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

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Diversity and host specificity of *Mycopsylla* species (Hemiptera: Homotomidae) and their *Psyllaephagus* parasitoids (Hymenoptera: Encyrtidae) on figs (*Ficus*).

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ABSTRACT

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

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

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Study species and insect sampling

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

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

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**

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

Statistical parsimony

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

Barcoding gap

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

Generalized Mixed Yule Coalescent (GMYC) model

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

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

RESULTS

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

Mycopsylla phylogenies

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

JModelTest2 indicated that the best models were HKY+I for *COI*, and JC for *Hist3* and *EF1α*. Mitochondrial ML and BI phylogenies showed the same topology (Fig. 2), with three highly supported clades (SI, SII, SIII). The nuclear genes were highly conserved, but the limited variation was congruent with the mtDNA clade structure. One fixed synonymous nucleotide 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*

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

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

Psyllaephagus phylogenies

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

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

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

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

Statistical parsimony

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

Barcoding gap

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

GMYC

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

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

Parasitoid morphology

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

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

Host-specificity of homotomids and parasitoids

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

DISCUSSION

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

Variable host-specificity across food web

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

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

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

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

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

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

Mycopsylla *and* Psyllaephagus *phylogeography varies across species*

Our study focused primarily on establishing the number of species of homotomids and 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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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 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 are the minimal and maximal values of pairwise divergences between species.

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**

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**

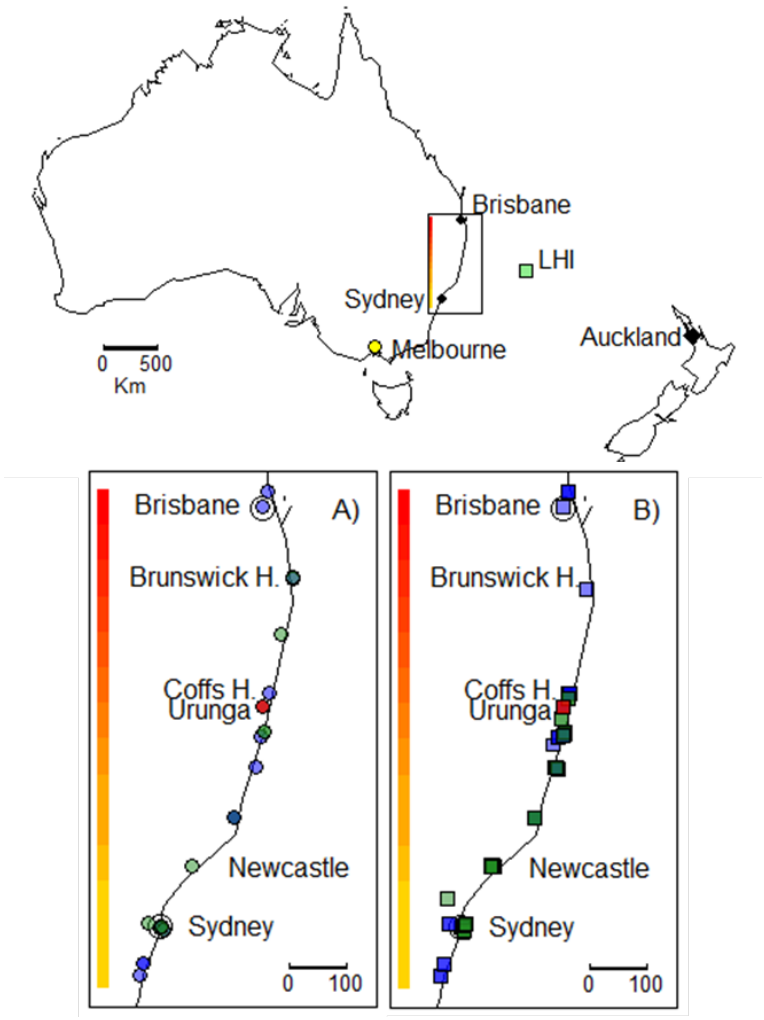
Figure 1 : Maps of the *Mycopsylla* and *Psyllaephagus* collections in Australia and New Zealand. Both *Mycopsylla* and *Psyllaephagus* were collected in LHI (light green square) and Auckland (black diamond) while only *Mycopsylla* were collected in Melbourne (yellow circle). Maps A) and B) represent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

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

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

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

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


Brisbane
LHI
Sydney
Melbourne
Auckland
0 500
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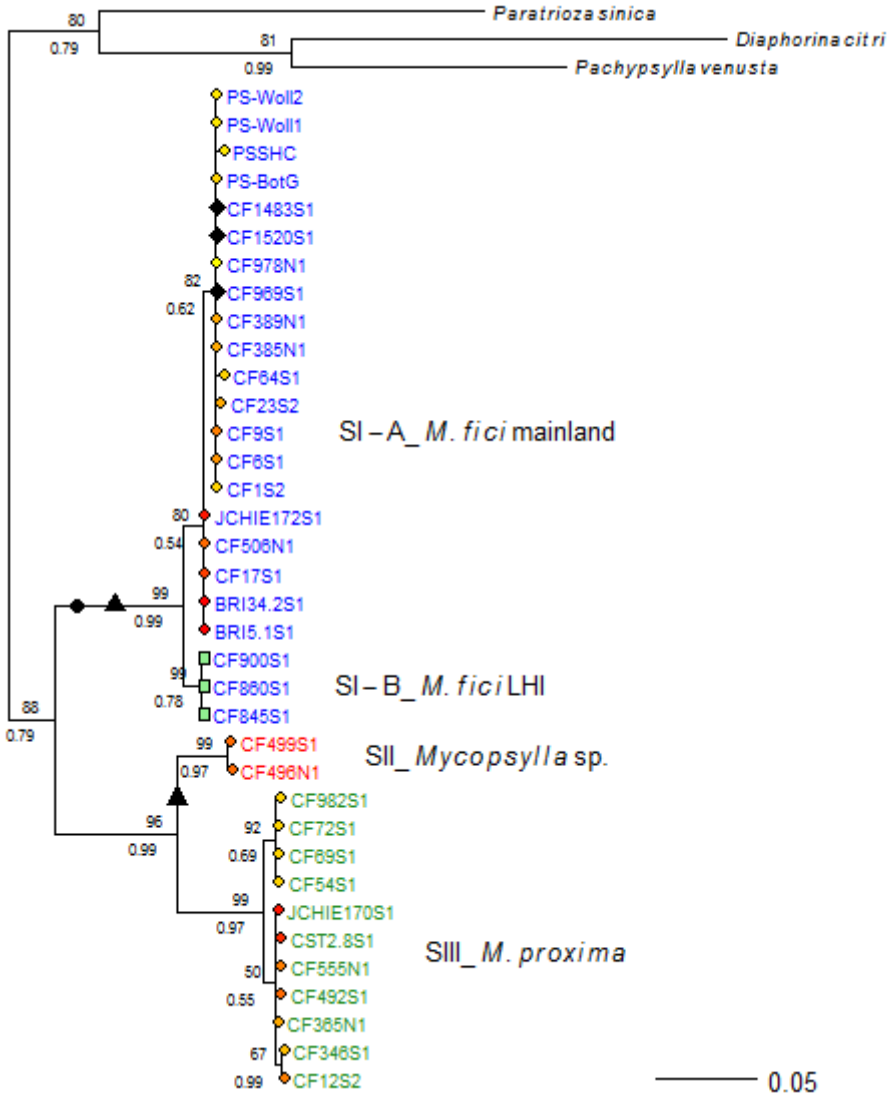
Brisbane
A)
Brunswick H.
Coffs H.
Urunga
Newcastle
Sydney
0 100
Brisbane
B)
Brunswick H.
Coffs H.
Urunga
Newcastle
Sydney
0 100

Fig. 1

719



720

721

722 Fig. 2

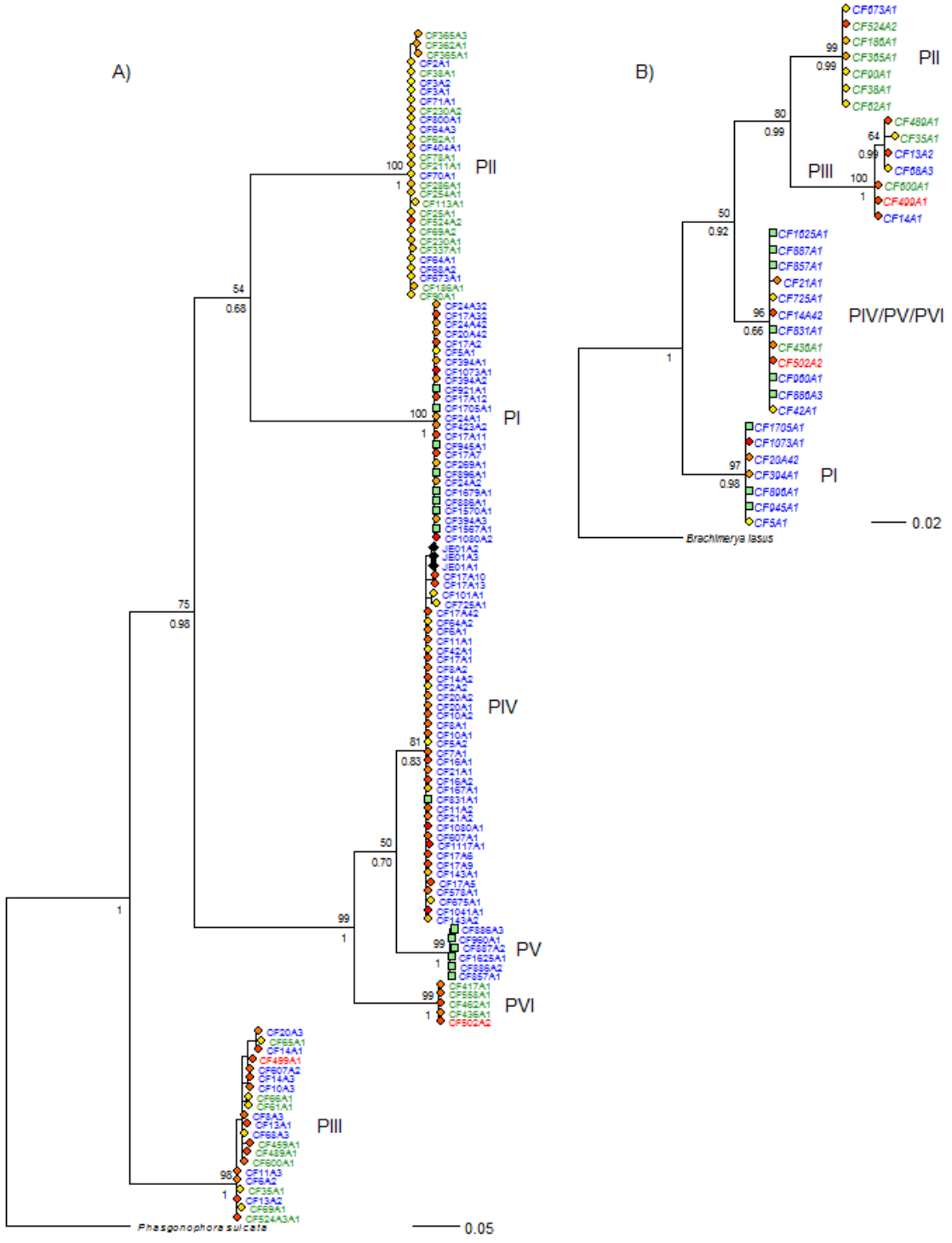

A)
PII
PI
PIV
PV
PVI
PIII
Phasgonophora sulcata
0.05
B)
PII
PIII
PIV/PV/PVI
PI
Brachimerya lasus
0.02

Fig. 3

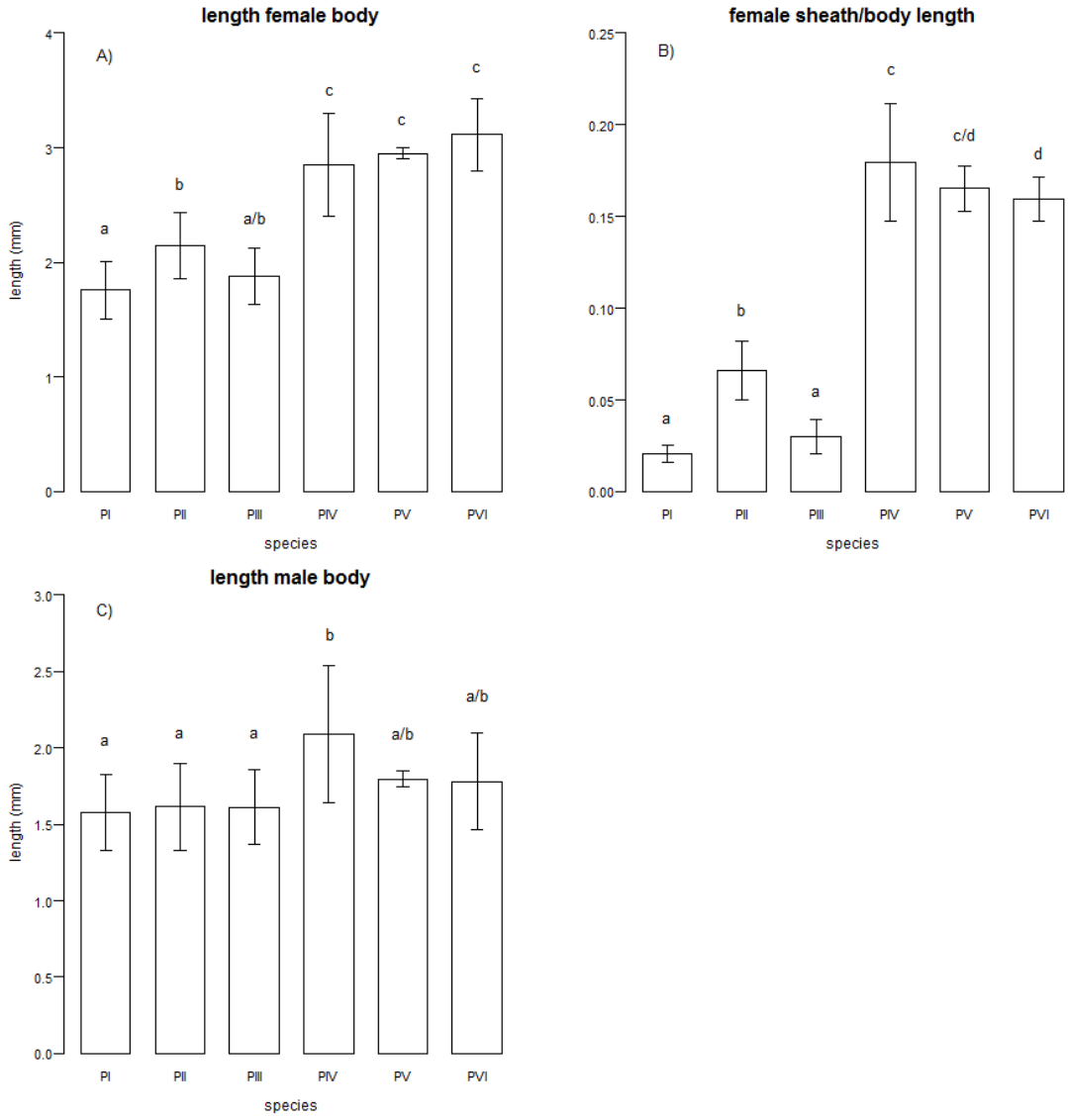

length female body
A)
length (mm)
species
PI
PII
PIII
PIV
PV
PVI
a
b
a/b
c
c
c
female sheath/body length
B)
a
b
a
c
c/d
d
length male body
C)
a
a
a
b
a/b
a/b

Fig. 4

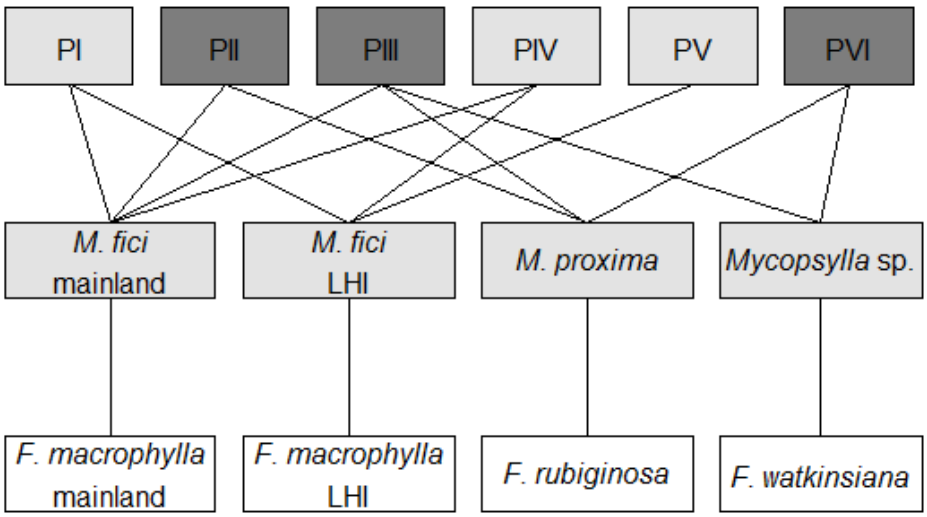

PI
PII
PIII
PIV
PV
PVI
M. fici mainland
M. fici LHI
M. proxima
Mycopsylla sp.
F. macrophylla mainland
F. macrophylla LHI
F. rubiginosa
F. watkinsiana

Fig. 5