1   **Gut microbial diversity in stingless bees is linked to host wing size and is influenced by**

2   **geography**

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18   **Running title**: Landscape of stingless bee gut microbiome

Originality-Significance Statement

Beneficial interactions between insects and their microbial symbionts are pivotal for their fitness. In this study, we analysed the gut microbiomes of two stingless bee species, *Tetragonula carbonaria* and *Austroplebeia australis*, that are widespread and important pollinators in Australia. We characterized their gut microbiomes and detected a significant positive correlation between gut bacterial richness and host forewing size for *T. carbonaria*; the first time that gut microbial diversity has been linked to a morphological trait in stingless bees. Furthermore, we found that host species' identity, management type (wild or managed) and geography all significantly influenced bee gut microbial diversity and composition, and were able to describe both bacterial and fungal core microbial taxa. This study reveals novel understandings of stingless bee gut microbiomes and provides the basis for utilizing microbial strategies to maintain colony health.

Summary

Stingless bees are globally important social corbiculate bees, fulfilling critical pollination roles in many ecosystems; however, their gut microbiota, especially fungal communities, are not well characterized to date. We collected 121 bee samples from two species, *Tetragonula carbonaria* and *Austroplebeia australis*, across a distance of 1,200 km of eastern Australia, and analysed their gut microbiomes. We found that the gut bacterial richness of *T. carbonaria* was influenced by geography (latitude and longitude) and positively correlated to an established fitness indicator in insects; namely, host forewing length/size that relates to flight capacity of stingless bees. We characterized the core microbiomes of the two bees and found that they consisted of the bacterial taxa *Snodgrassella*, *Lactobacillus*, Acetobacteraceae and *Bombella,* and the fungal taxa Didymellaceae, *Monocilium mucidum*, *Malassezia restricta* and *Aureobasidium pullulans*. Both host species identity and management (wild or managed) significantly influenced the gut microbial diversity and composition, and similarity between colonies declined as the geographical distance between them increased. This result was also supported by our co-existing network analyses. Overall,

47   we have thoroughly analysed stingless bee gut microbiomes, and provided novel evidence

48   that *T. carbonaria* bees with larger wings or from more southern populations have higher

49   microbial diversity in their guts.

50

51   **Key words**

52   *Austroplebeia australis*, bacterial and fungal communities, core microbiome, forewing size,

53   geographic variation, stingless bee, *Tetragonula carbonaria*

54 **Introduction**

55 Insect guts harbour many microorganisms across the three primary regions; foregut, midgut

56 and hindgut (Chapman and Chapman, 1998). These microorganisms have various host

57 functions that vary from aiding nutrient extraction from foods (Engel and Moran, 2013) to

58 detoxification of harmful compounds (Ceja-Navarro et al., 2015) and protection against

59 parasites and pathogens (Endt et al., 2010). Corbiculate bees in particular, are known to

60 possess characteristic gut microbiomes. Honey bee (*Apis* spp.) guts, for example, consist of a

61 core bacterial community including *Snodgrassella*, *Gilliamella*, *Lactobacillus* Firm-4 and -5

62 and *Bifidobacterium* (Koch and Schmid-Hempel, 2011; Kwong et al., 2017), which are

63 obtained through floral visits (McFrederick et al., 2017; Liu et al., 2019) and from within the

64 hive environment (e.g. through feeding on stored pollen). Increasing evidence shows that,

65 similar to other insects, corbiculate bees have co-evolved with their gut microbes to form

66 mutualistic relationships. The bees benefit from the microbiome primarily through defence

67 against enemies, regulation of growth and development and contributing to hive pollen

68 fermentation (Cano et al., 1994; Vásquez and Olofsson, 2009; Anderson et al., 2011; Koch

69 and Schmid-Hempel, 2011; Engel et al., 2012; Menezes et al., 2015; Kwong et al., 2017).

70 Conversely, changes to the gut microbiome composition of social bees, such as those caused

71 by antibiotic exposure, can lead to dysregulated immune systems and reduced ecological

72 fitness (Liu et al., 2019). Indeed, maintenance of a healthy gut microbiome is essential for

73 the colony and individual's homeostasis, nutrition and defence (Engel and Moran, 2013).

74

75 Among the corbiculate bees, stingless bees (tribe: Meliponini) comprise >500 species

76 globally (Michener, 2013), of which 11 recognised species occur in Australia, under two

77 genera: *Austroplebeia* and *Tetragonula* (Dollin and Dollin, 1997; Dollin et al., 2015). They

78 are important pollinators of natural plants and crops (Heard, 1999; Hall et al., 2020), and can

79 be harnessed by beekeepers either through rescuing colonies from felled trees, or

80 propagation in man-made hives (Halcroft et al., 2013). The sale of stingless bee hives for

81 crop pollination in Australia has increased over recent decades and, while natural

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82   populations exist across much of eastern Australia, most commercial colonies originate from

83   a narrow region of southern Queensland (Chapman et al., 2018). Thus, the origin of the

84   colony and whether it resides in a managed hive or is naturally occurring in a tree hollow,

85   may affect how microbiomes are shaped e.g., by the internal conditions within hives (Heard

86   2016). *Austroplebeia* and *Tetragonula* stingless bees are similar in body size and colour and

87   can occur sympatrically along the east coast of Australia. However, they belong to different

88   phylogenetic clades, and *Austroplebeia* tends to occur further inland in somewhat drier

89   habitats. Their behaviour also differs; for example, *T. carbonaria* is more active in flight and

90   perhaps collects more resin and pollen than *A. australis* (Leonhardt et al., 2014). In contrast,

91   *A. australis* colonies are more likely to focus on collecting high-quality nectar (e.g., of high

92   sugar concentrations) (Leonhardt et al., 2014). Such distinct behaviour, along with

93   differences in available floral resources within their habitats can thus shape different gut

94   microbiomes among stingless bee species (Vásquez et al., 2012). Previous studies have

95   shown that bacterial communities within the two Australian stingless bee genera can change

96   rapidly (Hall et al., 2021), and a novel clade of host-specific lactic acid bacteria

97   (*Lactobacillus*) has been found (Leonhardt and Kaltenpoth, 2014; Hall et al., 2021).

98   However, these studies used relatively few samples and to date there is limited comparison

99   of gut microbial communities across species and geographic ranges. Additionally, like other

100   animal gut microbiome studies, fungal communities in the guts of insects, including stingless

101   bees, have received little attention (de Paula et al., 2021). Insect-associated fungi including

102   moulds and yeasts can contribute to host nutrient provision (Menezes et al., 2015). For

103   instance, some fungi are involved in wood and food digestion in underground ant and termite

104   chambers (Zoberi & Grace, 1990). Similarly, the intracellular symbiotic fungi of beetles,

105   *Symbiotaphrina* spp., can both aid in food digestion and detoxify a variety of plant materials

106   (Dowd and Shen, 1990). Additionally, a Brazilian stingless bee *Scaptotrigona depilis* farms

107   specific strains from the fungal genus *Monascus* (Ascomycota) as a food source for its larvae

108   (Menezes et al., 2015). Despite the importance, fungal community composition and diversity,

109   their mutualistic interactions with the host and key drivers of the microbiome assembly

110   remain poorly understood.

111

112   The western (or European) honey bee (*Apis mellifera*) is one species for which a core

113   bacterial microbiome has been determined (Kwong et al., 2017). The bacteria *Snodgrassella*

114   *alvi*, *Gilliamella apicola*, *Lactobacillus sp.* and *Bifidobacterium* are ubiquitous and can be

115   found in essentially every adult worker honey bee, with each bacterial species showing a

116   specialized distribution in the gut (e.g., *S. alvi* and *G. apicola* dominate the ileum region of

117   hindgut) (Moran, 2015). Each of the core species can contain multiple strains, with a high

118   level of strain variation. Other social corbiculate bees, such as bumble bees and stingless

119   bees, may contain distinct strains of similar bacterial species to those found in honey bees

120   (Koch et al., 2013), but systematic studies of the core gut microbiome, in particular fungi

121   and their interactions with other microbial species within the microbiome, are yet to be done.

122

123   One of the aims of this study was to test if correlations exist between stingless bee gut

124   microbiome (bacteria and fungi) and morphological traits, such as flight capacity. Wing size

125   in insects is an essential functional trait for flight performance (Wootton, 1992), foraging,

126   dispersal and migration (Johansson et al., 2009). The wings of monarch butterflies (*Danaus*

127   *plexippus*) from migratory populations, for example, tend to be larger than those from non-

128   migratory populations, presumably because larger wings provide greater surface area for

129   soaring flight and energy preservation (Dockx, 2007; Altizer and Davis, 2010; Davis and

130   Holden, 2015). Insect wing length can predict flying capacity, and there is evidence that

131   honey bees from colonies infected with the ectoparasite *Varroa destructor* had shorter wing

132   lengths and flew shorter distances than healthy bees (Attisano et al., 2013; Blanken et al.,

133   2015). Maximum flight distances of stingless bees were also highly correlated with wing size

134   in six stingless bee species, suggesting that flight capacity is a function of their wing sizes,

135   and thus, bees with larger wings may be able to fly further to forage on more diverse plant

136   resources (Casey et al., 1985; Byrne et al., 1988; Araújo et al., 2004). However, to date no

137 link has been made between stingless bee morphological traits, such as wing, tibia and body

138 sizes, and gut microbial diversity.

139

140 Overall, despite the importance of stingless bees to pollination services, relatively little is

141 known of their gut microbiome, nor how sorting and dispersal limitation function in shaping

142 gut microbial communities; especially, how they are influenced by the host species,

143 management type and geography. Here, we collected 121 samples of stingless bee foragers

144 from two species; *Austroplebeia australis* and *Tetragonula carbonaria*, ranging 1,200 km

145 along the east coast of Australia (Queensland and New South Wales), and investigated the

146 composition of their gut microbiomes using 16S rRNA gene and fungal ITS amplicon

147 sequencing. We tested the following hypotheses: (i) a core microbiome exists in the gut of

148 stingless bee foragers; (ii) the gut microbiome structure is influenced by bee species,

149 geographic location and by whether bees are wild or managed (i.e. cultivated in hives); and

150 (iii) characteristics associated with flight and foraging capacities, such as wing length and

151 size, positively correlate to host gut microbiome diversity.

152

153 **Material and methods**

154 *Specimen collection, measurement and gut dissection*

155 We collected 121 stingless bee samples from the two most common and widespread

156 Australian stingless bee species, *T. carbonaria* and *A. australis,* across their distributional

157 ranges in QLD and NSW, Australia (**Fig.1a**). The *T. carbonaria* samples (n=80) were

158 collected between September 2018 and January 2020 across a range of 1,200 km in eastern

159 Australia. They comprised 43 samples collected from managed hives and 37 samples

160 collected from the wild (e.g. national parks). The *A. australis* samples (n= 41) had been

161 collected previously (Halcroft et al., 2013), in 2009 from wild tree-living colonies across a

162 250 km range across their natural distribution (**Fig.1a**). Consequently, only *T. carbonaria*

163 was used for analyses of the effect of management type (wild or managed) on gut microbial

164 composition and diversity. During sampling, geographic coordinates (longitude and latitude)

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165   were recorded, and all live specimens were immediately preserved in 70% ethanol, and

166   stored at -20 °C prior to gut dissection and morphological measurement. To minimize biases

167   due to inter-individual variability, three individuals from each sample were used for

168   microbiome analyses. The whole gut of each individual was dissected on a sterile Petri dish

169   under a microscope using sterilized forceps, and the gut materials were then pooled and

170   transferred to a 1.5 mL sterile centrifuge tube, and preserved at -20 °C prior to DNA

171   extraction. We took the following morphometric measurements to infer bee flight

172   performance and fitness (Wootton, 1992): forewing length, forewing area (forewing length

173   width), hind tibia length and total body length. Each trait was measured using a digital

174   microscope (Leica EZ4W, Leica Microsystems, Buffalo Grove, IL) for 1~3 bees per sample

175   (2~6 measurements of wings and hind tibia for each sample and an average of each trait was

176   used as the final result of the sample).

177

178   *DNA extraction from gut material and library preparation for high throughput amplicon*

179   *sequencing*

180   For each sample, the gut material of three bees were combined and used for DNA extraction

181   using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's

182   recommendations. DNA samples were quality checked and quantified using a Nanodrop

183   2000 (Thermofisher Scientific, USA) and Qubit (Thermofisher Scientific, USA) respectively,

184   before being stored at -20°C. Library preparation and bacterial and fungal amplicon

185   sequencing were then carried out at the Next Generation Sequencing Facility (Western

186   Sydney University, Australia). The 16S rRNA gene primers 341F (CCT ACG GGN GGC

187   WGC AG) and 805R (GAC TAC HVG GGT ATC TAA TCC) (Herlemann et al., 2011) and

188   the fungal ITS2 primers fITS7 (GTG ART CAT CGA ATC TTT G) and ITS4 (TCC GCT

189   TAT TGA TAT GC) (Ihrmark et al., 2012; White et al., 1990) were used for the

190   amplification and subsequent sequencing. All the samples were run on the MiSeq (Illumina)

191   platform, generating 300 bp paired end reads.

192

193　*Bioinformatics and statistics*

194　Sequencing files (FASTQ format) were processed using QIIME2 software and its plugins

195　(version 2019.7; http://qiime2.org/) (Bolyen et al., 2019). Briefly, the sequencing quality was

196　first assessed using FastQC (Andrews, 2010). Then, QIIME2 implementation of cutadapt

197　v2019.7.0 was used for removal of primer sequences, and DADA2 v2019.7.0 (Callahan et al.,

198　2016) was used for error-correction, quality filtering, chimera removal and constructing

199　feature tables and final sequence files. Sequencing reads were truncated at 260 bp and 240

200　bp for forward and reverse reads, respectively, resulting in sequence quality Q>20. The

201　amplicon sequence variants obtained were summarized and then assigned with taxonomic

202　information using the q2-feature-classifier. For the bacterial data, a Naïve Bayes classifier

203　pre-trained on the Greengenes 13_8 99% OTUs dataset was used to assign taxonomy to each

204　representative sequence. Bee- and plant-associated mitochondria and chloroplast sequences

205　were removed from the feature table to retain microbial features only. For the ITS fungal

206　dataset, the classifier was trained to UNITE v8.0 database (99%) (UNITE Community, 2019)

207　(DeSantis et al., 2006). The number of reads for the bacterial and fungal sequencing data was

208　rarefied to 7,125 and 944 sequences, respectively, per sample by re-sampling the OTU table.

209　The mean number of observed species (Obs), Chao1, Pielou_e, Simpson's and Shannon

210　diversity index values were calculated within QIIME2.

211

212　*Statistical analyses*

213　R version 4.0.3 (2020-10-10) was used for analyses unless otherwise stated. Correlations

214　between stingless bee traits and gut microbial alpha diversity were examined using linear

215　models (Pearson) and visualised in R. The effect of stingless bee species and management

216　types on gut microbial community composition and diversity were investigated using

217　permutational multivariate analysis of variance (PERMANOVA, permutation=9999), and

218　visualized with principal component analysis (PCA) using the Vegan package (v.2.5-6,

219　(Oksanen et al., 2013). Fitting bee traits onto PCA ordination was performed using function

220   *envfit* in Vegan (v.2.5-6). The ggplot2 package (version 3.3.3, (Ginestet, 2011)) was then

221   used to produce the stacked graph at phylum level.

222

223   The phylogenetic tree for the most abundant OTUs was calculated in QIIME2, and annotated

224   using Interactive Tree of Life (ITOL) (https://itol.embl.de). For core microbiome analysis

225   and random forest test, we used an online microbiome analyses tool following recommended

226   parameters (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml) (Chong et

227   al., 2020). The determination of the core microbiome identifies those members that occur

228   in >20% hosts at abundance of >0.1% within a defined host population, which is consistent

229   with previously used methods (Bereded et al., 2020). For analysing gut microbial community

230   variation over spatial gradients (latitude and longitude of each sample), we constructed

231   geographic and environmental distance-decay relationships based on our spatially highly

232   resolved set of samples (Soininen et al., 2007). This analysis reveals how the similarity in

233   host microbiome composition between communities varies with geographic distance. The R

234   package geosphere (1.5-10) (Hijmans, 2019) was used to calculate distance (km) between

235   locations based on geographic coordinates for each sample. The *vegdist* function in the

236   Vegan package (v.2.5-6) was used to calculate Bray-Curtis similarity (1-Bray-Curtis

237   dissimilarity). Distance-based multivariate analysis for a linear model was then performed to

238   investigate correlations between the Bray-Curtis similarity and geographic distance between

239   stingless bee samples.

240

241   Lastly, the microbial co-existing network analysis was used to explore the interactions

242   within the gut microbiomes. Co-existing network analysis reveals the potential interactions

243   between microbial symbionts coexisting in the gut environment (Faust and Raes, 2012).

244   Prior to microbial co-existence network calculation, OTUs present with <5 reads and in <3

245   samples in the normalized OTU tables were discarded to minimize spurious correlation that

246   can be caused by rare taxa. Benjamini-Hochberg corrections (based on 100 bootstraps) were

247   conducted to reduce the false discovery rate, and only strong (R>0.60) and robust (P<0.01)

248   correlations were maintained. Correlations were calculated using the SparCC-based

249   (Friedman and Alm, 2012) algorithm Fastspar (Watts et al., 2019), which was visualized and

250   edited in Gephi (Bastian et al., 2009). Structural equation models (SEM) were used to

251   evaluate the effects of morphometric traits and management approaches of bees on the

252   bacterial richness in their gut, which was conducted using AMOS17.0 (AMOS IBM, USA).

253   The maximum-likelihood estimation was fitted to the SEM model, and Chi-square and

254   approximate root mean square error were calculated to examine model fit. Adequate model

255   fits were determined according to a non-significant chi-square test ($P > 0.05$), high goodness

256   fit index (GFI) (> 0.90), low Akaike value (AIC) and root square mean error of

257   approximation (RMSEA) (< 0.05) as previously described (Delgado-Baquerizo et al., 2016).

258

259   **Results**

260   *The alpha diversity of stingless bee gut microbial communities*

261   The gut bacterial and fungal communities of the two stingless bee species across a

262   substantial geographic range were characterized using high throughput 16S rRNA gene and

263   ITS amplicon sequencing (**Fig.1a**). There was a significantly higher alpha diversity of the

264   bacterial communities in *A. australis* than *T. carbonaria* (Obs $P<0.0001$ **Fig.1b**, Chao1

265   $P<0.0001$ and Shannon $P=0.002$, but not Simpson $P=0.29$). Similarly, alpha diversity of the

266   gut fungal community of *A. australis* was significantly higher than that of *T. carbonaria*

267   (Shannon $P=0.017$, Simpson $P=0.033$, Pielou_e $P=0.0047$, but not Obs $P=0.82$ **Fig.1c** or

268   Chao1 $P=0.83$). For gut bacterial communities of *T. carbonaria*, wild bees had higher alpha

269   diversity than managed bees (Chao1 $P=0.04$, but not for Obs $P=0.063$). Conversely, alpha

270   diversity of the fungal community was higher in managed *T. carbonaria* bees than in wild

271   bees (Chao1 $P=0.003$, observed species $P=0.004$).

272

273   *Core microbiome analyses of the stingless bee gut microbial communities*

274   At the phylum level, gut bacterial communities of both stingless bee species were dominated

275   by Proteobacteria, Firmicutes and Actinobacteria, along with less abundant Bacteroidetes,

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276 Verrucomicrobia, Tenericutes, Acidobacteria, Gemmatimonadetes and other unidentified

277 taxa (**Fig.S1a**). The fungal community was dominated by Ascomycota and Basidiomycota,

278 with Chytridiomycota, Mucoromycota and some unidentified fungal phyla also common

279 (**Fig.S1b**). To obtain an overall picture, we combined samples of the two species and

280 analysed their phylogenetic traits and dominant phylotypes using the top 255 (in relative

281 abundance) bacterial sequences (**Fig.2a**). We found that, at the OTU level, the stingless bee

282 gut microbiome was largely dominated by five OTUs, namely g_*Snodgrassella* (16.2%),

283 f_Acetobacteraceae (10.3%), f_Acetobacteraceae (9.8%), g_*Lactobacillus* (8.6%) and

284 g_*Lactobacillus* (6.1%) (**Fig.2a**). The stingless bee gut microbiome composition appears to

285 be highly variable within host species and colonies. When the presence threshold was set at

286 20% for defining the core microbiome, we found seven core bacterial OTUs, including:

287 g_*Snodgrassella*, f_Acetobacteraceae, g_*Lactobacillus*, f_Acetobacteraceae, g_*Lactobacillus*,

288 g_*Bombella* and s_*Lactobacillus* sp. Thmro15 (**Fig.2b**), and six fungal OTUs:

289 f_Didymellaceae, s_*Monocilium mucidum*, s_*Malassezia restricta*, s_*Aureobasidium*

290 *pullulans*, s_*Alternaria alternata* and s_*Malassezia globosa* (**Fig.2c**). The core gut

291 microbiome of these Australian stingless bees consists of a distinct group of bacterial taxa

292 from that observed in honey bees and bumble bees (**Table 1**). However, there are currently

293 no available data on core fungal taxa of other social corbiculate bees to compare. Fungal

294 guild analyses identified the core fungal species in stingless bees as both litter/soil

295 saprotrophic and also invertebrate-associated taxa (**Table 1**).

296

297 *Co-existing network analyses of the stingless bee gut microbiomes*

298 Bacterial and fungal taxa, and geographic coordinates (latitude and longitude) of samples

299 were integrated for co-existing network analyses to detect potential microbe-microbe

300 interactions and predict the involvement of geography in the microbial network (**Fig.3**).

301 From 121 samples and 2,799 OTUs, correlation network analyses identified 81 strong

302 correlations ($R > 0.6$, $P < 0.01$) for managed *T. carbonaria* (**Fig.3a**), 95 for wild *T.*

303 *carbonaria* (**Fig.3b**) and 574 for wild *A. australis* (**Fig.3c**, **Table S1**). The three networks (*T.*

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304   *carbonaria* managed and wild and *A. australis* wild) were formed by six distinct major

305   modules, and each module represents a group of OTUs that are closely connected with each

306   other but less linked with other OTUs in the network. The average degree of correlation for

307   each of the three networks ranged from 2.76 to 8.43, with *A. australis* having the largest

308   degree (8.43) followed by *T. carbonaria* wild (3.84) and *T. carbonaria* managed (2.76)

309   (**Table S1**), suggesting that wild *A. australis* may have the most extensive microbial

310   interactions of the three groups.

311

312   The 10 microbial taxa with the most connections with other microbes were highlighted in

313   each network structure (**Fig.3a,b,c**). Such microbial taxa within the managed *T. carbonaria*

314   network included g_*Bombella*, g_*Zymobacter*, s_*Neophaeomoniella niveniae* (fungi),

315   s_*Alternaria alternata* (fungi) and g_*Lactobacillus* (**Fig.3a**). The most connected microbial

316   taxa in the network of wild *T. carbonaria* included s_*Bifidobacterium bombi*,

317   g_*Rosenbergiella*, g_*Lactobacillus*, g_*Acinetobacter* and g_*Snodgrassella* (**Fig.3b**). For wild

318   *A. australis,* these included f_Rhodobacteraceae, f_Burkholderiaceae, g_*Algoriphagus*,

319   g_*Hydrogenophaga*, and s_*Terrimonas* sp. 16-45A (**Fig.3c**). We also found a few microbes

320   with close connections to latitude and longitude, indicating a geographic influence in the

321   formation of the gut microbiome network. Among these, there were three taxa with

322   connections to latitude and longitude, for managed *T. carbonaria* (**Fig.3a**), 5 and 7

323   connections respectively for the wild *T. carbonaria* (**Fig.3b**). For *A. australis*, there were 2

324   taxa with connections to latitude and 33 to longitude (**Fig.3c**).

325

326   *Factors influencing stingless bee gut microbial community composition (beta-diversity)*

327   Bacterial and fungal community composition were influenced by both host species identity

328   (Bacterial: R^2=0.19, P=0.0001, fungal: R^2=0.03, P=0.0001) and management type

329   (managed/wild, assessed in *T. carbonaria* only) (Bacterial: R^2=0.025, P=0.023, fungal:

330   R^2=0.064, P=0.0001) (**Figs.4a,b**). When both bee species were considered together, the gut

331   bacterial community composition was closely associated with host forewing length

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332   (R^2=0.2047, P=0.0001), latitude (R^2=0.1420, P=0.0004) and longitude (R^2=0.5051,

333   P=0.0001) (**Fig.4a**). However, none of these factors were significant when the two species

334   were analysed independently. For the fungal community, host forewing and tibia lengths and

335   longitude were associated with the microbial community composition (R^2=0.18, R^2=0.40,

336   R^2=0.37 respectively, and P=0.0001 in all cases) (**Fig.4b**). When examining each species

337   separately, body length was associated with the fungal community composition of *A.*

338   *australis* (R^2=0.18, P=0.034), while longitude was associated with the fungal community

339   composition of *T. carbonaria* (R^2=0.16, P=0.004).

340

341   We then identified key microbial taxa distinguishing the gut microbiomes of the two species

342   using random forest tests (**Fig.S2**). Error rates for the bacterial and fungal communities

343   achieved stability when tree numbers for prediction reached 110 and 140 for bacterial and

344   fungal communities, respectively (**Fig.S2a,b**). The top ten bacterial OTUs that distinguished

345   between the two gut microbial communities were revealed; one belonging to g_*Gilliamella*,

346   six g_*Lactobacillus*, one g_*Mycobacterium* and one s_uncultured bacterium were associated

347   with *A. australis*, whereas one OTU belonging to f_Acetobacteraceae was associated with *T.*

348   *carbonaria*. For the fungal community, one OTU of s_*Zygosaccharomyces rouxii*, one

349   f_Didymellaceae and seven unidentified fungal OTUs were associated with *A. australis,*

350   whereas one OTU belonging to s_*Aureobasidium pulluans* was associated with *T.*

351   *carbonaria*.

352

353   We also used Bray-Curtis similarity index to determine the influence of geographic distance

354   (km) between samples on gut bacterial and fungal community composition (**Fig.S3**). A

355   significant linear decrease in composition similarity with increasing geographic distance was

356   seen between samples for *T. carbonaria* gut bacterial communities (P<0.0001). In addition,

357   there was a similarity decrease with increasing geographic distance for fungal communities

358   for both *T. carbonaria* (P<0.0001) and *A. australis* (P=0.002) (**Fig.S3a,c,d**). These results

359   show that the microbiome composition varies more between samples from geographically

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360   distant locations. However, the adjusted R^2 for each correlation was small (0.005~0.01),

361   indicating only a fairly weak link. No significant correlation was seen for gut bacterial

362   communities in *A. australis* (**Fig.S3b**). It is worth noting that the *A. australis* samples

363   covered a relatively smaller area, and so the power to detect small changes may have been

364   reduced compared to *T. carbonaria*.

365

366   *Correlation of stingless bee gut microbial richness with host morphological traits and*

367   *geography*

368   Linear models showed that forewing length and area, hind tibia length and body length were

369   all positively correlated (e.g. $R_{wing\ length–wing\ area}$= 0.86; $R_{wing\ length–body\ length}$=0.42; $R_{wing\ length–tibia}$

370   $_{length}$=0.78; N=110; P<0.0001 in all cases) (**Fig.S4a,b**). Additionally, hind tibia (P<0.0001)

371   and forewing length (P<0.0001) differed between the two bee species, but full body length

372   and forewing area did not. Interestingly, gut bacterial richness (Obs) in *T. carbonaria*

373   showed a significant positive correlation with host forewing length (R=0.44, N=71, P=0.001)

374   and forewing area (R=0.32, N=71, P=0.008) (**Fig.5a**), while full body length and hind tibia

375   length showed no correlation with gut bacterial richness or any other alpha diversity value.

376   The forewing area/body length ratio, which is believed to determine flight capacity, also

377   correlated with gut bacterial richness in *T. carbonaria* (R=0.26, P=0.033). For *A. australis*,

378   however, no correlation was seen between alpha diversity of gut microbiomes and host traits,

379   such as forewing length (bacterial R=-0.31, P=0.058; fungal R=0.12, P=0.49) or forewing

380   area (R=-0.16, P=0.33; R=0.07, P=0.48).

381

382   We then analysed the relationship between geography and host gut microbial richness.

383   Importantly, for *T. carbonaria*, longitude (R=-0.36, P=0.001) and latitude (R=-0.43,

384   P<0.0001) negatively correlated with the gut bacterial species richness, but not with fungal

385   species richness (**Fig.5b**). Longitude and latitude both influenced forewing length (Lon R=-

386   0.38, P=0.0008; Lat R=-0.44, P=0.0001) and forewing area (Lon R=-0.38, P=0.0008; Lat

387   R=-0.47, P<0.0001) (**Fig.5b**). For *A. australis*, neither latitude nor longitude influenced host

388   morphological traits, which is not surprising given the sampling range for this species was

389   considerably smaller than for *T. carbonaria*. Using SEM analysis that considered

390   morphological traits, geographical factors and management effects together, we found a

391   dominant effect of forewing length on gut bacterial richness, while longitude and latitude

392   effects on gut microbial richness were small (**Fig.S5**).

393

394   **Discussion**

395   We characterized the whole gut microbiome of two stingless bee species, *T.*

396   *carbonaria* and *A. australis*, from 121 samples spanning a large geographic range (1,200 km)

397   in eastern Australia. We tested whether gut microbiomes are linked to geographic location

398   (longitude and latitude), host species, management type (wild vs managed) and host traits.

399   For *T. carbonaria*, we observed a positive correlation between both forewing length and area

400   with host gut bacterial richness; samples collected in the south had both longer wings and

401   higher bacterial richness than those from the north of their range. For both species,

402   microbiomes consistently became more distinct from one another in their composition with

403   increasing geographic distance between samples. While this effect was relatively weak, it

404   suggests a role of geographical factors in shaping stingless bee gut microbiomes. We

405   analysed key microbial taxa using both core microbiome (abundance based) analyses and

406   microbial co-existing network analyses, which revealed key bacterial groups that show

407   differences from core microbes present in honey bee and bumble bee guts. This study is the

408   first comprehensive analysis of stingless bee gut microbiomes. Combining analyses of both

409   the bacterial and fungal communities with host traits and geography, we provide evidence of

410   a link between the gut microbiome and host morphological traits in stingless bees.

411

412   *Stingless bee gut microbial diversity correlates with host forewing size and geographic*

413   *location*

414   We observed a positive linear correlation between gut bacterial species richness and host

415   forewing length and size for *T. carbonaria*. Insect wing sizes are closely related to their

16

416   flight capacity (DeVries et al., 2010); longer wings favour wider variation in speed and

417   increase capacity for longer flight duration as well as perhaps also saving energy (DeVries et

418   al., 2010). These factors potentially increase the capacity of bees to collect diverse floral

419   resources. Furthermore, insects with larger wings are also more successful in host-seeking

420   and their location of oviposition sites (Berwaerts et al., 2002; Davis and Holden, 2015).

421   Therefore, larger wing size is likely to confer a fitness advantage. We did not, however, see

422   any correlation between gut bacterial/fungal richness with body/tibia size, so the correlation

423   with wing size is not an indirect consequence of a general correlation with body size. This

424   study therefore provides solid evidence of a correlation between wing traits with the host gut

425   microbiome. Additional research is now needed to determine whether increasing wing size

426   results in an increase to the diversity of the gut microbiome, or *vice versa*. Furthermore, it is

427   also important to consider the complexities of the tripartite interactions between wing size,

428   gut microbial diversity and geography, especially considering geography, as measured here

429   by latitude and longitude, actually involves a myriad of other environmental factors. For

430   example, much of Queensland experiences a tropical/subtropical environment, whereas

431   further south e.g., Western Sydney, experiences far greater temperature extremes. Thus, the

432   physiological requirements of the bees, and the floral resources available to them will vary

433   considerably.

434

435   *Bacterial communities*

436   In this study, Proteobacteria and Firmicutes were found to be the dominant bacterial phyla

437   in the stingless bee gut, followed by Actinobacteria; a pattern also observed in honey bees

438   and bumble bees (Kakumanu et al., 2016; Wang et al., 2019). Such microbial similarity

439   among species supports a strong host selection of the microbial environment by eusocial

440   bees. The core bacterial phylotype, *Lactobacillus*, has important functions in the host, such

441   as protection against pathogens, food digestion and pollen fermentation, as has been

442   demonstrated for honey bees in previous studies (Engel and Moran, 2013; Liu et al., 2019).

443   They are also common in the gut system of bumblebees worldwide (Kwong and Moran,

17

444   2016), suggesting that mutualisms with *Lactobacillus* exist throughout the eusocial bees

445   across different geographic regions. Our random forest model showed that *Lactobacillus* spp.

446   are the main indicator taxon distinguishing the stingless bee gut microbiomes of the two

447   species, with six of the ten key distinguishing OTUs belonging to this genus. This suggests a

448   great variance in phylogeny and/or abundance of the *Lactobacillus* genus at species/strain

449   levels between the two species. How this diversity of *Lactobacillus* relates to biological

450   differences and host function is yet to be studied.

451

452   *Snodgrassella spp.,* another core bacterial genus in the stingless bee gut microbiome, also

453   features in the core microbiome of both honey bees and bumble bees (Koch and Schmid-

454   Hempel, 2011). *Snodgrassella* spp. are saccharolytic fermenters and interestingly, have been

455   implicated in the protection of bumble bees against *Crithidia bombi* (Koch and Schmid-

456   Hempel, 2011). Furthermore, laboratory studies indicated that glyphosate (the primary

457   herbicide worldwide) can perturb the strain abundance of core gut *Snodgrassella alvi* in

458   honey bees, which led to higher rates of mortality when glyphosate-treated bees were

459   exposed to the opportunistic pathogen *Serratia marcescens*, highlighting the importance of

460   this bacterium in the maintenance of host health. As with *Lactobacillus*, the relevance of the

461   species/strain diversity of *Snodgrassella* spp. in the *stingless bee* gut microbiome is not yet

462   understood, but may correspond to differences in host metabolic capabilities. Interestingly, a

463   recent study surveyed gut microbiomes of Brazilian stingless bees by sampling multiple

464   species within the genus *Melipona,* and showed that stingless bees can lose the core

465   symbioses of *Snodgrassella* (Cerqueira et al., 2021). This suggests that strong ecological

466   shifts or functional replacements in the stingless bee gut microbiome can occur.

467

468   Stingless bee guts also contained bacterial genera such as *Pantoea, Sphingomonas* and

469   *Stenotrophomonas*, which commonly colonize all parts of flowering plants and are also

470   found in hive environments (Liu et al., 2017; Liu et al., 2020). *Snodgrassella,*

471   *Gilliamella* spp. (Graystock et al., 2017), *Saccharibacter* spp. (McFrederick et al., 2012),

18

472   *Massilia* spp. (Graystock et al., 2017) and *Acinetobacter* spp. (Graystock et al., 2017) are

473   commonly found on flowers (Liu et al., 2019), suggesting floral visits are key to the

474   microbial acquisition by stingless bees.

475

476   The gut bacterial species richness of *A. australis* was significantly higher than that of *T.*

477   *cabonaria*. Such microbial difference in gut may relate to the distinct foraging behaviour of

478   the two species. *T. carbonaria* evidently collects more protein enriched food (e.g. pollen)

479   than *A. australis* which likely focuses on high-quality nectar (carbohydrate enriched)

480   (Leonhardt et al., 2014). This higher level of carbohydrate foraging may be linked with the

481   higher bacterial richness seen in *A. australis* gut. Hive managed bees seem likely to possess

482   less gut bacterial diversity than wild bees, which may indicate a less diverse food

483   composition. A previous study also found that gut bacterial diversity of fruit fly (*Bactrocera*

484   *tryoni*) larvae was significantly lower in laboratory populations compared with field

485   populations (Deutscher et al., 2018). We also observed that stingless bee gut microbiomes

486   vary greatly among samples. This aligns with a previous study on whole-body bacterial and

487   fungal communities of managed *T. carbonaria* (Hall et al., 2021). Rapid temporal changes of

488   the bee microbiome composition observed in that study may also, to some extent, explain the

489   high variability of microbiome composition we saw across a geographic gradient. For

490   example, Hall et al. (2021) saw dramatic increases in the relative abundances of *Bombella*

491   and *Zymobacter* and almost complete depletion of *Snodgrassella* when colonies were moved

492   from a florally resource-rich site to a resource-poor site. Other studies have also shown

493   bacterial community diversity to vary considerably between bee colonies (Koch and Schmid-

494   Hempel, 2011; Leonhardt and Kaltenpoth, 2014). All the above findings suggest that

495   stingless bees are prone to compositional shifts, putatively influenced by food resources,

496   physiological status, origin of the colony and climate at different geographic locations.

497

498   *Fungal communities*

19

499   The dominant fungal phyla observed, Ascomycota and Basidiomycota, usually fulfil a

500   decomposing role in most land-based ecosystems, by breaking down organic materials such

501   as large molecules of cellulose or lignin, and in doing so play important roles in carbon and

502   nitrogen cycling (Dighton, 2016). It is logical then, to assume that these gut colonisers can

503   assist the animal host with food digestion (de Paula et al., 2021). We identified six core

504   fungal taxa in *T. carbonaria* and *A. australis*. Interestingly, two of these, *Malassezia*

505   *restricta* and *M. globosa*, are also among the most abundant fungal species in the human gut

506   (as indicated by their large presence in faecal samples and intestinal mucosa), and have been

507   identified in association with gut diseases including colorectal cancer (Coker et al., 2019).

508   This may make stingless bees a suitable animal model for investigation of the role of these

509   fungi in human gut health. Another core fungal genus, *Monocillium* spp. has previously been

510   isolated from soil, dead leaves and wood and some species (e.g., *M. curvisetosum*) originate

511   from aphids. There is evidence that *Monocillium* spp. are able to antagonise a plant parasitic

512   nematode by colonising their cysts (Ashrafi et al., 2017). *Aureobasidium pullulans* was

513   previously isolated from honey and honey bee guts (Good et al., 2014). The detected

514   *Alternaria alternata* can be an opportunistic fungal pathogen on plants causing leaf spots,

515   rots and blights (Tsuge et al., 2013); however, its function in the bee gut and whether it may

516   be vectored between plants by bees is currently unknown. Further investigations are needed

517   to determine how gut-colonising fungi interact with co-existing bacteria, and the

518   implications for host nutrition and fitness. Nevertheless, the present study is of considerable

519   value and interest in pollinator biology, providing insight into the gut-specific core fungi,

520   and will enable future investigations into the functional role of the core fungal community

521   associated with stingless bees.

522

523   *Distance-decay relationship between stingless bee gut microbiomes and geographic distance*

524   *of bee sampling*

525   Our data showed that microbial biogeographic patterns (a distance-decay relationship) could

526   be applied to stingless bee gut microbiomes on a geographic scale of 250~1,200 km. While

20

527   we predicted decreasing community similarity over distance due to dispersal limitation and

528   local adaptation of stingless bee microbiomes (Soininen et al., 2007; Nemergut et al., 2013),

529   evidence for such a relationship in bee microbiomes was previously lacking. An analysis

530   of relative abundances of *Snodgrassella* and *Gilliamella* across *Bombus* and *Apis* hosts found

531   poor correlation with geography (Koch et al., 2013). Previous studies found limited effects

532   of geographic location on microbiota composition probably due to small sample sizes and/or

533   geographic distances (Kwong et al., 2017). However, the bacterial and fungal distance-decay

534   relationship detected in our study, although significant, explained a small amount

535   (R^2=0.005~0.01) of observed variation, perhaps smaller than those typically observed for

536   plants and other animals. The number of samples, geographic area covered and sequencing

537   depth all could affect the differences, which highlights the need to couple high-throughput

538   sequencing methods with wide geographical coverage.

539

540   *Microbial interactions in stingless bee gut as estimated based on co-existing network*

541   *analyses*

542   The microbial network structure and complexity differed between the two stingless bee

543   species, which can reflect distinct microbial interactions in their gut environments. However,

544   whether these differences result in altered gut functions is not clear. The top five well-

545   connected nodes within the network structures in managed and wild *T. carbonaria* were

546   dramatically different from those identified in *A. australis*. The *T. carbonaria*-associated

547   bacterial taxa, *Bombella*, *Lactobacillus, Acinetobacter* and *Snodgrassella*, have functional

548   effects on nutrient acquisition and defence in honey bees and bumble bees (Kwong and

549   Moran, 2016). In contrast, the potential role of the top five nodes - f_Rhodobacteraceae,

550   f_Burkholderiaceae, g_*Algoriphagus*, g_*Hydrogenophaga* and s_*Terrimonas* sp. - in

551   microbial networks of *A. australis* are not well characterized.

552

553   Our sampling strategy covered large geographic gradients in habitat conditions (*T.*

554   *carbonaria* in particular), allowing for detection of robust co-existing patterns between

21

555   microbes and their geographic locations. We showed that the five modules in the networks

556   correlated with spatial variables, with each coordinate variable (latitude/longitude) being

557   correlated with at least one module, indicating a potential role of geography in the formation

558   of the network structure of the gut microbiome. To some extent, this supports our

559   observation of a significant distance decay of the gut microbiome similarity for stingless

560   bees. Whilst it does not necessarily imply cause, co-existing network analyses provide

561   evidence for the role of location in governing stingless bee microbiome assembly (Faust and

562   Raes, 2012).

563

564   **Conclusions**

565   We characterized the gut microbiomes of two stingless bee species from different genera

566   across 1,200 km thereby spanning large parts of their geographic ranges in eastern Australia.

567   We observed large variance in the microbiome structure between species, which was also

568   influenced by geographic location. Their gut microbial richness correlated to key host

569   morphological traits, namely wing length and area. We have also provided novel evidence of

570   the core fungal and bacterial species present in stingless bees, which appear to be distinct

571   from honey bees, bumble bees and other insects. The link between gut microbiomes and bee

572   fitness traits in *T. carbonaria* provide a novel framework to test functional interactions

573   between bees and gut microbiome, and to develop future solutions to conserve and manage

574   bees for crop pollination.

575

576   **Data availability**

577   The raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA)

578   under Bioproject code PRJNA698658. Datasets generated in this study are available at

579   10.6084/m9.figshare.14559801.

580

581   **Author contributions**

582   HL, BKS, JC, RS, MR and LB conceived the idea; Mark H, Megan H, SN and HL collected

583   wild and managed bee samples along eastern Australia; HL conducted bee gut dissections,

584   bee measurement and DNA extraction, and analysed the sequencing data; Mark H drew

585   Fig.1a and JW conducted the microbial co-existing network and SEM analyses; HL led

586   writing of the manuscript, and all authors contributed to manuscript edits and approved final

587   version for submission.

588

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772 **Table 1 Comparison of core gut microbiome in the three main eusocial bee groups.**

Honey bee (*Apis* spp.) (Kwong et al., 2017)		Bumble bee (*Bombus* spp.) (Kwong et al., 2017)		Stingless bee (*Tetragonula* and *Austroplebeia* spp.) (this study)		
Bacteria	**Fungi**	**Bacteria**	**Fungi**	**Bacteria**	**Fungi**	**Fungal guild**
Snodgrassella		*Snodgrassella*		*Snodgrassella*	Didymellaceae	NA
Gilliamella		*Gilliamella*		Acetobacteraceae	*Monocillium mucidum*	Litter saprotroph/ invertebrate-associated
Lactobacillus Firm-4		*Lactobacillus* Firm-4		*Lactobacillus*	*Malassezia restrita*	soil_saprotroph
Lactobacillus Firm-5	Not well characterized	*Lactobacillus* Firm-5	Not well characterized	*Lactobacillus* sp. Thmro15	*Aureobasiium pullulans*	Sooty mould
Bifidobacterium		*Bifidobacterium*		*Bombella*	*Alternaria alternata*	Plant pathogen
Bartonella apis		*Bartonella apis*			*Malassezia globosa*	Soil saprotroph
Frischella		*Frischella*				
		Bombiscardovia				
		Schmidhempelia				

773

774   **Figure captions**

775   **Fig.1 Sampling locations of stingless bee specimens across their known range in eastern**

776   **Australia (a) and alpha diversity of their gut microbiomes: bacterial communities (b) and**

777   **fungal communities (c)**. Solid black dots in (a) represent sampling locations for *T. carbonaria*

778   while orange dots represent locations for *A. australis*. Green shaded areas represent the

779   predicated range for *T. carbonaria,* while orange shading indicates the predicted east-coast range

780   of *A. australis*.

781

782   **Fig.2 The phylogenetic tree of the stingless bee gut microbiome and core microbiome**

783   **composition.** Phylogenetics and the abundance of the top 255 OTUs of the gut bacterial taxa (a).

784   The colour of each of the phyla was marked in different colours as indicated in the top label. The

785   dominant bacterial genera of *Lactobacillus* and *Acinetobacter* are marked by star and square,

786   respectively. Numbers in brackets represent [1] g_*Snodgrassella* (comprising 16.2% of total

787   taxa), [2] f_Acetobacteraceae (10.3%), [3] f_Acetobacteraceae (9.8%), [4] g_*Lactobacillus*

788   (8.6%) and [5] g_*Lactobacillus* (6.1%). Core microbial species (prevalence >20%) in the gut are

789   shown for bacterial species (b) and fungal species (c), respectively.
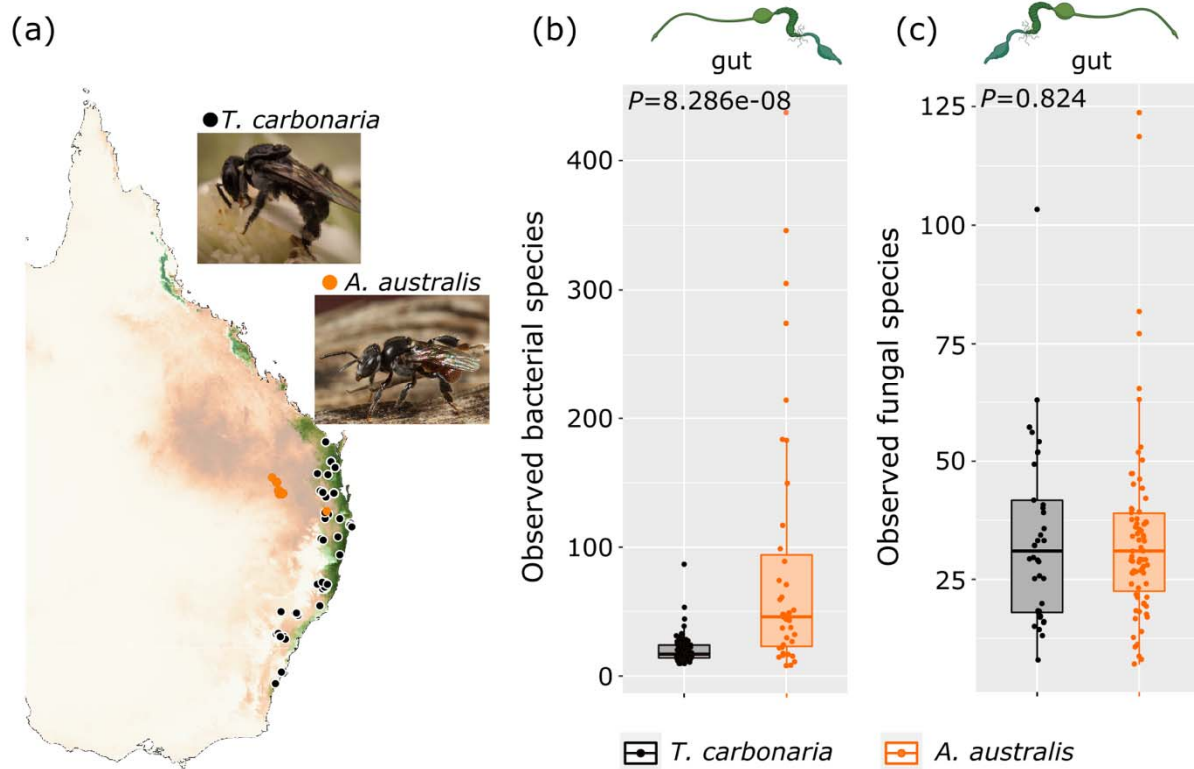
790

791   **Fig.3 Network analyses of stingless bee gut microbiomes**. Network analysis reveals the co-

792   existing patterns among bacterial and fungal taxa and geographic coordinate. Connections

793   represent a strong (Spearman's correlation coefficient $R> 0.6$) and significant ($P< 0.01$)

794   correlations. The size of each node is proportional to the number of connections. Networks

795   shown are for managed *T. carbonaria* (a), wild *T. carbonaria* (b) and wild *A. australis* (c)

796   colonies. Numbers [1]-[10] within each network represent the top 10 most connected

797   bacterial/fungal taxa to other OTUs in the gut microbiome: *T. carbonaria* managed: [1]

798   g_*Bombella*, [2] g_*Zymobacter*, [3] s_*Neophaeomoniella niveniae* (fungi), [4] s_*Alternaria*

799   *alternata* (fungi), [5] g_*Lactobacillus*, [6] g_*Weissella*, [7] s_*Pseudosydowia eucalypti* (fungi),

800   [8] g_*Hormonema* (fungi), [9] s_*Filobasidium oeirense* (fungi) and [10] g_*Gilliamella*; *T.*

801   *carbonaria* wild: [1] s_*T. carbonaria*, [2] g_*Rosenbergiella*, [3] g_*Lactobacillus*, [4]

802   g_*Acinetobacter*, [5] g_*Snodgrassella*, [6] s_*Lactobacillus* sp. Thmro15, [7] s_*Pseudosydowia*

803   *eucalypti* (fungi), [8] s_*Quambalaria cyanescens* (fungi), [9] g_*Zymobacter*, and [10] longitude

804   (geographic). *A. australis*: [1] f_Rhodobacteraceae, [2] f_Burkholderiaceae, [3] g_*Algoriphagus*,

32

805   [4] g_*Hydrogenophaga*, [5] longitude (geographic), [6] s_*Terrimonas* sp. 16-45A, [7]

806   g_*Cyanobium* PCC-6307, [8] g_*Porphyrobacter*, [9] g_*Snodgrassella* and [10] g_*Alishewanella*.

807   Lon: longitude, Lat: latitude.

808

809   **Fig.4 Principal component analyses (PCA) of the gut microbial community composition**

810   **associated with *T. carbonaria* and *A. australis*: bacterial communities (a), and fungal**

811   **communities (b).** Ellipses show the standard error of the mean (SE). Blue arrows represent the

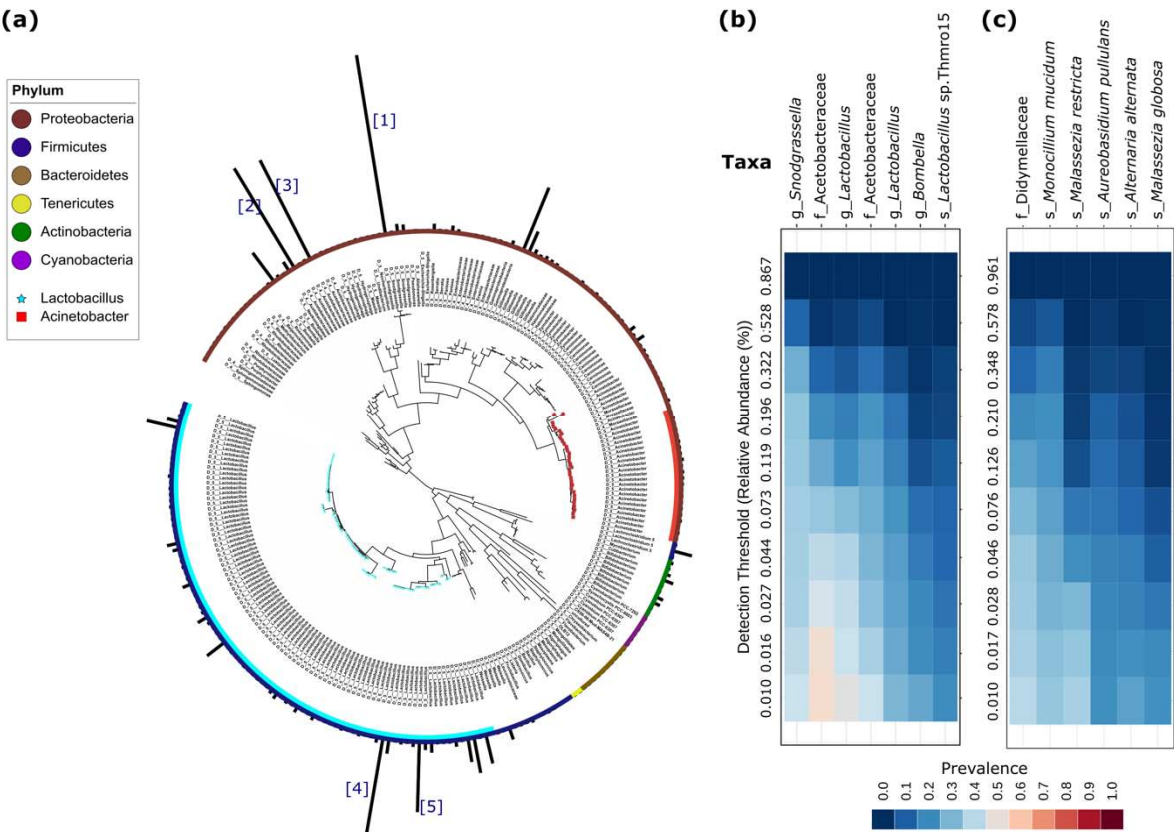812   direction of environmental gradients, with length proportional to strength of the correlation.

813

814   **Fig.5 Linear correlation between gut bacterial species richness in *T. carbonaria* with host**

815   **forewing length (a) and geographical location (b)**. Shown are regression (black line), 95%

816   confidence intervals (shading) and data points (black dots). Wing length analyses shown at log10
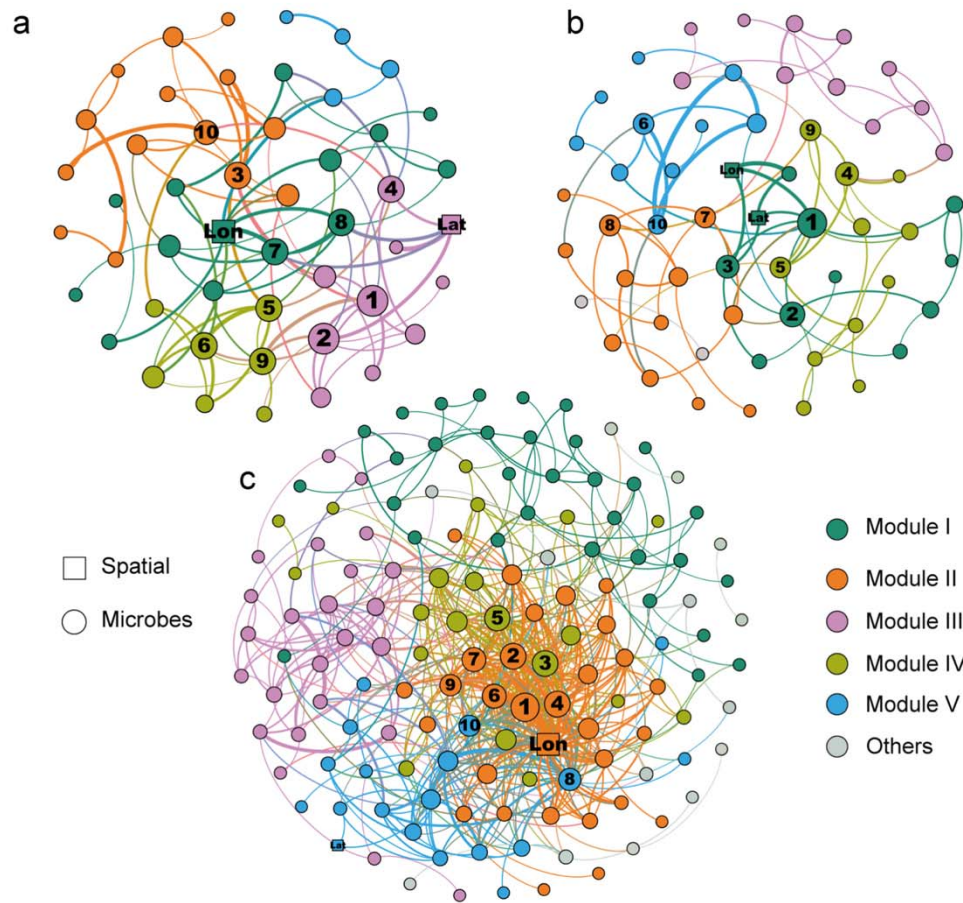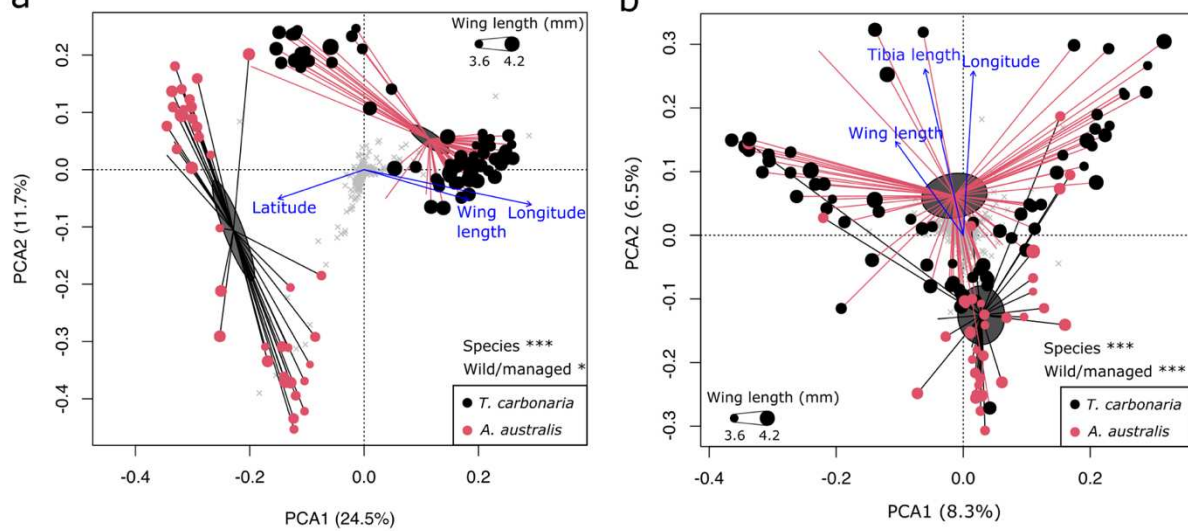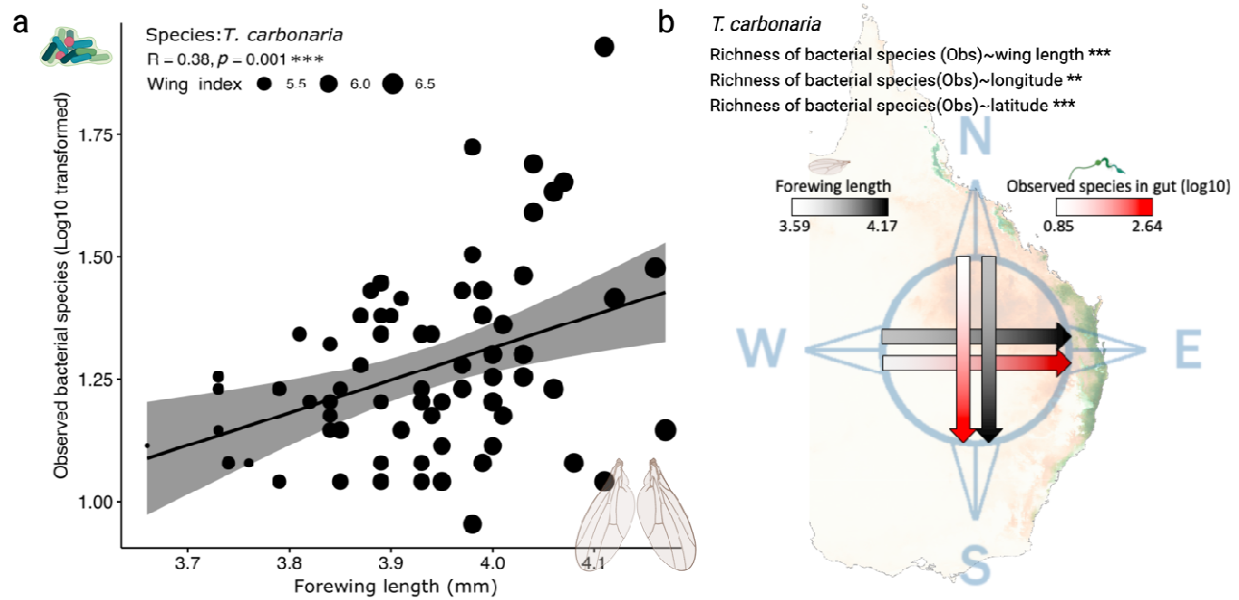
817   scale.

818 **Fig.1**

819

820   **Fig.2**

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822   **Fig.3**

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824 **Fig.4**

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826 **Fig.5**

827