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Diversity and specificity of sap-feeding herbivores and their parasitoids on Australian fig trees

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1 **Diversity and host specificity of *Mycopsylla* species (Hemiptera: Homotomidae) and their**

2 ***Psyllaephagus* parasitoids (Hymenoptera: Encyrtidae) on figs (*Ficus*).**

3

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22 **ABSTRACT**

23 1. The ecology, diversity and parasitoid complex of plant-sap feeding insects of the family
24 Homotomidae (Hemiptera: Psylloidea) specialised on fig trees (*Ficus*) have so far received little
25 research attention. However, they are ecologically important, as occasional outbreaks of the
26 homotomid *Mycopsylla fici* may cause complete defoliation of its host plant, the Moreton Bay
27 fig (*Ficus macrophylla*). *Mycopsylla proxima*, the only other species reported from Australia,
28 feeds on *F. rubiginosa* without any recorded outbreaks.

29 2. We searched for homotomids and their parasitoids on eight *Ficus* species on the east coast of
30 Australia, Lord Howe Island (LHI) and in Auckland, New Zealand, and detected them on three
31 *Ficus* species. Using mitochondrial and nuclear DNA sequences, we delimited three *Mycopsylla*
32 species, including a putative new species on *F. watkinsiana*. We also characterised six
33 (including one previously described) parasitoid species of the genus *Psyllaephagus*
34 (Hymenoptera: Encyrtidae) based on congruent morphological characters and molecular data.

35 3. Each of the homotomid species was highly host-specific to a single fig species, while
36 parasitoid species varied in host-specificity: three host-specific to *M. fici* and three host-
37 generalists. Geographic distribution varied among parasitoid species; e.g. one host-specific
38 species was found on both the mainland and LHI, but a second species only on LHI.

39 4. Our study revealed previously unrecognised diversity in fig homotomids and especially in
40 their parasitoids. The herbivores and parasitoids showed contrasting patterns of host-specificity.
41 Interestingly, *M. fici*, the only outbreak species, had the highest diversity of associated
42 parasitoid species and was the only species with host-specific parasitoids.

43

44

45 **INTRODUCTION**

46 Any given insect species is typically involved in complex interactions with several other
47 species, as part of a food web that characterises feeding interactions through sets of links
48 between species (Pimm *et al.*, 1991). For example, an insect herbivore acts both as a consumer
49 of its host plant(s), and as a host for parasitoids or prey for predators. Correct assessment of host
50 specificity and trophic links first requires accurate delimitation of the species (host plant, its
51 herbivores and their parasitoid species) involved, which may be complicated by the existence of
52 cryptic species. Furthermore, a crucial step in understanding food web structure is the study of
53 the degree of specialisation, i.e. the number of host species, for each species involved. This is of
54 importance for assessment of community dynamics (van Veen *et al.*, 2006) and global species
55 diversity (Mora *et al.*, 2011), as well as for more applied purposes, such as biological control
56 (Stiling & Cornelissen, 2005).

57 Fig trees (*Ficus*, Moraceae) form a large plant genus (Frodin, 2004), comprising approximately
58 750 species worldwide, with the highest diversity (>500 species) in Asia and Australasia
59 (Rønsted *et al.*, 2008). Fig trees may be keystone species (Terborgh, 1986) and Janzen (1979)
60 noted that they support a large diversity of frugivores and other herbivores. Amongst the
61 insects, various flies and beetles, as well as diverse fig wasp lineages, rely on fig fruit resources
62 (Basset *et al.*, 1997). Fig trees are intensively studied for their mutualistic interaction with tiny
63 pollinator wasps (Hymenoptera: Agaonidae), with which they show high reciprocal partner
64 specificity (Cruaud *et al.*, 2012). Despite intense interest in fig – wasp symbiosis, far less
65 research has been undertaken on other insect herbivores feeding on fig trees (Basset *et al.*, 1997;
66 Basset & Novotny, 1999; Novotny *et al.*, 2005). Fig trees are host plants for *Mycopsylla* spp.,
67 sap-sucking insects of the family Homotomidae (Hemiptera: Psylloidea). These homotomids are
68 sometimes referred to as “fig psyllids”, but they do not belong to the family Psyllidae and their
69 diversity and ecology has been far less studied than other families of the Psylloidea superfamily.

70 *Mycopsylla* spp. appear to feed only on *Ficus* and their nymphs produce a sticky covering on the
71 lower surface of fig leaves, a 'lerp', under which they develop (Newman, 2004). The biology of
72 *Mycopsylla* has been relatively little studied and their diversity and host relations are poorly
73 understood. However, the ecological importance of *Mycopsylla fici* (Tryon) cannot be
74 neglected, as it experiences occasional massive population outbreaks as observed in Sydney in
75 1996 (Newman, 2004) and on Lord Howe Island (LHI, volcanic remnant located ~ 600 km off
76 the east coast of Australia) in 2013/2014 (CF, JLD & JMC, pers. obs.). Outbreaks may result in
77 complete defoliation of its host plant, *Ficus macrophylla* (Nicholls, 1939; Newman, 2004),
78 limiting the number of leaves and fruits available to support other animals that feed or shelter on
79 the tree. More generally, several species of the superfamily Psylloidea are known for major
80 outbreaks on various plant species that can result in significant damage to host plants and
81 ecosystems (e.g. *Bactericera cockerelli*, Hill, 1947; *Cardiaspina* sp., Hall *et al.*, 2015;
82 *Cardiaspina fiscella* Gherlenda *et al.*, 2016).

83 In Australia, *Ficus* species diversity is highest in north Queensland and the Northern Territory,
84 but several species are also widespread in southern Queensland and coastal New South Wales,
85 with diversity decreasing southwards. While most areas have several co-occurring fig species,
86 only two *Mycopsylla* species, *M. fici* (Tryon) and *M. proxima* Froggatt*,* have been described in
87 Australia, on *Ficus macrophylla* Desf. ex. Pers. (Moreton Bay fig) and *Ficus rubiginosa* Desf.
88 ex.Vent. (Port Jackson fig), respectively (Froggatt, 1901; Hollis & Broomfield, 1989).
89 *Mycopsylla fici* is found on the two forms of its host *F. macrophylla*: f. *macrophylla* is native to
90 wet forests in Eastern Australia, from the South Coast of New South Wales (NSW) to southern
91 Queensland, while f. *columnaris* is endemic to LHI (Dixon, 2001). Outside their natural
92 distribution, *F. macrophylla* trees have also been planted in numerous parks and gardens across
93 Australia (e.g. in Melbourne and Perth), and overseas, e.g. in Auckland, New Zealand, since the
94 19th century. *Mycopsylla fici* is also present in Auckland, where it was first recorded in 1995
95 (Bain, 2004). The distribution of *F. rubiginosa,* the host of *M. proxima*, overlaps the smaller
96 range of *F. macrophylla*, and is continuous from near Eden in southern NSW to Cape York

97 Peninsula in far north Queensland. In contrast to *M. fici*, *M. proxima* has not been reported as an
98 outbreak species or as causing complete defoliation of its host. In addition to these two
99 Australian species, three *Mycopsylla* species have been described from India (although Newman
100 (2004) suggests that they are only a single species), one from Papua New Guinea, and three
101 from New Caledonia, including one from the Loyalty Islands (Hollis & Broomfield, 1989).

102 Nymphs of Psylloidea species are attacked by various parasitoid wasps and most of these
103 belong to the genera *Psyllaephagus* Ashmead (Hymenoptera: Encyrtidae) and *Tamarixia*
104 Mercet (Hymenoptera: Eulophidae) (Riek, 1962; LaSalle, 1994). Newman (2004) studied the
105 basic biology of *M. fici* during a major outbreak in Sydney in the late 1990s and recorded the
106 presence of *Psyllaephagus* wasps, noting two different size classes of females. These may have
107 represented two different species, but this was not explored further. In fact, there have been no
108 detailed descriptions of any parasitoid species attacking *Mycopsylla* species in Australia.
109 Interestingly, one species (*Psyllaephagus cornwallensis*) attacking *M. fici* has been described
110 from New Zealand, where the host tree and homotomid were introduced (Berry, 2007). It is not
111 yet known if this parasitoid species is native to Australia, although this is highly likely. In
112 Australia, the diversity and host specificity of psyllid parasitoids besides the ones feeding on
113 *Eucalyptus* specialised psyllids (Riek, 1962) are not well described.

114 More extensive sampling is needed to assess the diversity and host specificity of *Mycopsylla*
115 spp. and their associated parasitoids in eastern Australia. In this study, we focussed on a food
116 web that comprises Australian *Ficus* species in the section *Malvanthera*, their homotomids
117 (*Mycopsylla* spp.), and associated parasitoids (*Psyllaephagus*). We addressed three key
118 questions: 1) Are homotomid and parasitoid diversity higher than previously described due to
119 the existence of un-sampled or cryptic species? 2) How host-specific are fig homotomid and
120 parasitoid species? 3) Do closely related parasitoid/homotomid species utilise the same, or
121 closely related, homotomid/*Ficus* species?

122

123 **MATERIALS AND METHODS**

124 **Study species and insect sampling**

125 Multiple fig species are found on the eastern coast of Australia and we searched for homotomids
126 on two dioecious species from the *Ficus* section *Sycidium* (*Ficus coronata* (n = 30-40 trees) ,
127 *Ficus fraseri* (n=14)) and six monoecious species from two *Ficus* sections – *Malvanthera* (*F.*
128 *macrophylla* (n>100), *F. rubiginosa* (n>100), *Ficus obliqua* (n>60) and *F. watkinsiana* (n = 40-
129 50) and *Conosycea* (*Ficus microcarpa* (n>100), *Ficus benjamina* (n>100)). In Australia,
130 *Mycopsylla* spp. have previously only been recorded from the two malvantheran fig species, *F.*
131 *macrophylla* and *F. rubiginosa.* Multiple collections were made between March 2013 and
132 December 2014 to sample fig homotomids and parasitoids along the eastern coast of NSW and
133 Queensland from Wollongong to Brisbane, as well as in Melbourne (Victoria), on LHI and in
134 Auckland, New Zealand (Fig. 1 and Table S1 in Supporting Information).

135 Infested leaves were only found for three *Ficus* species. Leaves with lerps (solidified excretions
136 by the nymphs forming a sticky protective covering, Newman, 2004) were collected from
137 multiple branches of infested *F. macrophylla, F. rubiginosa* and *F. watkinsiana* trees and kept
138 in Petri dishes at ambient room temperature (~20° C) until adult insects (homotomids and
139 parasitoids) emerged. In addition, homotomid nymphs were collected directly from lerps soon
140 after field sampling. Specimens were preserved in absolute ethanol and stored at -18°C until
141 DNA extraction. For the analysis, we then chose 36 homotomids (23 individuals from *F.*
142 *macrophylla*, 11 from *F. rubiginosa* and 2 from *F. watkinsiana*) and 128 parasitoids (95 from
143 homotomids on *F. macrophylla*, 31 from homotomids on *F. rubiginosa* and 2 individuals from
144 homotomids on *F. watkinsiana*), representing the morphological, host and geographic diversity
145 of the adult specimens collected (Fig. 1). However, only homotomid nymphs were collected
146 from *F. watkinsiana*.

147

148 **Morphometric measurements**

149 We first grouped all homotomids and parasitoids into distinct morphotypes, with *Psyllaephagus*
150 morphotypes based on the descriptions by Froggatt (1901) and Hollis and Bromfield (1989). In
151 addition, we measured and assessed several parasitoid morphological traits, following Noyes &
152 Hanson (1996) and Berry (2007). Prior to molecular analysis, all parasitoids were photographed
153 using a stereomicroscope and the INFINITY ANALYZE software (Lumenera corp., Ottawa,
154 ON). Body and antenna lengths were measured for male and female parasitoids. Ovipositor
155 sheath length was measured for females, and antennal morphology was recorded for males and
156 females. We compared the measured traits (i.e. body length, sheath length:body length and
157 antenna length:body length) between species, using a Kruskal-Wallis test with the Benjamini
158 and Hochberg (1995) correction and multiple comparison of treatments as implemented in the R
159 package 'agricolae' (De Mendiburu, 2014; R Development Core Team, 2014).

160

161 **DNA extraction and sequencing**

162 DNA was extracted from the entire body of individual homotomids and parasitoids using a
163 Chelex method (Walsh *et al.*, 1991). Individuals were placed into 100 µL homogenization
164 solution (5% Chelex, 0.01% proteinase K), crushed with a pestle, incubated at 56 °C for 35 min
165 then at 96 °C for 15 min and centrifuged for 5 min at 13,000 rpm.

166 We sequenced three homotomid gene fragments – mitochondrial *Cytochrome Oxidase 1* (*COI*),
167 and nuclear *Histone 3* (*Hist3*) and *Elongation Factor 1α* (*EF1α*). For the parasitoids, we
168 sequenced two mitochondrial (*cytochrome b* and 16S rDNA) and one nuclear (D2 region of the
169 28S rDNA) gene fragments (Table S2 in Supporting Information).

170 PCR for *COI* was performed in a total volume of 25 µL containing 1x buffer, 3 mM of $MgCl_2$,
171 0.1 mM of dNTPs, 0.5 µM of each primer, 1 unit of *Taq* DNA (Promega, Madison, WI) and 1
172 µL of genomic DNA (Table S3 in Supporting Information). PCR for the other genes (i.e. *EF1α*,
173 *Hist3*, *cytb*, 16S rDNA and 28S rDNA) followed the same general protocol as *COI* with the
174 exception of $MgCl_2$ concentration and PCR amplification conditions that differed between

175 genes (Table S3 in Supporting Information). PCR fragments were sequenced directly in one
176 direction at Macrogen (Korea) using BigDye Terminator v.3.1. The sequence data (homotomid
177 *COI*, *EF1α*, *Hist3* and parasitoid *cytb*, 16S rDNA, 28S rDNA) sequences were deposited in
178 GenBank under accession numbers KT273227-KT273238 and KU522537-KU522595 and
179 aligned sequence are archived at http://doi.org/10.4225/35/57a95a900f19a.

180

181 **Phylogenetic analyses**

182 Sequences for each locus were aligned using the Muscle alignment tool in Geneious 6.1.7.
183 Alignment of the protein-coding genes was checked by translating the sequences into amino
184 acids using MEGA v 6.06 (Tamura *et al.*, 2013). No evidence for the presence of pseudogenes
185 (i.e. no stop codons or frameshifts) was detected. We used the nucleotide substitution model
186 selected by JModelTest2 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012), based on the
187 Bayesian Information Criterion (BIC). When needed the shape parameter of the Gamma
188 distribution (G) and the proportion of invariant sites (I) were estimated in MEGA.

189 Sequence data of each gene were analysed using Maximum Likelihood (ML) in MEGA v 6.06.
190 ML branch support was tested with 1,000 bootstrap pseudo-replicates. Nodes with bootstrap
191 values >70% were considered supported, and those with a value >90% well-supported.
192 Sequence data were also analysed using Bayesian Inference (BI) in MrBayes v 3.2.2 (Ronquist
193 & Huelsenbeck, 2003; Ronquist *et al.*, 2012). Two runs of four Monte Carlo Markov Chain
194 (MCMC) chains (3 "heated" and 1 "cold") were run in parallel in MrBayes for $2x10^6$
195 generations and sampled every 5,000 generations. Tracer v1.6 (Drummond *et al.*, 2012), as well
196 as the standard deviation of split frequencies, were used to assess stationarity of the Markov
197 chains.

198

199 **Species delimitation using *COI* for *Mycopsylla* and *cytb* for the parasitoids**

200 We explored species boundaries and delimited species using a range of common approaches:

201 *Statistical parsimony*

202 This method partitions the data into independent networks that link haplotypes using statistical
203 parsimony based on a 95% confidence interval (Templeton *et al.*, 1992) and this can be seen as
204 an initial step to visualize likely species boundaries. We used TCS v1.21 (Clement *et al.*, 2000)
205 and POPART (Leigh & Bryant, 2015) to perform a statistical parsimony analysis on homotomid
206 *COI* data and parasitoid *cytb* data.

207 *Barcoding gap*

208 The 'barcoding gap' is a discontinuity between pairwise mtDNA distances of conspecific and
209 heterospecific individuals (Hebert *et al.*, 2003; Čandek & Kuntner, 2015) that often allows
210 simple visual detection of species boundaries. We used the Kimura 2 parameter (K2P) distance
211 model (Kimura, 1980) to calculate genetic distances in MEGA v 6.06. While the use of K2P has
212 been questioned (Srivathsan & Meier, 2012), it is widely adopted and facilitates comparison
213 with other studies. TaxonDNA (Meier *et al.*, 2006) was then used to cluster mtDNA sequences
214 using the observed barcoding gap.

215 *Generalized Mixed Yule Coalescent (GMYC) model*

216 A GMYC model is a common statistical approach to single-locus species delimitation. It is
217 based on the differentiation of branching rates resulting from a speciation process (Yule pure-
218 birth model) from those resulting from an intra-specific process (neutral coalescent model)
219 (Pons *et al.*, 2006). The number of species present in the dataset was determined using *COI* and
220 *cytb* data with the single threshold method in the package 'splits' (Ezard *et al.*, 2009) in R
221 v3.1.0. This requires ultrametric trees, which were generated using Beast v1.8.0 (Drummond &
222 Rambaut, 2007; Drummond *et al.*, 2012). Based on comparison of the Ln likelihood generated
223 by DNAml and DNAmlk implemented in Phylip v3.6 (Felsenstein, 1989), a strict clock model
224 was applied. A coalescent prior set to a constant population size was used, as it is thought to be

225 more conservative than a Yule prior (Monaghan *et al.*, 2009). All other priors for the model
226 parameters were kept as default values. The MCMC chain was run in Beast for 10 million
227 generations and sampled every 1000 generations. Tracer v1.6 was used to visualize the
228 estimated sample size and stationarity of the parameters.

229

230 **RESULTS**

231 Adults *Mycopsylla* collected from *F. macrophylla* and *F. rubiginosa* grouped into two distinct
232 morphospecies, as described in Hollis and Broomfield (1989) and Froggatt (Froggatt, 1901).
233 Only nymphs of *Mycopsylla* were collected from *F. watkinsiana*. Parasitoids from all three
234 *Mycopsylla* species were grouped into four morphotypes, although the delimitation was clearer
235 for the males, due to variation in their antennal morphologies, than for the females (see
236 parasitoid morphology section). Based on the identification keys they all appeared to belong to
237 the genus *Psyllaephagus* (Riek, 1962; Noyes & Hanson, 1996; Berry, 2007).

238

239 ***Mycopsylla* phylogenies**

240 After trimming of incomplete ends, 414 nucleotides of *COI*, 279 of *EF1α* and 285 of *Hist3* were
241 kept for analysis. Across the 36 homotomid individuals used for *COI*, 16 haplotypes with 68
242 polymorphic sites were found. For *EF1α* and *Hist3*, 19 and 23 individuals were sequenced and
243 we found 3 alleles with 2 polymorphic sites, and 2 alleles with only one polymorphic site,
244 respectively.

245 JModelTest2 indicated that the best models were HKY+I for *COI*, and JC for *Hist3* and *EF1α*.
246 Mitochondrial ML and BI phylogenies showed the same topology (Fig. 2), with three highly
247 supported clades (SI, SII, SIII). The nuclear genes were highly conserved, but the limited
248 variation was congruent with the mtDNA clade structure. One fixed synonymous nucleotide
249 substitution differentiated *Hist3* sequences of individuals collected on *F. macrophylla* from

250 those collected on *F. rubiginosa* and *F. watkinsiana*. For *EF1α*, two nucleotide positions varied
251 between clades. One synonymous substitution allowed differentiation of *Mycopsylla* collected
252 from *F. macrophylla* from those collected from *F. rubiginosa* and *F. watkinsiana*, while another
253 allowed differentiation of *Mycopsylla* collected from *F. watkinsiana* from those collected from
254 *F. macrophylla and F. rubiginosa* (Fig. 2). Only two species of *Mycopsylla* have been described
255 previously in Australia: *M. fici* from *F. macrophylla* and *M. proxima* from *F. rubiginosa*. No
256 species has been previously described from *F. watkinsiana* and our data support a putative new
257 *Mycopsylla* species (referred to as *Mycopsylla* sp.) on this host plant.

258

259 ***Mycopsylla* species delimitation using *COI* sequences**

260 *Statistical parsimony*

261 Eight steps (base differences), corresponding to the 95% cut-off, were set as the connection
262 limit between haplotypes. We distinguished three independent networks for the *COI* data for
263 *Mycopsylla* collected on *F. macrophylla*, *F. rubiginosa* and *F. watkinsiana*, respectively, with
264 16 haplotypes of which 10 were present with one individual only (Fig. S1 in Supporting
265 Information).

266 *Barcoding gap*

267 Genetic differences between pairs of individuals varied from 0% to 12.6% for *COI*. For *COI* the
268 barcoding gap occurred between 2.2% (i.e. maximum intraspecific variation) and 5.8% (i.e.
269 minimum interspecific divergence) (Fig. S2). It led to the delimitation of three species, i.e. *M.*
270 *fici, M. proxima* and *Mycopsylla* sp. from *F. watkinsiana* (Table 1). Intraspecific divergences
271 ranged from 5.8-6.5% between *Mycopsylla* sp. and *M. proxima* to 11.4-12.6% between *M.*
272 *proxima* and *M. fici* (Table 1).

273 *GMYC*

274 The GMYC model that assigned individuals into five clusters was preferred over the null model
275 of uniform branching rate, i.e. assuming one species (GMYC maximum likelihood= 273.9, null
276 model likelihood= 270.8, *p*=0.04). The five clusters were 1) *M. fici* from the Australian
277 mainland and New Zealand, 2) *M. fici* from *F. macrophylla* from LHI, 3) *M. proxima* from *F.*
278 *rubiginosa* from Sydney, 4) *M. proxima* from *F. rubiginosa* from Northern NSW and 5)
279 *Mycopsylla* sp. from *F. watkinsiana*.

280 In summary, two analyses (statistical parsimony and barcoding gap) using mtDNA sequences
281 and nuclear sequences both recognized three homotomid species corresponding strictly to the
282 three different fig species. However, GMYC further split: a) homotomids from *F. macrophylla*
283 into mainland/New Zealand and LHI populations; and b) homotomids from *F. rubiginosa* into
284 Sydney and northern NSW populations.

285

286 ***Psyllaephagus* phylogenies**

287 After trimming for incomplete ends 367, 190 and 290 nucleotides of the mitochondrial *cytb* and
288 16S rDNA, and nuclear 28S rDNA sequences, respectively, were kept for analysis. Across the
289 128 individuals tested, the *cytb* sequences displayed 147 polymorphic sites and 31 haplotypes
290 were found. The 16S rDNA sequences had 71 polymorphic sites across 19 haplotypes for the 39
291 individuals sequenced. The 28S rDNA sequences displayed 37 polymorphic sites across seven
292 alleles in the 33 individuals sequenced. *Cytb* had the highest polymorphism (π= 0.17), followed
293 by 16S rDNA (π= 0.11) and the much less variable nuclear 28S rDNA (π= 0.04).

294 JModelTest2 indicated that mitochondrial *cytb* and 16S rDNA followed a HKY+G model, and
295 the nuclear 28S rDNA a K80+G model. ML and BI phylogenies showed the same topology for
296 each gene. Although the phylogenies differed across the three genes, they did not conflict with
297 each other in terms of clade membership, but represented different levels of resolution likely
298 reflecting the difference in mutation rates of the genes used. When using nuclear 28S rDNA to
299 build the phylogeny, only four clades, each with high support, were observed (Fig. 3B). In

300 contrast, *cytb* phylogenies split one 28S rDNA clade into three highly supported sub-clades,
301 PIV, PV and PVI (Fig. 3A); these clades were also supported by 16S rDNA presented in
302 supporting information (Fig. S3). However, we observed some conflicts in terms of tree
303 topology (Fig. 3), e.g. PII and PIII are sister clades when using 28S and 16S rDNA, but PII and
304 PI are sister clades when using *cytb*. Individuals collected in New Zealand clustered with clade
305 PIV, suggesting that clade PIV is *P. cornwallensis*.

306

307 ***Psyllaephagus* species delimitation using *cytb* sequences**

308 *Statistical parsimony*

309 Six independent networks were found using *cytb* sequences. Eight steps (base differences),
310 corresponding to the 95% cut-off, were set as the connection limit between haplotypes. Only
311 one network comprised a single haplotype, which grouped just five individuals (corresponding
312 to PVI). The six networks corresponded to the same groups (PI, PII, PIII, PIV, PV, PVI)
313 delineated with the phylogenetic tree (Fig. S4 in Supporting Information).

314 *Barcoding gap*

315 Genetic differences between pairs of individuals varied from 0% to 27.2% and the barcoding
316 gap occurred between 1.7% (i.e. maximum intraspecific divergence) and 6.7% (i.e. minimum
317 interspecific divergence). Using TaxonDNA and the previously found threshold percentages, six
318 species were delimited (Fig. S2 in Supporting Information). Interspecific divergence of *cytb*
319 ranged from 6.7-7.6% between PV and PIV to 26.9-27.2% between PI and PV (Table 1).

320 *GMYC*

321 The GMYC model that assigned individuals into six clusters was preferred over the null model
322 of uniform branching rate (GMYC maximum likelihood= 719.9, null model likelihood= 694.2,
323 $p<0.001$). This means that all the clades delimited in the *cytb* tree constitute distinct species
324 according to the GMYC method.

325 *Cytb* sequences suggested the existence of six parasitoid species, regardless of the delimitation
326 method used. In contrast, when using 28S rDNA sequences, only four groups were evident.
327 Clades PIV, PV and PVI were grouped together, separately from clades PI, PII and PIII.
328 Interestingly, Clade PVI grouped individuals collected on *F. rubiginosa* and *F. watkinsiana* on
329 the mainland of Australia while clade PV contained individuals found on *F. macrophylla* on
330 LHI. Clade PIV grouped individuals found on *F. macrophylla* on the mainland of Australia and
331 in New Zealand. However, one individual collected on LHI was also found in this clade.
332 *Psyllaephagus* sp. PI was a specialist of *M. fici* found on both the mainland and LHI, while PII
333 and PIII were host generalists. We concluded that these six taxa are most likely all different
334 species, varying in host specificity. Within species, there was no obvious geographic sub-
335 structure.

336

337 **Parasitoid morphology**

338 All species had characteristics of the genus *Psyllaephagus* as described in Noyes & Hanson
339 (1996) and Berry (2007). Female body size differed significantly between some species
340 (Kruskal-Wallis, χ^2= 43.4, p= 3.08e^{-8}), with females of species PIV, PV and PVI being larger
341 than those from species PI, PII and PIII (Fig. 4). The same was true for the ovipositor sheath to
342 body length ratio, which was higher in PIV, PV and PVI than in PI, PII and PIII. In addition, PII
343 had a higher ratio than PI and PIII, and PIV a higher ratio than PVI (Kruskal-Wallis, χ^2= 60.9,
344 p= 7.8e^{-12}). The shape of the antennal scape also differed between species; females of species
345 PII, PIV, PV and PVI have an expanded scape while those from PI and PIII have a narrower,
346 only slightly expanded scape (Fig. S5 in Supporting Information). Male body size also differed
347 between species (Kruskal-Wallis, χ^2= 27.3, p= 5.06e^{-5}), with species PIV bigger than species PI,
348 PII and PIII (Fig. 4). Males of PV and PVI were not significantly different in size to males of
349 the other species. The ratio of antenna to body length ratio did not differ between species in
350 males or females. Male antennae also differed in form between some species (Fig. S5 in

351 Supporting Information). Species PII, PIV, PV and PVI had filiform antennae without hairs,
352 whereas species PI had filiform antennae but the flagellum was covered with hairs. Species PIII
353 had flagellate antennae.

354

355 **Host-specificity of homotomids and parasitoids**

356 Each of the three *Mycopsylla* species appeared completely host-specific to one fig species (Fig.
357 2). Given this, we assumed that parasitoids collected from one *Ficus* species developed in the
358 appropriate host-specific *Mycopsylla* species. Parasitoid species showed different levels of host
359 specificity (Fig. 3), with three species (PI, PIV and PV) highly host specific to *M. fici* (on *F.*
360 *macrophylla*), while the other three were polyphagous (PII attacked *M. fici* and *M. proxima*, PIII
361 attacked all three *Mycopsylla* species, and PVI attacked *M. proxima* and *Mycopsylla* sp.) (Fig.
362 5). However, we were only able to sample a few individuals belonging to *Mycopsylla* sp. from
363 *F. watkinsiana* and additional sampling may yield further information on its associated
364 parasitoids (e.g. PII). In our sampling, only *M. fici* had host-specific associated parasitoids (Fig.
365 3).

366

367 **<u>DISCUSSION</u>**

368 We characterised three *Mycopsylla* species from three *Ficus* species by using genetic
369 approaches and extensive field surveys of eight *Ficus* species in Australia and New Zealand.
370 One of the three *Mycopsylla* species is a new undescribed species from *F. watkinsiana*.
371 Furthermore, we characterised six parasitoid species of the genus *Psyllaephagus*, including five
372 new species (Froggatt, 1901; Newman, 2004), that attack the *Mycopsylla* species. The three
373 *Mycopsylla* species appeared highly host-specific, but host specificity patterns were more
374 complex for *Psyllaephagus*, which included both specialists and generalists. Interestingly, only
375 *M. fici* appeared to support specialist parasitoid species.

376

377 *Higher species diversity than previously described*

378 The new *Mycopsylla* sp. on *F. watkinsiana* showed 5.8-6.5% divergence in *COI* sequence from
379 the closest species, *M. proxima.* Percy (2003) found that intraspecific mitochondrial divergence
380 varied between 1 and 10% for psyllid species collected on different islands, but was restricted to
381 an upper limit of 3% for continental species. Taylor et al. (2016) identified a 5-6% divergence
382 as the threshold that best matched morphological and ecological characteristics for their triozid
383 species delimitation. In addition, we found one fixed synonymous nucleotide difference
384 between *EF1α* sequences between *Mycopsylla* sp. and *M. proxima*. Overall, these molecular
385 data suggest a new *Mycopsylla* species, but as only nymphs were found, description of adult
386 morphology was not possible.

387 Previously, only one *Psyllaephagus* species (*P. cornwallensis,* here *Psyllaephagus* sp. IV)
388 associated with *M. fici* has been described and this was from New Zealand - outside the native
389 range of its host (Berry, 2007). We also collected this species in Australia. Our molecular
390 delimitation of parasitoid species supports the existence of at least four species using the slow-
391 evolving nuclear 28S rDNA data, but more likely the six species suggested by using the faster
392 evolving mitochondrial *cytb* data sequences (Lin & Danforth, 2004). Other studies such as the
393 ones on the pollinator wasp species on *F. rubiginosa* found a similar situation with additional
394 species discovered based on cytb relative to 28S sequences. However, the status of these
395 additional species was then further supported by nuclear microsatellite markers (Haine et al.,
396 2006, Darwell et al., 2014). Interestingly, while *Psyllaephagus* sp. PIV, PV, PVI, shared very
397 similar features in terms of size, sheath length and antennal morphology, they were collected on
398 two different land masses (PV/PIV) or from different hosts (PV-PIV/PVI). In addition, their
399 mitochondrial sequences were at least 6% different. The lack of differentiation in nuclear DNA
400 suggests relatively recent divergence, but it is possible that PIV and PV are strongly diverged
401 populations of a single species, as observed for their host species *M. fici*.

402

403 *Variable host-specificity across food web*

404 We found different levels of host-specificity across the fig *Mycopsylla* food web (Fig. 5). Here,
405 we established that the herbivore species were highly host specific while their associated
406 parasitoid species had various degrees of specialisation. Interestingly, host specificity reflects
407 host availability; host tree species that occur at high densities are common and may therefore be
408 a relatively stable resource for homotomids (and parasitoids) while trees with lower species
409 abundance may be considered as a fluctuating resource for homotomids (and parasitoids).

410 *Mycopsylla fici* and *M. proxima* appeared highly host specific to *F. macrophylla* and *F.*
411 *rubiginosa*, respectively. With only a few *Mycopsylla* individuals collected from *F.*
412 *watkinsiana*, it is difficult to draw strong conclusions, but, given the high host specificity of *M.*
413 *fici* and *M. proxima,* and the absence of homotomids from other *Ficus* species we surveyed, it
414 seems likely that this putative new *Mycopsylla* sp. is specific to *F. watkinsiana*. Far more
415 studies are available for insects from other families within Psylloidea and most of these
416 described psyllids as highly host specific at the tree species level (Hodkinson, 2009; Burckhardt
417 *et al.*, 2014; Ouvrard *et al.*, 2015). In addition, closely related psyllid species tend to develop on
418 closely related plant species (Hollis & Broomfield, 1989), as we found with Australian
419 *Mycopsylla* species feeding only on *Ficus* species belonging to section *Malvanthera*.

420 The *Psyllaephagus* species detected in our study had different levels of host specificity. Three
421 were highly host-specific to *M. fici* (two on the mainland and one on LHI), but none was
422 specific to *M. proxima* or *Mycopsylla* sp. from *F. watkinsiana*. Generalist species attacked *M.*
423 *fici* and *M. proxima* (PII) or all three *Mycopsylla* species (PIII). Nonetheless, more extensive
424 sampling of *Mycopsylla* sp. may lead to the discovery of new parasitoid species that could be
425 host-specific. The fact that only *M. fici* is currently known to have host-specific parasitoids may
426 again reflect host availability, with high abundance of *M. fici* and much lower abundance for the
427 other *Mycopsylla* species. This is consistent with the 'resource fragmentation hypothesis'

428 (Janzen, 1981), which suggests that rare host species tend to not support specialist parasitoid
429 species. Indeed, other studies have found that the number of specialist parasitoid species is
430 positively correlated with host density (e.g. Dawah *et al.*, 1995). *Mycopsylla fici* lerps are
431 usually much bigger (up to 30 individuals in a lerp), and in higher abundance within and
432 between trees (pers. obs.), than those of *M. proxima* (rarely more than two individuals per lerp).
433 In addition, major outbreaks of homotomids have only been reported for *M. fici*.

434 Another interesting point is the absence of generalist parasitoids on LHI. This raises multiple
435 questions regarding the host preferences and dispersal abilities of the generalist *Psyllaephagus*
436 species. It could also indicate that *Psyllaephagus* PI, PIV and PV are better competitors than the
437 other generalist species. When *M. fici* outbreaks occur, host resources may be abundant enough
438 for all parasitoid species to coexist on this host. However, between outbreaks, populations of *M.*
439 *fici* are far smaller and, extrinsic and intrinsic competition between parasitoid species may be
440 intense (Harvey *et al.*, 2013) and favour the stronger competitors (see for instance Patil *et al.*,
441 1994; Feng *et al.*, 2015). On the mainland, other *Mycopsylla* species may provide refuges for
442 populations of the weaker competitors amongst generalist parasitoid species. However, they
443 lack alternative hosts on LHI so may be driven to extinction by specialists when hosts are rare
444 and competition is intense (Paranhos *et al.*, 2013).

445 One important aspect that we were unable to investigate here is whether some of the parasitoid
446 species are hyperparasitoids. Hyperparasitoids appear common in systems where the primary
447 hosts are hemipteran (e.g. in aphids - Muller *et al.*, 1999) and this will be an interesting topic for
448 further investigation. It could also explain the restriction of some species (e.g. PII or PIII) to the
449 mainland if they attack parasitoid species also present only on the mainland.

450

451 Mycopsylla *and* Psyllaephagus *phylogeography varies across species*

452 Our study focused primarily on establishing the number of species of homotomids and
453 parasitoids and patterns of host specificity. However, our sequence data also provided some

454 interesting preliminary phylogeographic insights. *Mycopsylla fici* clustered into two well-
455 supported clades on the Australian mainland/New Zealand and on LHI, suggesting that the LHI
456 population may be genetically discrete. While within-species phylogeographic patterns were
457 recovered for *M. fici*, among-species phylogeographic patterns can be discussed for the
458 parasitoid species. Interestingly, the genetically close and morphologically similar parasitoids
459 PIV and PV, both host specific to *M. fici*, show different distribution patterns, with PIV mainly
460 on the mainland and PV only recorded on LHI. On the other hand, PI, also host specific to *M.*
461 *fici*, was collected repeatedly on both mainland and LHI. This may suggest more recent or
462 ongoing exchange of some parasitoid species between LHI and the mainland without any
463 mixing of *M. fici*. Surprisingly, only one individual from LHI was found in clade PIV. This
464 could indicate occasional dispersal between island and mainland. These observations are
465 interesting as they imply that different parasitoid species attacking the same host may have
466 different dispersal abilities. These preliminary observations should be followed up with targeted
467 population genetic studies of focal species within this system.

468

469 *Conclusions*

470 In this study, our data support a putative new species of *Mycopsylla* homotomids and five new
471 species of *Psyllaephagus* parasitoids associated with *Ficus* species in Australia. Revealing
472 unrecognised species diversity is a crucial step towards understanding species interactions and
473 food webs, and may be of particular importance for parasitoids, for which diversity is often
474 underestimated due to the existence of numerous cryptic species. In addition, sampling a host-
475 parasitoid system across the geographic range of the host plant can provide insights into the
476 different phylogeographic patterns of interacting species, their relative dispersal abilities and
477 how geographic barriers may impact species in various ways.

478

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488

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661

662

663 Table 1: Percentage of mitochondrial pairwise divergence for A) *COI* of *Mycopsylla* and B) *cytb* of *Psyllaephagus*. All codons were used for the analysis for a
664 total of 414 and 357 bases for the homotomid *COI* and parasitoid *cytb*, respectively. In bold, intra-specific divergence. The percentages presented in the table
665 are the minimal and maximal values of pairwise divergences between species.

666

A)	*M. fici*_mainland	*M. fici*_LHI	*Mycospylla* sp.	*M. proxima*
*M. fici*_mainland	**1.2**			
*M. fici*_LHI	**1.5-2.2**	**0**		
Mycospylla sp.	9.9-10.7	10.2-10.4	**0.2**	
M. proxima	11.4-12.6	11.6-12.1	5.8-6.5	**0.2-1.2**

667

B)	PI	PII	PIII	PIV	PV	PVI
PI	**0**					
PII	22.7-23	**0.6**				
PIII	21.8-23.2	21.6-22.1	**1.7**			
PIV	24.4-24.6	22.7-23.5	22.1-23.5	**0.8**		
PV	26.9-27.2	22.7-23.2	21.8-23.2	6.7-7.6	**0.3**	
PVI	26.1	23.8-24.1	22.4-23.2	11.2-11.8	12.9-13.2	**0**

668

669

670 Figure 1 : Maps of the *Mycopsylla* and *Psyllaephagus* collections in Australia and New Zealand.
671 Both *Mycopsylla* and *Psyllaephagus* were collected in LHI (light green square) and Auckland
672 (black diamond) while only *Mycopsylla* were collected in Melbourne (yellow circle). Maps A)
673 and B) represent the collections of *Mycopsylla* (circle) and *Psyllaephagus* (square), respectively,
674 made in Australia. Colours correspond to the tree species from which collections were made
675 (blue from *F. macrophylla*, green from *F. rubiginosa* and red from *F. watkinsiana*). The
676 intensity of the colours corresponds to the sampling effort - the darker the colour, the higher the
677 number of insects collected. On the left side, the yellow to red scale corresponds to the colour
678 used for the phylogenetic trees. Brunswick H. is Brunswick Heads and Coffs H. is Coffs
679 Harbour. Scales are in km.

680

681 Figure 2: 50% majority rule consensus tree constructed using BI of *Mycopsylla COI* sequences.
682 The colour of the tip name corresponds to the host tree of collected *Mycopsylla*, blue: *F.*
683 *macrophylla*, green: *F. rubiginosa* and red: *F. watkinsiana*. The colour of the circle in front of
684 the tip name corresponds to the location where the homotomid was collected. The gradient S/N
685 on the mainland of Australia is represented by a gradient from yellow to red, LHI individuals
686 are represented by light green squares and Auckland individuals by black diamonds. Black
687 triangles and circles indicate a nucleotide change between *EF1α* and *Hist3* sequences,
688 respectively, of the three species. Topologies of ML and BI were identical. Numbers at the
689 nodes are posterior probabilities from BI analysis (lower number) and ML bootstrap values
690 (upper number), estimated from 1000 bootstrap replicates. Scale represents the number of
691 substitutions per site.

692

693 Figure 3: 50% majority rule consensus tree constructed using BI of A) *cytb* sequences and B)
694 28S rDNA for *Psyllaephagus*. The colour of the tip name corresponds to the host tree of
695 collected *Psyllaephagus*, blue: *F. macrophylla*, green: *F. rubiginosa* and red: *F. watkinsiana*.
696 The colour of the circle in front of the tip name corresponds to the location where the parasitoid
697 was collected. The gradient S/N on the mainland of Australia is represented by a gradient from
698 yellow to red, LHI individuals are represented by light green squares and Auckland individuals
699 by black diamonds. PIV is *P. cornwallensis.* Topologies of ML and BI were identical. Numbers
700 at the nodes are posterior probabilities from BI analysis (lower number) and ML bootstrap
701 values (upper number), estimated from 1000 bootstrap replicates. Scale represents the number
702 of substitutions per site.

703

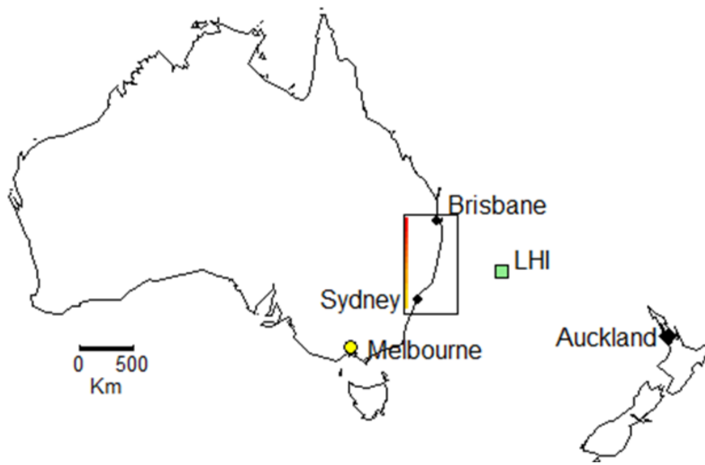
704 Figure 4: Comparison of the length of body (A) and ratio ovipositor sheath/body length (B) of
705 female and size of body (C) of male of different *Psyllaephagus* species. The letters correspond
706 to the result of the post-hoc test: species with the same letter are not statistically different.

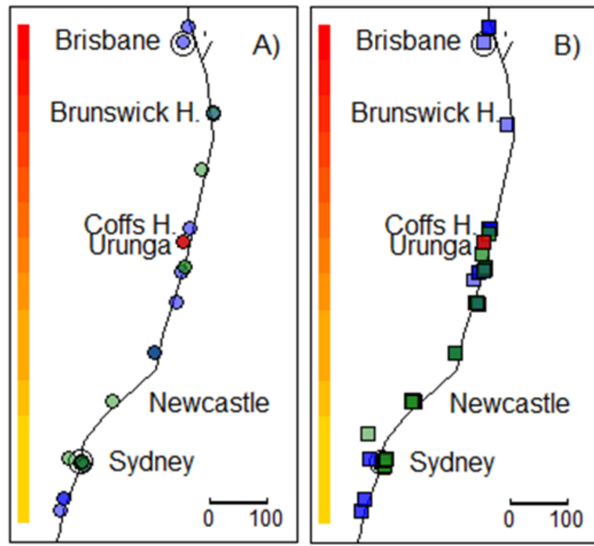
707

708 Figure 5: Food web of the *Mycopsylla* homotomids and their associated *Psyllaephagus*
709 parasitoids found on three species of fig trees. The lower level corresponds to the tree species
710 the insects were collected from. The second level corresponds to the *Mycopsylla* spp. collected,
711 *M. fici* collected on the mainland of Australia and on LHI are separate as they have different
712 parasitoid species attacking them. The third level corresponds to *Psyllaephagus* that emerged
713 from the different *Mycopsylla* spp. The boxes are coloured according to the specialisation of the
714 insect species: light grey for specialists and dark grey for generalists.

715

716

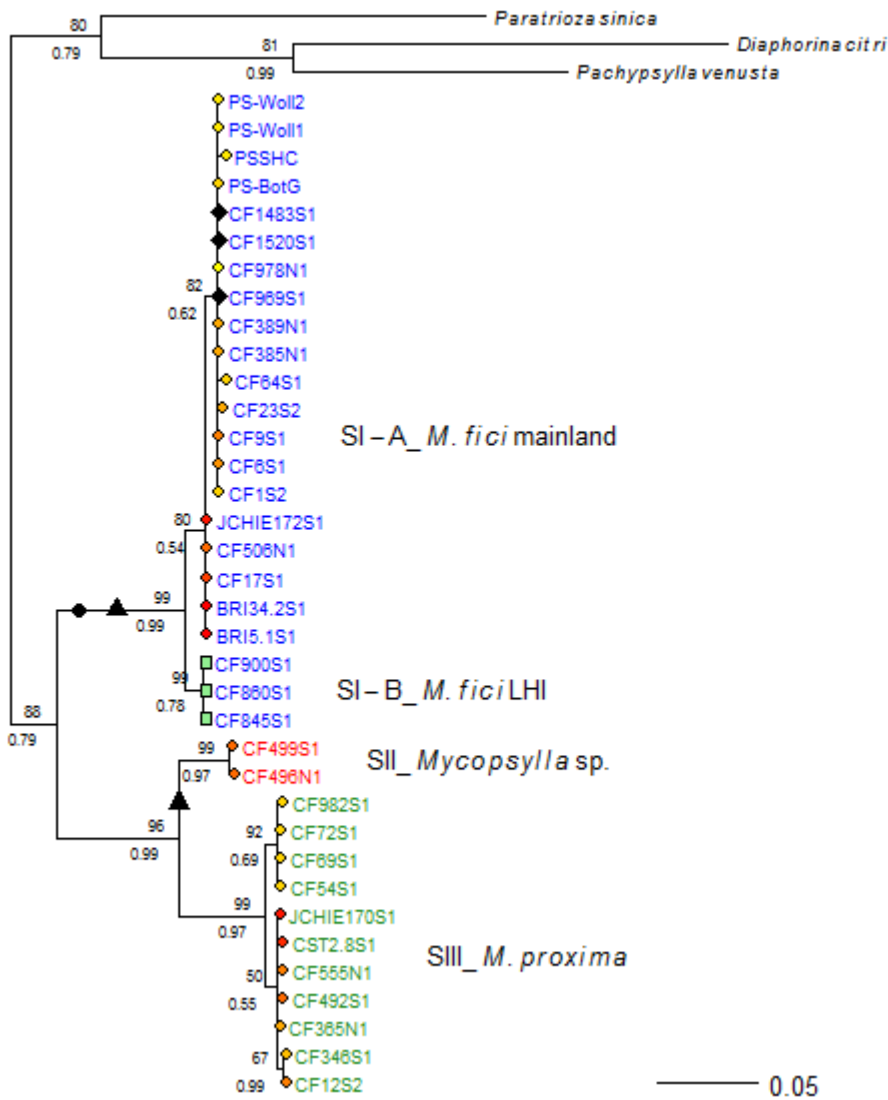




717

718 Fig. 1

719

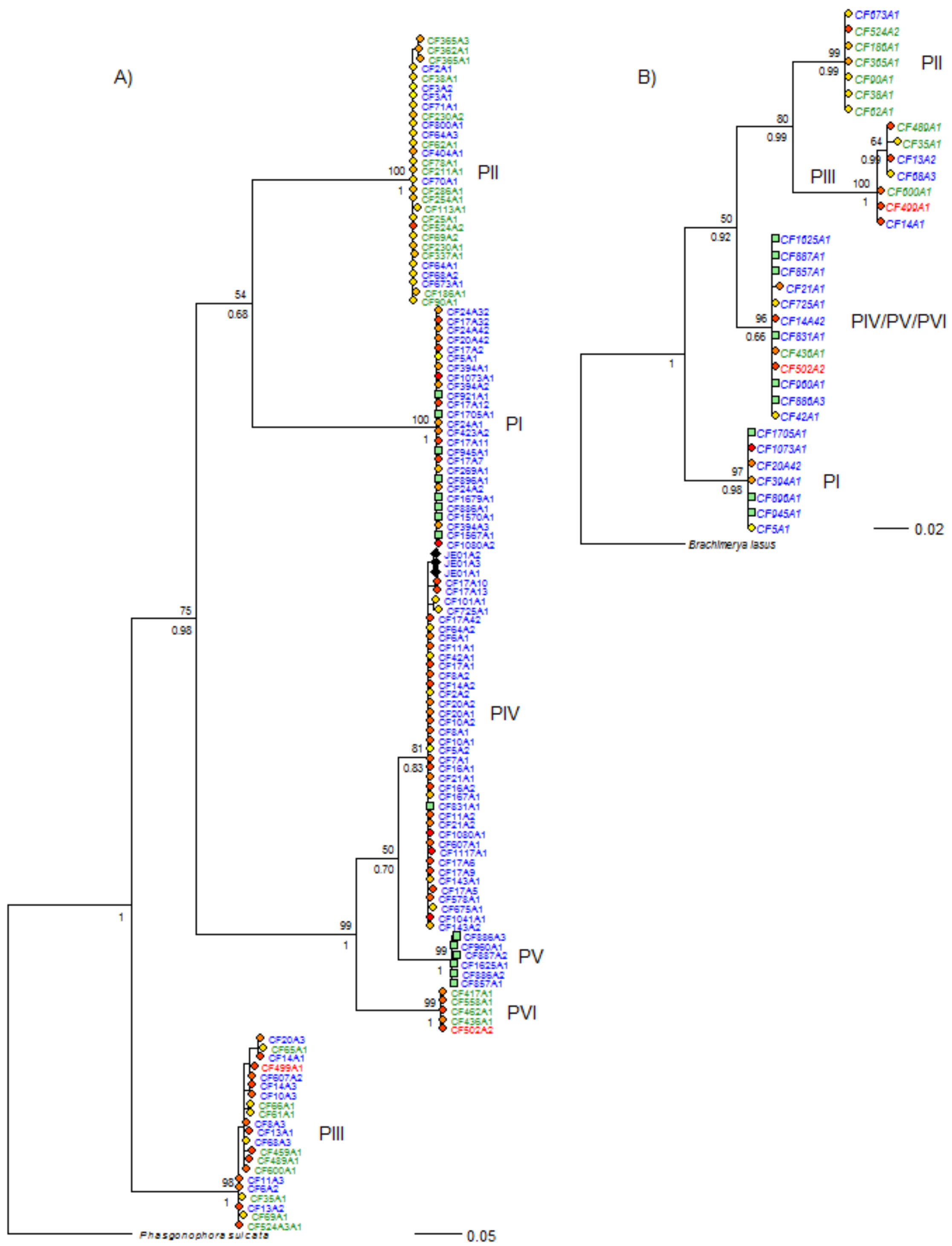


720

721

722 Fig. 2

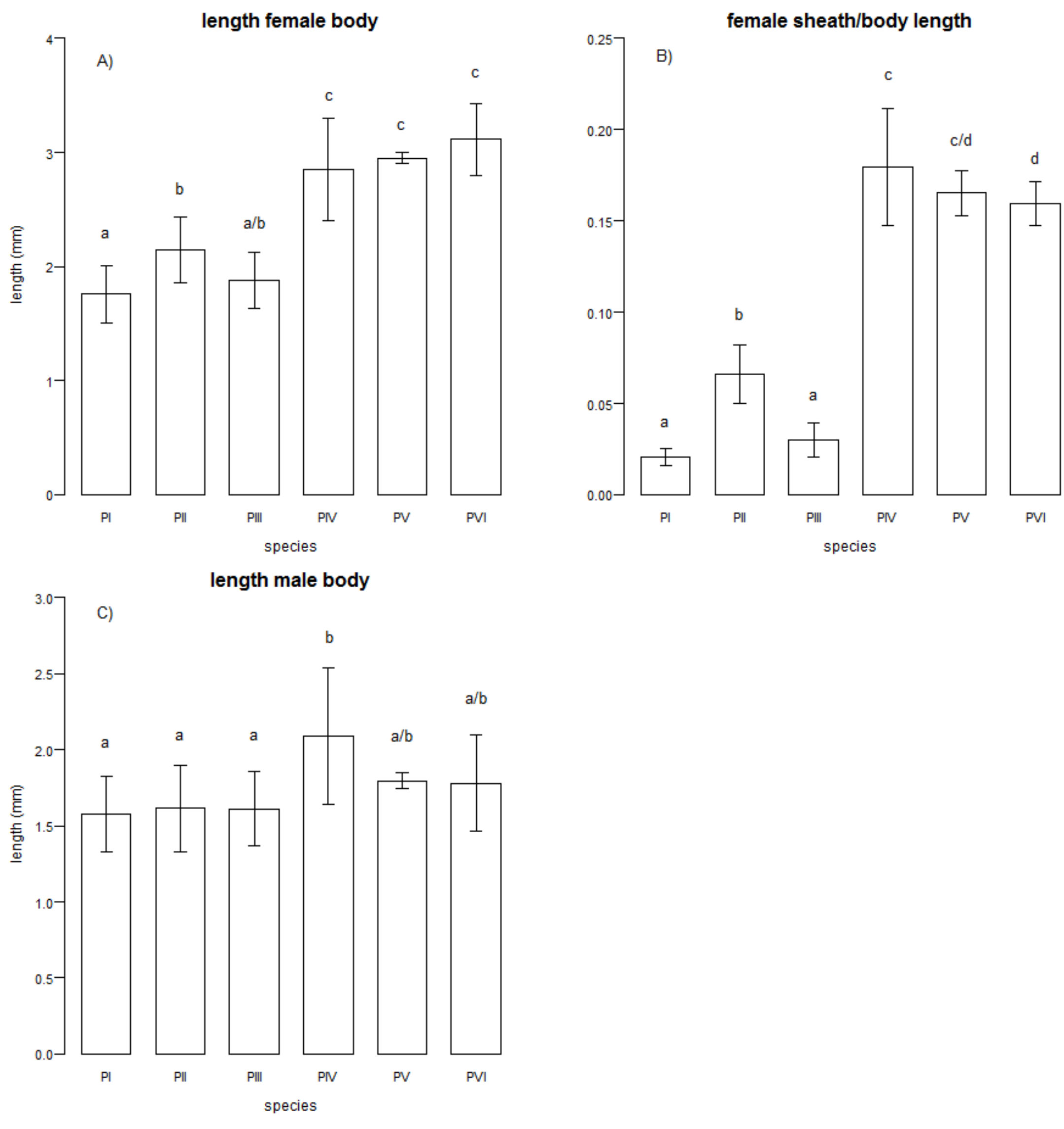
723



724

725 Fig. 3

726



727

728

729 Fig. 4

730

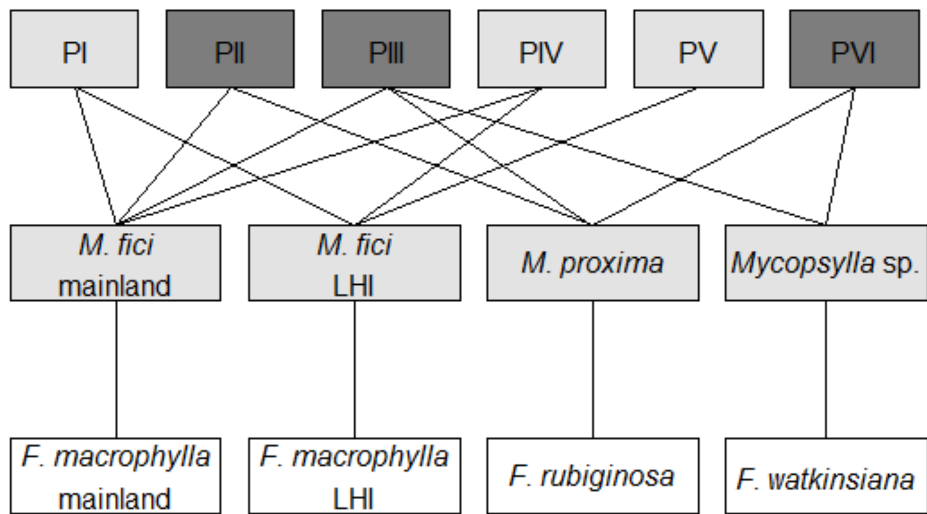


Fig. 5