS reads in our dataset, which may result from the differences in methodology. Our phylogenetic analysis of ITS sequences showed that the most abundant fungi in planthoppers were associated with plants, such as *Sarocladium* (Hypocreales: Sarocladiaceae). Additionally, they were more abundant in guts than in other tissues. It is possible that fungi are acquired by planthoppers from direct feeding. The correlation analysis showed bacterial abundance and diversity were not correlated with those of fungi in general, suggesting that their biology may be distinct in planthoppers.

In conclusion, our results demonstrate that bacterial and fungal microbiota in rice planthoppers vary significantly among different species. The bacterial diversity and abundance have no correlations with those of fungi in rice planthoppers. Although planthoppers are dominated by *Wolbachia*, the bacterial diversity differs significantly among sexes, tissues, and host environments. The abundant fungi in planthoppers are likely associated with plant symbionts or pathogens. However, this study has many limitations. Our

method may have resulted in missing taxa. The greater diversity of symbionts does not necessarily mean their greater absolute abundance. More detailed characterizations and functional analyses of microbes in rice planthoppers should be unraveled by specifically designed experiments. Additionally, the mechanisms responsible for symbiont-host interactions should be explored.

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Disclosure

The authors declare that there is no conflict of interest in this work.

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Table 1 Comparison of bacterial and fungal community within *L. striatellus* and *S. furcifera.*

			Alpha diversity				Beta diversity			
			Observed		Shannon		Unweighted-Unifrac		Weighted-Unifrac	
			χ^2	*P*	χ^2	*P*	R^2	*P*	R^2	*P*
Bacteria	*L. striatellus*	Gender	0.71	0.4	6.327	**0.012**	0.005	0.779	0.001	0.975
		Location	10.02	0.075	8.411	0.135	0.058	0.451	0.135	**0.004**
		Tissue	3.34	0.503	17.187	**0.002**	0.038	0.615	0.067	0.098
	S. furcifera	Gender	5.17	**0.023**	11.581	**0.001**	0.041	**0.004**	0.075	**0.002**
		Location	14.93	**0.011**	2.742	0.74	0.134	**0.007**	0.058	0.204
		Tissue	2	0.736	5.368	0.252	0.058	0.263	0.276	**0.001**
Fungi	*L. striatellus*	Gender	0.261	0.609	0.078	0.781	0.014	0.255	0.019	0.254
		Location	5.481	0.36	11.001	0.051	0.087	**0.001**	0.05	0.828
		Tissue	11.534	**0.021**	12.674	**0.013**	0.065	**0.002**	0.075	0.132
	S. furcifera	Gender	2.772	0.096	0.97	0.325	0.024	0.276	-0.015	0.975
		Location	2.085	0.837	2.891	0.717	0.15	**0.001**	0.196	0.086
		Tissue	15.507	**0.001**	19.171	**0.000**	0.091	**0.001**	0.08	0.311

Figure legends

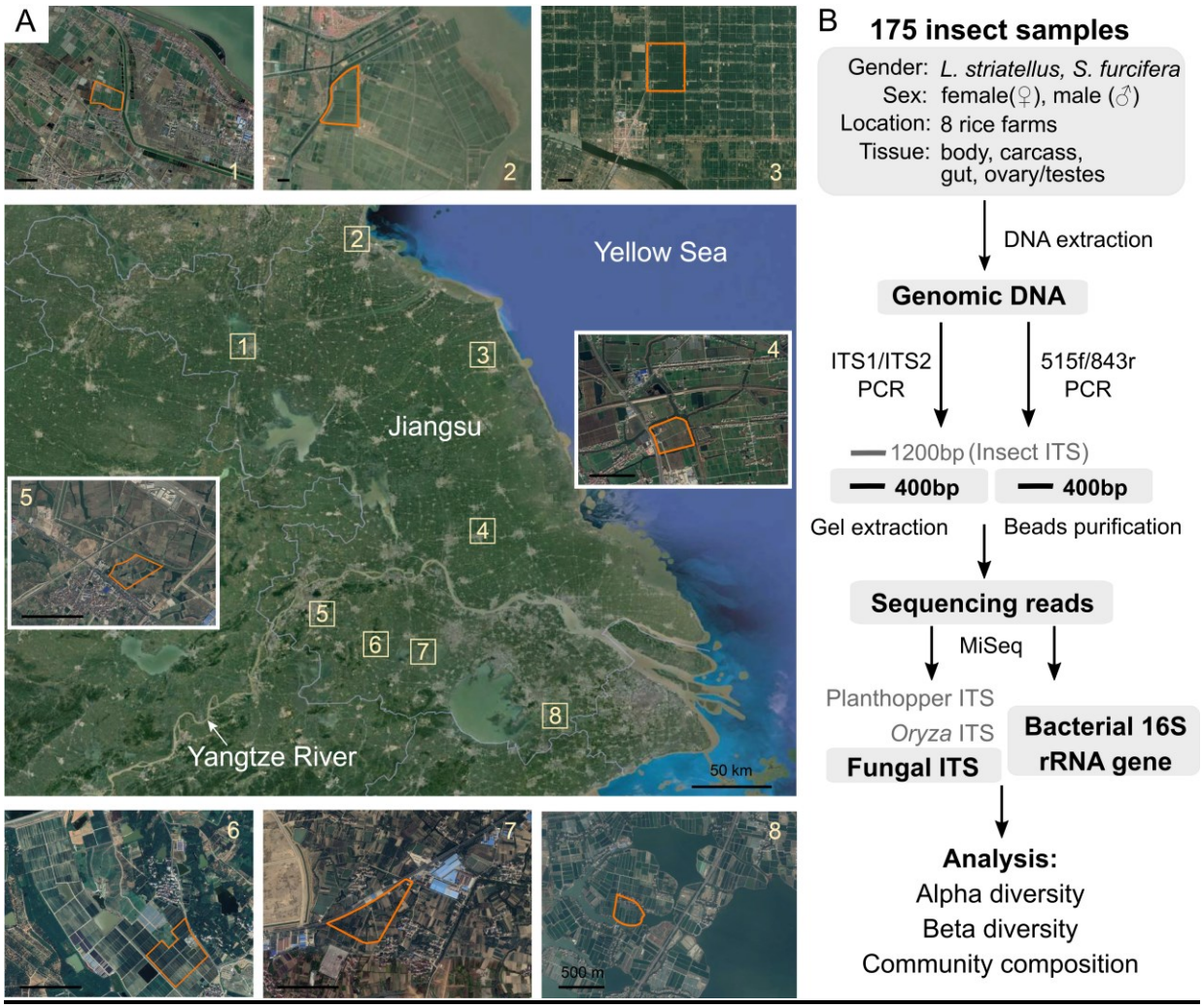

Fig. 1 Collection localities for wild planthoppers (A) and workflow of microbiota analysis (B). The map was downloaded from Google Earth. The locations of sampling sites are noted with numbers on the center big map. The farms where planthoppers were collected are labeled with red boxes. The scale bars for small maps were for 500 m.

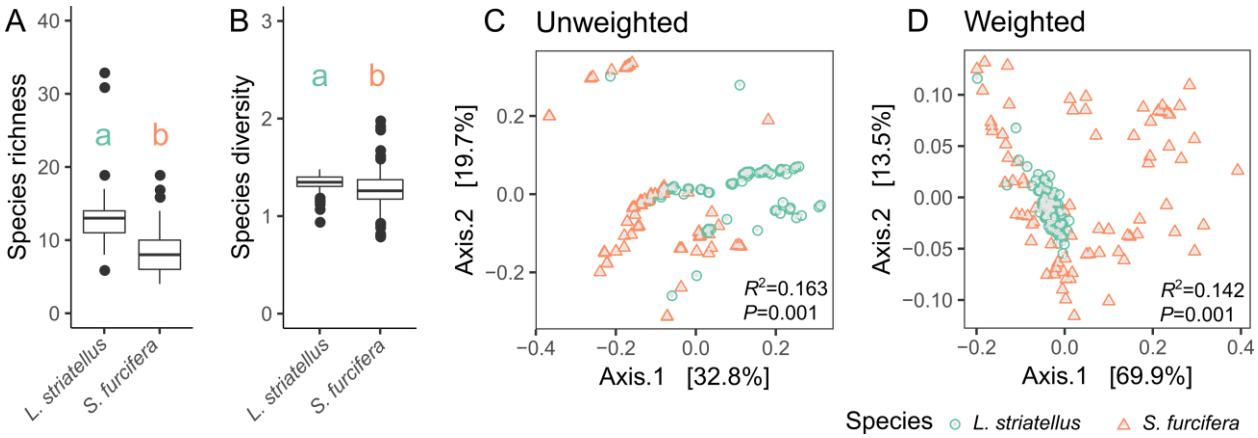

Fig. 2 Bacterial community variation between *L. striatellus* and *S. furcifera*. (A–B) Boxplots of (A) species richness (observed OTUs) and (B) species diversity (Shannon index). The significant differences of alpha diversities were analyzed using Kruskal-Wallis test. (C–D) Unweighted (C) and Weighted (D) UniFrac-based PCoA plots of bacterial communities. The significant differences in beta diversities were analyzed using adonis analysis with 999 Monte Carlo permutations. Different letters indicate significant statistical difference at $P < 0.05$.

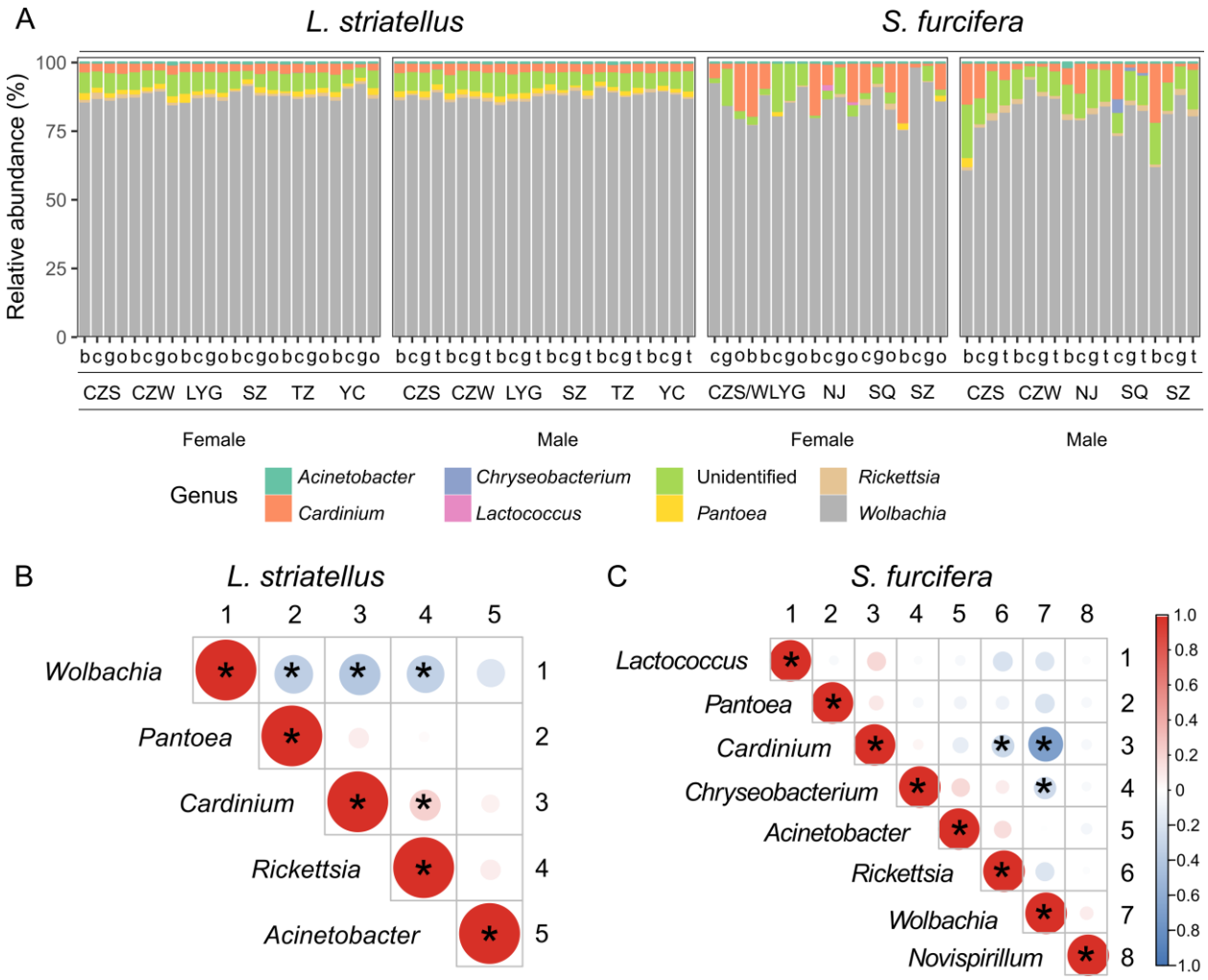

Fig. 3 Taxonomic composition of bacterial communities associated with *L. striatellus* and *S. furcifera*. Relative abundance of each bacterial genus (A). Each bar is indicated by a different color at genus level. Sampling depth was set to 1500 to remove samples with low sequence reads. OTUs that could not be classified into genus or were < 0.05% of average relative abundance in groups are summarized as "unidentified." (B–C) Spearman correlations of samples with the OTU abundance of genus in *L. striatellus* (B) and *S. furcifera* (C). Positive correlations are displayed in red and negative correlations in blue color. Color intensity and the size of the circle are proportional to the correlation coefficients. * means the significant level $P < 0.05$.

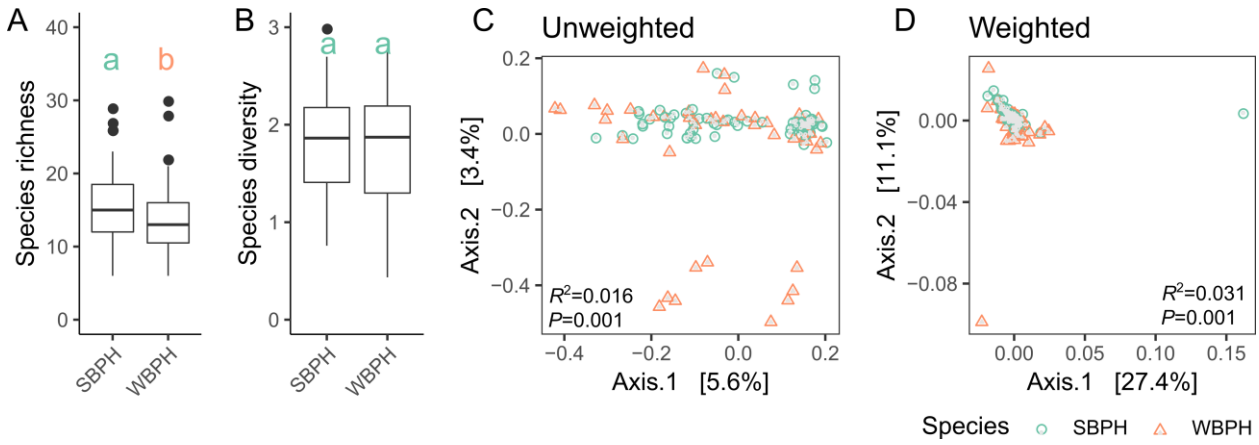

Fig. 4 Fungal community variation between *L. striatellus* and *S. furcifera.* (A–B) Boxplots of (A) species richness (observed OTUs) and (B) species diversity (Shannon index). The significant differences of alpha diversities were analyzed using Kruskal-Wallis test. (C–D) Unweighted (C) and Weighted (D) UniFrac-based PCoA plots of fungal communities. The significant differences in beta diversities were analyzed using adonis analysis with 999 Monte Carlo permutations. Different letters indicate significant statistical difference at *P* value < 0.05.

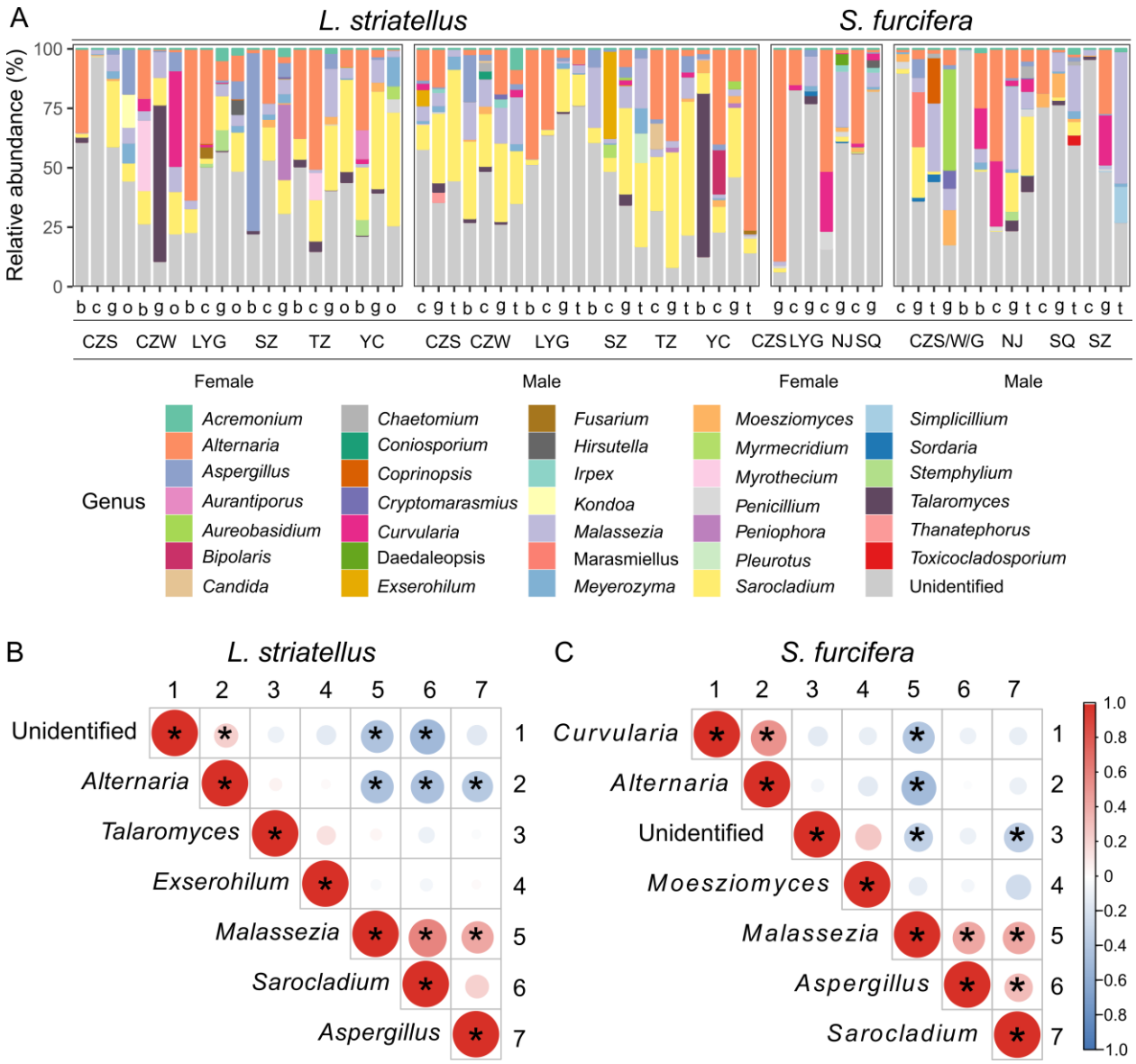

Fig. 5 Taxonomic composition of fungal communities associated with *L. striatellus* and *S. furcifera*. Relative abundance of each fungal genus (A). Each bar is indicated by a different color at genus level. Sampling depth was set to 500 to remove samples with low sequence reads. OTUs that could not be classified into genus or were < 0.1% of average relative abundance in groups are summarized as "unidentified." (B–C) Spearman correlations of samples with the OTU abundance of top 7 genus in *L. striatellus* (B) and *S. furcifera* (C). Positive correlations are displayed in red and negative correlations in blue color. Color

intensity and the size of the circle are proportional to the correlation coefficients. Significant correlations are shown as * ($P < 0.05$).

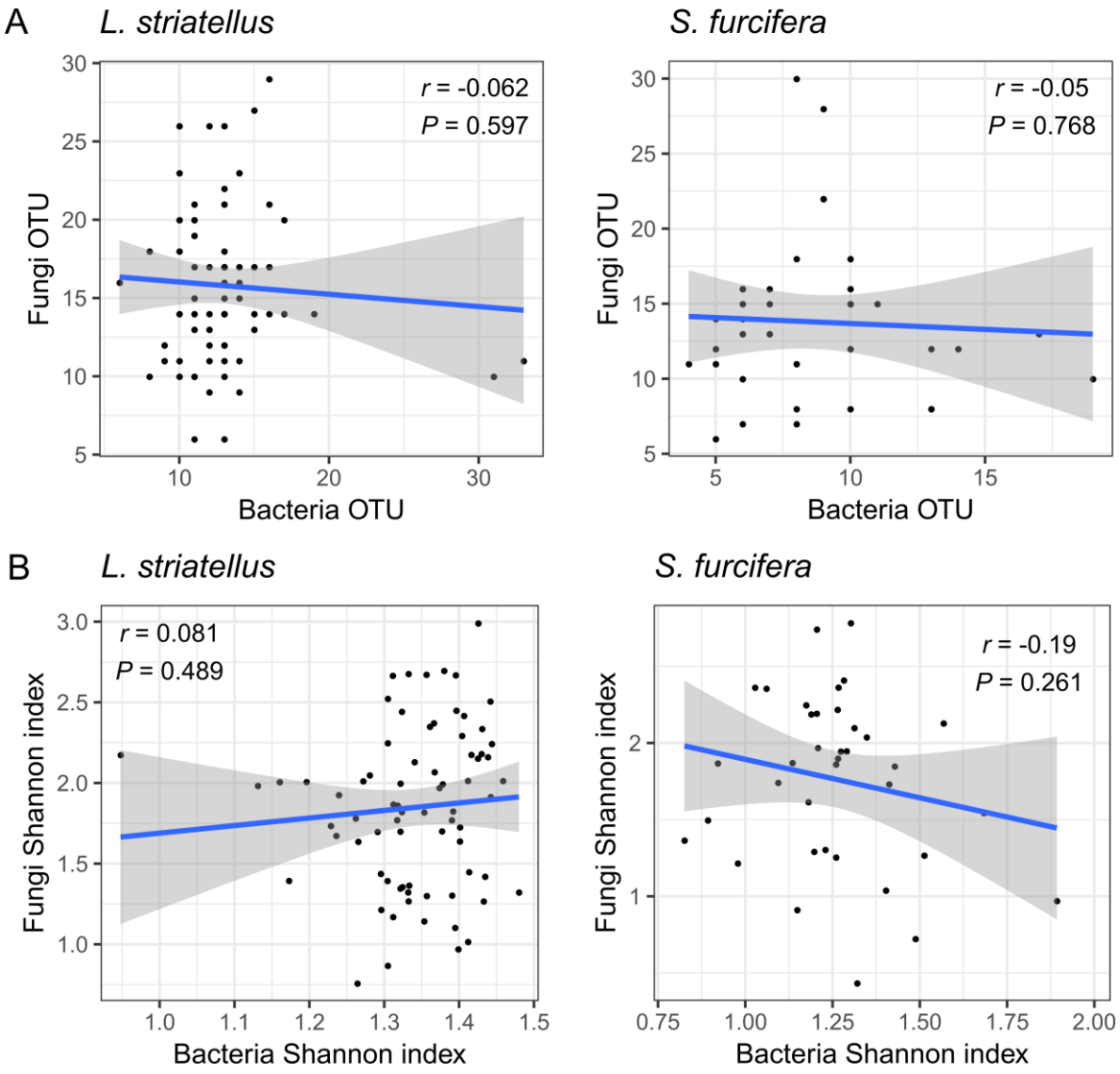

Fig. 6 Correlation of bacterial and fungal alpha diversity indexes in *L. striatellus* and *S. furcifera*. The Pearson correlation coefficient (r) and the significance level (P value) are shown on plot figures.