Comparison of the bacterial diversity and abundance between both sexes of *Leptocybe invasa* Fisher & La Salle (Hymenoptera: Eulophidae)

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Comment [1]: Title change suggested as above

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Abstract

Background. Insects harbor a myriad of microorganisms, many of which can affect the sex ratio and manipulate the reproduction of the host. *Leptocybe invasa* is an invasive pest that causes serious damage to eucalyptus plantations, and both a female-biased sex ratio and thelytokous parthenogenesis in *L. invasa* contribute to the rapid invasion and fast growth of the population. However, the internal bacterial composition and abundance of *L. invasa* and the differences between both sexes remain unclear.

Results. The Illumina MiSeq platform was used to compare the composition of the bacterial community in adult females and males of *L. invasa* by sequencing the V3-V4 region of the 16S ribosomal DNA gene. The results showed that 1320 operational taxonomic units (OTUs) were obtained in total. These OTUs were subdivided into 24 phyla, 71 classes, 130 orders, 245 families and 501 genera. At the genus level, the dominant bacteria in females and males were *Rickettsia* and *Rhizobium*, respectively.

Conclusion. The bacteria living in *L. invasa* females and males were highly diverse. There were differences in the bacterial community in *L. invasa* between both sexes, and the bacterial diversity in male specimens was more abundant than that observed in female specimens. This study presents a comprehensive comparison of bacterial communities living in *L. invasa* . Bacterial endosymbionts are thought to play a significant role in the reproductive strategy, sex regulation and the invasive mechanism of *L. invasa* and provides a basis for follow-up studies on the coevolution and interaction between *L. invasa* and its predominant bacteria.

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Comment [3]: This female-biased sex ratio occurs as a result of thelytokous reproduction – change sentence to clarify this.

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Comment [4]: There are also other factors such as the fact that Leptocybe is a gall-former and protected within the gall and its small size making it difficult to detect early on. These would also need to be mentioned here.

Comment [5]: Do you mean the abundance of the bacterial endosymbionts? If so you need to state this more clearly. Furthermore – do you also mean the differences of endosymbiotic species harbored by males and females? This sentence needs a lot of clarification.

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Comment [6]: This portion of the sentence requires substantial clarification – as it currently stand the authors suggest that differences between male and female specimens is unclear, which is not the case at all. The two sexes can be clearly distinguished based on their antennae as well as other morphological characteristics.

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Introduction

There are numerous microorganisms living in insects, including bacteria, fungi, yeast and viruses, that play a vital role in the growth and reproduction of host insects (*Dillon & Dillon, 2004; Doğanlar, 2005; Crotti et al., 2012; Frago et al., 2012; Engel & Moran, 2013; Hammer & Bowers, 2015*). In the course of long-term coevolution, microorganisms have a close relationship with host insects, which may have an effect on reproduction, survival, community interaction, and the ability to resist predators and vectors (*Oliver et al., 2003, 2010; Moran, 2007; Clark et al., 2008; Moran et al., 2008; Moya et al., 2008*). Bacterial diversity and function have been well studied in some insects. For instance, the bacteria in termites focus on *Bacteroidetes, Firmicutes* and *Actinobacteria* and could assist their hosts in breaking down lignocellulose and promoting the nitrogen cycle (*Warnecke et al., 2007; Brune, 2014).* The bacteria in *Aphis gossypii* improve the resistance and adaptation of the host (*Łukasik et al., 2013a, b*).

In addition, previous investigations have shown that sex is an important factor affecting bacterial diversity. For example, due to different attacking behaviors, the overall diversity and richness of bacterial communities associated with female *Dendroctonus valens* are relatively higher than those associated with male beetles (*Xu et al., 2016*). The bacterial composition of mosquitoes was also affected by the different sexes (*Minard et al., 2013; Zouache et al., 2011*). Different anatomies and life histories of male and female flies could provide differential opportunities for bacterial colonization (*Tang et al., 2012*).

The blue gum chalcid *Leptocybe invasa* Fisher & LaSalle (Hymenoptera: Eulophidae: Tetrastichinae) is a cosmopolitan pest that damages many *Eucalyptus* species (*Mendel et al., 2004; Le et al., 2018*). *L. invasa*, originated in Australia, was first recorded in 2000 and has been discovered in 45 countries of Asia, Europe, Africa, Oceania and America thus far (*Le et al., 2018; Zheng et al., 2014*). Every delicate twig, vein and petiole of Eucalyptus trees may provide a spawning ground for this pest, and galls ultimately lead to the stunted growth of the trees, causing great losses in local eucalyptus plantations (*Mendel et al., 2004; Zheng et al., 2014; Huang et al., 2018*).

Until now, few studies have reported on the overall interior bacteria of *L. invasa,* which is an invasive and gall insect. Only a few studies have reported their interior bacteria completely. *Wang et al. (2018)* cultured 11 strains in female adults of *L. invasa* in winter using traditional methods and classified them into 3 phyla (*Firmicutes, Actinobacteria, Proteobacteria*), 3 classes

Comment [9]: In my opinion the introduction is too short and does not adequately introduce the topic of bacteria in insects and too little information is given on Leptocybe to put the need for this work into context.

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(*Bacilli, Actinobacteria, Gammaproteobacteria*) and 4 orders (*Bacillales, Micrococcales, Lactobacillales, Enterobacterales*) that were related to growth, development, nutrition metabolism and immunity. *Nugnes et al. (2015)* researched the bacteria living in adults among different populations through denaturing gradient gel electrophoresis (DGGE) analysis and found that *Rickettsia* occurred in the reproductive tissues of female *L. invasa*, resulting in the speculation of a relationship with its thelytokous parthenogenesis. *L. invasa* harbors a myriad of bacteria (*Wang et al., 2018; Nugnes et al., 2015*), and bacterial differences between sexes have a large effect on insects. Therefore, the overall interior bacterial composition and abundance of *L. invasa* and the differences between both sexes are important to study.

Comment [10]: Such as….

In this study, the interior bacteria in female and male adults of *L. invasa* were sequenced by 16S rDNA from the V3-V4 region to shed light on the interior bacterial composition. Adult females and males were also compared to address sexual differences in the interior bacteria. These results would provide valuable bacterial pool of *L. invasa* and would further contribute to understanding their productive strategies and invasion mechanisms.

Materials & Methods

Comment [11]: In general, the materials and methods requires a lot more detail and editing of grammar is essential.

Insect sampling

L. invasa female and male adults were captured from *Eucalyptus* plantations located at the Teaching and Experiment Base of Forestry College, Guangxi University (108°17′ E, 22°51′ N), Nanning City, Guangxi Zhuang Autonomous Region. The host plant in this survey was DH201-2 (*Eucalyptus grandis* × *E. tereticornis*) (Myrtales: Myrtaceae). A few specimens should have been sequenced using COI region and their identity confirmed by comparison to GenBank sequences of Leptocybe.

Comment [12]: How were these specimens identified? It is also important to distinguish between Leptocybe Haplogroup A and Haplogroup B (see Dittrich-Schroder et al., 2018 and Nugnes et al., 2015) as these are very divergent lineages

Comment [13]: When and how?

Total DNA extraction

Adults of both sexes of *L. invasa* newly emerged into 12 h were fasted for 6 h, and each sex included 50 adults. Then both samples sterilized externally with 75% ethanol for 2-5 min, and rinsed third times in sterilized water to remove microbes on the surface. Total bacterial DNA of each samples were extracted using the Power Soil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions. DNA quality and quantity were assessed by the ratios of 260 nm/280 nm and 260 nm/230 nm. Then the qualified DNA was stored at -80 □ until further processing.

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PCR amplification and cloning of bacterial 16S rDNA gene

Amplification of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed using bacteria-universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GACTACHVGGGTWTCTAAT-3'). The PCR reactions were carried out in a 50 μL solution containing 10 μL 10 × buffer, 0.2 μL Q5 High-Fidelity DNA Polymerase, 10 μL High GC Enhancer, 1 μL dNTP, 10 μM of each forward and reverse primer, 60 ng genome DNA and up to 50 μL with dd H_2O. The amplifications were performed in an ABI Applied Biosystems 9902 thermal cycler with an initial denaturation step at 95 □ for 5 min, followed 35 cycles of annealing and extending (each cycle occurred at 95 □ for 1 min, followed by 50 □ for 1 min and an extension step at 72 □ for 1 min) and the final extension at 72 □ for 7 min. The PCR products were checked by electrophoresis on an agarose gel (1.8% agarose, 1 × TBE) followed by staining with ethidium bromide and visualization under ultraviolet light. The PCR products from the first step PCR were purified through VAHTSTM DNA Clean Beads. A second round PCR was then performed in a 40 μL reaction which contained 20 μL 2 × Phμsion HF MM, 8 μL ddH_2O, 10 μM of each forward and reverse primer and 10 μL PCR products from the first step. The second PCR was run under the following conditions: an initial denaturation at 98 □ for 30s, followed by 10 cycles at 98 □ for 10 s, 65 □ for 30 s and 72 □ for 30 s, with a final extension at 72 □ for 5 min. Finally, all PCR products were quantified and pooled together by Quant-iT™ dsDNA HS Reagent. High-throughput sequencing analysis of bacterial rRNA genes was performed on the purified, pooled sample using the Illumina Hiseq 2500 platform at Biomarker Technologies Co., Ltd, Beijing, China.

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Bioinformatics and statistical analysis

After sequencing, PE Reads obtained from HiSeq sequencing were merged by overlapping to obtain raw tags. To obtain clean tags, the raw tags were de-noised, sorted and separated using Trimmomatic (version 0.33). The remaining sequences were filtered for redundancy, and all unique sequences for each sample were then clustered into operational taxonomic units (OTUs) at similarities of 97%. Low-abundance OTUs were identified and eliminated using UCHIME v4.2. The taxonomic notes of the OTUs were conducted in the Silva reference database. Species abundance tables were generated by QIIME, and community structures in every taxon category were plotted by R software. The relative abundances of the bacteria were determined as percentages. The relatively high abundances at the genus level were selected to construct the phylogenetic tree.

Alpha diversity based on Chao1 richness and ACE richness estimators, as well as Simpson and Shannon diversity indices, was evaluated using the Mothur v.1.11.0 program. Among them, Chao1 and ACE measured species richness in the samples, Shannon reflected community diversity, Simpson reflected the concentration degree of dominant species in the community, and coverage index reflected whether the sequencing results represented the real situation of microorganisms in the samples. A higher Chao1, ACE and Shannon index and a lower Simpson index indicates that the species in a sample are more abundant. A higher coverage indicates a higher probability of a detected species and a lower probability of an undetected species.

Results

Sequencing and Classification

A total of 533266 raw tags (370680 from males and 162586 from females) were obtained from *L. invasa,* and 476235 effect tags (328833 from males and 147402 from females) were generated (Table S1), which were classified into different OTUs based on the identity level at 97%. Among the 476235 effect tags, a total of 1320 OTUs were obtained; of these 1320 OTUs, 154 OTUs were common to both sexes, and there were 38 and 1128 specific OTUs belonging to female and male adults, respectively (Fig 1).

Analysis of Alpha Diversity

Alpha diversity was estimated by five indices: Chao1, Shannon, Simpson, ACE and coverage. The results in Table 1 show that the bacteria in *L. invasa* adults were diverse between both sexes. Among them, the Chao1 (229.50 vs 1282.00) and ACE estimators (212.84 vs 1282.28) were lower in the females than in the males. Good agreement was also observed between Simpson and Shannon indices. The Shannon index (0.59 vs 6.13) was lower in the females than in the males, while the Simpson index (0.85 vs 0.01) was higher in the female wasps than in the male wasps, indicating that the diversity of the bacterial community in males was higher than that in females. The coverage was near 100% for both males and females, illustrating a higher probability of bacteria that were detected and a lower probability of bacteria that were undetected.

The Analysis of Community Composition and Species Abundance

The bacterial community composition and species abundance in both sexes of *L. invasa* were analyzed (abundance more than 0.1%) based on the results of the OTUs (Table 2, Fig 2). At the phylum level, a total of 24 phyla were detected and classified in the samples. *Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Cyanobacteria, Saccharibacteria, Fusobacteria,*

Comment [16]: The figures need to have some labels – I was not able to understand what A – E represented. I assume Phylum, Class, Order….etc. This should be very clear

Acidobacteria and *Chloroflexi* were the dominant bacteria annotated in females, and of them, *Proteobacteria* was the highest, accounting for 95.63% of the total. Males not only had the same bacteria as females but also had *Gemmatimonadetes, Nitrospirae, Spirochaetae* and *Tenericutes*. Among them, Proteobacteria was also the dominant bacteria in males, with an abundance of 34.99%, and *Firmicutes* was the subdominant bacteria, accounting for 33.06% of the total. At the class level, 71 classes were annotated, including *Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, Clostridia, Bacteroidia, Bacilli, Fusobacteria, Actinobacteria, Deltaproteobacteria, Sphingobacteria, Erysipelotrichia, Gemmatimonadetes, Spirochaetes, Flavobacteria, Acidimicrobia, Solibacteres, Negativicutes,* and *Epsilonproteobacteria*. *Alphaproteobacteria* were the dominant bacteria in females, with an abundance of 94.45%. The dominant and subdominant bacteria in males were *Clostridia* (abundance was 22.95%) and *Alphaproteobacteria* (abundance was 16.28%), respectively. There were 130 orders detected and classified, including *Rickettsiales, Clostridiales, Bacteroidales, Rhizobiales, Lactobacillales,* and *Fusobacteriales*, and among them, 40 orders were common to both sexes. The difference was that *Rickettsiales* had the highest abundance in females, accounting for 93.72%, but *Clostridiales*, *Bacteroidales* and *Rhizobiales* were the most abundant in males, accounting for 22.90%, 13.16% and 10.59%, respectively. At the family level, 245 families were detected and classified. The dominant bacteria were *Rickettsiaceae* in females, with an abundance of 93.67% in total, and the dominant and subdominant bacteria in males were *Ruminococcaceae* and *Lachnospiraceae* with abundances of 10.43% and 8.65%, respectively. At the genus level, 501 genera were classified, including *Rickettsia*, *Rhizobium*, *Fusobacterium*, and *Sphingomonas*. *Rickettsia* (an abundance of 93.67%) and *Rhizobium* (an abundance of 5.73%) were the dominant bacteria in females and males, respectively. In addition, it was noteworthy that the abundance of *Rickettsia* was less than 1% in males (Table 3). The phylogenetic relationship of bacteria in both sexes of *L. invasa* is shown in Fig 3.

Discussion

Insects harbor various bacteria, some of which influence the reproduction of host insects over a long period of coevolution (*Dillon & Dillon, 2004; Frago et al., 2012*). Indeed, bacteria that manipulate the sex rate and reproduction of *L. invasa* could exist (*Nugnes et al., 2015*). Previous studies have suggested that the reproductive mode of *L. invasa* is mainly thelytokous parthenogenesis, but male adults have also been found in Turkey (*Doğanlar, 2005*), China

(*Zheng et al., 2014*) and India (*Kumari et al., 2010*). The sex ratio (male: female) was 1: 2.1-1: 5.5 in some areas and up to 1: 23.2-1: 195 in others (*Doğanlar, 2005; Zheng et al., 2014, 2018*). Undoubtedly, the rapid spread and fast growth of populations of *L. invasa* are closely related to the female-biased sex ratios and thelytoky (*Zheng et al., 2014*). Therefore, comparing the bacterial communities harbored by the various sexes in this paper may be very important for the reproductive strategies and biocontrol of *L. invasa*.

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Differences in the bacteria between female and male adults

Comment [17]: More discussion and reference to the literature is needed in this section. Currently this section lists bacteria in males and bacteria in females with minimal – no interpretation etc. This reads more like a results section.

This research revealed that the bacteria harbored in *L. invasa* have high diversity. The microorganisms found in the female adults were classified into 10 phyla, 26 classes, 44 orders, 76 families, and 122 genera, and those in the male adults were classified into 24 phyla, 69 classes, 127 orders, 238 families, and 487 genera (Table 2). The diversity of the bacterial community in males was higher than that in females, which also appeared in the Alpha diversity analysis (Table 1). Furthermore, the bacterial phylotypes and their relative abundances differed significantly between male and female wasps of *L. invasa*. The abundance of *Proteobacteria* varied at the phylum level, although *Proteobacteria* was the dominant bacteria in both sexes. The dominant bacteria in both sexes of *L. invasa* were dissimilar at other levels. In females, the dominant bacteria were *Alphaproteobacteria, Rickettsiales, Rickettsiaceae* and *Rickettsia*, while *Clostridia, Clostridiales, Ruminococcaceae* and *Rhizobium* were the dominant bacteria in males (Fig 2). In addition, sequences of *Gemmatimonadetes, Spirochaetae, Sphingobacteria, Rhizobiaceae*, *Chitinophagaceae*, *Xanthomonas* and *Vibrio* were detected in males. The variation of bacterial communities between males and females may be partly explained by the different physiological structure between the two sexes of *L. invasa,* namely, that the female wasps have ovaries, which harbor an abundance of *Rickettsia*, and occupy different bacterial niches than the males (*Nugnes et al., 2015*). Another possibility is that insects could also launch innate and systematic immune responses to cope with the colonization of microbes (*Leulier & Royet, 2009*), and females have stronger immune systems than males (*Kurtz et al., 2000*).

Comparison of the bacteria with other insects

The bacterial community analysis at the phyla level demonstrated that *Proteobacteria* was the most dominant group in female and male wasps, and *Firmicutes, Bacteroidetes, Actinobacteria* and *Fusobacteria* were also annotated. Previous studies revealed that *Proteobacteria* were dominant in many insects, such as *Bactrocera tau* (*Prabhakar et al., 2012*), *Lutzomyia* sand fly

(*Sant'Anna et al., 2012*), *Schistocerca gregaria* (*Dillon et al., 2010*) and *Anopheles stephensi* (*Rani et al., 2009*). Moreover, the major bacteria were also *Proteobacteria* in *Bactrocera minax* (*Wang et al., 2004*), ground beetles (*Jonathan et al., 2007*), *Helicoverpa armigera* larvae (*Priya et al., 2012*) and *Holotrichia parallela* larvae (*Huang et al., 2013*). Furthermore, *Proteobacteria* or *Firmicutes* were the dominant bacteria in *Plutella xylostella* larvae (*Xia et al., 2013*), *Aedes albopictus* and *A. aegypti* (*Zouache et al., 2011*). In contrast, *Firmicutes* and *Bacteroidetes* were the major bacteria phyla detected in the guts of termites (*Xiang et al., 2012*) and bees (*Mohr & Tebbe, 2006*).

Functional prediction of dominant bacteria

Several of the bacteria detected in this study are commonly described in insects at the genus level, and some have been found in Hymenoptera, such as honeybees (*Mohr & Tebbe, 2006*) and termites (*Xiang et al., 2012*). Intriguingly, two genera, *Staphylococcus* and *Escherichia*, were known to contain cultivable species (*Wang et al., 2018*). Gloverin and lysozyme gene expression was upregulated when silkworm larvae were fed *Escherichia* and *Staphylococcus*, indicating that the two bacteria are closely related to the immune signaling pathway of the silkworm (*Douglas, 2015*). We hypothesized that *Escherichia* and *Staphylococcus* may also be involved in the immunoreaction of *L. invasa*. Functions have been suggested for some of the other bacterial genera detected in this study. The *Enterobacteriaceae* that are associated with insects help with digestion, the detoxification of toxic substances, resistance to pathogens and enhance the adaptability of the host (*Anand et al., 2010*). Adding *Enterobacter* in feed could extend the life span of Mediterranean flies (*Behar et al., 2005, 2008*). Similarly, *Enterobacteriaceae* (*Hongoh & Ishikawa, 2000*) and *Acinetobacter* (*Broderick et al., 2004*) could facilitate carbon-nitrogen metabolism and accelerate the growth and development of host insects, e.g., the *Acinetobacter* belonging to termites have a nitrogen-transforming function according to Warnecke's *(2007)* research. *Enterobacteriaceae* and *Acinetobacter* have significant effects on the growth of *L. invasa*, and carbon, nitrogen and other elements play a very important role in nutrition as essential amino acids rely on these elements to build central carbon skeletons. Some bacteria associated with immunization were also discovered in *L. invasa*, such as *Lactobacillus*. *Lactobacillus* had some positive effects on insect resistance (*Xia et al., 2013*). In addition, *Bacillales* were also detected in this study and may be insect pathogens, such as *Bacillus thuringiensis* and *B. cereus* (*Broderick et al., 2004; Raymond et al., 2010; Song et al., 2014*). In contrast, some *Bacillus* in termites might be involved in the degradation of cellulose and hemicellulose (*Konig, 2006*). In this study, *Bacillales* were detected in both genders, and their specific functions need further study. Nevertheless, *Acinetobacter* was detected in *L. invasa,* and previous research showed that *Acinetobacter* produces an antiviral compound that inhibits a tobacco mosaic virus (*Lee et al., 2009*). Moreover, members of *Bacteroidetes* are specialized in the degradation of complex organic matter, including lignocellulosic compounds (*Yuki et al., 2015*). *Bacteroidetes* are also involved in the decomposition and metabolism of polysaccharides (*Xu et al., 2003; Sonnenburg et al., 2010*), which are beneficial to the absorption and digestion of the host (*Liu et al., 2011*). In addition, the *Bacteroidetes* also include some *Azotobacter*, such as *Azobacteroides pseudotrichonympha*, which could provide a host with amino acids for nutrition (*Doda et al., 2009; Desai & Brune, 2012*). *Bacteroidetes* related to degradation and fermentation of phytomass could influence the nutrient absorption of *L. invasa*, but further studies are needed. Many other groups of bacteria with undefined functions were detected in *L. invasa* for the first time in this study. A better knowledge of the bacteria associated with *L. invasa* will allow researchers to investigate their role in host biology.

A sequence similarity search revealed that *Rhizobium* was the dominant bacterium in male adults (Fig 2, Table 3). *Rhizobium* produces a variety of enzymes with cellulose- and pectin-hydrolyzing activities that can hydrolyze the glycoside skeleton of the plant cell wall and play a very important role in the symbiosis between *Rhizobium* and leguminous plants (*Robledo et al., 2008; Huang et al., 2018*). *Rhizobium* is an endosymbiont detected in the gut of some phytophagous insects and can help the host synthesize nitrogen-containing substances that are lacking in food (*Russell et al., 2009*).

Rickettsia (an abundance of 93.67%) was the dominant bacteria present in female adults, while less than 1% was present in males (Fig 2, Table 3). *Rickettsia* is a maternally inherited intracellular bacterium in a wide range of arthropods and is capable of controlling populations by reproductive manipulations, such as parthenogenesis inducing (PI) (*Hagimori et al., 2006; Adachi-Hagimori et al., 2008; Giorgini et al., 2010*) and male killing (Lawson *et al*., 2001; Schulenburg *et al*., 2001; Majerus & Maherus, 2010). Moreover, *Rickettsia* affects the fitness in the host and avoids adverse environmental conditions (*Oliver et al., 2003; Sakurai et al., 2005; Chiel et al., 2009; Himler et al., 2011; Brumin et al., 2011*). For instance, preadult development of *Bemisia tabaci* B-biotype was faster with *Rickettsia* infection than without (*Chiel et al., 2009*).

Comment [18]: More information on exactly how Rickettsia manipulates the host is necessary

Compared with uninfected whiteflies, *Himler et al. (2011)* found that *Rickettsia*-carrying whiteflies produced more offspring, developed faster, had a higher rate of survival to adulthood, and produced a higher proportion of daughters. *Nugnes et al. (2015)* found that *Rickettsia* is located in reproductive tissues in females and passed to the next generation through vertical transmission; thus, a possible reason for thelytokous parthenogenesis in *L. invasa*. The female *L. invasa* is dominant and plays an important role in invasion and colonization (*Zheng et al., 2014*). The results of the current investigation could explain why the sex ratio in wasps is female-biased and support the hypothesis that *Rickettsia* can induce thelytokous parthenogenesis in *L. invasa*. However, both explanations need further testing. In this research, a low level of *Rickettsia* was present in males. A previous investigation suggested that *Rickettsia* could pass to the offspring by vertical transmission (*Nugnes et al. 2015*), and a threshold density of *Rickettsia* bacteria in eggs is required to trigger the development of female embryos (*Giorgini et al., 2010*). Although no evidence has shown that the *Rickettsia* living in *L. invasa* can be transmitted horizontally (*Gualtieri et al., 2017*), we cannot rule out the possibility that male-*Rickettsia* is obtained through horizontal transmission in some way. Removing *Rickettsia* by feeding antibiotics could produce more male offspring. *Giorgini et al. (2010)* found that *Rickettsia*-infected *Pnigalio soemius* only generate female progeny, and after 24 h, when the *Rickettsia* were removed by 20 mg/mL rifampin, adults produced almost all male offspring. *Hagimori et al. (2006)* declared that *Rickettsia* was related to the thelytokous parthenogenesis of *Neochrysocharis formosa*, a dominant parasite of leaf miner, and after removing *Rickettsia* from the adults by feeding tetracycline, female offspring without *Rickettsia* were present. Therefore, future studies should clarify whether *Rickettsia* is involved in the reproductive manipulation of *L. invasa* through feeding with antibiotics.

Comment [19]: Clarify what you mean by this

Conclusions

The results in this study characterize the bacterial diversity and differences between both sexes in *L. invasa* by high-throughput sequencing, suggesting that the interior bacterial community was abundant and that the majority of these species remained uncultivated. Moreover, the males harbored a more diverse bacterial community than the females, and the bacterial communities of *L. invasa* varied between the two sexes. These results enrich the information of microbial information of *L. invasa*, help research the reproductive strategy, sex control and invasive mechanism, and lay the foundation for further studies on the excavation and utilization of

Comment [20]: This is the first mention of bacteria that are unculturable. This should be discussed earlier.

microbes for the biological control of *L. invasa*.

Acknowledgements

The authors thank Prof. Yongqiang He for sharing his knowledge of bacteria, the State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, and the members of the Guangxi Key Laboratory of Forest Ecology and Conservation.

Comment [21]: If the reader does not have sufficient background knowledge on Leptocybe it would be unclear why the implementation of biological control would need to be considered or is even important. More background on Leptocybe, its importance globally and details on exactly how these bacteria could be used to aid in biological control need to be discussed.

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