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

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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 the thelytokous parthenogenesis, low temperature
resistance, protection in galls, generation overlap and small body of *L. invasa* contribute to its
rapid invasion and population growth. However, the endosymbiotic bacterial composition,
abundance and sex differences of *L. invasa* remain unclear. Therefore, this research aimed to
identify the bacterial communities in *L. invasa* adults and compare them between the sexes of *L.
invasa* lineage B.

Results. The Illumina MiSeq platform was used to compare bacterial community composition
between females and males of *L. invasa* by sequencing the V3-V4 region of the 16S ribosomal
RNA gene. A total of 1320 operational taxonomic units (OTUs) were obtained. 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 endosymbiotic bacteria of *L. invasa* females and males were highly diverse.
There were differences in the bacterial community of *L. invasa* between sexes, and the bacterial
diversity in male specimens was greater than that in female specimens. This study presents a
comprehensive comparison of bacterial communities in *L. invasa* and these data will provide an
overall view of the bacterial community in both sexes of *L. invasa* with special attention on sex-
related bacteria.

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*). Over the course of long-term coevolution, microorganisms develop a close relationship with host insects, which may have an effect on the reproduction, survival, community interactions, and the ability to resist predators and vectors of the hosts (*Oliver et al., 2003, 2010; Moran, 2007; Clark et al., 2008; Moran et al., 2008; Moya et al., 2008*). In light of the significant functions of the microorganisms, they have received much attention from the international academic community. In some insects, the diversity and function of endosymbiotic bacteria have been well studied. For instance, the bacteria in termites are mainly *Bacteroidetes, Firmicutes* and *Actinobacteria* and can assist their hosts in breaking down lignocellulose and promoting the nitrogen cycle (*Warnecke et al., 2007; Brune, 2014*). The bacteria in *Aphis gossypii* improve its resistance and adaptation (*Łukasik et al., 2013a, b*). In recent years, manipulating endosymbionts for pest control has raised wide concern, and its theory and methods have been applied successfully to some extent. Introduction of antimalarial endosymbionts into the mid gut of host pests could inhibit the breeding of plasmodia and in turn reduce the efficiency of mosquito transmission of malaria (*Wang et al., 2012*). Mixed application of antibiotics and insecticides effectively reduced the quantity of endosymbionts in *Nilaparvata lugens* while improving the control effect of insecticides (*Shentu et al., 2016*). Based on research on the related incompatible insect technique (IIT), researchers used the maternally inherited endosymbiotic bacterium *Wolbachia* for sterilization, which had good effects on eliminating the fecundity of mosquitoes (*Zheng et al., 2019*). Obviously, it is necessary to clarify the bacterial composition and diversity in insects, which are the bases of manipulating endosymbionts for pest control. In addition, previous investigations have shown that sex is an important factor affecting bacterial diversity. For example, due to different attack behaviors, the overall diversity and richness of bacterial communities associated with female *Dendroctonus valens* are higher than those associated with males of this beetle species (*Xu et al., 2016*). The bacterial composition of mosquitoes was also affected by sex (*Minard et al., 2013; Zouache et al., 2011*). Different anatomies and life histories between male and female flies could provide differential opportunities for bacterial colonization (*Tang et al., 2012*).

85   The blue gum chalcid *Leptocybe invasa* Fisher & LaSalle (Hymenoptera: Eulophidae:

86   Tetrastichinae) is a cosmopolitan pest that damages many *Eucalyptus* species (*Mendel et al.,*

87   *2004; Le et al., 2018*). *L. invasa*, originating in Australia, was first recorded in 2000 and has

88   since been discovered in 45 countries of Asia, Europe, Africa, Oceania and America (*Le et al.,*

89   *2018; Zheng et al., 2014a*). A new study demonstrated that an increasing number of areas will

90   become suitable for *L. invasa* due to climate warming (*Huang et al., 2019*). Every delicate twig,

91   vein and petiole of eucalyptus trees may provide a spawning ground for this pest, and galls

92   ultimately lead to stunted growth of the trees, causing great losses in local eucalyptus plantations

93   (*Mendel et al., 2004; Zheng et al., 2014a; Huang et al., 2018*). DNA barcode data indicated that

94   *L. invasa* includes two genetically separate lineages (lineages A and B). Researchers considered

95   the Italian, Argentinean and Tunisian populations to belong to lineage A and the Chinese

96   population to belong to lineage B (*Le et al., 2018; Dittrich-Schröder et al., 2018*). The absence

97   of natural enemies, presence of large amounts of suitable host plants, small size, protection in

98   galls, strong resistance to low temperature and thelytokous parthenogenesis of *L. invasa* caused

99   its rapid invasion and growth of in China (*Zheng et al., 2014a*). As a result, it has become one of

100   the most difficult pests to control (*Zheng et al., 2014a; Huang et al., 2018; Le et al., 2018*). It is

101   important in tems of theory and application to study the endosymbiotic bacterial diversity of *L.*

102   *invasa* and then control the wasps by using these endosymbiotic bacteria.

103   To date, few studies have reported on the overall endosymbiotic bacteria of *L. invasa,*

104   which is an invasive gall-inducing insect. Only a few studies have comprehensively examined

105   the endosymbiotic bacteria in this species. *Wang et al. (2018)* cultured 11 strains from female

106   adults of *L. invasa* in winter using traditional methods and classified them into 3 phyla

107   (*Firmicutes, Actinobacteria,* and *Proteobacteria*), 3 classes (*Bacilli, Actinobacteria,* and

108   *Gammaproteobacteria*) and 4 orders (*Bacillales, Micrococcales, Lactobacillales,* and

109   *Enterobacterales*) that were related to growth, development, nutrition metabolism and immunity.

110   *Nugnes et al. (2015)* researched the bacteria living in adults among different populationsvia

111   denaturing gradient gel electrophoresis (DGGE) analysis and found that *Rickettsia* occurred in

112   the reproductive tissues of female *L. invasa*, suggesting a relationship with its thelytokous

113   parthenogenesis. *L. invasa* harbors a myriad of bacteria, and bacterial differences between sexes

114   have strong effects on insects, such as effects on reproductive regulation (*Wang et al., 2018;*

115   *Nugnes et al., 2015*). Therefore, the overall endosymbiotic bacterial composition and abundance

116   of *L. invasa* and the differences between sexes are important to study.

117   In this study, the endosymbiotic bacteria in female and male adults of *L. invasa* were

118   indentified by 16S rRNA sequencing of the V3-V4 region to shed light on their internal bacterial

119   compositions. The females and males were also compared to address sexual differences in the

120   endosymbionts. These results will provide a valuable bacterial pool for *L. invasa* and will further

121   contribute to understanding its reproductive strategies and invasion mechanisms.

Materials & Methods

Insect sampling

124   Branches of DH 201-2 (*Eucalyptus grandis* × *E. tereticornis*) (Myrtales: Myrtaceae) harboring

125   galls of *L. invasa* were removed from the Teaching and Experiment Base of Forestry College,

126   Guangxi University (108°17′ E, 22°51′ N), Nanning city, Guangxi Zhuang Autonomous Region

127   from July to August 2018. The branches were placed in a plastic bottle filled with water to retain

128   freshness and transferred into a sealed net cage (40 cm × 40 cm × 80 cm) at room temperature to

129   keep the adults from escaping. The water in the plastic bottle was renewed daily until the

130   emergence of *L. invasa* adults. Sexes were identified by morphological observation (*Zheng et al.,*

131   *2014b*).

DNA extraction

133   Fifty adults of each sex of *L. invasa* newly emerged within 12 h were fasted for 6 h. Then, both

134   samples were sterilized externally with 75% ethanol for 2-5 min and rinsed 3 times with

135   sterilized water to remove microbes on the surface. The total bacterial DNA of each sample was

136   extracted using a Power Soil DNA Isolation Kit (MO BIO Laboratories) according to the

137   manufacturer's instructions. The quality and quantity of DNA were assessed by the ratios of 260

138   nm/280 nm and 260 nm/230 nm. Then, the qualified DNA was stored at -80 ℃ for further

139   processing. The DNA of each individual was extracted by using a Chelex-100 and proteinase K-

140   based method (*Gebiola et al.,* 2009).

PCR amplification and cloning of the bacterial 16S rRNA gene

142   Amplification of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed

143   by using the universal bacterial primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R

144   (5'- GACTACHVGGGTWTCTAAT-3'). PCRs were carried out in 50 μL solutions containing 10

145   μL of 10× buffer, 0.2 μL of Q5 High-Fidelity DNA Polymerase, 10 μL of High GC Enhancer, 1

146   μL of dNTPs, 10 μM each forward and reverse primer, 60 ng of genomic DNA and enough

147   ddH$_2$O to reach 50 μL. The amplifications were performed in a ABI Applied Biosystems 9902

148   thermal cycler with an initial denaturation step at 95 ℃ for 5 min, followed by 35 cycles of

149   annealing and extension (each cycle consisted of 95 ℃ for 1 min, 50 ℃ for 1 min and extension

150   at 72 ℃ for 1 min) and a final extension at 72 ℃ for 7 min. The PCR products were checked by

151   electrophoresis on an agarose gel (1.8% agarose, 1× TBE), stained with ethidium bromide and

152   visualized under ultraviolet light. The products from the first round of PCR were purified with

153   VAHTS™M DNA Clean Beads. The second round of PCR was then performed in a 40 μL

154   reaction containing 20 μL of 2 × Phμsion HF MM, 8 μL of ddH$_2$O, 10 μM each forward and

155   reverse primer and 10 μL of PCR product produced in the first round. The second round of PCR

156   was run under the following conditions: initial denaturation at 98 ℃ for 30 s, followed by 10

157   cycles at 98 ℃ for 10 s, 65 ℃ for 30 s and 72 ℃ for 30 s and a final extension at 72 ℃ for 5 min.

158   Finally, all PCR products were quantified and pooled by Quant-iT™ dsDNA HS Reagent. High-

159   throughput sequencing analysis of bacterial rRNA genes was performed on the purified, pooled

160   sample by using the Illumina HiSeq 2500 platform at Biomarker Technologies Co., Ltd, Beijing,

161   China.

162   **Bioinformatics and statistical analysis**

163   After sequencing, PE reads obtained with HiSeq sequencing were merged by overlapping to

164   obtain raw tags. To obtain clean tags, the raw tags were denoised, sorted and separated by using

165   Trimmomatic (version 0.33). The remaining sequences were filtered for redundancy, and all

166   unique sequences in each sample were clustered into operational taxonomic units (OTUs) on the

167   basis of 97% similarity. Low-abundance OTUs were identified and eliminated by using

168   UCHIME v4.2. Taxonomic assignment of the OTUs was conducted with the Silva reference

169   database. Species abundance tables were generated by QIIME, and community structures in

170   every taxon category was plotted by R software. The relative abundances of the bacteria were

171   determined by percentages.

172   Alpha diversity based on Chao1 richness and ACE richness estimators, as well as the

173   Simpson and Shannon diversity indices, was evaluated by using the mothur v.1.11.0 program.

174   Among these measure, Chao1 and ACE reflected species richness in the samples, the Shannon

175   index reflected community diversity, the Simpson index reflected the dominance of species in

176   the community, and the coverage index reflected the degree to which the sequencing results

177   represented the actual composition of the microorganisms in the samples.

178   **Molecular characterization and phylogenetic analyses**

179   COI was amplified by using the forward primer LCO1490 and reverse primer HCO2198

180   (*Nugnes et al., 2015*). The 16S rRNA gene of *Rickettsia* was amplified by using the primers

181   listed in Table S1. The PCR program for both genes (COI and 16S rRNA) was as follows: 3

182   min of initial denaturation at 94 ℃, 30 cycles at 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 1

183   min and a final extension of 5 min at 72 ℃. PCR products were observed by using 1.0%

184   agarose gel electrophoresis, and the amplified fragments were directly sequenced by

185   TsingKe Biological Technology Co., Ltd, Beijing, China. Representative sequences of other

186   regions were downloaded from GenBank, and sequence alignment was completed by using

187   Clustal X. The neighbour-joining method was used to construct a consensus phylogenetic

188   tree with MEGA7 software. To evaluate the branch support of the phylogenetic tree,

189   bootstrap analysis of 1000 replicates was performed.

190   **Accession numbers**

191   Data is available at NCBI SRA, accession numbers: SRR9591039, SRR9591038. The COI and

192   16S rRNA sequences determined in this study have been deposited in the GenBank database

193   with Accession number MN524231 and MN524230, respectively.

194   # Results

195   **Sex of *L. invasa* specimens in this study**

196   All female and male specimens were identified on the basis of morphology. In this study, a total

197   of 656 females and 51 males were collected (Table S2). The materials were deposited at the

198   Forest Conservation Laboratory, College of Forestry, Guangxi University, Nanning 530004,

199   China.

200   **Sequencing and classification**

201   A total of 533266 raw tags (370680 from males and 162586 from females) were obtained for *L.*

202   *invasa,* and 476235 clean tags (328833 from males and 147402 from females) were generated

203   (Table S3), which were classified into different OTUs based on 97% similarity. Among the

204   476235 clean tags, a total of 1320 OTUs were obtained; of these 1320 OTUs, 154 were common

205   to both sexes, and 38 and 1128 were specific to female and male adults, respectively (Fig. 1).

206   **Analysis of Alpha diversity**

207   Alpha diversity was estimated by five indices: Chao1, the Shannon index, the Simpson index, the

208   ACE and coverage. The results in Table 1 show that the bacteria in *L. invasa* adults were diverse

209   in both sexes. The Chao1 (229.50 vs 1282.00) and ACE (212.84 vs 1282.28) values were lower

210   in the females than in the males. Good agreement was also observed between the Simpson and

211   Shannon indices. The Shannon index (0.59 vs 6.13) was lower in the females than in the males,

212   while the Simpson index (0.85 vs 0.01) was higher in the female wasps than in the male wasps,

213   indicating that the diversity of the bacterial community in males was higher than that in females.

214   The coverage was near 100% for both males and females, illustrating a higher probability of

215   bacteria being detected than of bacteria being undetected.

216   **The analysis of community composition and species abundance**

217   The bacterial community composition and species abundance in both sexes of *L. invasa* were

218   analyzed (abundances greater than 0.1%) based on the results of the OTUs (Table 2, Fig. 2). A

219   total of 24 phyla were detected and classified in the samples. *Proteobacteria* was the dominant

220   bacterial phylum annotated in females and males, accounting for 95.63% and 34.99% of bacteria,

221   respectively. At the genus level, *Rickettsia* (with an abundance of 93.67%) and *Rhizobium* (with

222   an abundance of 5.73%) were the dominant bacteria in females and males, respectively. In

223   addition, it was noteworthy that the abundance of *Rickettsia* was less than 1% in males (Table 3).

224   **Molecular characterization and phylogenetic analyses**

225   After comparison with Genebank, the identification of *L.invasa* in this research was lineage B

226   and the phylogenetic tree of COI also indicated that the population of this research belonged to

227   the lineage (Fig. 3). The phylogenetic analysis of 16S rRNA genes revealed that the *Rickettsia* of

228   *L.invasa* symbionts belonged to the *Rickettsia* transitional group (Fig. 4).

229   # Discussion

230   **Differences in bacteria between female and male adults**

231   This research revealed that the bacteria harbored in *L. invasa* had high diversity, and many of the

232   endosymbiotic bacteria were annotated in this species for the first time. Based on alpha diversity

233   analysis, the diversity of the endosymbiotic bacteria in males was higher than that in females

234   (Table 1). The variation in bacterial communities between males and females may be partly

235   explained by differences in physiology structure and between the two sexes of *L. invasa*;

236   specifically, the female wasps have ovaries, which harbor an abundance of *Rickettsia*, allowing

237   the genus to occupy different bacterial niches than in males (*Nugnes et al., 2015*). Another

238   possibility is that insects launch innate and systematic immune responses to cope with microbe

239   colonization (*Leulier & Royet, 2009*) and females have stronger immune systems than males

240 (*Kurtz et al., 2000*).

241 **Comparison of the bacteria with those in other insects**

242 Bacterial community analysis at the phylum level demonstrated that *Proteobacteria* was the

243 dominant group in female and male wasps, and *Firmicutes, Bacteroidetes, Actinobacteria* and

244 *Fusobacteria* were also annotated. Previous studies revealed that *Proteobacteria* were dominant

245 in other Hymenoptera, such as *Apis cerana* and leaf-cutter ants (*Ahn et al., 2012*; *Zhukova et al.,*

246 *2017*) In contrast, *Firmicutes* and *Bacteroidetes* were the major bacterial phyla detected in the

247 guts of termites (*Miyata et al., 2007*; *Xiang et al., 2012*) and bees (*Mohr & Tebbe, 2006*).

248 *Firmicutes* and *Actinobacteria* were the dominant bacteria in *A. mellifera* and bumblebees (*Ahn*

249 *et al., 2012*; *Praet et al., 2018*).

250 **Putative Functions of dominant endosymbiotic bacteria in *L. invasa***

251 Several of the bacteria detected in this study are commonly described in insects at the genus level,

252 and some have been found in Hymenoptera, such as honeybees (*Mohr & Tebbe, 2006*) and

253 termites (*Xiang et al., 2012*). Intriguingly, two genera, *Staphylococcus* and *Escherichia*, are

254 known to contain cultivable species (*Wang et al., 2018*). Gloverin and lysozyme gene expression

255 was upregulated when silkworm larvae were fed *Escherichia* and *Staphylococcus*, indicating that

256 the two bacteria were closely related to the immune signaling pathway of the silkworm (*Douglas,*

257 *2015*). We hypothesized that *Escherichia* and *Staphylococcus* may also be involved in the

258 immunoreaction of *L. invasa*. Functions have been suggested for some of the other bacterial

259 genera detected in this study. The *Enterobacteriaceae* that are associated with insects help with

260 digestion, the detoxification of toxic substances, and resistance to pathogens and enhance the

261 adaptability of the host (*Anand et al., 2010*). Adding *Enterobacter* to feed extended the life span

262 of Mediterranean flies (*Behar et al., 2005, 2008*). Similarly, *Enterobacteriaceae* (*Hongoh &*

263 *Ishikawa, 2000*) and *Acinetobacter* (*Broderick et al., 2004*) facilitated carbon-nitrogen

264 metabolism and accelerated the growth and development of host insects; e.g., the *Acinetobacter*

265 belonging to termites have a nitrogen-transforming function according to Warnecke's *(2007)*

266 research. Some bacteria associated with immunization were also discovered in *L. invasa*, such as

267 *Lactobacillus*. *Lactobacillus* had some positive effects on insect resistance (*Xia et al., 2013*). In

268 addition, *Bacillales* were also detected in this study and may be insect pathogens, such as

269 *Bacillus thuringiensis* and *Bacillus cereus* (*Broderick et al., 2004; Raymond et al., 2010; Song et*

270 *al., 2014*). In contrast, some *Bacillus* in termites might be involved in the degradation of

271   cellulose and hemicellulose (*Konig, 2006*). In this study, *Bacillales* were detected in both sexes,

272   and their specific functions require further study. Nevertheless, *Acinetobacter* was detected in *L.*

273   *invasa,* and previous research showed that *Acinetobacter* produces an antiviral compound that

274   inhibits tobacco mosaic virus (*Lee et al., 2009*). Moreover, members of *Bacteroidetes* specialize

275   in the degradation of complex organic matter, including lignocellulosic compounds (*Yuki et al.,*

276   *2015*). *Bacteroidetes* are also involved in the decomposition and metabolism of polysaccharides

277   (*Xu et al., 2003; Sonnenburg et al., 2010*), which are beneficial for host absorption and digestion

278   (*Liu et al., 2011*). In addition, *Bacteroidetes* also include some *Azotobacter*, such as

279   *Azobacteroides pseudotrichonympha*, which can provide the host with amino acids for nutrition

280   (*Doda et al., 2009; Desai & Brune, 2012*). *Bacteroidetes* involved in the degradation and

281   fermentation of phytomass could influence the nutrient absorption of *L. invasa*, but further

282   studies are needed. Many other groups of bacteria with undefined functions were detected in *L.*

283   *invasa* for the first time in this study. Better knowledge of the bacteria associated with *L. invasa*

284   will allow researchers to investigate their role in host biology.

285   A sequence similarity search revealed that *Rhizobium* was the dominant bacterium in male

286   adults (Fig. 2, Table 3). *Rhizobium* produces a variety of enzymes with cellulose- and pectin-

287   hydrolyzing activities that can hydrolyze the glycoside skeleton of the plant cell wall and play a

288   very important role in the symbiosis between *Rhizobium* and leguminous plants (*Robledo et al.,*

289   *2008; Huang et al., 2018*). *Rhizobium* is an endosymbiont detected in the gut of some

290   phytophagous insects and can help the host synthesize nitrogen-containing substances that are

291   lacking in food (*Russell et al., 2009*).

292   *Rickettsia* (with an abundance of 93.67%) was the dominant bacterial genus present in

293   female adults (Fig. 2, Table 3). *Rickettsia* is a maternally inherited intracellular bacterium in a

294   wide range of arthropods and is capable of controlling populations by reproductive manipulation,

295   such as parthenogenesis inducing (PI) (*Hagimori et al., 2006; Adachi-Hagimori et al., 2008;*

296   *Giorgini et al., 2010*) and male killing (MK) (*Lawson et al., 2001; Schulenburg et al., 2001;*

297   *Majerus & Maherus, 2010*). During female gamete formation in *Rickettsia*-carrying

298   *Neochrysocharis formosa*, meiotic cells underwent only one equatorial division, and meiotic

299   recombination was absent, which demonstrated that *Rickettsia* could induce parthenogenesis by

300   changing the meiosis of wasps (*Adachi-Hagimori et al., 2008*). *Rickettsia* also induced male

301   embryo death in *Adalia bipunctata* and *A. decempunctata* (*Hurst GDD et al., 1993, 1996;*

302 *Werren JH et al., 1994*). Moreover, *Rickettsia* affects the fitness of the host and protects it

303 against adverse environmental conditions (*Oliver et al., 2003; Sakurai et al., 2005; Chiel et al.,*

304 *2009; Himler et al., 2011; Brumin et al., 2011*). For instance, preadult development of the

305 *Bemisia tabaci* B-biotype was faster with *Rickettsia* infection than without (*Chiel et al., 2009*).

306 *Himler et al. (2011)* found that *Rickettsia*-carrying whiteflies produced more offspring,

307 developed faster, had a higher rate of survival to adulthood, and produced a larger proportion of

308 daughters than did uninfected whiteflies. Males have never been recorded in Italy, Tunisia and

309 Argentina, and rarely in Turkey (sex ratio 0-0.5%) (*Nugnes et al., 2015*). These results show that

310 *L. invasa* reproduces by thelytokous parthenogenesis. In contrast, males appeared more

311 frequently in China, India and Thailand. In this study, the sex ratio was 7.2%. In addition,

312 *Nugnes et al. (2015)* found that *Rickettsia* was located in reproductive tissues in females and

313 passed to the next generation via vertical transmission, representing a possible reason for

314 thelytokous parthenogenesis in *L. invasa*. Female *L. invasa* play an important role in invasion

315 and colonization (*Zheng et al., 2014a*). The results of the current investigation could explain why

316 the sex ratio in wasps is female-biased and support the hypothesis that *Rickettsia* can induce

317 thelytokous parthenogenesis in *L. invasa*. However, both explanations require further testing. In

318 addition, a low abundance of *Rickettsia* was present in males in this research. For Hymenoptera,

319 the dominant reproductive mode is arrhenotoky; that is, diploid females develop from fertilized

320 eggs, and haploid males develop from unfertilized eggs (*van Wilgenburg et al., 2006)*. A

321 previous investigation suggested that *Rickettsia* could be passed to the offspring by vertical

322 transmission (*Nugnes et al., 2015*), and a threshold density of *Rickettsia* bacteria in eggs is

323 required to trigger the development of female embryos (*Giorgini et al., 2001; 2010*). Removing

324 *Rickettsia* by feeding antibiotics could lead to the production of more male offspring. *Giorgini et*

325 *al. (2010)* found that *Rickettsia*-infected *Pnigalio soemius* generated only female progeny, and

326 after 24 h, when the *Rickettsia* was removed by 20 mg/mL rifampin, adults produced almost all

327 male offspring. *Hagimori et al. (2006)* declared that *Rickettsia* was related to the thelytokous

328 parthenogenesis of *N. formosa*, a dominant parasite of leaf miners, and after removing *Rickettsia*

329 from the adults by feeding the adults tetracycline, female offspring without *Rickettsia* were

330 present. Therefore, future studies should clarify whether *Rickettsia* is involved in the

331 reproductive manipulation of *L. invasa* accomplished via feeding with antibiotics. Furthermore,

332 environmental factors could also influence the density of the bacteria, and endosymbiont

333 densities and functions may change with space, time and season (*Bordenstein & Bordenstein,*

334 *2011*; *Nugnes et al., 2015*). A previous study indicated that the sex ratio of the Chinese

335 population could change with temperature, presumably because the relationship between

336 *Rickettsia* strain and Chinese population is weaker than that of Western population, which could

337 be more susceptible to temperature (*Zhu et al., 2014*; *Nugnes et al., 2015*). In addition, another

338 plausible explanation may be the use of different host plants (the host of lineage A is *E.*

339 *camaldulensis* but in this research, it was DH201-2), which has been demonstrated in other

340 systems (*Ferrari et al., 2007*; *Biere and Tack 2013*). Therefore, it is also essential to compare the

341 differences in bacteria between *L. invasa* that parasitize different hosts.

342 **Conclusions**

343 The results of this study obtained by high-throughput revealed the bacterial diversity and

344 differences between sexes in *L. invasa*, suggesting an abundant endosymbiotic bacterial

345 community, and some bacteria were reported in *L. invasa* for the first time. Moreover, the males

346 harbored a more diverse bacterial community than did the females. The next research should

347 focus on the bacteria found in this study to identify their specific ecological functions and the

348 specific sex-based regulatory mechanism of *Rickettsia* occurrence in *L. invasa*.

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Figure 1

Venn diagram of OTU distribution in *Leptocybe invasa* female and male adults.

Numbers within compartments indicate OTU counts of according to mathematical sets.

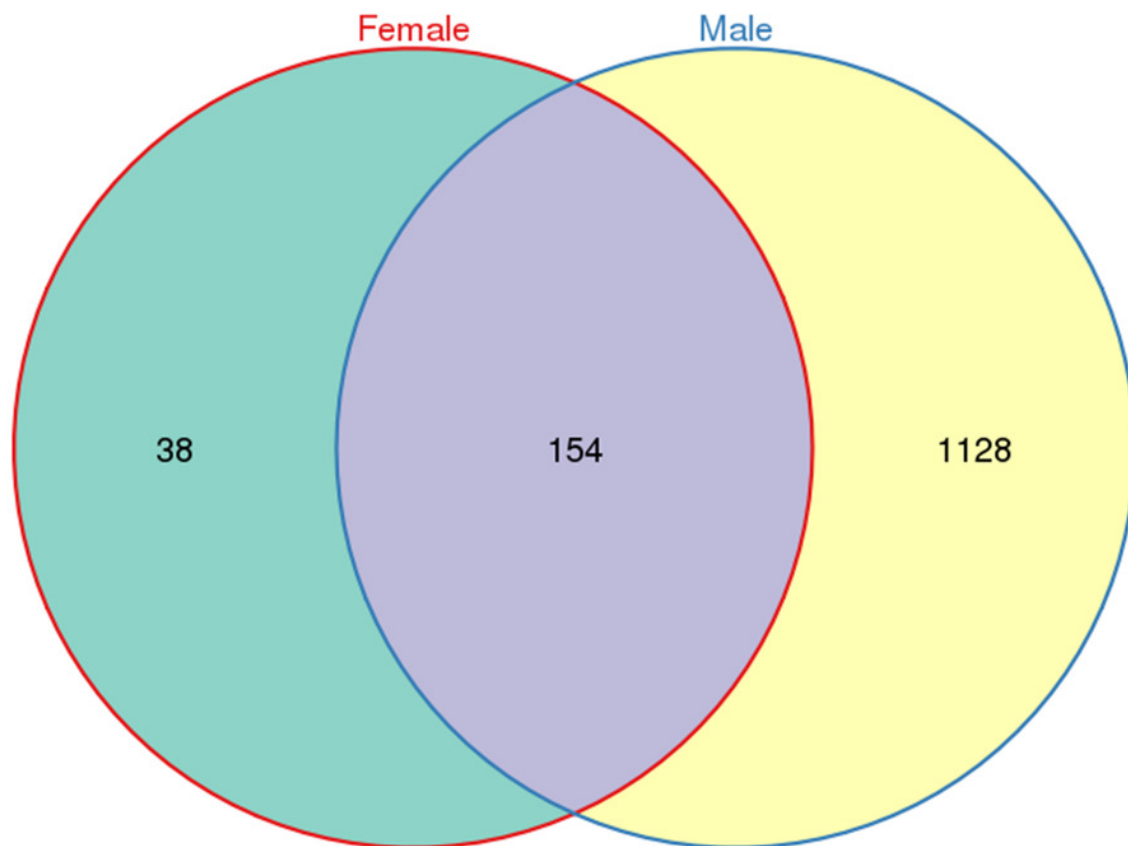



Figure 2

Relative abundance of top 10 bacteria at the levels of phylum (A), class (B), order (C), family (D) and genu (E) in females and males of *Leptocybe invasa*.

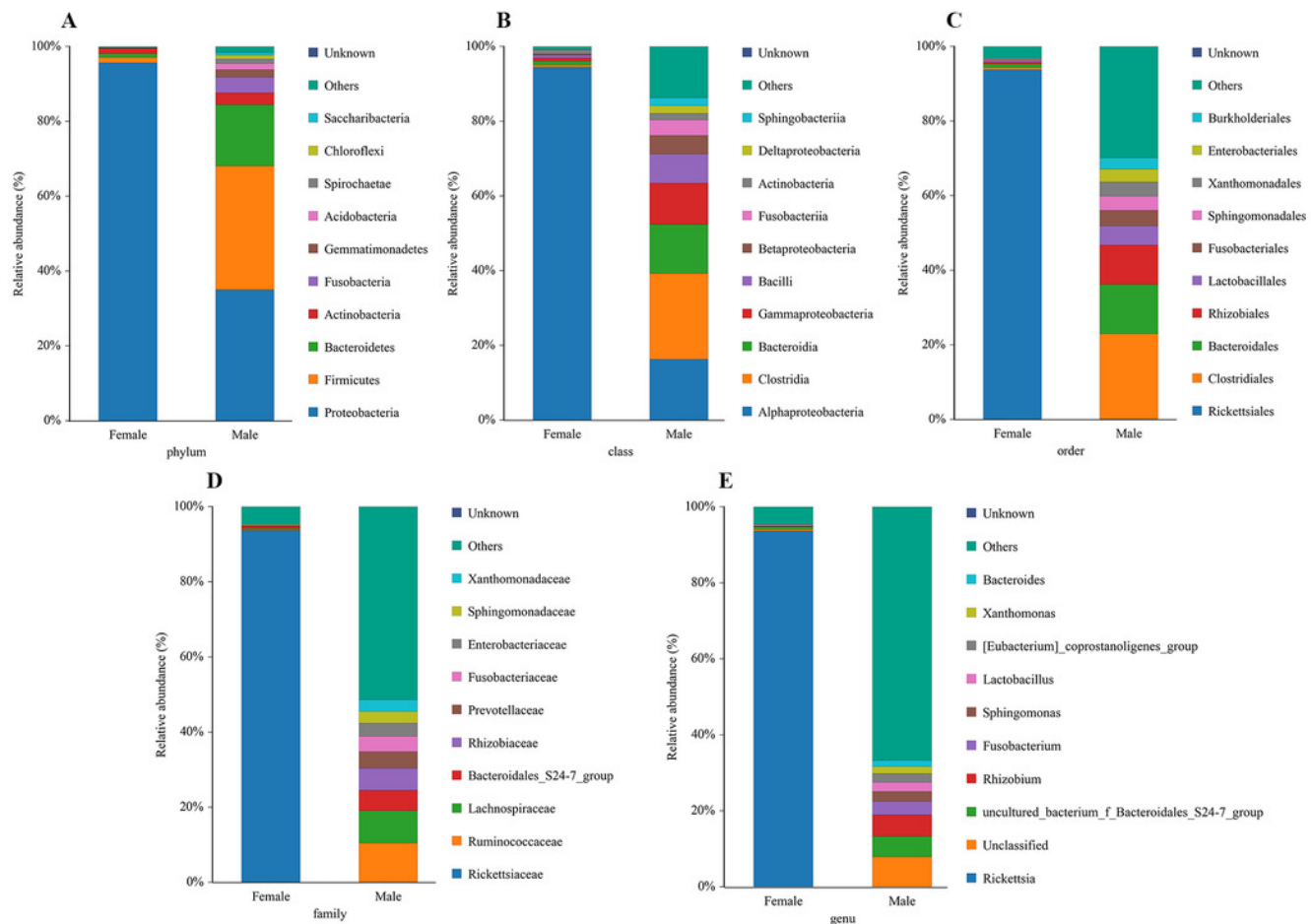



Figure 3

Phylogenetic tree of different *Leptocybe invasa* populations based on COI sequences.

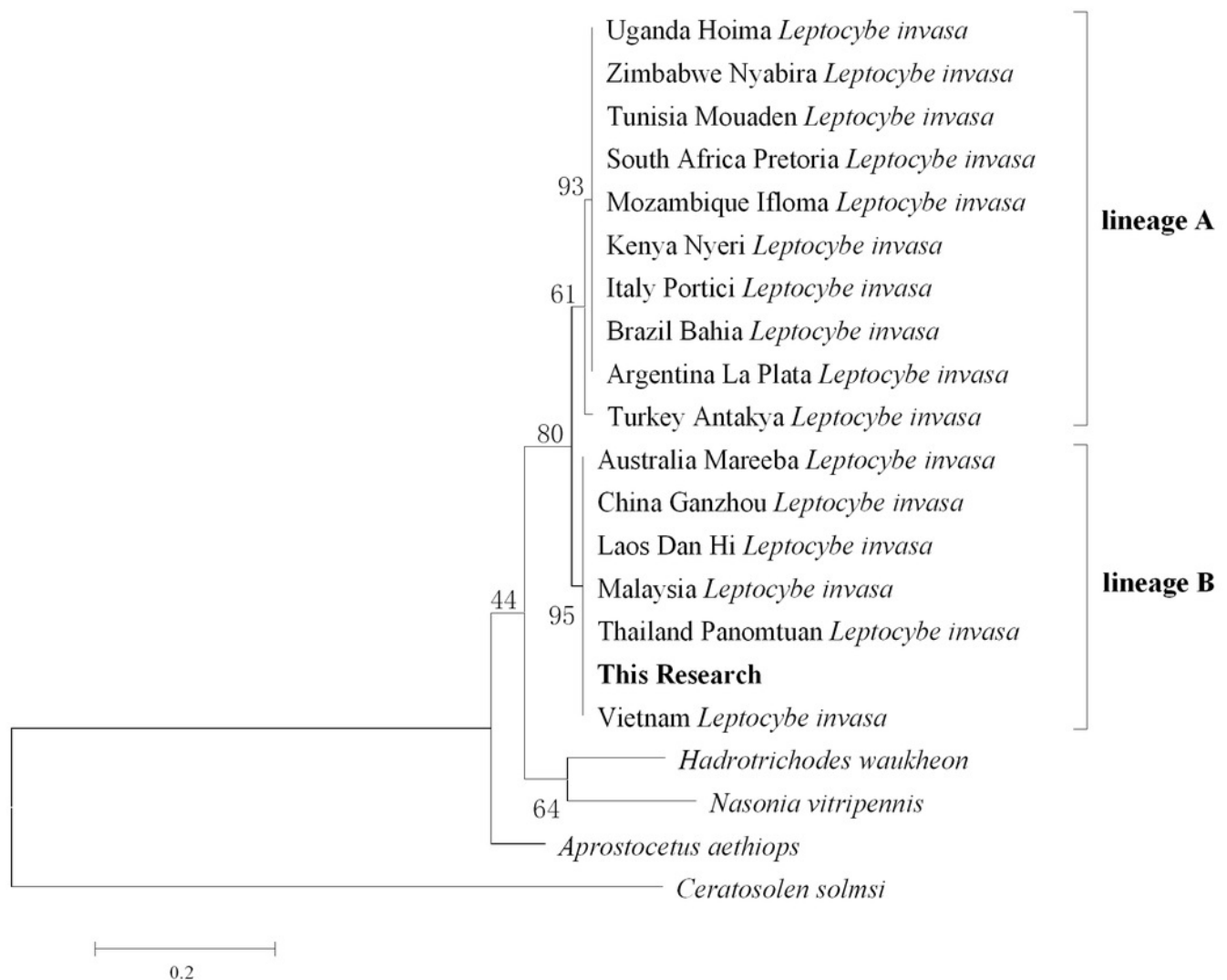
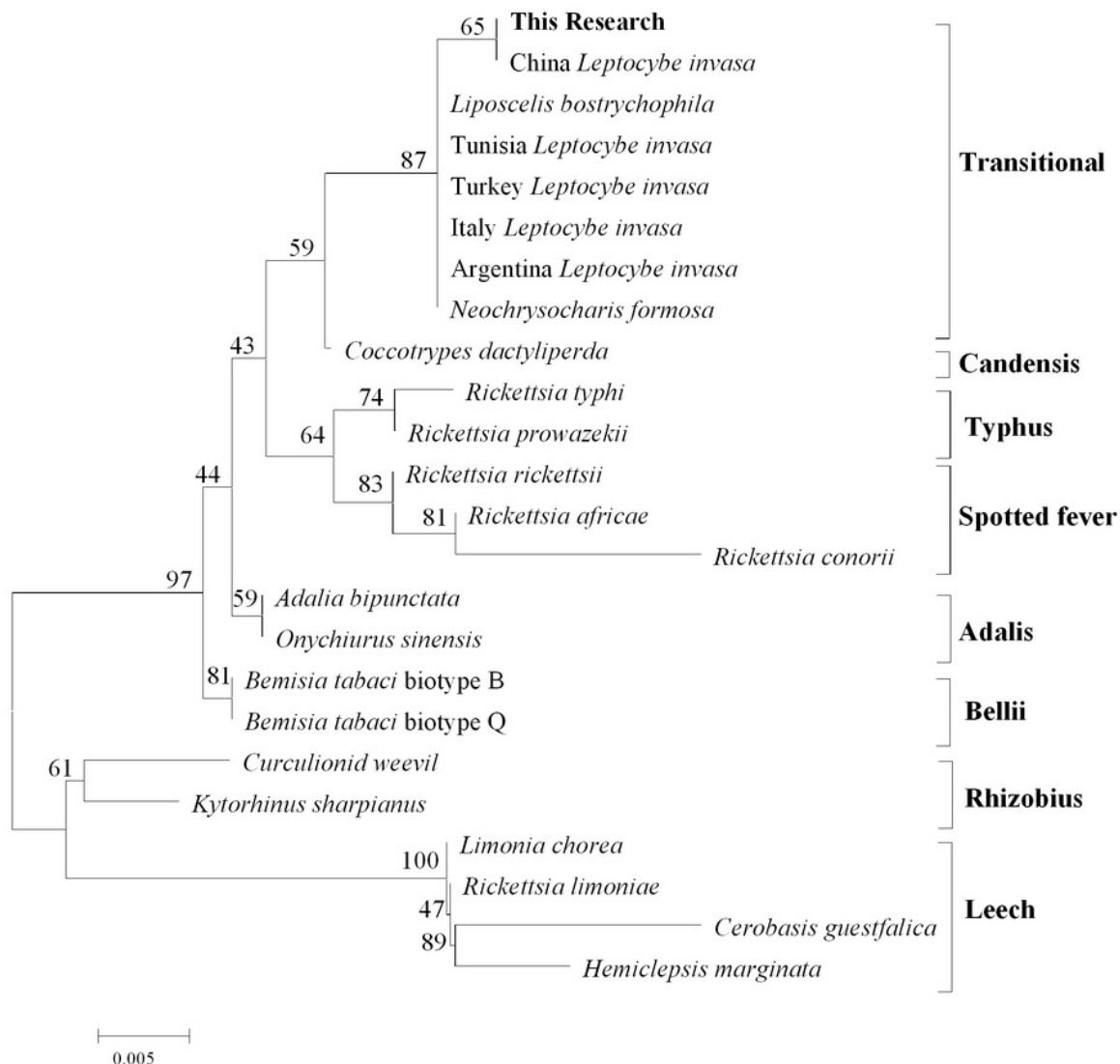



Figure 4

Phylogenetic analysis of different *Rickettsia* groups based on their 16S rRNA sequences.

Statistics of alpha diversity indices of the bacteria in female and male adults of *Leptocybe invasa*.

1 **Table 1:**

2 **Statistics of alpha diversity indices of the bacteria in female and male adults of *Leptocybe invasa***

Sample	ACE	Chao1	Simpson	Shannon	Coverage
Female	212.84	229.50	0.85	0.59	1.00
Male	1282.28	1282.00	0.01	6.13	1.00

Basic composition of the bacterial colonies in female and male adults of *Leptocybe invasa*.

1 **Table 2:**

2 **Basic composition of the bacterial colonies in female and male adults of *Leptocybe invasa***

Sample	Phylum	Class	Order	Family	Genus
Female	10	26	44	76	122
Male	24	69	127	238	487
Female-specific	0	2	3	7	14
Male-specific	14	45	86	169	379
Sex-in common	10	24	41	69	108
Total	24	71	130	245	501

Relative abundance of dominate bacteria at the levels of genus in female and male adults of *Leptocybe invasa*.

1 **Table 3:**

2 **Relative abundance of dominate bacteria at the levels of genus in female and male adults of**

3 *Leptocybe invasa*

Genus	Female (%)	Male (%)
Rickettsia	93.67	0.04
uncultured_bacterium_f_*Bacteroidales*_S24-7_group	0.71	5.37
Lactobacillus	0.31	2.38
Sphingomonas	0.25	2.62
Bacteroides	0.11	1.65
Fusobacterium	0.04	3.49
[Eubacterium]_*coprostanoligenes*_group	0	2.34
Rhizobium	0	5.73
Unknown	0	0.01
Xanthomonas	0	1.83
Others	4.48	66.68
Unclassified	0.44	7.86