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1 **Faster speciation of fig-wasps than their host figs leads to decoupled speciation**
2 **dynamics: snapshots across the speciation continuum**

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25 **Abstract**

26 Even though speciation involving multiple interacting partners, such as plants and their
27 pollinators, has attracted much research, most studies focus on isolated phases of the process.
28 This currently precludes an integrated understanding of the mechanisms leading to co-
29 speciation. Here, we examine population genetic structure across six species-pairs of figs and
30 their pollinating wasps along an elevational gradient in New Guinea. Specifically, we test
31 three hypotheses on the genetic structure within the examined species-pairs and find that the
32 hypothesized genetic structures represent different phases of a single continuum, from
33 incipient co-speciation to the full formation of new species. Our results also illuminate the
34 mechanisms governing co-speciation, namely that fig wasps tend to accumulate population
35 genetic differences faster than their figs, which initially decouples the speciation dynamics
36 between the two interacting partners and breaks down their one-to-one matching. This
37 intermediate phase is followed by genetic divergence of both partners, which may eventually
38 restore the one-to-one matching among the fully formed species. Together, these findings
39 integrate current knowledge on the mechanisms operating during different phases of the co-
40 speciation process. They also reveal that the increasingly reported breakdowns in one-to-one
41 matching may be an inherent part of the co-speciation process. Mechanistic understanding of
42 this process is needed to explain how the extraordinary diversity of species, especially in the
43 tropics, has emerged. Knowing which breakdowns in species interactions are a natural phase
44 of co-speciation and which may endanger further generation of diversity seems critical in a
45 constantly changing world.

46

47 **Keywords:** Fig and fig-wasp mutualism, population genomics, population structure, co-
48 speciation, altitudinal gradient, Papua New Guinea

49 **1 Introduction**

50 How species originate has been one of the central questions in biology, and we still only have
51 an incomplete answer, especially when the process involves interactions between multiple
52 species. These interactions both generate and maintain biodiversity, where interacting species
53 complexes promote or constrain diversification of each other (Forister & Feldman, 2011).
54 Some of the most suitable systems for research are those following tight one-to-one
55 matching, such as those between some plants and their pollinators. Among the most widely
56 studied, is the mutualistic interaction between figs (genus *Ficus*, Moraceae) and their
57 pollinating fig-wasps (Agaonidae) (Cruaud et al., 2012; Kiestler, Lande, & Schemske, 1984;
58 Van der Niet, Peakall, & Johnson, 2014; Weiblen, 2004). Yet, recent studies have begun to
59 challenge the notion that interactions within this model system are tight, and growing
60 evidence suggests that the posited one-to-one matching between figs and their pollinating
61 wasps often breaks down (Cook & Rasplus, 2003; Haine, Martin, & Cook, 2006; Rasplus,
62 2011). How much these breakdowns repudiate current models of co-speciation (Hembry,
63 Yoder, & Goodman, 2014; Rasplus, 2011), and how much they might be an inherent part of
64 the co-speciation process is currently unknown (Bronstein, Dieckmann, & Ferrière, 2009).
65 The resultant gaps and conflicts in current literature and theory demonstrate pressing need for
66 a more complete and integrated knowledge of the mechanisms that govern co-speciation
67 across the comprehensive continuum, from its incipient phases toward the full formation of
68 new species.

69 While individual phases of co-speciation are well-documented in the literature (Cruaud et al.,
70 2012; Weiblen, 2004), few studies have investigated co-speciation within a comparative
71 framework to integrate previous results along the different stages of the speciation
72 continuum. In the case of *Yucca* plants and their pollinating moths, it has been reported that
73 congruence between the phylogenies of the interacting partners may result from the

74 geographic tracking between the pollinators and their host plants, rather than from strict co-
75 speciation (Althoff, Segreaves, Smith, Leebens-Mack, & Pellmyr, 2012; Hembry et al., 2014).
76 In figs and fig wasps, wasp diversification is typically constrained by the host plants
77 (Jousselin et al., 2008), which produces predominantly one-to-one patterns of co-speciation
78 (Cruaud et al., 2012), despite the role that occasional duplications and host switches have also
79 played in the mutualisms co-evolution (Cook & Rasplus, 2003; Jousselin et al., 2008;
80 Rasplus, 2011). Still, contrary to the expectation under strict co-speciation, where speciation
81 events of interacting lineages occur at the same time (Huelsenbeck, Rannala, & Larget,
82 2002), Moe & Weiblen (2012) and Yu et al. (2019) have proposed faster speciation rates for
83 wasps than their host figs, suggesting that occasional breakdowns in the one-to-one matching
84 might be inevitable. While each of these studies provides useful insights, the results are hard
85 to compare, as they span different regions, taxa, and different phases of the co-speciation
86 process. To achieve a synthesis, we could benefit from a comparative approach that captures
87 multiple phases of co-speciation within the same system.

88 **1.1 Co-speciation process and its underlying mechanisms**

89 Theoretical and empirical work suggests that the divergence patterns of one of the interacting
90 partners could be associated with divergence from the other, progressing from increasing
91 genetic divergence toward the complete formation of genetically well-defined species
92 (Forister & Feldman, 2011; Futuyma & Agrawal, 2009). Specifically, pollinators have been
93 hypothesized to promote reproductive isolation between plant populations during the initial
94 phases of co-speciation (Hembry et al., 2014), which holds especially for pollinators with
95 limited dispersal abilities that modulate gene flow between their host plant populations
96 (Kiestler et al., 1984; Souto-Vilarós et al., 2018; Van der Niet et al., 2014). These effects
97 should be particularly evident in obligate nursery pollination systems, where pollinators are
98 the sole means of pollen transfer between the host flowers. The pollinators, in turn, depend

99 exclusively on the flowers that serve as oviposition site and food resource for their
100 developing larvae (Althoff et al., 2012; Galil & Eisikowitch, 1968; Kawakita, 2010).
101 Growing genetic divergence within the host-plant populations should therefore be matched
102 with the corresponding divergence across the populations of their pollinators (or vice versa),
103 producing the characteristic pattern of co-speciation. Despite the extreme specificity of
104 nursery pollinating systems, the one-to-one matching between host plants and their
105 pollinators has been often reported to break down (Darwell, Al-Beidh, & Cook, 2014; Li,
106 Wang, & Hu, 2015; Pellmyr, 1999; Yu et al., 2019). The causes of these breakdowns remain
107 unclear. But one largely overlooked possibility is that the breakdowns represent an inherent
108 part of the co-speciation process.

109 Cross-elevational comparisons may afford snapshots of genetic differentiation among the
110 populations of figs and their pollinating wasps, representing different phases of the co-
111 speciation process. Even though fig wasps are generally highly mobile dispersers, relying on
112 passive dispersal by wind (Ahmed, Compton, Butlin, & Gilmartin, 2009; Kobmoo, Hossaert-
113 Mckey, Rasplus, & Kjellberg, 2010; Liu, Compton, Peng, Zhang, & Chen, 2015), their
114 mobility, and thereby the gene flow between their host plants, may sometimes be impaired.
115 For instance, fig wasps associated with dioecious and under-canopy fig species have limited
116 flight ability (Venkateswaran, Kumble, & Borges, 2018), which is further compounded by the
117 fact that dioecious fig trees tend to cluster into dense local populations (Dev, Kjellberg,
118 Hossaert-Mckey, & Borges, 2011; Wachi, Kusumi, Tzeng, & Su, 2016). In contrast, wide-
119 ranging monoecious species have little genetic structure over much of their ranges (Bain et
120 al., 2016) and their pollinating wasps have been shown to be good dispersers (Venkateswaran
121 et al., 2018). Additionally, differences in forest type, vegetation density, and dispersal
122 capacity owing to wind, typical of different elevations within a montane system, likely
123 produce elevational differences in gene flow. We expect varying levels of differentiation

124 between the pollinator species from distinct evolutionary lineages (sections) of *Ficus* that
125 colonized Mt Wilhelm during different stages of its orogeny. Consequently, each *Ficus*
126 lineage likely represents a semi-independent radiation along the gradient. This makes cross-
127 elevation comparisons of population genetic structure between distinct lineages appealing for
128 co-speciation research, as these may represent different stages along the speciation process.

129 **1.2 Gene flow and speciation along elevational gradients**

130 Elevational gradients have long been regarded as natural laboratories for the study of
131 speciation (Körner, 2007; McCain & Grytnes, 2010; Rahbek & Museum, 1995). Tropical
132 mountains in particular are often portrayed as ‘speciation pumps’, generating a
133 disproportionate amount of the world’s biodiversity (Kreft & Jetz, 2007). The Andes of South
134 America, Mount Cameroon in western Africa, Mount Kinabalu in Borneo and New Guinea’s
135 central range are among the most species-rich places on Earth and frequent subjects of
136 macroevolutionary research (Rull, 2011; Toussaint et al., 2014). Restricted gene flow along
137 the slopes of mountains, owing to environmental heterogeneity and non-random mating,
138 likely promote population isolation, genetic differentiation, local adaptation and, eventually,
139 parapatric speciation, explaining the high turnover of species with elevation (Byars, Parsons,
140 & Hoffmann, 2009; Caro, Caycedo-Rosales, Bowie, Slabbekoorn, & Cadena, 2013;
141 Kirkpatrick & Barton, 1997; Reis, Ciampi-Guillardi, Bajay, de Souza, & dos Santos, 2015).

142 **1.3 Study system**

143 Here, we investigate the population genetic structure of six *Ficus* species (from four lineages,
144 or sections) and their corresponding wasp pollinators along the Mount Wilhelm elevational
145 gradient in New Guinea (NG). Fig species were selected to represent different stages of the
146 co-speciation process: from continuous species with a wide elevational distribution, to
147 parapatric, botanically recognized, sub-species, and finally, parapatric monophyletic species
148 complexes; each set representing an additional step along the speciation continuum. Because

149 of their tight mutualism with pollinating fig wasps, our choice of host plant species therefore
150 predetermined the choice of pollinating wasps, following the classic one-to-one relationship
151 characteristic of this mutualism. We placed the fig species along the putative continuum
152 according to existing phylogenetic, chemical (Souto-Vilarós et al., 2018), population genetic
153 (Segar et al., 2017), and morphological information (Berg & Corner, 2005). Further, the
154 species were selected to be representative of an identifiable pattern of wide-ranging species
155 (e.g. *Ficus dammaropsis* Diels), and recognized lowland and highland (sub)species pairs (e.g.
156 *F. saccata* Corner/*F. megalophylla* Diels and *F. trachypison* K. Schum. /*F. quercetorum*
157 Corner), in line with extensive field observations (Segar et al., 2017; L. Sam, unpublished
158 data) and literature surveys (e.g. Berg and Corner, 2005). Our field collections started widely
159 but focused in on species with high local abundance amenable to comparative analyses.
160 While the selected fig species may not always be separated by known reproductive isolation
161 barriers, the criteria detailed above indicate that they lie along different stages of the
162 speciation continuum, which makes them suitable and of interest for population genetic
163 comparisons. Such an approach, to date, is unique for this system and our study represents the
164 only case where multiple fig and wasp species are studied together within a comparative
165 framework.

166 **1.4 Hypothesized scenarios**

167 Within our study, we aim to identify genetic structure of populations of figs and their
168 pollinating wasps in order to gauge the connectivity of individuals along their elevational
169 ranges. Under the presumed one-to-one species specificity, we expect to find well connected
170 population structure in both figs and wasps along their entire elevational distribution (Figure
171 1A). Alternatively, wasp local adaptation to the varying conditions along both extremes of
172 the gradient, may promote more defined population structure. This structuring would in turn
173 limit gene flow between figs, resulting in matching population structure for both interacting

174 partners. Incomplete wasp specialization in the intermediate contact zone may be a
175 mechanism by which genetic differentiation between highland and lowland fig
176 subpopulations is minimized since even rare migrants can maintain gene flow between
177 populations (Figure 1B; Ellstrand, 2014). This mechanism can be further reinforced by
178 localized seed dispersal, which may additionally magnify the genetic structuring of fig
179 populations.

180 Finally, further isolation between these parapatrically formed wasp clusters would continue to
181 promote genetic distance between fig populations. Under this scenario, the accumulation of
182 genetic dissimilarity is decoupled, such that parapatric wasp clusters (or incipient species)
183 pollinate and thereby mediate the gene flow between fig subpopulations. This process
184 eventually leads to the fig's speciation, which consequently restores of the one-to-one species
185 matching between the figs and their wasps (Figure 1C).

186 For our study, we chose the New Guinean figs in the sub-genera *Sycomorus* and *Sycidium*.
187 This system is typified by very rare shifts of the pollinating wasps between their host plants
188 (Moe, Rossi, & Weiblen, 2011; Silvius, Clement, & Weiblen, 2008), such that co-speciation,
189 rather than host shifts, typically explains the patterns of genetic matching between the plants
190 and their pollinators. To rule out the possibility that host shifts might explain some of the
191 detected patterns in population genetic structure, we built local phylogenies that included all
192 con-generics sequenced to date. If host switching were common within this system, we would
193 expect our focal species complexes to be non-monophyletic within this wider sampling
194 context.

195 Even though our study system has the obvious advantage that associations between the
196 interacting partners are clearly defined, given that one pollinator only enters one fig during its
197 life time (female wasps often lose wings and antennae as they enter a fig, such that they
198 cannot leave, and entering the 'wrong' species of fig consequently results in zero fitness), we

199 cannot exclude the possibility that any limitation to genetic exchange between parapatric
200 populations (as mediated by phenology and degrees of selfing, for example) could further
201 influence the genetic structure of the studied populations. Nevertheless, our principal
202 objective here is not to definitively identify the causes of the detected genetic differentiation.
203 Rather, we use the differentiation patterns to capture the co-speciation continuum, integrate
204 the currently fragmentary results on co-speciation mechanisms, and evaluate previously
205 formulated hypotheses on co-speciation along elevational gradients (Section 1.4), inspired by
206 prominent literature in the field.

207

208 **2 Materials and Methods**

209 We chose six species-pairs of figs and their pollinating wasps and studied the genetic
210 structure of their respective populations across elevations in the tropical montane system of
211 New Guinea (200m – 2,700m; all elevations are given above sea level). DNA was extracted
212 from 285 fig individuals and corresponding fig wasps and sequenced, using the NextRAD
213 approach (Russello, Waterhouse, Etter, & Johnson, 2015). This approach is suitable for
214 samples with very low concentrations of DNA (as is typical of tiny fig wasps). The sequences
215 were filtered to generate a dense SNP matrix for each species-pair, which we then processed
216 through MCMC-based clustering algorithms to uncover the genetic structure of the examined
217 populations. The uncovered clusters were compared against the predictions following from
218 each of the three hypotheses on co-speciation dynamics (Figure 1). The comparisons
219 consequently illuminated the mechanisms through which co-speciation operates within the
220 fig-wasp system, at least within the examined tropical mountains.

221 **2.1 Study sites**

222 The present study was carried out at six sites along Mount Wilhelm in Papua New Guinea
223 (PNG). All sites are spaced by approximately 500 m vertical increments, from 200 meters to

224 2,700 metres, ranging from lowland alluvial forest up to lower montane forest. Site names
225 and locations are described in Table 1. Forest types, broad species composition and general
226 climatic conditions have been described in detail elsewhere (McAlpine, Keig, Falls, &
227 CSIRO, 1983; Sam, Koane, Jeppy, Sykorova, & Novotny, 2017; Toussaint et al., 2014). We
228 note that environmental conditions change rapidly along the transect, for example there is a
229 15°C temperature difference between our lowest and highest sites and a semi-permanent
230 cloud layer at around 2,000 m.

231 **2.2 Focal Species**

232 Previous surveys of *Ficus* diversity carried out along the gradient (Segar et al., 2017; L. Sam,
233 unpublished data) reported approximately 70 of the ca. 150 *Ficus* species present in PNG;
234 however, pollinating fig-wasp species composition is unknown. Here, we studied five species
235 and one taxonomically defined subspecies complex, all of which are endemic to New Guinea
236 and adjacent islands (Berg & Corner, 2005). We selected two, wide ranging, described
237 species: *F. wassa* Roxb. (Sycidium) and *F. arfakensis* King (Sycocarpus), as representatives
238 of species with wide elevational distributions. Their corresponding pollinating wasps are
239 *Kradibia wassae* Wiebes and *Ceratosolen solitarius* Wiebes, respectively, however, we
240 identified multiple distinct wasp entities responsible for pollinating these fig species (see
241 Results); *F. trichocerasa* Diels (Adenosperma) is a botanically recognized subspecies
242 complex that replace each other in parapatry at approximately 1,700 m (Berg & Corner,
243 2005); this complex represents an intermediate step along the speciation continuum where
244 sub-species are not yet fully differentiated. Pollinating wasps of both subspecies are
245 undescribed. Finally, we studied a monophyletic complex of three species (Souto-Vilarós et
246 al., 2018) belonging to the section *Papuacyse* that follow a lowland, mid-elevation, and
247 highland distribution, henceforth referred to as *F. itoana* species complex which includes: *F.*
248 *itoana* Diels pollinated by *Ceratosolen armipes* Wiebes; *F. microdictya* Diels pollinated by

249 *C. sp. "kaironkensis" (nom.nud)* (Weiblen et al., 2001); and an as of yet undescribed fig-
250 wasp species-pair here referred to as *Ficus sp. "IMI"* and *Ceratosolen sp. "IMI"* (see also
251 Souto-Vilarós *et al.* 2018). This complex represents a further step along the speciation
252 continuum where each species-pair has differentiated enough to constitute a separate entity.
253 Volatile organic compounds from both the *Ficus itoana* and *F. trichocerasa* complexes have
254 been shown to differ between (sub)species, as well as pollinator preference for each of these
255 emissions (Souto-Vilarós et al., 2018), which implicates co-evolution as the likely
256 mechanism of cospeciation within these species (as opposed to, e.g., similar responses to
257 geographic isolation, shared histories of immigration and colonization). Species names,
258 breeding systems and distributions are detailed in Table 1, a map including collection sites
259 and each individual tree sampled is presented in Figure 2.

260 We conducted sampling between August 2015 and November 2016. At each site we geo-
261 referenced and tagged between 10 and 15 individual trees of each locally available focal taxa.
262 Nevertheless, in some instances, we were unable to find enough individuals for certain
263 species at certain locations (i.e. *F. itoana* at the 200m site). Similarly, due to differential
264 rearing success of fig wasps, sampling size is in some cases imbalanced (see Table 1 for
265 details). For each tree, we collected 10 to 15 leaf discs of 2.4 cm in diameter using a cork-
266 borer and stored them in colour changing silica gel for later DNA extraction and molecular
267 analysis. In order to sustain wasp populations, fig trees fruit year round. Adult (and gravid)
268 female wasps emerge from the trees in search of a fig in which to lay her eggs, however,
269 fruiting is asynchronous within trees and so wasps regularly move away from their natal trees
270 (Galil & Eisikowitch, 1968). For each male tree tagged, we monitored fig development
271 weekly or daily as necessary. Upon ripening to D-stage (the developmental stage when fig-
272 wasps have hatched but not yet emerged from the fig; Galil & Eisikowitch, 1968), we
273 collected 10-15 figs and stored them in plastic pots covered with fine mesh to allow wasps to

274 emerge. Once emerged, we collected ten female and ten male pollinating wasps for storage in
275 100% ethanol. A second collection of five males and five females was done using tubes filled
276 with colour changing silica gel topped with cotton wool as per (Moe et al., 2011). For each
277 tree, we used a single female fig-wasp from the silica gel collections for DNA extraction. We
278 considered pooling of individuals inappropriate for such a fine-scale study and given that
279 each fig typically contains many sisters (pollinating foundresses rarely leave a fig once inside
280 and small figs are often pollinated by only one or two wasps) we maximised sampling above
281 the fig level where possible. Finally, we collected and stored all remaining wasps in 100%
282 ethanol. All samples were stored in a -20°C freezer and shipped to the Czech Academy of
283 Sciences, Czech Republic. Fig vouchers are stored in the National Herbarium in Lae, PNG,
284 the New Guinea Binatang Research Centre (BRC), PNG, and the Czech Academy of
285 Sciences, Czech Republic. Wasp vouchers are stored at the BRC and the Czech Academy of
286 Sciences and Harper Adams University, UK.

287 **2.3 DNA extraction and nextRAD sequencing**

288 We performed DNA extractions from leaf discs using the CTAB protocol (Doyle & Doyle,
289 1987) followed by an additional cleaning step using a silica column (Souto-Vilarós et al.,
290 2018). From each individual fig, a single female pollinating wasp stored and dried in silica
291 gel was used for DNA extraction. Isolations were performed using DNeasy Blood & Tissue
292 kits (Qiagen) following the manufacturers' protocol with the following modifications: Initial
293 lysis was conducted at 37°C overnight followed by 30 minute incubation with 1 µL of RNase
294 (Qiagen) per sample. Final yield was further enhanced by using a total of 200 µL of
295 deionized water in two rounds of column washing. For both fig and wasp DNA samples, 1
296 µL of the final extract was used for QuBit quantification (QuBit 3 ThermoFisher Scientific)
297 and 2 µL were loaded on a GelRed® Nucleic Acid Gel Stain (Biotium) pre-stained 2%
298 agarose gel ran at 120 V for 70 minutes for visual inspection. Finally, fig DNA solutions

299 were diluted to a total of 200 ng in 40 μ L of EB buffer (Qiagen), while wasp DNA solutions
300 (due to significantly lower yields) were vacuum dried and resuspended in 35 μ L of EB buffer
301 to increase concentration to ~20 ng of DNA per sample.

302 Isolations were sent to SNPSAURUS, LLC for genotyping-by-sequencing (GBS) using
303 Nextera-tagmented reductively amplified DNA sequencing (NextRAD) as per Russello et al.,
304 (2015). Genomic DNA was first fragmented with the Nextera reagent (Illumina, Inc), which
305 also ligates short adapter sequences to the ends of the fragments. The Nextera reaction was
306 scaled for fragmenting 5 ng of genomic DNA for figs and 3 ng for wasps, although 17.5 ng
307 and 6 ng of genomic DNA for figs and wasps respectively was used as input to compensate
308 for the amount of degraded DNA in the samples and to increase fragment sizes.

309 For fig samples, fragmented DNA was then amplified for 26 cycles at 73°C, with one of the
310 primers matching the adapter and extending 9 nucleotides into the genomic DNA with the
311 selective sequence GTGTAGAGC. For wasp samples, fragmented DNA was amplified for 25
312 cycles at 72°C, with one of the primers matching the adapter and extending 8 nucleotides into
313 the genomic DNA with the selective sequence GTGTAGAG. Thus, only fragments starting
314 with a sequence that can be hybridized by the selective sequence of the primer was efficiently
315 amplified. The nextRAD libraries were sequenced single end on a HiSeq 4000 with four
316 lanes of 150 bp reads (University of Oregon).

317 Variant called format (VCF) files were generated by SNPSAURUS, LLC using custom scripts.
318 First, reads from each sample were trimmed using the BMAP tool *bbduk* (Bushnell, 2014).
319 Trimming removed the Nextera adapter sequences and also quality trimmed the end of the
320 reads as needed. Next, an equal number of reads were collected from each sample to create a
321 catalogue of 10 million reads total. Read collection was done with BBTOOLS (BMAP) with
322 randomly selected reads. The catalogue was then analyzed to find sequences which may
323 represent haplotypes in the population, by counting the instances of each sequence and

324 keeping sequences with 10-1000 instances. These sequences were collapsed by aligning the
325 sequences to each other with the BBTOOLS program, using an alignment threshold of 85%
326 identity. Reads that aligned to each other were considered different haplotypes of the same
327 locus, and a single sequence of the aligning group was selected to represent that locus. Each
328 sample's trimmed sequences were mapped to this *de novo* reference (using BBMAP), and the
329 alignment piped to SAMTOOLS *view* to convert to a BAM file followed by a sorting step
330 (using SAMTOOLS *sort*) to create a sorted BAM file for each sample (Heng Li et al., 2009).
331 Genotype calling was done using *mpileup* (SAMTOOLS), using the *de novo* reference as a
332 reference sequence and the list of sorted BAM files as input. This genotype table was further
333 filtered using VCFTOOLS version 0.1.13 (Danecek et al., 2011) to remove alleles with low
334 frequency in the population, which helps remove sequence error artefacts. The VCF file was
335 also filtered by removing loci with all heterozygous genotypes or loci with more than 2
336 alleles at a single position, both of which suggest distinct loci had been collapsed together
337 inappropriately. Specific commands for each step as well as additional dataset details (i.e.
338 number of SNPs per species/individual) are provided as supplementary material, VCF files
339 for each species used in our analysis are available from the Dryad Digital Repository
340 (doi:10.5061/dryad.5m4dn73). Raw demultiplexed NextRAD sequences are available from
341 the Short Read Archive (NCBI) BioProject number PRJNA555181.

342 We generated molecular barcodes for 86 wasps (representative of each identified genomic
343 cluster; see Results) using cytochrome oxidase *b* (*cyt-b*), a marker with high specificity and
344 amplification rates in fig wasps which has the ability to delimit closely related fig wasp
345 species (Lopez-Vaamonde et al., 2009; Segar, Lopez-Vaamonde, Rasplus, & Cook, 2012).
346 Due to unsuccessful amplification of *cyt-b* for individuals of *Ceratosolen* sp. "IMI," we
347 generated COI sequences of 4 individuals to compare them against previously published
348 sequences for wasps of the *F. itoana* complex. PCR primers and conditions for both

349 fragments are outlined in Cruaud et al. (2010). Similarly, in order to establish the role of host
350 shifting in our system we tested the monophyly of wasp clades (see Results) through the
351 reconstruction of wider molecular phylogenies using the F2 copy of elongation factor 1-
352 alpha, (*ef1-a*) and *cyt-b*. We amplified *ef1-a* using the primers reported by Cruaud et al.
353 (2011) and concatenated both markers for a total of 92 individuals (50 *Kradibia* and 42
354 *Ceratosolen*) spread across the genetic clusters identified. We provide a breakdown of data
355 collected for each individual wasp (nextRAD-seq, *cyt-b* and *ef1-a*) in the supplementary
356 material, when all DNA was used for NextRAD sequencing of alternative individuals were
357 made.

358 **2.4 Data analysis**

359 Population genetic structure was inferred using FINERADSTRUCTURE (Malinsky, Trucchi,
360 Lawson, & Falush, 2018), a pipeline specifically designed for the analysis of RAD data using
361 the FINESTRUCTURE Markov chain Monte Carlo (MCMC) clustering algorithm (Lawson,
362 Hellenthal, Myers, & Falush, 2012). Without previous assumptions of populations, the
363 program generates a “co-ancestry matrix” between all individuals and collection sites which
364 is then combined with MCMC sampling to identify clusters of individuals which share the
365 highest degree of estimated relatedness. We converted VCF files to the required format using
366 custom python scripts (supplementary material). The software RADPAINTER, as implemented
367 in the FINERADSTRUCTURE package, was then used to calculate the co-ancestry matrix.
368 Individuals were assigned to populations using 100,000 MCMC replications and a burn-in of
369 10,000 as per default settings (Malinsky et al., 2018). Tree building was conducted using the
370 default parameters and results were visualized using R scripts (available at
371 <http://cichlid.gurdon.cam.ac.uk/fineRADstructure.html>). Because our data is not sorted
372 according to genome coordinates, linkage disequilibrium (LD) between RAD loci is rather
373 strong and can lead to an overestimation of population splits. In order to retrieve a

374 conservative number of identifiable population clusters, prior to calculating the co-ancestry
375 matrix, RAD loci were reordered according to LD using the sampleLD.R script implemented
376 in the package (Malinsky et al., 2018). An initial analysis was done including all samples to
377 generate a co-ancestry matrix for all figs (Figure S1; supplementary material), and fig-wasps
378 (Figure S2; supplementary material) in order to identify the relative relatedness of all
379 samples. Further analyses were done per species in order to increase resolution of the
380 identified groups.

381 Levels of genetic differentiation between collection sites were estimated by pairwise F_{ST}
382 from the VCF files using the Weir and Cockerham (Weir & Cockerham, 1984) method as
383 implemented in VCFTOOLS version 0.1.13 (Danecek et al., 2011).

384 We used the *cyt-b* barcodes to obtain molecular operational taxonomic units (MOTUs) for
385 wasps and used jMOTU v.1.0.7 (Jones, Ghoorah, & Blaxter, 2011) and the web version of
386 ABGD (Automatic Barcode Gap Discovery for primary species delimitation; Puillandre,
387 Lambert, Brouillet, & Achaz, 2012) in order to distinguish whether or not retrieved wasp
388 clusters could constitute different species (see Results). Mitochondrial DNA can be used to
389 delimit fig wasp species (Yu *et al.* 2019); although we note that additional evidence is
390 required to address reproductive isolation between them and that ongoing gene flow (e.g.
391 mtDNA introgression) may prevent complete resolution in many cases (Whitworth, Dawson,
392 Magalon, & Baudry, 2007). Nevertheless, this method provides an objective way of defining
393 the cut-off point that should be used to distinguish between intra- and inter-specific variation
394 and can be used in combination with coalescence or phylogenetic assignment of MOTUs to
395 provide more robust delimitation. jMOTU derives genetic distances from pair-wise
396 Needleman–Wunsch alignments, selecting representatives of each resultant cluster for all-
397 against-all comparisons. The number of clusters (MOTUs) is reported for a given set of
398 similarity thresholds and plotted, such that an inflection and plateau are expected at the

399 barcode gap, after which increasing genetic dissimilarity results in a stable number of
400 clusters. The barcode gap lower limit represents the genetic distance just beyond the upper
401 limits of intra-specific variation. ABGD derives a threshold by using a range of genetic
402 similarities to determine the extent of intra-specific variation and cluster MOTUs, subsequent
403 recursive partitioning is used to partition that data and determine the best estimate of the
404 barcode gap as well as the corresponding number of MOTUs. Using jMOTU we set the low
405 BLAST identity filter to 97 but otherwise settings were left as default. For ABGD we set the
406 minimum genetic distance to 0.001 and the maximum to 0.4. The number of steps was set to
407 200 and the relative gap width was set to 0.75. The number of bins was set to 40. We used the
408 Kimura K80 distance with the TS/TV ratio set to the default 2.0. The barcode gap was
409 identified using jMOTU by plotting the number of MOTUs vs. the percentage cut-off and
410 finding the value over which the MOTU numbers plateau following a sharp decrease which
411 results from intra-specific clustering (the results of both analyses are presented as
412 supplementary material). This was compared to the number of MOTUs found from the
413 barcode gap as estimated by ABGD, the barcode gap is well represented by the histogram of
414 genetic distances between sequences: the barcode gap is the region between intra-specific and
415 inter-specific distances (Puillandre et al., 2012).

416 Phylogenetic analyses were performed separately for *Ceratosolen* and *Kradibia* wasps and
417 alignments of both *cyt-b* and *ef1-a* markers were concatenated. We included only the
418 individuals for which we had sequences for *ef1-a*, and complemented them with *cyt-b*
419 sequences. In addition to the generated sequences, we included published sequences for
420 pollinating fig wasps which may co-occur at our study site obtained by Cruaud et al. (2012).
421 Alignment was conducted using MUSCLE (Edgar, 2004) and tree building using maximum
422 likelihood (ML) as implemented in RAxML-HPC BlackBox (Stamatakis, 2014) using
423 HKY+G and HKY+I+G substitution models for each species (*Ceratosolen* and *Kradibia*

424 respectively) selected according to Bayesian information criterion as per JMODELTEST 2
425 version 0.1.10 (Darriba, Taboada, Doallo, & Posada, 2012). The final alignments for
426 *Ceratosolen* included 76 individuals while the *Kradibia* alignment included 67 sequences.
427 Sequences from the genus *Dolichoris* were used as outgroup in both instances.

428

429 **3 Results**

430 Co-ancestry heat maps generated using FINERADSTRUCTURE provide detailed population
431 structure for both figs and fig wasps. For *F. wassa* and *F. arfakensis*, we were able to detect
432 highland and lowland populations of figs, often with mid-elevation contact zones. Low
433 pairwise fixation index (F_{ST}) values suggest these to be well connected populations indicating
434 that some of these species do have wide elevational distributions. Contrastingly, our analysis
435 on fig-wasps reveal more tightly clustered entities along the gradient with very high F_{ST}
436 values suggesting infrequent exchange of genes; supplementary genetic barcoding analyses
437 show inter-specific distances in mitochondrial DNA commensurate with species level splits,
438 however, whether or not these entities are reproductively isolated from each other remains
439 unknown. For the two widely distributed fig species, our analysis suggests multiple highly
440 differentiated genetic entities along the gradient while for the subspecies and sister species
441 complex, it is likely that individual wasp species are responsible for pollinating each of the
442 fig (sub)species maintaining the classic one-to-one species specificity. Importantly, within
443 our study, we place emphasis on the comparative approach that draws on multiple species,
444 while exercising caution when interpreting the results of any one comparison on its own,
445 especially when sample sizes are low.

446 **3.1 *Ficus wassa*: two populations of figs with a contact zone and more than three** 447 **clusters of wasps**

448 Figure 3 illustrates the level of co-ancestry between *F. wassa* (Figure 3A) as well as its
449 *Kradibia* pollinators (Figure 3B). *Ficus wassa* has a wide elevational distribution from sea
450 level to 2,700 m (Berg & Corner, 2005). At our sites we find highland and lowland
451 population clusters, while the mid-elevations (here defined as 1,700 m) appear to represent a
452 contact zone. F_{ST} values (Figure 5A), however, reveal little genetic differentiation between
453 sites (F_{ST} between sites <0.01), suggesting these clusters to be populations of a wide-ranging
454 species. Given that *F. wassa* has been traditionally cultivated by humans, it seems possible
455 that this pattern may have been, at least partly, influenced by human-mediated dispersal.
456 Pollinating wasps display a contrasting pattern with a very closely related and sub-structured
457 lowland cluster composed by most wasps from the 200 m site and a second group from the
458 700 and 1,200 m sites. There is further evidence of a deep split between lowland and
459 highland pollinator clusters. F_{ST} values between wasp populations are correspondingly high
460 (Figure 5B) while F_{ST} values within the clusters show moderate genetic diversity (700 m vs.
461 1,200 m $F_{ST} <0.09$; between highland populations $F_{ST} <0.14$). According to our mtDNA
462 analyses, these three clusters are certainly distinct enough to constitute separate MOTUs
463 (with pairwise distance above 2.5% likely representing the barcode gap according to jMOTU;
464 Figure S3). Nevertheless, the co-ancestry analysis (Figure 3B) shows one individual from the
465 200 m site grouping with the 700 m and 1,200 m cluster, and one 1,200 m individual with the
466 highland group. This may be due to incomplete wasp specialization and consequently
467 maintaining a certain level of gene flow between fig populations, particularly at the mid-
468 elevation contact zone. However, the exact interpretation remains uncertain due to the small
469 sample sizes involved. Finally, mtDNA suggests even deeper splits and an extra fourth (an
470 additional highland cluster), or even fifth MOTU (an additional lowland cluster, according to
471 ABGD). Although the barcoding gap is ambiguous within this clade perhaps reflecting recent
472 ancestry and incomplete lineage sorting or mtDNA introgression (Figure S3).

473 **3.2 *Ficus arfakensis*: three populations of figs and four clusters of wasps**

474 Equivalent plots are given for *F. arfakensis* (Figure 3C) and its corresponding pollinator(s)
475 (Figure 3D). These plots suggest a split between highland (1,700 m) and lowland fig
476 populations (below 700 m) and a distinct third entity at mid-elevations (1,200 m). The
477 situation seems comparable to that of *F. wassa* as F_{ST} values are moderate ($F_{ST} < 0.15$ for all
478 comparisons; Figure 5C) suggesting that *F. arfakensis* occupies a broad elevational
479 distribution with a low degree of gene-flow between otherwise distinct populations. The wasp
480 data shows four clear groups: two sympatric wasp clusters occurring in the lowlands
481 (between 200 and 700 m), a third cluster at 1,200 m and a final cluster at 1,700 m. F_{ST} values
482 between these wasp clusters retrieve high genetic diversity for each comparison (Figure 5D)
483 and jMOTU analysis retrieved four distinct MOTUs (a result corroborated by ABGD), with a
484 distinct barcoding gap at 2.5% (Figure S3). As in the case above, wasps typical of the mid-
485 elevation site (1,200 m) are found at lower elevations (Figure 3D), suggesting that incomplete
486 pollinator specialisation occurs.

487 **3.3 *Ficus trichocerasa*: two subspecies of figs and three clusters of wasps**

488 Figure 4 shows the co-ancestry matrix for *F. trichocerasa* subsp. *trichocerasa* and *F.*
489 *trichocerasa* subsp. *pleioclada* (Figure 4A) and their corresponding fig-wasps (Figure 3B).
490 *Ficus trichocerasa* has two clear and genetically defined clusters which correspond to the
491 described ranges of both subspecies (Berg & Corner, 2005; Souto-Vilarós et al., 2018). F_{ST}
492 values between subspecies show high genetic dissimilarity ($F_{ST} > 0.24$) while there appears to
493 be very little dissimilarity within subspecies at different sites ($F_{ST} < 0.07$; Figure 5C).
494 Similarly, wasp individuals cluster according to the populations of their host subspecies.
495 Unfortunately, due to a combination of limited rearing success and tree availability at the
496 1,200 m site, we were only able to retrieve information from three individual wasps which
497 appear to constitute a third cluster or contact zone between subspecies, which makes any

498 clear generalizations for this species unwarranted. However, F_{ST} values suggest these to be
499 closely related to the 700 m population ($F_{ST} = 0.07$) while they appear to be quite distinct
500 from the highland (1,700-2,200 m) population ($F_{ST} > 0.80$; Figure 5C). Our barcoding results
501 from both approaches supported this finding of three wasp clusters (Figure S3). We
502 successfully barcoded four individuals of *Ceratosolen* associated with *F. trichocerasa* at
503 1,200 m with three of these individuals clustering alongside all individuals from 700 m, and
504 one forming a singleton cluster at a barcode threshold of 1%; thus, while more samples at this
505 site would further clarify the relationship between wasps at this contact zone, there is a clear
506 distinction between lowland and highland pollinators (Figure S3).

507 **3.4 *Ficus itoana* complex: three species of figs and three species of wasps**

508 Finally, Figure 4 shows the co-ancestry matrix for all species in the *F. itoana* complex (*F.*
509 *itoana*, *F.* sp. “IMI” and *F. microdictya*; Figure 4C) and their corresponding fig-wasps
510 (*Ceratosolen armipes*, *C.* sp. “IMI” and *C. “kaironkensis”*; Figure 4D). Our analyses
511 recover three distinct clusters corresponding to each of the three species. According to the co-
512 ancestry heat map, *Ficus* sp. “IMI” appears to be closely related to its sister species *F.*
513 *microdictya* ($F_{ST} = 0.16$), however, these two species have distinct breeding systems and
514 pollinating fig-wasps (Souto-Vilarós et al., 2018). Interesting to note is that one *F.* sp. “IMI”
515 individual appears to have shared ancestry between *F. microdictya* and *F.* sp. “IMI”
516 suggesting it to be a hybrid between both. Pairwise F_{ST} comparisons between species clusters
517 show moderate to high genetic differentiation (F_{ST} between 0.16 and 0.34; Figure 5D). In the
518 case of the pollinating fig-wasps of this complex, we identify three clear clusters
519 corresponding to each of the host species and F_{ST} values between comparisons show high
520 genetic differentiation between pollinating fig-wasps ($F_{ST} > 0.63$; Figure 5D). We were
521 unable to amplify *cyt-b* sequences for all but one pollinating wasp of *F.* sp. “IMI,” however,
522 the morphological and behavioural differences described by Souto-Vilarós et al. (2018)

523 provide compelling evidence that this is indeed a separate species. Similarly, a BLAST
524 (Madden, 2003) search for highly similar sequences (megablast) on the NCBI nucleotide
525 collection database of our COI sequences for these individuals found that these sequences are
526 equally dissimilar to those from *C. armipes* and *C. "kaironkensis"*, which are the closest
527 matches. This final comparison is the only one where incomplete pollinator specialisation
528 was not found (but see Souto-Vilarós et al., 2018).

529 **3.5 Molecular phylogenies**

530 We recovered well resolved molecular phylogenies (Figure S4) that were in broad agreement
531 with those in published studies (e.g. Cruaud et al., 2012), albeit using fewer taxa and markers.
532 The main result was that the monophyly of our molecular clusters was maintained, with no
533 evidence for host shifting in this system. Furthermore, we were able to recover the major
534 RAD-seq derived clusters, adding further support for their validity and demonstrating that the
535 distances between described species are commensurate with those between our genetic
536 clusters.

537

538 **4 Discussion**

539 Our findings reveal a co-speciation continuum that spans six species of figs and their
540 pollinating wasps within the tropical mountains of New Guinea. The genetic structure of the
541 examined populations is consistent with a scenario where wasps diverge faster than their host
542 figs, generating locally adapted clusters along the elevation gradient during the early stages of
543 co-speciation. The newly formed clusters of wasps then likely restrict pollen movement,
544 which contributes to the observed genetic structure of the fig populations. Restricted pollen
545 movement, potentially further compounded by restricted seed dispersal, may lead to
546 reproductive isolation between the fig populations leading to their eventual speciation. Under
547 this scenario, one-to-one matching breaks down during the intermediate phases of co-

548 speciation when the same species of a fig may be pollinated by multiple localized species of
549 wasps. The matching is later restored, as the wasp clusters impose novel reproductive barriers
550 to gene flow among fig populations culminating in their speciation. These findings uncover
551 the mechanisms of co-speciation in a hallmark study system, which could be informative of
552 the broader dynamics operating among plants and their pollinators.

553 While other explanations for the detected patterns in our genetic data are possible, our
554 interpretation is consistent with classic work on mathematical modelling which suggests that
555 any small pool of figs, which differ enough to restrict gene flow, could co-evolve with their
556 pollinating wasps and thereby promote co-speciation (Kiestler et al., 1984). Further, we found
557 that wasps experience far greater limitations to gene flow along the gradient than do figs, as
558 evidenced by the tight clustering of wasp populations, higher F_{ST} values between populations
559 than within them, and confirmed by DNA barcoding and phylogenetic analyses. These wasp
560 clusters may in turn promote character divergence within fig ecotypes (for example through
561 volatile preference). Importantly, our findings held across multiple pairs of interacting
562 species and across different stages of the speciation process. Still, further work is required
563 both in terms of taxa and regions studied. In particular, future studies across the central range
564 of New Guinea would illuminate if the same differentiation emerges in parallel along
565 different mountain slopes. Recent work has reported nine species of pollinating wasps,
566 distributed parapatrically within the geographic range of their host, *Ficus hirta* Vahl (Yu et
567 al., 2019), suggesting that different wasp species might be pollinating the same fig species at
568 different locations, even when every location shows only one pollinator. Should the co-
569 speciation dynamics we found operate more broadly across different montane systems, there
570 may be far more pollinating fig-wasps per host fig than typically presumed.

571 Our results integrate and extend previously reported findings on co-speciation. In line with
572 the accumulating evidence against the one-to-one species specificity in the fig and fig wasp

573 mutualism (Cook & Rasplus, 2003; Rasplus, 2011), we find that the one-to-one matching
574 occasionally breaks down. We also identify the circumstances when the breakdowns occur,
575 and illustrate that they represent an inevitable intermediate phase of the co-speciation process
576 (Fig. 3B, D). Such process of “split and sort speciation” has been previously proposed in the
577 literature (Cook & Segar, 2010). Our results indicate that the breakdowns may not be
578 exceptions to the rule, but rather a transitional, and therefore only rarely reported, phase of
579 co-speciation. Further, we find that speciation rates in host plants may be decoupled from
580 speciation rates of their pollinators, which likely holds in other systems as well (e.g. *Yuccas*
581 and *Yucca* moths), especially when the interacting partners have dramatically different life-
582 cycles, generation times, and genetic divergence rates (Moe, Clement, & Weiblen, 2012).
583 Finally, our results connect the previous evidence for different phases of speciation into one
584 process (Nosil, Harmon, & Seehausen, 2009) and thereby provide mechanistic insights about
585 the progression of co-speciation, from its incipient phases toward the complete formation of
586 new species (Figure 1). Further research into the mechanisms, however, remains warranted.
587 In particular, a definitive proof of co-speciation resulting from co-evolution (e.g. rather than
588 from similar responses to geographic isolation) would require demonstrating a match
589 between the signal genes in figs (scent pathways) and receiver genes in wasps (olfactory
590 receptors) (Segar, Volf, Sisol, Pardikes, & Souto-Vilarós, 2019). While the genomic data for
591 such analyses are becoming increasingly available (Mori et al., 2017; Xiao et al., 2013) they
592 have yet to be compiled for our study system. Genomic matching of the corresponding genes
593 in plants and pollinators stands out as the necessary next step toward the mechanistic
594 understanding of co-speciation patterns (Segar et al., 2019).

595 **4.1 Caveats and alternative explanations**

596 Our results may have been influenced by multiple classes of confounding factors. It is
597 possible that the genetic differentiation is influenced by co-occurring species of figs and their

598 pollinating wasps. For example, host-switching could represent an alternative explanation for
599 some of the detected patterns in our genomic data. While we cannot exclude such effects
600 completely, our phylogenetic results dispute that such effects could be prominent, given that
601 we found strong support for the monophyly of the retrieved wasp clusters. Co-speciation,
602 rather than host switching, therefore seems to act as a plausible explanation for the genetic
603 patterns detected. This interpretation corresponds with much of the previous work that has
604 shown that switching, although important in the evolution of current associations, is rare
605 where the survival and reproduction of both interacting partners are coupled (Jousselin et al.,
606 2008). In line with our phylogenetic results, evidence from extensive studies including *F.*
607 *arfakensis*, *F. wassa* and twenty additional sympatric fig species both within a phylogenetic
608 (Silvieus et al., 2008) and at the population level (Moe et al., 2012) recover congruent fig and
609 wasp relationships and only limited evidence for host switching within this system.
610 Admittedly, a comprehensive phylogeny including all sympatric figs and wasps is the only
611 definitive way to rule out host switching. Still, we placed our wasp species into the most
612 densely sampled phylogeny available to date. We would therefore expect to find at least some
613 evidence for host shifts if these played a relevant role in our system. Yet, we found none. In
614 addition to host shifts, immigration followed by colonization could also explain the genetic
615 patterns observed. However, our fig species have been reported throughout the entire island
616 of New Guinea (Berg & Corner, 2005) and genetic analyses confirmed that the species are
617 not dispersal limited (Segar et al., 2017). This indicates that adaptation (e.g. to climate at
618 different elevations), rather than dispersal, limits the geographic distribution of figs and their
619 co-speciation with fig wasps.

620 Further, while our sample sizes were limited, especially for some species, they were
621 sufficient to recover robust support for some of the evaluated hypotheses (Figure 1) and
622 identify the speciation continuum across the wasps and their host figs in a comparative

623 context. Nevertheless, there are a few instances where additional wasp samples would
624 certainly strengthen our conclusions. Specifically, more wasp samples from the *F.*
625 *trichocerasa* complex at the 1,200m site would help clarify whether or not this constitutes a
626 third cluster, as retrieved by jMOTU analysis, or if it represents a hybrid zone between
627 pollinators of both subspecies (as revealed by the ancestry analysis). Similarly, increasing
628 wasp sample size for the highland *F. wassa* wasps could help refine the structuring of these
629 populations. We further acknowledge that our results cannot definitively establish the
630 presence of reproductive isolation barriers among the studied wasp clusters. Still,
631 phylogenetic and mtDNA results tentatively indicate that the wasp clusters were divergent
632 enough to constitute separate species. Moreover, we argue that the relevance of our results
633 lies in comparing different phases of the co-speciation process, and such comparisons are
634 interesting in their own right, regardless of whether the process has produced reproductive
635 isolation. Finally, we note that low rearing success in mid- and high elevations had biological
636 foundations, including low pollination rates in mid-elevation *F. itoana* (potentially a sign of
637 hybrid depression) and exceptional (non-wasp) parasite loads at the higher range edge of *F.*
638 *wassa* (2,700m) as has been reported also for other fig species (Piatscheck, Van Goor,
639 Houston, & Nason, 2018).

640 **4.2 Mechanisms of co-speciation in figs and their pollinating wasps**

641 Within montane systems, environmental conditions change dramatically even over short
642 distances, which typically imposes steep reproductive barriers in both plants and animals
643 (Bachman, Baker, Brummitt, Dransfield, & Moat, 2004; Körner, 2007; Reis et al., 2015;
644 Rieseberg & Willis, 2007). Figs and fig pollinating wasps are known to have co-evolved over
645 millions of years (Cruaud et al., 2012; Jousselin et al., 2008), and still, it has been proposed
646 that speciation dynamics in nursery pollination mutualisms may sometimes be decoupled
647 (Hembry & Althoff, 2016). Currently, it is uncertain whether speciation events are due to

648 strict co-speciation, host shifts, and/or duplications (Cook & Segar, 2010). It appears that
649 when ecological gradients are less pronounced, plants do not diversify while their pollinating
650 wasps might. For example, *Ficus hirta* appears to form a cline across its large continental and
651 oceanic range and in this case, a recent study found up to nine pollinating wasp species along
652 its range (Yu et al., 2019). Contrastingly, Kobmoo *et al.* (2010) found that a single population
653 of *Ceratosolen fusciceps* Mayr pollinates *F. racemosa* L. throughout most of South-East
654 Asia, a relatively homogeneous, but large biogeographic region. Similarly, the authors also
655 found a genetically distinct wasp entity in mainland Australia pollinating the same fig species
656 suggesting two pollinating species. Likewise, studies on *Pleistodontes imperialis* Saunders
657 (the pollinator of *F. rubiginosa* Desf. ex. Vent.) found limited gene flow and possible cryptic
658 wasp species over a wide latitudinal gradient in eastern Australia (Darwell et al., 2014). Our
659 results reveal a similar pattern, albeit at a far smaller scale, with structured fig populations
660 across an elevational gradient, and multiple pollinating wasps for species with broad
661 elevational distributions. It appears that environmental heterogeneity and discrete geographic
662 barriers play an important role in the local isolation of fig populations giving rise to rapid
663 isolation of pollinating wasps resulting in the formation of new species, without the necessity
664 of a new host fig. We suggest that both temperature gradients and cloud cover will pose
665 problems to dispersing fig wasps, indeed the largest genetic turnover is found between 1,200
666 m and 2,200 m. Horizontal distances are generally small between our study sites (< 10km)
667 and isolation by distance is not found in *F. arfakensis* across larger distances (~80km; Segar
668 et al., 2017). Due to their short life-span, assortative mating within the fig before emergence,
669 and weak dispersal abilities, specifically for below-canopy pollinators (Dev et al., 2011;
670 Wachi et al., 2016), fig wasps seem to speciate along their host's range, perhaps aided by a
671 resistance to Allee effects and genetic incompatibilities driven by *Wolbachia* (Yu et al.,
672 2019). This structuring within wasps may eventually restrict pollen movement, promoting fig

673 population structure and ultimately, host fig speciation (Cook & Segar, 2010). Competitive
674 exclusion of less well adapted fig wasps (Liu et al., 2014) might also restore one-to-one
675 pollination similar to sympatric non-pollinating fig wasps diverging in ovipositor length, and
676 likely niche (Darwell & Cook, 2017). Of more immediate significance is the fact that
677 pollination within evolving species groups is not always entirely specific, and our results
678 suggest that mid-elevation pollinator individuals can move one site up or down the transect.
679 This renders the exact reason for connectivity between populations of figs open for debate,
680 and suggests that species sorting of wasps may occur before ancestral fig species fully split.

681 **4.3 Insights from the speciation continuum**

682 We base our conclusions on the comparisons across the species examined. Such comparative
683 approach holds the promise of revealing generalities while being less sensitive to the possible
684 differences in species' genetic makeup, dispersal history, and the employed sample size. Our
685 data shows that for broadly ranging fig species (i.e. *F. wassa* and *F. arfakensis*), populations
686 at different elevations host different species of wasps. In both cases, pollinator specificity and
687 dispersal abilities may restrict gene flow between fig populations while a certain degree of
688 connectivity between them maintains species integrity, possibly through seed dispersal.
689 Speciation may be at a particularly early stage in *F. wassa*, with the exchange of wasps and
690 genes between populations a distinct possibility. It has been reported that immigrant seed
691 gene flow may have evolutionary significant consequences over wide distances, maintaining
692 otherwise isolated entities as a single species (Ellstrand, 2014). Historical cultivation of *F.*
693 *wassa* could also have contributed to the increased connectivity between the populations.
694 However, we specifically avoided abandoned gardens and restricted our sampling to
695 undisturbed forests to mitigate the effects of human-mediated dispersal.
696 The *F. trichocerasa* subspecies complex represents an additional step in the speciation
697 continuum, where divergent populations have developed distinct volatile profiles (Souto-

698 Vilarós et al., 2018) and morphological differences (which become less clear at the contact
699 zone, between 1,200 – 1,500 m ; Berg & Corner, 2005). The development of such differences
700 suggests these taxa are in the early stages of ecological speciation, even though they are not
701 yet totally differentiated (Nosil et al., 2009; Wu, 2001). Since scent is believed to be a major
702 trait in pollinator attraction in the fig-wasp mutualism (Hossaert-McKey et al., 2016; Proffitt
703 et al., 2009), ecological isolation due to pollinator preference for each subspecies may
704 eventually lead to the formation of fully separate fig species. The *F. itoana* complex
705 represents an advanced stage in the speciation process where all three species are pollinated
706 by a corresponding wasp species and all three pairs are separated parapatrically following a
707 lowland (*F. itoana*), mid-elevation (*F. sp.* “IMI”) and highland (*F. microdictya*) distribution.
708 In this case, the formation of new fig species potentially restored the classic one-to-one
709 species specificity of such specialized mutualisms.

710 **4.4 Restricted dispersal of both pollen and seeds as promoters of plant speciation**

711 Besides pollen movement, in our system restricted to pollinating fig wasps, a key mechanism
712 maintaining plant genetic connectivity is the movement of seeds across the landscape. Our
713 focal species have a variety of fruit syndromes (Table 1) which suggest different seed
714 dispersing guilds (Lomáscolo, Levey, Kimball, Bolker, & Alborn, 2010; Shanahan, 2000;
715 Shanahan, So, Compton, & Corlett, 2001). Seed dispersal by birds may have a different effect
716 depending on the bird species. Previous bird surveys at the Mt. Wilhelm elevational gradient
717 have demonstrated that bird communities change with elevation and only a few species are
718 found throughout the entire gradient (Marki et al., 2016; Sam & Koane, 2014; Sam et al.,
719 2017). Similarly, fruit bats have been shown to disperse fig seeds (Shilton, Altringham,
720 Compton, & Whittaker, 1999; Thornton et al., 2001), but, the range over which they disperse
721 differs according to species. Bat abundance decreases sharply with elevation at our transect
722 (P. Amick, unpublished data) suggesting that dispersal by bats may be restricted to lower

723 elevations, while dispersal by small understory animals, such as bandicoots (M.S. pers. obs.)
724 is restricted to their home ranges. Studies in *Prunus mahaleb* L. (Rosaceae) found different
725 relative contributions of seed dispersers to genetic connectivity in the studied population
726 between open and closed microhabitats. Small birds were shown to be responsible for short-
727 distance dispersal while larger birds and mammals account for long-distance dispersal
728 demonstrating that different frugivores contribute differently to seed-mediated gene flow
729 (Jordano, Garcia, Godoy, & Garcia-Castano, 2007). It has been suggested that limited seed
730 dispersal in turn limits pollen dispersal due to higher local tree density further isolating
731 already diverging populations (Hardy et al., 2006). We propose that the next step to better
732 understand the drivers of fig population structure and speciation could be to couple our
733 results with collecting information on seed dispersers and the distances at which they are able
734 to disperse seeds.

735 **4.5 Barriers to gene flow in continuous populations**

736 Speciation can unfold across environmental gradients, even in the absence of discrete
737 barriers. Gene flow along a gradient is often mediated by the interplay of biotic and abiotic
738 factors, which may eventually lead to speciation. For instance, continuous populations of
739 *Anthoxantum odoratum* L. (Poaceae) growing along a pollution gradient across a heavy metal
740 mine boundary experience limitations to gene flow, particularly at the contact zone, through
741 differences in flowering time (Antonovics, 2006). These phenological differences promote
742 assortative mating between metal tolerant and non-tolerant plants, effectively maintaining
743 genetic divergence (Antonovics, 2006). Animal pollination can further reinforce these
744 barriers promoting local adaptation and avoid swamping of maladapted genes (Kirkpatrick &
745 Barton, 1997). This can be seen in the insect pollinated alpine grass *Poa hiemata* Vickery
746 (Poaceae) in which flowering time differs between highland and lowland populations, leading
747 to reduced opportunities for pollinators, causing genetic structuring between elevations

748 (Byars et al., 2009). Even in plants pollinated by a wide range of insects, genetic structuring
749 between lowland and highland species has been described and attributed, in part, to effective
750 pollinator availability at different elevations (Reis et al., 2015). Feedbacks, whereby insects
751 become adapted to local conditions and limit the flow of maladapted genotypes between plant
752 individuals, may result in even stronger barriers to gene flow, and enhance the rates of
753 diversification (Armbruster & Muchhala, 2009). Specialised pollination mutualisms offer an
754 even more tractable system to measure the influence of biotic and abiotic mechanisms
755 promoting plant divergence. In the case of *Yucca brevifolia*, the two varieties studied by
756 Godsoe *et. al.* (2009) at different geographic distributions appear to be the result of co-
757 evolution with their corresponding moth pollinators rather than abiotic conditions (Godsoe et
758 al., 2009). Our results for figs and their pollinating wasps extend this growing spectrum of
759 examples. They also demonstrate how biotic interactions along the continuum of
760 environmental conditions (temperature, productivity, changing vegetation types, etc.) can
761 result in speciation.

762 **4.6 Conclusion**

763 Figs and wasps present a remarkable system with to study co-speciation, from the early
764 phases of genetic differentiation to the establishment of gene flow barriers. Our results
765 demonstrate that mutualistic interactions, often implicated in speciation bursts and the
766 generation of new diversity (Schemske, Mittelbach, Cornell, Sobel, & Roy, 2009), tend to
767 break down occasionally. We also demonstrate the evolutionary consequences of the
768 breakdowns in mutualistic interactions, and reveal how they reciprocally foster co-speciation
769 across the interacting partners. Knowing which of the previously reported breakdowns
770 represent an intermediate step in co-speciation and which an adverse side effect of other
771 perturbations (e.g. human-induced changes in the biotic milieu) may be relevant for
772 conservation. Further, we find that speciation tends to be imbalanced, even within the

773 constraints of a tight mutualism. Specifically, pollinators, owing to their faster life-cycles,
774 shorter generation times, and faster genetic differentiation, may speciate significantly faster
775 than their host plants. Together, these findings demonstrate how new diversity of figs and
776 their pollinating wasps is generated within an understudied system famous for its
777 extraordinary diversity, the mountain slopes of tropical New Guinea. They also pave the way
778 toward a more integrated and nuanced understanding of the speciation process involving
779 multiple interacting partners and, consequently, illuminate the mechanisms behind the
780 interplay of species interactions and environmental gradients, whose confluence presumably
781 contributes significantly to the accumulation of species diversity.

782

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1087 **Data availability**

1088 Data used for this study are available from the Dryad Digital Repository [http://](http://10.5061/dryad.5m4dn73)
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1091 BMN169018 (broken down per individual in supplementary material). Demultiplexed
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1093 PRJNA555181.

1094 **Author’s contributions**

1095 V.N., S.T.S. and D.S.V. planned the research with guidance of G.D.W. and B.I. for suitable
1096 focal species. S.T.S., D.S.V., M.S., B.I. and T.K. conducted the fieldwork and managed all
1097 field assistants while not on site. D.S.V. and J.M. conducted and managed all aspects of the
1098 molecular laboratory while C.T.D. assisted with the NGS data management and analysis.
1099 D.S.V., A.M. and S.T.S. analysed the data and wrote the manuscript with substantial input
1100 from all authors. All authors contributed and approved the final version of the manuscript.

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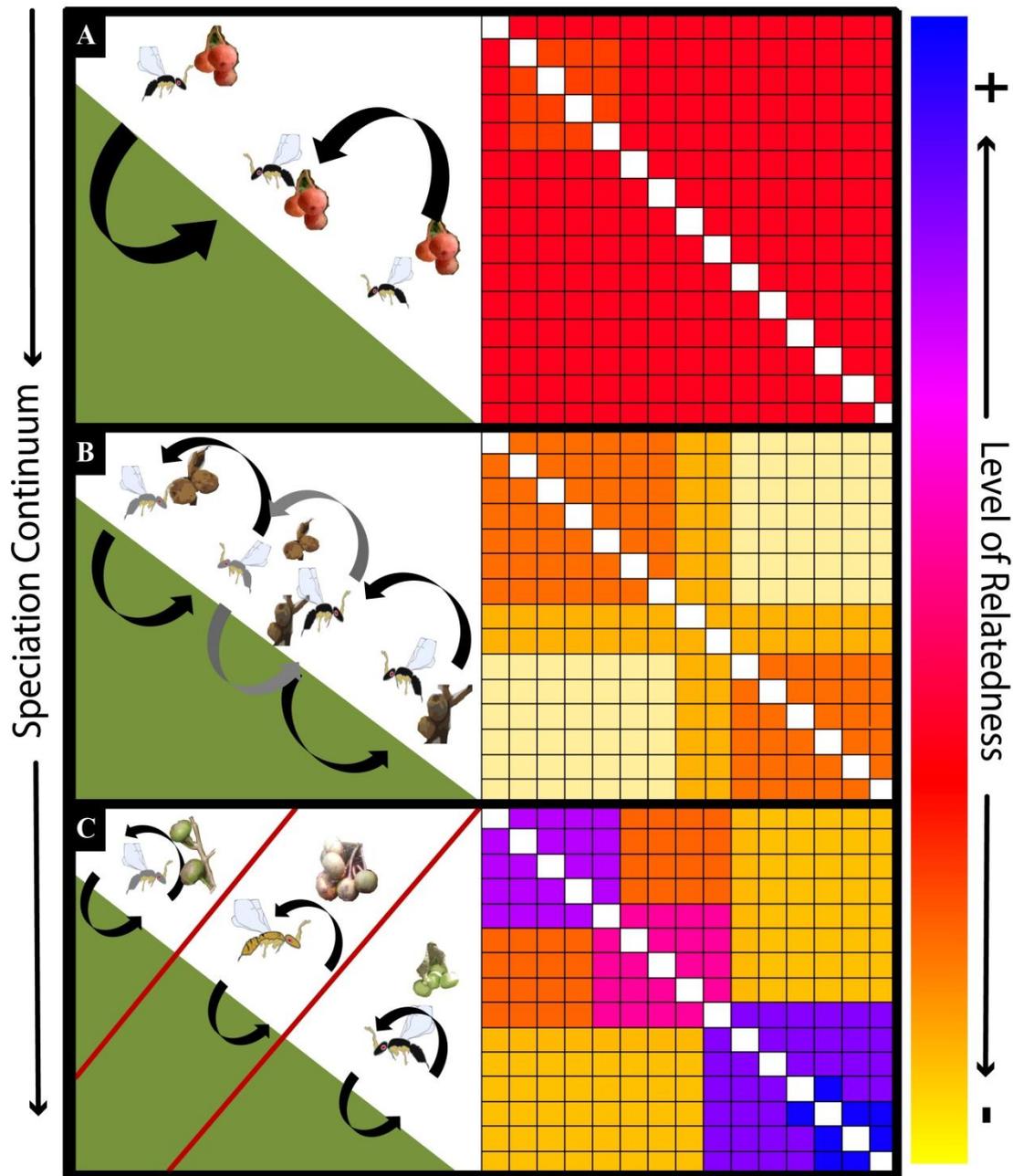
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Table 1. Collection sites, focal *Ficus* species and corresponding wasps collected from each location. Numbers within parenthesis for pollinators indicate which population cluster they belong to as revealed by FINERADSTRUCTURE analysis. Dispersal syndrome is derived from fruit morphology

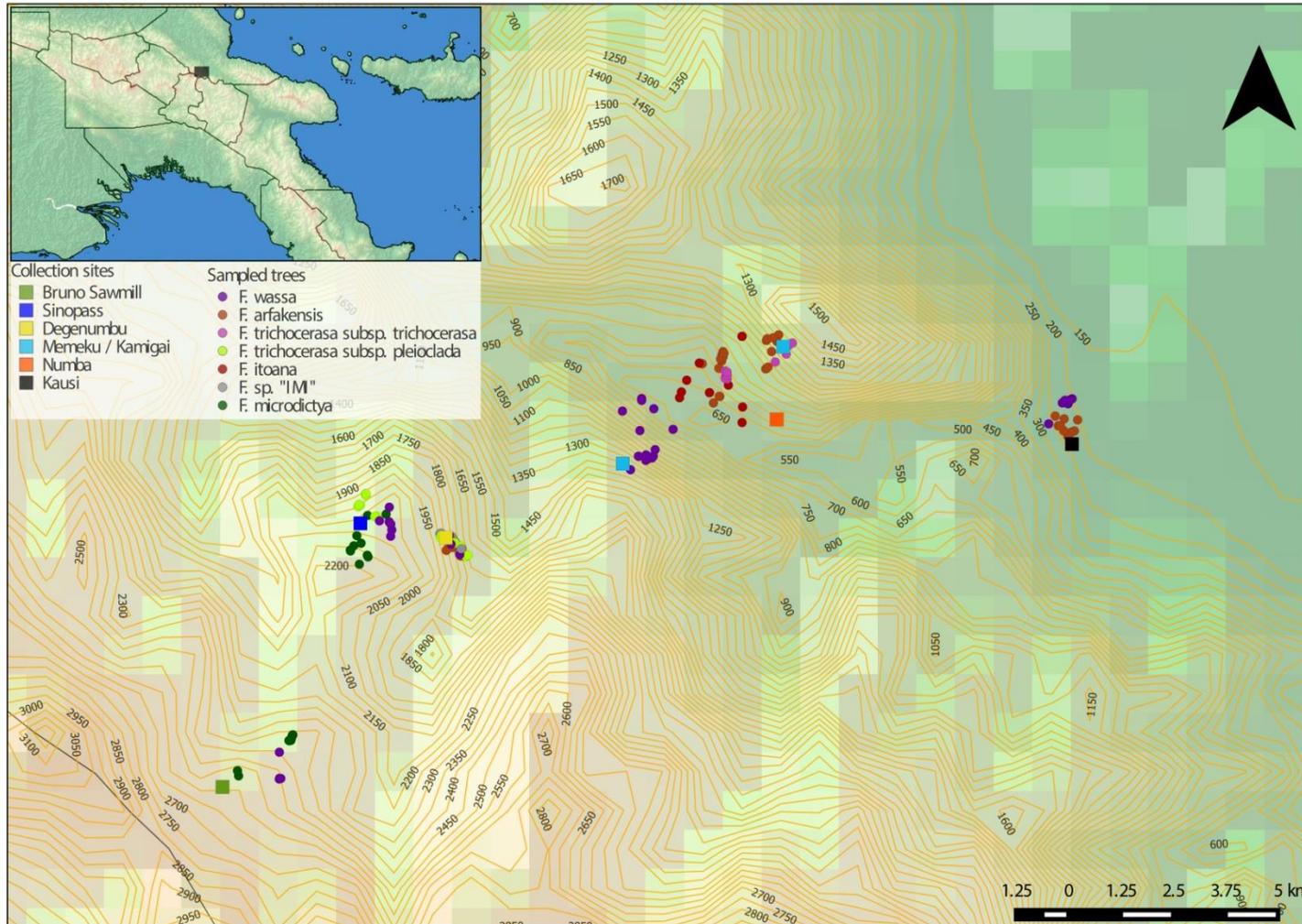
Sampling Site	Elevation	Species	<i>n</i>	Dispersal syndrome	Breeding system	Elevational range*	Pollinator	<i>n</i>
Kausi	200	<i>F. wassa</i>	10	Bird	Dioecious	0 - 3000	<i>Kradibia wassae</i> (1)	17
		<i>F. arfakensis</i>	10	Bird	Dioecious	0 - 1600	<i>Ceratosolen solitarius</i> (1 & 2)	20
		<i>F. itoana</i>	3	Bat	Dioecious	0 - 1800	<i>Ceratosolen armipes</i>	na
Numba	700	<i>F. wassa</i>	15	Bird	Dioecious	0 - 3000	<i>Kradibia wassae</i> (2)	9
		<i>F. arfakensis</i>	9	Bird	Dioecious	0- 1600	<i>Ceratosolen solitarius</i> (1 & 2)	18
		<i>F. trichocerasa</i> <i>subsp. trichocerasa</i>	15	Bat	Dioecious	0 - 1400	<i>Ceratosolen</i> "TRI" (1)	11
		<i>F. itoana</i>	11	Bat	Dioecious	0 - 1800	<i>Ceratosolen armipes</i>	15
Memeku	1200	<i>F. arfakensis</i>	15	Bird	Dioecious	0 - 1600	<i>Ceratosolen solitarius</i> (3)	13
		<i>F. trichocerasa</i> <i>subsp. trichocerasa</i>	7	Bat	Dioecious	0 - 1400	<i>Ceratosolen</i> "TRI" (1)	3
		<i>F. itoana</i>	5	Bat	Dioecious	0 - 1800	<i>Ceratosolen armipes</i>	4
Kamigai	1200	<i>F. wassa</i>	8	Bird	Dioecious	0 - 3000	<i>Kradibia wassae</i> (2)	5
Degenumbu	1700	<i>F. wassa</i>	14	Bird	Dioecious	0 - 3000	<i>Kradibia wassae</i> (3)	4
		<i>F. arfakensis</i>	16	Bird	Dioecious	0 - 1600	<i>Ceratosolen solitarius</i> (4)	11
		<i>F. trichocerasa</i> <i>subsp. pleioclada</i>	14	Bat	Dioecious	1500 - 2600	<i>Ceratosolen</i> "TRI" (2)	18
		<i>F. sp. "IMI"</i>	13	Bandicoot**	Andromonoecious	1700**	<i>Ceratosolen sp. "IMI"</i>	30
Sinopass	2200	<i>F. wassa</i>	14	Bird	Dioecious	0 - 3000	<i>Kradibia wassae</i> (3)	6
		<i>F. trichocerasa</i> <i>subsp. pleioclada</i>	13	Bat	Dioecious	1500 - 2600	<i>Ceratosolen</i> "TRI" (2)	14
		<i>F. microdictya</i>	14	Bird	Monoecious	2000 - 2600	<i>Ceratosolen "kaironkensis"</i>	18
Bruno Sawmill	2700	<i>F. wassa</i>	16	Bird	Dioecious	0 - 3000	<i>Kradibia wassae</i> (3)	3
		<i>F. microdictya</i>	15	Bird	Monoecious	2000 - 2600	<i>Ceratosolen "kaironkensis"</i>	16

* Species distribution taken from Berg & Corner 2005

** personal observation

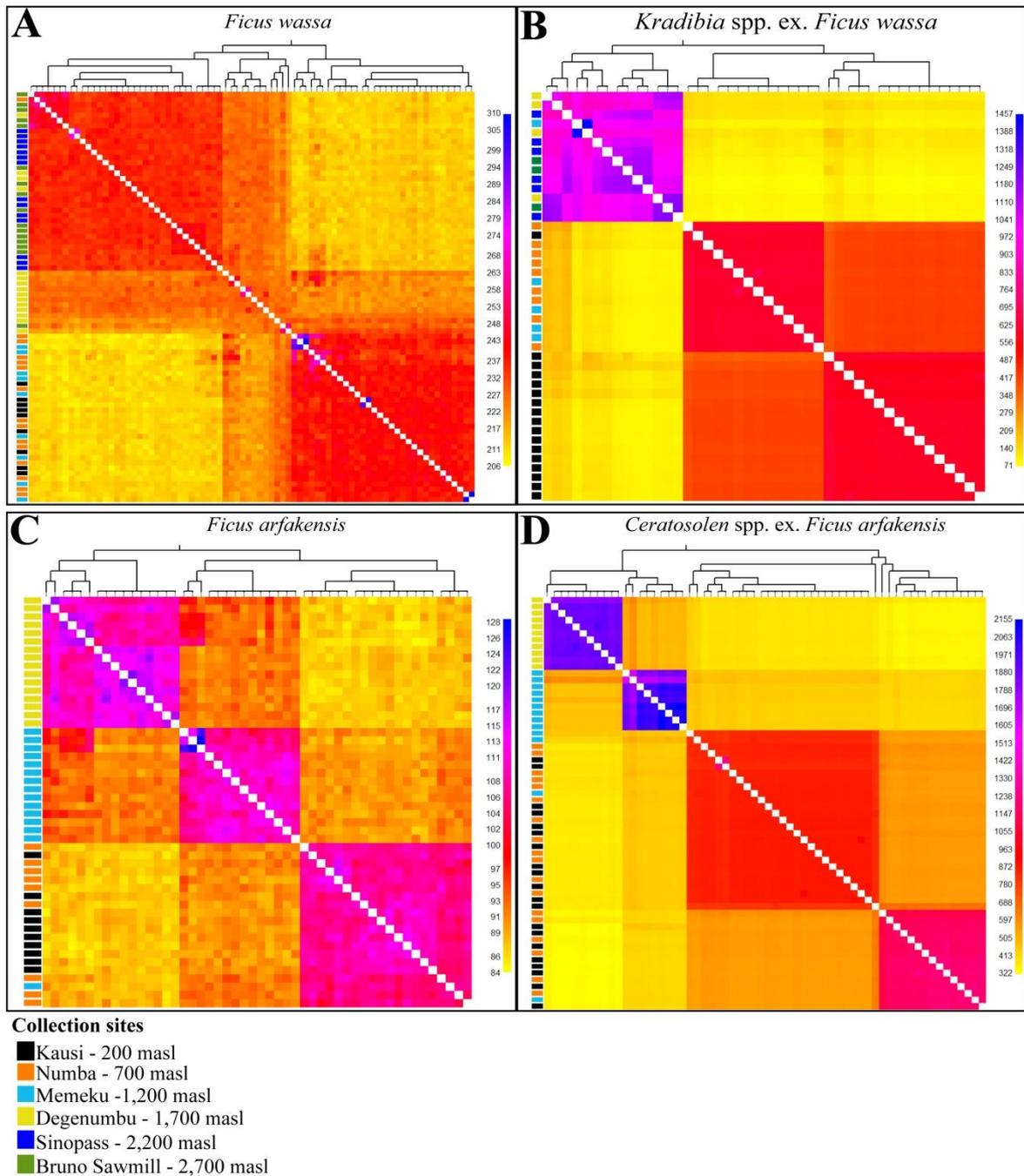


1104
 1105 **Figure 1.** Three stages of the co-speciation process and the genetic patterns expected under
 1106 each of them. Fig wasps in the classic one-to-one scenario (A) maintain genetic connectivity
 1107 and population structure of their fig host across its distribution along an elevational gradient.
 1108 Fig wasps at both extremes of the gradient adapt locally (B) and thus, influence host fig
 1109 population structure; some degree of occasional gene flow at the contact zone due to
 1110 incomplete fig wasp specialization, maintains limited host fig connectivity. Increasing
 1111 reproductive isolation between fig populations lead to their speciation (C), restoring the one-
 1112 to-one scenario for parapatrically distributed fig species. Heat maps mimic the co-ancestry
 1113 matrix, darker colours indicate higher level of relatedness among populations. Black arrows
 1114 indicate pollen flow, gray arrows indicate occasional pollen flow between fig ecotypes or
 1115 subspecies; red lines represent barriers to gene flow.
 1116



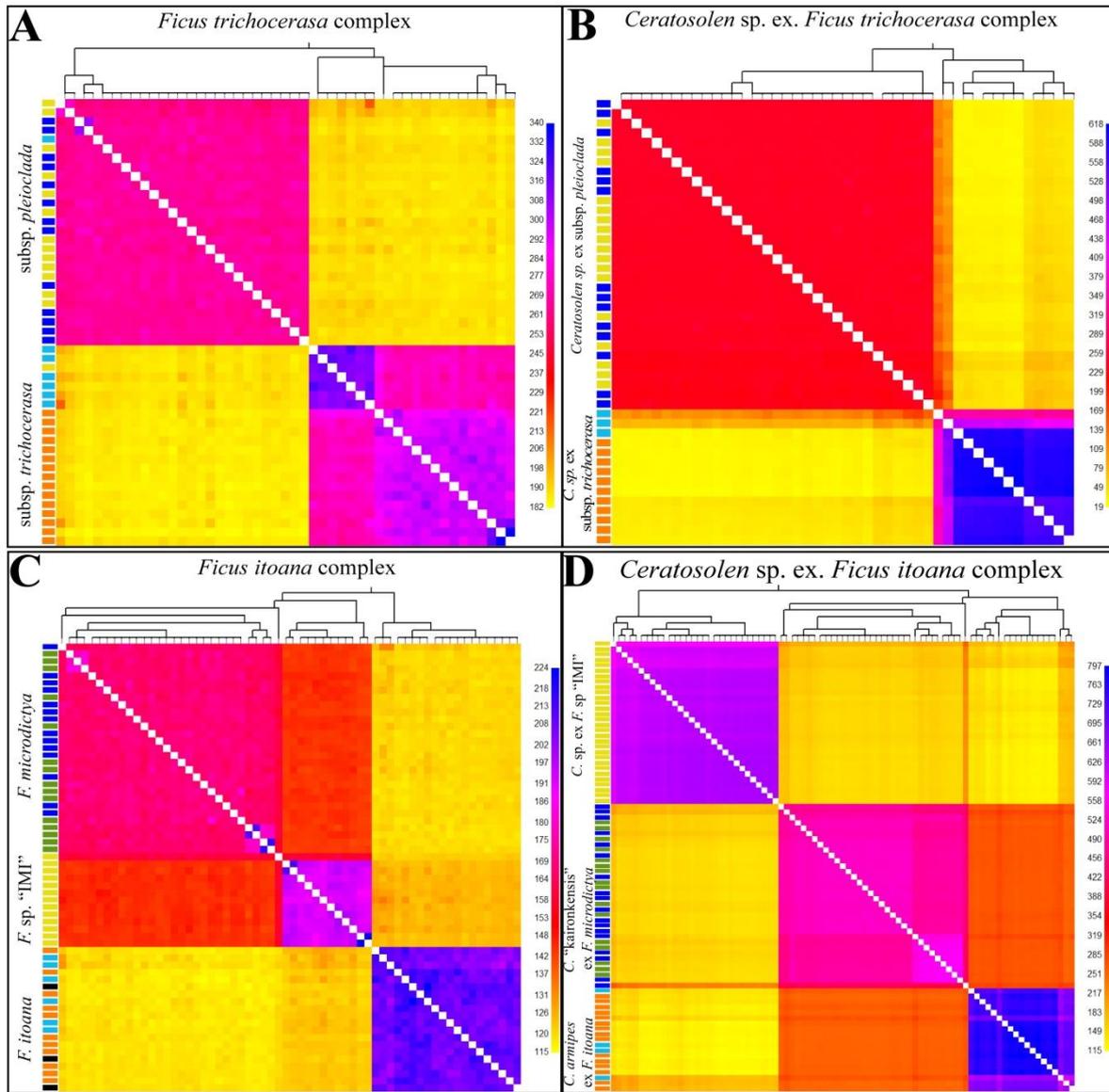
1117
 1118 **Figure 2.** Geographic location of field sites (squares) and sampled trees (circles) along the Mount Wilhelm elevational gradient in Madang
 1119 province, Papua New Guinea (note that there are two sites at the 1,200 m elevation due to a lack of *F. wassa* individuals at the original Memeku
 1120 site).

1121



1122

1123 **Figure 3.** Co-ancestry matrix for figs (A: *F. wassa*; C: *F. arfakensis*) and their pollinating
 1124 fig-wasps (from genera *Kradibia* and *Ceratosolen*: panels B and D respectively). The results
 1125 show substantial population structure among the figs. They also reveal multiple wasp species
 1126 responsible for pollination of the different fig populations. Values on the scale bar to the right
 1127 of each matrix represent the estimated level of co-ancestry based on shared RAD loci.
 1128 Highest levels of relatedness are indicated in dark blue. Lowest values are indicated in
 1129 yellow. The population tree shows coalescent relationships between the populations, based on
 1130 their inferred co-ancestry. Coloured boxes on the left indicate the collection site for each
 1131 individual (legend on the bottom left).

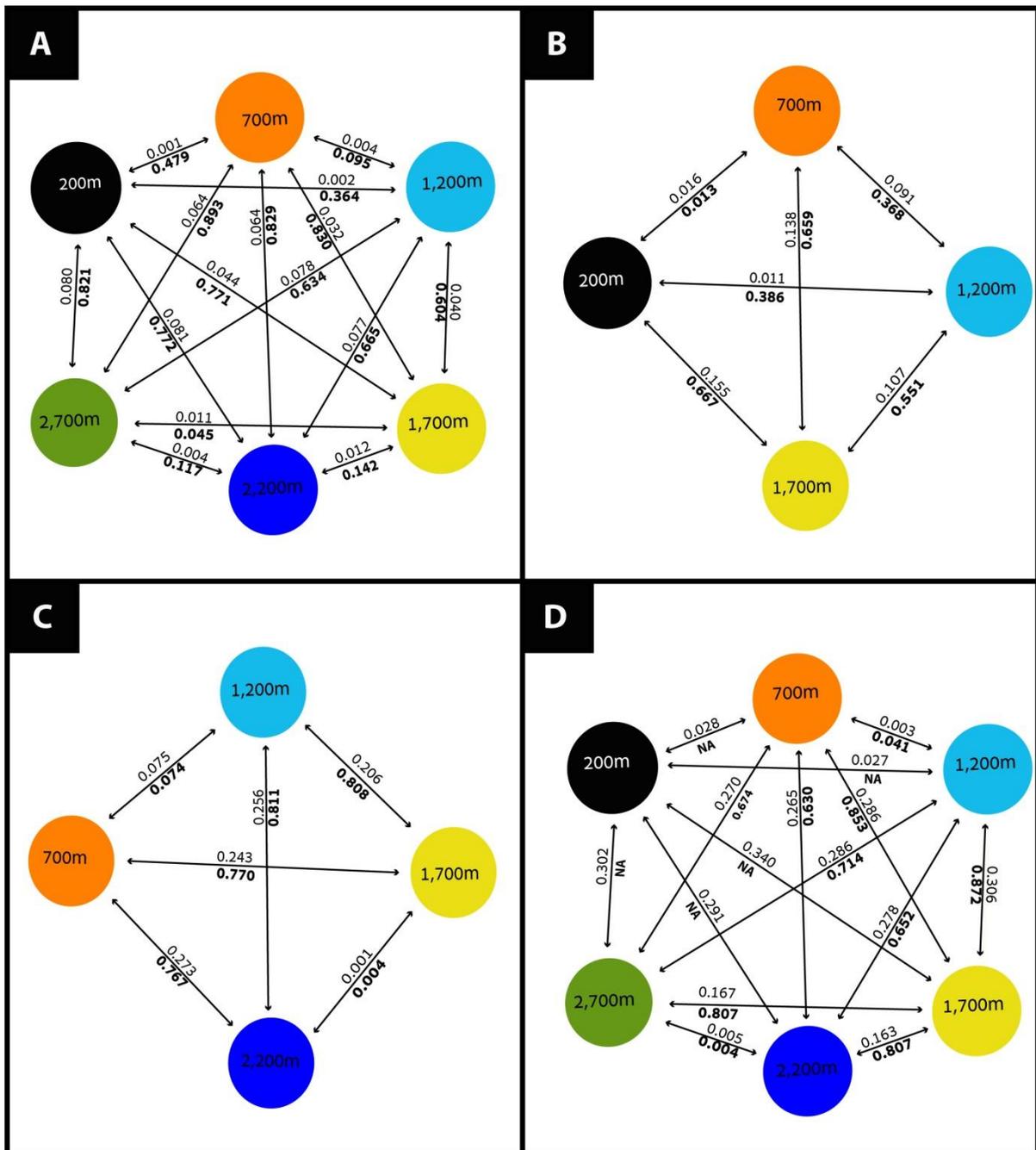


Collection sites

- Kausi - 200 masl
- Numba - 700 masl
- Memeku - 1,200 masl
- Degenumbu - 1,700 masl
- Sinopass - 2,200 masl
- Bruno Sawmill - 2,700 masl

1132

1133 **Figure 4.** Co-ancestry matrix for figs (A: *F. trichocerasa*; C: *F. itoana* complex) and their
 1134 pollinating fig-wasps (panels B and D respectively). The results show clear clustering by
 1135 (sub)species for both the figs and the wasps except for *F. trichocerasa* pollinators from the
 1136 mid-elevations (1,200 – 1,700 m) where both species appear to converge in a contact zone.
 1137 Values on the scale bar to the right of each matrix represent the estimated level of co-ancestry
 1138 based on shared RAD loci. Highest levels of relatedness are indicated in dark blue. Lowest
 1139 values are indicated in yellow. The population tree shows coalescent relationships between
 1140 the populations, based on their inferred co-ancestry. Coloured boxes on the left indicate the
 1141 collection site for each individual (legend on the bottom left).



1142

1143 **Figure 5.** F_{ST} comparisons between sites for *F. wassa* (A), *F. arfakensis* (B), *F. trichocerasa*
 1144 (*F. itoana* complex) (D) and their corresponding pollinating fig-wasps. Mean F_{ST}
 1145 values above the lines connecting the nodes (collection site) are for comparisons between figs
 1146 while below the line in bold are mean F_{ST} values for corresponding species of pollinating
 1147 wasps.