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Why so specious? The role of pollinators and symbionts in plant population structure and speciation along elevational gradients

Ph.D Thesis

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Annotation

This thesis explores the role mutualist pollinators and their symbionts play in the genetic structuring and speciation of their host plants along an elevational gradient in Papua New Guinea. Using the fig and fig-wasp mutualism as a model system, we employed high-throughput sequencing techniques to explore fine-scale population genomics of both fig and wasps along their elevational range. We found there to be clear lowland and highland clustering of tree populations along the gradient, often with a mid-elevation contact zone. In the case of the pollinating wasps, we retrieved the same clustering except in this case, the genetic difference between clusters was high enough as to conceder them as separate species. This result supports evidence from other studies challenging the cospeciation paradigm of one wasp species per fig species. In addition, we explore ecological traits which may promote, or at least, maintain, reproductive isolation between fig (sub)species along with behavioural preference tests from pollinating wasps. In order to further investigate the mechanisms promoting wasp speciation along the gradient, we describe *Wolbachia* infection status as well as strain type. *Wolbachia*-induced cytoplasmic incompatibility (CI) is often invoked as a possible speciation agent since it can rapidly provoke and maintain reproductive isolation between otherwise freely interbreeding insect populations. Finally, we explore non-pollinating fig wasp (NPFW) diversity along the gradient for a subset of our focal species. Our study reveals that there is a tight relationship between NPFW diversity and host species, and a mid-elevation peak.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 7th of March, 2019

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Daniel Souto-Vilarós

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List of papers and authors' contribution

The thesis is based on the following papers:

I. Segar, S.T., Volf, M., Zima Jr., J., Isua, B., Sisol, M., Sam, L., Sam, K., **Souto-Vilarós, D.** & Novotny, V. 2017. Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus*. Journal of Evolutionary Biology, 30(3), 512-523. https://doi.org/10.1111/jeb.13020
[IF: 2.53]
[STS, MV & DSV collected data in the field with assistance from BI and MS and guidance for suitable species from LS and KS. MV, JZ & DSV performed lab work and analyzed microsatellite data. STS wrote the manuscript with substantial input from all authors.]

II. **Souto-Vilarós, D.,** Machac, A., Michalek, J., Darwell, C.T., Sisol, M., Kuyaiva, T., Isua, B., Weiblen, G.D., Novotny, V. & Segar, S.T. 2019 Faster speciation of fig-wasps than their host figs leads to decoupled speciation dynamics. (Manuscript)
[VN, STS & DSV planned the research with guidance of GDW and BI for suitable focal species. STS, DSV, MS, BI & TK conducted the fieldwork and managed all field assistants while not on site. DSV and JM conducted and managed all aspects of the molecular laboratory while CTD assisted with the NGS data management and analysis. DSV, AM and STS analysed the data and wrote the manuscript with substantial input from all authors.]

III. **Souto-Vilarós, D.,** Proffit, M., Buatois, B., Rindos, M., Sisol, M, Kuyaiva, T., Isua, B., Michalek, J., Darwell, C.T., Hossaert-McKey, M., Weiblen, G.D., Novotny, V. & Segar, S.T. 2018. Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. Journal of Ecology, 106(6), 2256-2273. https://doi.org/10.1111/1365-2745.12995
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GDW suggested suitable species for the study. DSV & M.R. designed the Y-tube experiment and photographed the specimens. DSV, MS, BI and TK conducted fieldwork, with initial assistance from STS. MP, BB and MH-M assisted with the GCMS and volatile analysis and all aspects of sample collection in the field. CTD and JM assisted with NGS data management and phylogenetic analysis. DSV analysed the data and wrote the manuscript with substantial input from all authors.]

IV. **Souto-Vilarós, D.**, Darwell, C.T., Michalek, J., Sisol, M., Isua, B., Kuyaiva, T., Weiblen, G.D., Novotny, V. & Segar, S.T. 2019. Non-random and predictable distribution of *Wolbachia* strains along an elevational gradient in Papua New Guinea. (Manuscript)

[VN, STS, & DSV planned the research and provided input at all stages. GDW and BI suggested suitable species for the study. DSV, MS & TK conducted and managed all fieldwork aspects with initial assistance from STS. JM conducted and managed all aspects of the molecular laboratory.CTD, STS & DSV analysed and interpreted the results. DSV wrote the manuscript with substantial help from all authors]

V. **Souto-Vilarós, D.**, Houadria, M., Michalek, J., Sisol, M., Isua, B., Kuyaiva, T., Weiblen, G.D., Novotny, V. & Segar, S.T. 2019. Contrasting patterns of fig was communities along Mt. Wilhelm, Papua New Guinea. (Manuscript)

[VN, STS, & DSV planned the research and provided input at all stages. GDW and BI suggested suitable species for the study. DSV, MS & TK conducted and managed all fieldwork aspects with initial assistance from STS. JM assisted with data analysis and management of fig dissections. DSV & MH analysed and interpreted the results. DSV wrote the manuscript with substantial help from all authors]

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Co-author agreement

Simon T. Segar, the supervisor of this Ph.D thesis and co-author of chapter I, fully acknowledges the major contribution of Daniel Souto-Vilarós to this manuscript.

Simon T. Segar, Ph.D

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Introduction

The origin of species has been a central question in biology even before Darwin's seminal book on the subject (1859). Since then, there has been much debate on the definition of a species, and such definitions have even posed fundamental barriers to the advancement of our knowledge on the planet's biodiversity (Rieseberg & Willis 2007). Nowadays, speciation is regarded as a continuous process where species found today represent a 'snap-shot' of a very slow process rather than the end-product of an event (Coyne & Orr 2004). In general, speciation involves an initial genetic differentiation among populations of the same species, followed by reproductive isolation, resulting in reproductive incompatibility between individuals from diverged populations (Rieseberg & Willis 2007; Givnish 2010). Finally, the rise of ecological differences between these now closely related species allows for long-term coexistence, increasing the overall species pool.

The presence of geographic barriers is considered an initial step towards genetic differentiation as these pose an obstacle to dispersal and disrupt gene flow patterns, spatially isolating populations and eventually leading to their speciation (Mayr 1970; Coyne & Orr 2004; Coyne 2011; Ferris, Sexton & Willis 2014). Geographic distance appears to play an important role in restricting gene flow even between populations of the same species (Ferris *et al.* 2014; Reis *et al.* 2015). The reduction of gene flow represents a critical step not only in allopatric speciation, but also in parapatric and sympatric models and can be studied through patterns of genetic structure found in present day populations (Kiester, Lande & Schemske 1984; Coyne & Orr 2004; Givnish 2010; Coyne 2011; Ferris *et al.* 2014). Even without complete isolation, gene flow patterns may facilitate the accumulation of genetic differences, widening the gap between populations, thus allowing adaptive traits to arise. This local adaptation is likely to reinforce initial barriers to gene flow, and so facilitate ecological speciation (Kiester *et al.*

1984; Givnish 2010; Andrew & Rieseberg 2013; Van der Niet, Peakall & Johnson 2014; Seehausen *et al.* 2014; Marques *et al.* 2016; Souto-Vilarós *et al.* 2018a). For instance, parasitic fig-wasps of the genus *Apocryptophagus* seem to have diverged in sympatry (within the same fig host) by having different ovipositor lengths. This length variation allows these two different wasp species to oviposit in the same fig, at different stages of its development (Weiblen & Bush 2002). Insect-plant interactions offer an ideal system to study the role of insects in plant diversification and vice versa, whether through pollination, herbivory or parasitism.

Pollinator-mediated plant diversification

Outnumbered only by insect species, flowering plants began to diversify relatively recently and display spectacular examples of adaptive radiation, co-evolution, and myriad of speciation mechanisms acting in synchrony (Kiester *et al.* 1984; Rieseberg & Willis 2007; Givnish 2010; Armbruster, Shi & Huang 2014; Givnish *et al.* 2015). Plants vary dramatically in mating systems, chromosome number (ploidy), seed and pollen dispersal and life history, all of which contribute to genetic connectivity and differentiation, and in many cases act as mechanisms that promote reproductive isolation (Kiester *et al.* 1984; Rieseberg & Willis 2007; Ferris *et al.* 2014; Van der Niet *et al.* 2014). Since gene flow in most flowering plants is intrinsically linked to their pollination system, angiosperms can be used to study the influence of gene flow in the speciation process, and how differences in pollinator behaviour and morphology may affect genetic connectivity between populations (Kiester *et al.* 1984; Van der Niet *et al.* 2014; Givnish *et al.* 2015). It has even been suggested that geography, pollinator type, and mating system barriers are crucial in promoting isolation between sister species, however, there are few examples providing estimates of such isolation (Rieseberg & Willis 2007; Van der Niet *et al.* 2014; Reis *et al.* 2015; Gervasi & Schiestl 2017).

The idea that "*the various contrivances by which flowers are fertilized by insects*" is a major driver of angiosperm diversity can again be traced back to Darwin (1862). Although his book focused on the fertilization of orchids, Darwin identified several mechanisms of pollinator-mediated selection, and mounting evidence suggests that these mechanisms may be fundamental in the diversification of angiosperms (Kiester *et al.* 1984; Fenster *et al.* 2011; Schiestl & Johnson 2013; Armbruster *et al.* 2014; Van der Niet *et al.* 2014). Flowers attract pollinators using different visual and chemical cues, while pollinators visit flowers to collect pollen, nectar, oils, brooding sites and are even sometimes lured by the flower to perceived mating opportunities (Kiester *et al.* 1984; Renner 2006; Fenster *et al.* 2011; Cruaud *et al.* 2012; Whitehead & Peakall 2014; Sun *et al.* 2015). Since pollinators directly influence a plant's reproductive success (fitness), pollinators are thought to be agents of selection, and ultimately, fundamental players in the speciation process (Kiester *et al.* 1984; Sapir & Armbruster 2010; Cruaud *et al.* 2012; Van der Niet *et al.* 2014; Trunschke, Sletvold & Ågren 2017; Souto-Vilarós *et al.* 2018b). In fact, pollinator-mediated reproductive isolation has been observed in several angiosperm lineages such as *Mimulus* (Phrymaceae; Schemske & Bradshaw, 1999), *Aquilegia* (Ranunculaceae; Whittall & Hodges, 2007), *Penstemon* (Plantaginaceae; Wilson, Wolfe, Armbruster, & Thomson, 2007), not only supporting the association between pollinator groups and floral traits (so called pollination syndromes), but also that plant-pollinator mutualisms may promote speciation. In fact, such intricate relationships between pollinators and plants result in some cases of co-evolution such as orchids and orchid bees (Jersáková, Johnson & Kindlmann 2006), figs and fig-wasps (Machado *et al.* 2005; Cruaud *et al.* 2012), yucca and yucca-moths (Smith *et al.* 2008) and passion flowers and hummingbirds (Abrahamczyk, Souto-Vilarós & Renner 2014).

Van der Niet & Johnson (2012) carried out an extensive review on pollinator-mediated diversification which included approximately 1.4% of all extant angiosperms (~3,500 species). The authors found that plant lineages with frequent pollinator shifts are characterized by specialized morphological features. This agrees with the general assumption that the more generalized the pollination system is, the more likely that changes in pollinators are rather subtle and less important in the role of diversification (Kiester *et al.* 1984; Van der Niet & Johnson 2012). Van der Niet *et al.* (2014) proposed a model for assessing the likelihood of evolution of pollinator isolation. Firstly, pollinator specialization determines whether or not there will be an overlap between a novel pollination system and the original. The more specialized the system is, the higher the likelihood that assortative mating will occur in sympatry. Second, diverging traits which focus on pollinator attraction could quickly cause ethological isolation, a clear example of this occurs in sexually deceptive orchids, where minor chemical changes can cause complete pollinator shift followed by assortative mating (Jersáková *et al.* 2006). Third, a change to different pollinator group (i.e. moth to hummingbird shift) causes pollinator isolation when compared to flowers which rely on the same pollinator group (i.e. only moths). Finally, geographic context may play a major role in the evolution of pollinator-mediated reproductive isolation. In general, for flowers with pollinator-specialized morphology, if pollinator shifts occur in sympatry, the likelihood that pollinator isolation may evolve is greatest simply because those shifts which lead to reproductive isolation are the only ones which can remain in the face of gene flow (Van der Niet *et al.* 2014). Depending on these tree factors, pollinator-mediated reproductive isolation may or may not evolve over large geographic distances.

Grant & Grant (1965) suggested that geographic variation in the pollinator 'climate' (that is, changes in local abundance and distribution of pollinators) is likely to be a primary driver of plant diversification. Indeed, it has been demonstrated in *Bathysa australis* (Rubiaceae) in Brazil, that highland and lowland populations along an altitudinal gradient show more genetic differentiation between populations than within populations (Reis *et al.* 2015). This population structure suggests that even plants with generalist pollination systems are subject to pollinator abundance and dispersal abilities. Similarly, Segar *et al.* (2017) in a study of two *Ficus* (Moraceae) species along the Mount Wilhelm elevational gradient in Papua New Guinea (PNG) revealed distinct highland and lowland clusters. The fig and fig-wasp mutualism is amongst the most specialized pollination systems, and here too, fig population structure may be subject to the dispersal abilities of these small insects. The development of reproductive isolation remains as one of the least well understood steps in ecological speciation, but there is enough evidence to suggest that pollinator abundance in an environmental mosaic may promote such isolation and lead to speciation (Rieseberg & Willis 2007; Van der Niet & Johnson 2012; Nosil 2012).

Certainly, plant diversification cannot be attributed exclusively to pollinator-driven processes and as such, should be analysed in a rather comprehensive way, including alternative biotic and abiotic factors. Mountains have long been regarded as speciation 'pumps' due to varying environmental conditions over short vertical distances (Bachman *et al.* 2004; Körner 2007). It is evident that environmental conditions across altitudinal gradients change drastically, and biological diversity is well adapted to particular conditions along the gradient. Increased diversity along altitudinal gradients is often thought of as a consequence of parapatric speciation, where species ranges overlap but gene flow is reduced due to ecological and/or environmental factors (Coyne & Orr 2004; Gavrilets

2004). Such landscape heterogeneity has been viewed as one of the most important reproductive barriers in plants (Rieseberg & Willis 2007). More distant populations are expected to have less genetic exchange as a function of limited pollen or seed dispersal across space, thus generating spatial genetic structure. In view of this, pollen and seed vectors directly influence genetic structure of plant populations (Reis *et al.* 2015). Integrating community trait information and population genetic data of populations along a 'speciation continuum' (i.e. species with wide ranging distributions, subspecies complexes and sister species) could elucidate patterns of gene flow, local adaptation, and population structure. In mountains, elevational gradients may act as barriers to gene flow since such gradients may prevent movement of pollinators and/or seed dispersers together with other abiotic factors such as temperature, humidity, solar radiation, precipitation etc. (Körner 2007).

New Guinea as a hotspot for biodiversity

As one of the most biologically diverse areas of the world, the island of New Guinea has an estimated 6% of the world's plant and animal species distributed in roughly 1% of the earth's total land area (Barrows *et al.* 2009). Its impressive diversity and high levels of endemism are attributed to a wide range of different habitats, from seasonal savannahs and lowland rainforests to alpine areas along high mountain ranges and diverse climatic conditions (Mittermeier *et al.* 1998; Novotny *et al.* 2010; Toussaint *et al.* 2014). The island is at the intersection of five tectonic plates giving rise to its ever-changing topology. Besides its impressive diversity, the New Guinean highland cordillera is one of the largest and most remote highland regions in the world, with extensive spans of virtually untouched tropical forest (Toussaint *et al.* 2014). This cordillera is made up several mountain ranges running from east to west with mountains over 4,800m in West Papua (Indonesia) and Mount Wilhelm (4,509 meters) in the Bismarck

Range, PNG. Botanical surveys estimate over 15,000 vascular plant species with the most varieties of sugarcane, orchids and figs found in the world (Barrows *et al.* 2009). Similarly, the few insect groups which have been studied in detail are extremely diverse, and total estimates for the island approximate 300,000 insect species (for a recent project inventorying insect diversity along Mt. Wilhelm see Robillard, Legendre, Villemant, & Leponce, 2016). Due to its biological richness and diverse landscape, New Guinea and the surrounding archipelago have served as a natural laboratory for the study of speciation, community assemblage, and biogeography (e.g. Diamond, 1973; Novotny et al., 2005; Plowman et al., 2017; Sam, Koane, Jeppy, Sykorova, & Novotny, 2017).

All collections used for the present project came from the Mt. Wilhelm elevational gradient located on its north-east slope (-5.44, 145.20; -5.47, 145.03). The gradient ranges from 200 m above sea level (a.s.l.) up to 3,700 m a.s.l. Study sites are regularly spaced every 500 m in elevation and habitats range from lowland alluvial forest in the floodplains of the Ramu River to upper montane forests and grassland at its summit. The gradient experiences steady climatic changes uphill with an average temperature of 27.4˚C in the lowlands and decreasing ~0.54˚C with every 100 m of elevation down to 8.37˚C at the timberline. Mean annual precipitation is approximately 3,288 mm in the lowlands and up to 4,400 mm at the 3,700 m a.s.l. site. Forest type, typical vegetation composition and climatic conditions are described in detail in Paijmans (1976) and McAlpine, Keig, Falls, & CSIRO (1983). As the focal species of this thesis are in the genus *Ficus*, limited in distribution from 200m to 2,700m a.s.l., collections were restricted to six sites along the gradient.

The figs and the fig wasps

Of all specialized plant-pollinator mutualisms, *Ficus* and their corresponding pollinating wasps are one of the most striking examples of co-diversification (Kiester *et al.* 1984; Cruaud *et al.* 2012). With over 750 fig species, the pollination mutualism is such an intimate affair that, generally speaking, a single species of chalcid wasp (Agaonidae) is responsible for the pollination of a single *Ficus* species and both depend entirely on each other for reproduction (Cruaud et al., 2012; Weiblen, 2002; see Cook & Segar, 2010 for a review of the exceptions). The pollination of figs, and the life-cycle of these wasps has been extensively described (Galil & Eisikowitch 1971; Janzen & Agaonidae 1979) but to briefly summarize, the wasp's life begins and ends within the syconia (enclosed inflorescences, commonly known as 'figs') of its host *Ficus*. After developing, wasps mate within the fig and the wingless male wasps proceed to chew a tunnel from which gravid, and pollen laden female wasps emerge in search for another receptive fig in which to lay her eggs. Species specific volatile cues attract her to a receptive fig, and thanks to specialized head morphology she crawls inside through the ostiole (a tight, bract-covered opening at the apex of the syconia); this is typically a fatal decision, since after passage through the ostiole, wasps are unable to exit the fig. After gaining access, wasps oviposit down the styles of the flowers within, at the same time actively or passively depositing pollen on to stigmas. In the case of monoecious trees, some flowers will become seeds, while the others will house the wasp's larvae; which flowers become which depend on wasp ovipositor and floral style length compatibility (Weiblen 2004). In functionally dioecious figs, the life-cycle is identical except that foundresses either enter all female florets, in which case, due to a mismatch in ovipositor and style length, wasps only pollinate the flowers producing seeds, but are unable to lay their eggs. Functionally male figs are located in different trees in which the wasps enter and lay eggs in the shorter-styled flowers thus serving as a wasp

nursery from which emerging wasps collect pollen and the cycle begins again (Hossaert-McKey *et al.* 2016).

Since the foundress decision to enter a fig is irreversible, there should be strong selection for them to make the correct choice. Though beneficial for the fig, entering a female fig represents the ultimate fitness cost to the wasp, no offspring. From the wasps' perspective, selection should favour avoidance of female figs, although this would be detrimental to the fig. Similarly, from the fig's perspective, selection should favour female figs to become more attractive to wasps in order to produce more seeds, similarly destabilizing the mutualism. This suggests strong volatile mimicry between both male and female trees in order to maintain the stability of dioecious systems in figs. Similarly, it is thought that volatile signatures are responsible for maintaining species specificity within the system since wasps entering wrong host trees would be detrimental for both parties (Janzen & Agaonidae 1979; Weiblen 2002; Hossaert-McKey *et al.* 2016). Finally, in order to sustain wasp populations, fig trees must produce fruits year-round. This continuous fruit production consequently feeds large populations of frugivores (up to 1,200 recorded species), and the trees themselves host many herbivorous insects and associated predators, making *Ficus* a truly keystone genus in tropical forests worldwide (Shanahan *et al.* 2001; Novotny *et al.* 2010).

The largest molecular phylogenetic study to date, performed on both figs and pollinating fig wasps, dated the co-diversification event at ~75 million years ago, making it the first significant case of long-term co-diversification of an insect-plant association (Cruaud *et al.* 2012). Unlike other documented cases of mutualisms, where speciation seems to be driven mostly by host-shifts, phylogenetic congruence in several of the fig and wasp lineages support the idea of co-speciation (Jousselin *et al.* 2008).

Furthermore, studies have shown that both plant volatile cues and morphological fit between wasps and figs may be key traits for co-adaptation, further explaining this pattern (Cruaud *et al.* 2012; Souto-Vilarós *et al.* 2018b). Janzen (1979) attempted to explain the astounding diversity of figs suggesting that fig and fig-wasp speciation must occur in allopatry, since otherwise there would be no pool of generalist pollinating wasps to visit mutant figs. On the other hand, Ramirez (1970) suggested the opposite, where evolution of mechanical and chemical barriers between different populations, coupled with adaptations of pollinators, may contribute to the speciation of both interactants, with each fig population acting as "*an island in space and time*" (Ramírez 1970). Similarly, based on mathematical modelling, Kiester *et al.* (1984) suggest that any small pool of figs which differs sufficiently from neighbouring populations to restrict gene flow between them, could co-evolve with their locally adapted pollinating wasps and promote diversification. Although small and short lived, these tiny wasps are effective pollinators over hundreds of kilometres in continuous habitats (Ahmed *et al.* 2009; Kobmoo *et al.* 2010; Liu *et al.* 2015), however, discrete geographic barriers seem to limit their dispersal (Sutton, Riegler & Cook 2016). Similarly, long distance dispersal is particularly true for monoecious trees (approximately half of all *Ficus* species) while wasps associated with dioecious and under-canopy figs do not seem to disperse over such distances and appear to be clustered into local populations (Dev *et al.* 2011; Wachi *et al.* 2016). Such localized tree and wasp populations, coupled with the temperature sensitivity of fig wasps (Jevanandam, Goh & Corlett 2013) could potentially set the stage for population divergence and speciation along environmental (i.e. elevation) gradients.

Wolbachia and the fig-wasp

An estimated 40% of insect species are infected with the endobacterium *Wolbachia;* among other consequences, one of the most important effects of infection is the imposition of cytoplasmic incompatibility (CI) on their hosts (Stouthamer, Breeuwer & Hurst 1999; Shoemaker *et al.* 2002; Engelstädter & Telschow 2009; Yang *et al.* 2012). CI causes mortality in the offspring of incompatible matings through unidirectional (infected vs. non-infected matings) or bidirectional (individuals infected with different strains) incompatibility. *Wolbachia* is normally transmitted through the egg cytoplasm and so is maternally inherited. Its reproductive effects such as male killing and feminization or distorting the offspring sex-ratio to in favour of females allows it to spread through a population (Shoemaker *et al.* 2002; Engelstädter & Telschow 2009). Infected females have a selective advantage since they can mate with both infected and uninfected males of a population and so can influence the evolutionary history of its host population (Werren 1997; Rokas 2000; Engelstädter & Telschow 2009). Because CI can rapidly induce complete reproductive isolation between populations with different infection status, *Wolbachia* can lead to, or strengthen genetic divergence and ultimately, speciation. Nevertheless, empirical evidence of how *Wolbachia* acts as a speciation agent is rather limited. One example comes from the *Nasonia* species complex (*N. vitripennis, N. giraulti* and *N. longicornis*). Dated phylogenetic studies date this complex to have arisen ~500,000 years ago and all appear to have speciated in sympatry (sometimes even emerging from the same parasitized host; Werren, 1997). All three species are infected with species-specific *Wolbachia* strains and are unable to hybridize, however, when treated with antibiotics, crosses between *N. vitripennis* and *N. giraulti* produce fertile hybrids demonstrating that bidirectional CI is due to contrasting *Wolbachia* strains (Werren 1997; Bordenstein & Werren 2007). Nevertheless, whether *Wolbachia* is the primary cause for reproductive isolation remains uncertain

but at least in laboratory conditions, *Wolbachia* is in fact responsible for the maintenance of such isolation.

Fig wasps seem an appropriate candidate for studying CI and in general *Wolbachia* infection and transmission since in most cases, figs host pollinating wasps, but also a diverse community of non-pollinating fig wasps (NPFW). Both of these groups have a certain degree of host-specificity, they are phylogenetically and/or ecologically closely related, and unlike other systems, they interact in a closed community within the syconia. Additionally, it has been shown that fig wasps have amongst the highest incidences of *Wolbachia* compared to other insects (Shoemaker *et al.* 2002; Yang *et al.* 2012). Coupled with evidence of multiple pollinator species per fig host, and pollinating wasps visiting more than one fig species (Cook & Rasplus 2003; Cook & Segar 2010), these characteristics make fig wasps ideal candidates for exploring patterns of *Wolbachia* infections and its potential role on their speciation.

Methods and scope of this thesis

Taking these factors into account, beyond serving as a model system for studying co-evolution, *Ficus* and corresponding fig wasps may in fact prove to be a model system for studying pollinator-driven speciation. Pollinator isolation must be taken into account together with other biotic and abiotic isolating mechanisms such as CI-inducing bacteria and environmental gradients. As mentioned before, one of the greatest challenges for studying pollinator-driven speciation is to identify if pollinator-driven divergence can lead to isolation (Van der Niet & Johnson 2012). One approach would be to confirm whether ecotype boundaries, such as morphological and/or chemical variation correspond to barriers to gene flow. The rapid advancement of molecular tools allows us to study evolutionary processes at the genomic scale using non-model organisms in natural populations.

Current methods allow for the discovery of hundreds to thousands of genetic markers for individuals, paving the way for the field of population genomics (Davey & Blaxter 2010; Hohenlohe *et al.* 2010; Russello *et al.* 2015). One such method is Restricted Site-Associated DNA sequencing (RADseq) which can identify thousands of markers randomly distributed along the entire genome, allowing the study population genomics at high resolution (Davey & Blaxter 2010; Davey *et al.* 2011; Russello *et al.* 2015). As with many other tropical genera perhaps due to recent radiation of some lineages within *Ficus* and/or historical hybridization (Cruaud *et al.* 2012), genetic differences between closely related *Ficus* species are hard to detect using traditional molecular markers, thus making it an ideal candidate for using high-throughput sequencing technology. Similarly, volatile compound specificity is well understood across many fig species (Hossaert-McKey *et al.* 2016), however, little is known regarding volatile variation across gradients and between different populations of the same species. Investigating the patterns of *Wolbachia* infections in pollinating wasps along an elevational gradient is also helpful in order to understand the distribution of infection status and strains across different wasp populations and species. As evidence of cryptic pollinator species continues to arise, it will be of great interest to understand the mechanisms which maintain reproductive isolation between these. *Wolbachia* infection, if not a direct agent of speciation, could strengthen the isolating barriers between incipient wasp species.

Focal species

This study focuses on a subset of the ~75 *Ficus* species found along the Mt. Wilhelm gradient, endemic to New Guinea and adjacent islands (Berg & Corner 2005). *Ficus wassa* Roxb. (range: 200masl to 2700masl) and *Ficus arfakensis* King (range: 200masl to 1700masl) are two wide ranging species occurring without any clear morphological variation along their range. Their

pollinating fig-wasps have been described (*Kradibia wassae* Wiebes, and *Ceratosolen solitarius* Wiebes, respectively); however, it is unknown whether or not these species are capable of pollinating figs throughout their host's range. *Ficus trichocerasa* Diels subsp. *trichocerasa* and *F. trichocerasa* subsp. *pleioclada* (range: 200masl to 1200masl and 1700masl to 2700masl respectively) are a species complex with described lowland and highland subspecies. Their pollinating wasp is an undescribed species in the *Ceratosolen* genus, it is unknown if the same species of fig wasp pollinates both fig subspecies. Finally, we focus on a three species complex: *F. itoana* Diels, *F. microdictya* Diels and a third undescribed species, all belonging to the Papuacyse section. *F. itoana* (range: 200masl to 1200masl) and *F. microdictya* (range: 2000masl to 2700masl) appear to be sister species presumably diverging parapatrically with a lowland and highland lineage. *F. microdictya* is one of the few known cases of *Ficus* reversal to its ancestral monoecious state (Weiblen 2004). The pollinating wasps of these species have been described (*Ceratosolen armipes* Wiebes and *"Ceratosolen kaironkensis"* Weiblen [*nom. nud.*], respectively). During the field work of this project, a third undescribed species occurring at the mid-elevation site (Degenumbu, 1,700 m a.s.l.) was collected on the grounds that it seemed to be a hybrid between *F. itoana* and *F. microdictya*. Analysis carried out in this thesis revealed this to be a genetically distinct species pollinated by an undescribed relative of both *C. armipes* and *"C. kaironkensis"* (see chapters II and III).

In **Chapter I** we used microsatellite data to identify barriers to gene flow in two widespread *Ficus* species along the Mt. Wilhelm elevational gradient (*F. arfakensis* and *F. hahliana* Diels). Both species have a relatively wide elevational distribution, and considering the environmental changes experienced at different elevations begs the question of how these changes affect population structure and gene flow patterns. Using 10 polymorphic

microsatellite loci, we demonstrate strong barriers to gene flow between 1,200m and 1,700m a.s.l. This suggests that environmental limitations on pollinator and/or seed dispersers may be responsible for limited gene flow between fig populations. This study served as a premise to explore in further detail population structure and connectivity of more fig species along the transect, as well as their pollinating wasps.

In **Chapter II** we expanded our focal species to include all six species previously described and in order to recover more robust data, we used Nextera-based RAD-seq methods for both figs and wasps. This method allowed us to retrieve a dense single nucleotide polymorphism (SNP) dataset which was used to infer ancestry between samples of each species along the transect. Our results recovered similar patterns to **chapter I,** however, at a much finer scale. We were able to discern fig lowland and highland populations, often with a third cluster at the mid elevations. Similarly, fig wasps clustered with a high degree of shared ancestry along the gradient. Further analysis using fixation index statistics (Fst) revealed that these wasp clusters have very large genetic differentiation, enough to consider them as distinct species. These results suggest that wasps speciate faster than their host figs and so mediate fig population structure.

Chapter III further explores the mechanisms which are responsible for the maintenance of reproductive isolation between fig species. For this chapter we collected and analysed volatile organic compounds (VOCs) from a subset of our focal species. Using Gas Chormatography / Mass Spectrometry (GCMS) analysis we quantified the difference in the scent signatures of a subset of the focal species. We discovered that species indeed vary in their scent profiles, but a certain degree of overlap exists amongst them. Additionally, we found that scent profiles within the same species but at different collection sites vary from each other, however,

whether or not pollinating wasps are able to distinguish between them remains unknown. Additionally, through common 'Y-tube' experiments, we recorded wasp choice by presenting them with receptive figs from their own host species or figs from close relatives in order to observe if scent alone could be used by the wasps to distinguish between fig species. We found that for at least one species, fig wasps are equally attracted to their host fig as well as its sister species. Further morphological analyses of these wasp species revealed that in this case, even if a fig wasp is attracted to the 'wrong' fig species, morphological incompatibilities would prevent the wasp from successfully entering and/or ovipoisting in the flowers within.

In **Chapter IV** we screened 284 pollinating fig wasps from all focal species for *Wolbachia* infection. We further strain typed all infected individuals and mapped infection status on an SNP based wasp phylogeny. In this chapter we found that the distribution of *Wolbachia* strain and infection status along the phylogeny followed a non-random distribution, supporting some role of *Wolbachia* in wasp speciation, or at least the maintenance of reproductive isolation between incipient species. In order to explore the data in more detail, we built a machine learning algorithm which was able to accurately predict *Wolbachia* infection status and strain in approximately 70% of the cases, based exclusively on the wasps' fig host species and elevation (in metres above sea level). This chapter revealed interesting patterns in *Wolbachia* infection status which generally match the retrieved wasp clusters from **chapter II** allowing us to speculate on the role *Wolbachia* may have either as a speciation agent or reinforcing reproductive isolation through uni- and bidirectional cytoplasmic incompatibility.

Finally, besides being involved in a tight pollination mutualism, figs also house a wide diversity of parasitizing wasps which have also been suggested as responsible for bringing *Wolbachia* into this enclosed system.

In **Chapter V** we compare non-pollinating fig wasp (NPFW) communities for a subset of our focal species along the gradient. For this chapter we sorted through the emerged wasps of several individual fig trees for each species at each collection site along the transect and sorted individual wasps to genus level. We identified a peak in diversity of NPFW at the mid-elevation site (1,700m a.s.l.), and these communities differed between host species and elevation. NPFWs are known to be able to tolerate a broader range of environmental heterogeneity than do pollinating wasps and so it remains to confirm whether NPFWs found throughout the host's range are indeed the same species, or if they, like pollinators, form subspecies along the gradient.

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Chapter I

Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus*

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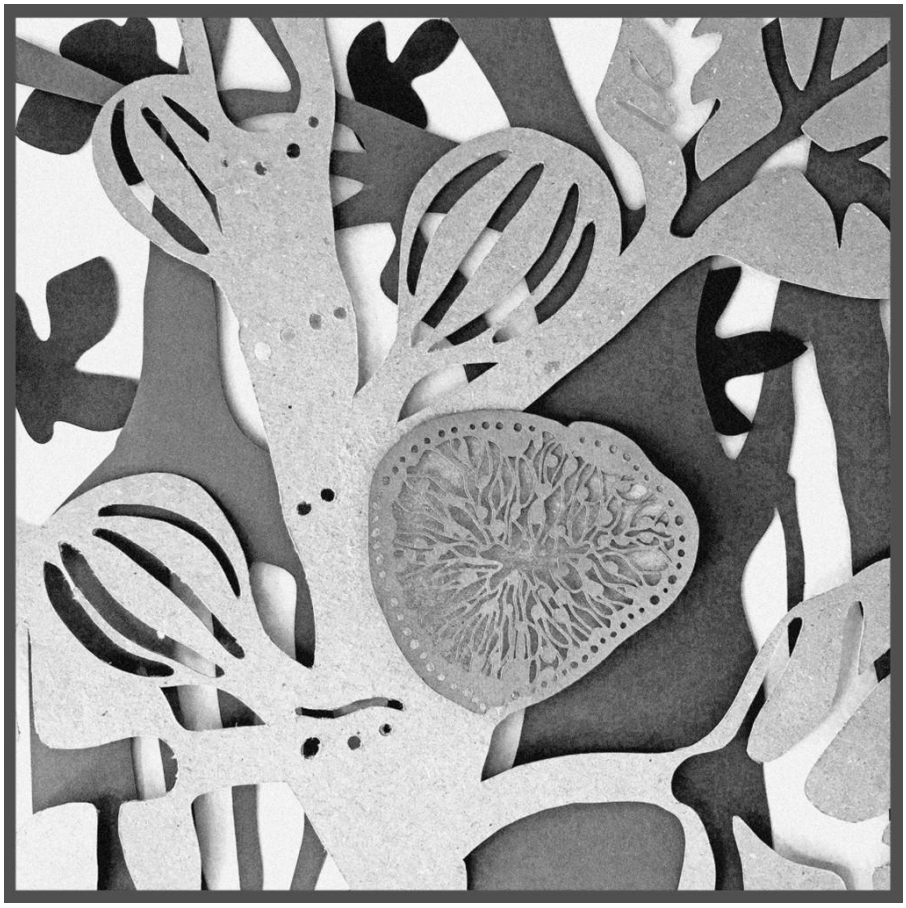

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Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus*

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Abstract

Much of the world's insect and plant biodiversity is found in tropical and subtropical 'hotspots', which often include long elevational gradients. These gradients may function as 'diversity pumps' and contribute to both regional and local species richness. Climactic conditions on such gradients often change rapidly along short vertical distances and may result in local adaptation and high levels of population genetic structure in plants and insects. We investigated the population genetic structure of two species of *Ficus* (Moraceae) along a continuously forested elevational gradient in Papua New Guinea. This speciose plant genus is pollinated by tiny, species-specific and highly coevolved chalcid wasps (Agaonidae) and represented by at least 73 species at our study gradient. We present results from two species of *Ficus* sampled from six elevations between 200 m and 2700 m a.s.l. (almost the entire elevational range of the genus) and 10 polymorphic microsatellite loci. These results show that strong barriers to gene flow exist between 1200 m and 1700 m a.s.l. Whereas lowland populations are panmictic across distances over 70 km, montane populations can be disjunct over 4 km, despite continuous forest cover. We suggest that the limited gene flow between populations of these two species of montane *Ficus* may be driven by environmental limitations on pollinator or seed dispersal in combination with local adaptation of *Ficus* populations. Such a mechanism may have wider implications for plant and pollinator speciation across long and continuously forested elevational gradients if generalist insect pollinators and vertebrate seed dispersers also form populations based on elevation.

Introduction

Many of the world's biodiversity 'hotspots' include long tropical or subtropical elevational gradients (Myers *et al.*, 2000; Mittermeier *et al.*, 2004; Mutke & Barthlott, 2005). Rapidly changing environmental conditions along such elevational gradients can lead them to function as 'diversity pumps' which may contribute to the origin of a large proportion of the world's biodiversity (Robin *et al.*, 2010; Schultheis *et al.*, 2012; Toussaint *et al.*, 2014). Phylogeographic studies of insects indicate that the formation of species in parapatry, where species ranges abut but do not overlap (Gavrilets, 2004), in montane habitats, can create speciation 'cradles' that fuel lowland diversity (Hall, 2005). Studies of plant communities also reveal high levels of species turnover at mid-elevations in large, species-rich tropical families (e.g. Burger, 1995). Local adaptation (and the filtering of maladapted genotypes) and limitations to insect mediated gene flow are likely to be

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especially important in insect pollinated flowering plants (Reis *et al.*, 2015), which represent the majority of tropical forest floras. Given that over 75% of species in terrestrial ecosystems is involved in a plant based food web (Price, 2002), it is not unreasonable to suggest that understanding the mechanisms of speciation in plants along elevational gradients is an important research goal, especially given our rapidly changing climate.

Montane habitats surrounded by lowland forest (called 'sky islands') have a clear role in promoting peripatric speciation in the hyperdiverse Australasian region (Toussaint *et al.*, 2013), with species of montane origin feeding back into the overall lowland species pool. The region's highlands provide an excellent natural laboratory in which to investigate ongoing or incipient speciation. Within the wider region, Papua New Guinea (PNG) is recognized as being particularly biodiverse. Indeed, 5% of the world's animal and plant species are found in PNG (an area representing 0.5% of the world's total land mass), and two-thirds of these species are endemic. The country is also known for its dramatic and geologically active topography which may contribute considerably to its high levels of endemism and biodiversity. Many ecologically important plant genera have diversified considerably in PNG, acting as important host islands for insect herbivores (Weiblen *et al.*, 2001, 2006; Novotny *et al.*, 2010). One such genus is *Ficus* (Moraceae). This pantropical genus is extraordinarily species-rich, containing over 800 species, 157 of which occur in PNG (Berg & Corner, 2005; Cruaud *et al.*, 2012). In PNG, *Ficus* species are overrepresented amongst plant species with wide elevational ranges and represent one of the key genera in forest communities along elevational gradients (Novotny *et al.*, 2005). Pollination in *Ficus* is performed exclusively by wasps in the chalcid family Agaonidae (Wiebes, 1979). These tiny wasps are usually species-specific and can act as effective pollinators over tens to hundreds of kilometres (Nason *et al.*, 1996; Ahmed *et al.*, 2009), whereas seed dispersal is carried out by a wide range of vertebrates, including bats and birds (Shanahan *et al.*, 2001). As such, both fig pollen and seeds can be transported over large distances (by pollinating wasps and frugivores, respectively). However, little is known about gene flow in *Ficus* along ecological gradients, for instance within populations of *Ficus* species with wide elevational ranges. Whereas there are documented examples of lowland and highland varieties or subspecies in at least three sections of *Ficus* (Berg & Corner, 2005), several examples of extremely close relatives occupying lowland and highland habitats can be found within the Papuan species in section *Sycocarpus* (which is pollinated by wasps from the genus *Ceratosolen*). A section is an infrageneric clade of species and each *Ficus* section is often, but by no means exclusively, pollinated by one genus of wasp (Cook & Segar, 2010). The endemic Papuan species in section *Sycocarpus* have a relatively recent origin (around 15 MY) (Cruaud *et al.*, 2012) and are represented by several complexes, with some species still capable of hybridizing (Moe & Weiblen, 2012). We studied gene flow in two understorey species of *Ficus* from section *Sycocarpus*, *F. arfakensis* King and *F. hahliana* Diels, along a continuously forested elevational gradient from 200 m a.s.l. to the local elevational limit of the genus at 2700 m a.s.l. in Papua New Guinea's Central Range.

Given that wasp-mediated gene flow between populations of *Ficus* in lowland habitats can cover tens to hundreds of kilometres (Nason *et al.*, 1996; Ahmed *et al.*, 2009), we might expect to see a similar pattern in montane populations. This would be evidenced by panmixia in the populations of both species studied here. However, whereas forest cover can be continuous, environmental conditions vary dramatically across elevational gradients, and may lead to limitations on pollinator and/or seed dispersal and even local adaptation followed by phenotypic isolation. This would result in genetic structure corresponding to gradual or sudden climactic changes in vertical distance. We expect genetic diversity in *Ficus* to decrease with elevation. This is because lowland populations are connected to a large gene pool through long-distance wasp migration (Nason *et al.*, 1996), whereas highland allelic diversity would be a nested subset of lowland diversity if vertical transmission is limited (mountains acting as bottlenecks). Mechanistically, the above canopy winds that facilitate long-range dispersal of wasps in lowland habitats are likely to be a less effective method of pollinator dispersal to higher elevations. This may be especially true for understorey tree species (Harrison, 2003), like *F. arfakensis* and *F. hahliana*. Furthermore, major genetic bottlenecks may occur at climatic interfaces, for example at the 'cloud layer' (the site of near constant cloud immersion resulting from relief precipitation). These interfaces may limit gene flow between elevations and exacerbate the genetic disparity between adjacent populations, allowing population specific alleles to accumulate. We combined extensive surveys of local *Ficus* species and population genetic data to address the hypothesis that limitations to gene flow occur along our study gradient.

Materials and methods

Survey of local *Ficus* diversity

A detailed survey of all local *Ficus* species was carried out at six of seven study sites along an elevational gradient focused on Mt. Wilhelm in Papua New Guinea (excluding Degenumbu, see Table 1 and Fig. S1 for site locations). At each elevational study site, teams of researchers (led by L. Sam) and paraecologists tagged all *Ficus* trees having a d.b.h. (diameter at breast height)

Table 1 Names of sample sites, their elevation (m a.s.l), their GPS coordinates, distance in a straight line to the gradient site with the lowest elevation (distance to lowest elevation (DLE)), and sampled species. Mean syconial volume (cm^3) at each site is given for *Ficus hahliana*.

Site name	Elevation (m)	Latitude	Longitude	DLE (km)	Sampled *Ficus* species	*N* = 49	Syconia volume (cm^3) ± SE (*N*)	Sampled *Ficus* species	*N* = 58
Ohu	200	05°14′00″S	145°41′00″E	70	*F. hahliana*	4	NA	*F. arfakensis*	1
Kausi	200	05°44′33″S	145°20′01″E	0	*F. hahliana*	10	0.99 ± 0.03 (158)	*F. arfakensis*	14
Numba	700	05°44′14″S	145°16′12″E	7	*F. hahliana*	5	0.89 ± 0.02 (154)	*F. arfakensis*	15
Memeku	1200	05°43′18″S	145°16′17″E	7	*F. hahliana*	10	1.17 ± 0.03 (179)	*F. arfakensis*	13
Bananumbu	1700	05°45′21″S	145°14′11″E	11	*F. hahliana*	5	NA	*F. arfakensis*	5
Degenumbu	1700	05°45′45″S	145°11′55″E	15	*F. hahliana*	10	2.88 ± 0.44 (9)	*F. arfakensis*	10
Sinopass	2200	05°45′34″S	145°10′49″E	17	*F. hahliana*	5	4.48 ± 0.43 (30)		
Bruno Sawmill	2700	05°48′57″S	145°09′02″E	22	*F. hahliana*	5	5.45 ± 0.28 (60)		

>1 cm within ten 500 × 10 m transects; transects were located at least 200 m from each other. Each tree was identified to species level and given a unique tree identifier number. We summarized species turnover along the gradient by calculating the percentage dissimilarity for each elevation in comparison with the 200 m site, using the Chao-Sorensen distance based on abundance data.

Focal species, plant tissue collection and genotyping

The genus *Ficus* is very species-rich at this site, and after a detailed survey of local *Ficus* diversity, we selected two species with wide elevational ranges for our population genetic study. Our study species are also both endemic to PNG and form part of a recent radiation, such that any population genetic patterns found are more likely to have occurred *in situ*, rather than as a result of multiple long-distance colonizations. Indeed, PNG itself is relatively young (Toussaint *et al.*, 2014) with the Central Range likely to be between 5 and 10 MY old. *Ficus arfakensis* has a recorded range of up to 1600 m in elevation (Berg & Corner, 2005), and it is widespread in PNG. As with many members of section *Sycocarpus*, *F. arfakensis* grows as a small understorey tree and is often locally abundant in secondary forest (Berg & Corner, 2005). *Ficus hahliana*, described by Berg & Corner (2005) as a lowland species, is often found close to rivers throughout PNG. Morphologically, *F. hahliana* is easily confused with *F. bernaysii* King (up to 1800 m a.s.l.). Both species form a species complex including also the highland species *F. iodotricha* Diels (700–2900 m a.s.l.), and as such, we genotyped four individuals of the latter two species.

For clarity, we use the term *population* solely for inferred biological clusters, we use the term *site* exclusively for sampling sites (each comprising 10 transects) and we use the term *elevation* for combining sites with the same elevation along our gradient. All elevations are given in metres above sea level (a.s.l.). We refer to our main sampling location as the 'elevational gradient'. There are eight sampling sites in total (Table 1). One elevation (1700 m) was sampled across two sites. We had to relocate our 1700-m sampling site during the project but after our survey (Table 1) due to land ownership disputes at our original site. The Ohu site was located in Ohu village (145°41′ E, 5°14′ S) near Madang (around 70 km north-east of our elevational gradient). Hence, there are seven sites and six elevations along the gradient, and one site outside of the gradient. Although Ohu is a lowland site, it is not part of our elevational gradient; we therefore do not group it with Kausi (also 200 m) when *a priori* assumptions are needed. *Ficus arfakensis* is present at five sites and four elevations (Ohu and between 200 and 1700 m) and absent at the two highest elevations (2200 and 2700 m), and we sampled an average of 14 individual trees per elevation. *Ficus hahliana* is present at all eight sites (Ohu and between 200 and 2700 m), and we sampled an average of eight individual trees per elevation (Table 1).

We have no *a priori* information on what constitutes a population for species with such wide elevational ranges. We therefore sampled evenly spaced sites that likely represent discrete within population samples, with some expectation that populations will comprise multiple sites. This means that site sample size is usually smaller than population size (see Results). We initially aimed to sample at least one tree per transect, so that the major barriers to gene flow could be identified. We selected only male trees so that pollinating wasps could be subsequently collected and associated with a given host tree (Souto-Vilarós *et al.*, in prep). Our selective sampling criteria and the naturally low density of mature trees meant that we effectively sampled haphazardly across transects at each site. The distance between trees sampled at a given site therefore ranged from 20 m to 1 km but was always less than the distance between sites, so that sampling at each site was representative of the local population. GPS location and voucher specimens were collected for a subset of the trees.

We sampled leaf discs, which were only collected from male trees large enough to bear fruit, at least 20 m was left between individuals and clonal individuals were avoided. Leaf discs (collected using a cork borer of 2.4 cm in diameter) were dried in the field in ziplock plastic bags containing two table spoons of colour indicating silica gel, which was replaced when necessary. All samples were then stored at −20 °C until needed for analysis. We isolated DNA from one leaf disc per individual using Invisorb Spin Plant Mini Kits (STRATEC Molecular, Germany). Due to polyphenol and secondary metabolite carry-over through the spin column in some samples (in particular for *F. hahliana*), we also extracted DNA using a modified CTAB protocol (Doyle & Doyle, 1987) with an additional cleaning step through a silica spin column or agarose gel. This removed all traces of contaminants yielding highly concentrated and pure DNA as measured by both Qubit Fluorometer (Invitrogen, OR, USA) and NanoDrop (Thermo Scientific, Wilmington, DE, USA). The syconia of *F. hahliana* clearly vary in size with elevation, and this may influence both wasp entry and seed dispersal. We quantified this variation by collecting a total of 590 mature syconia across six sites (Table 1) and measured both height and width to the nearest 0.01 mm using vernier callipers. Volume (cm^3) was calculated using a standard cone volume formula: $V = \pi r^2(h/3)$.

To analyse population genetic structure, we selected 11 microsatellite loci previously published for the genus *Ficus* (Moe & Weiblen, 2011; Garcia *et al.*, 2012), which were amplified in three multiplex sets (Table 2). Each PCR was composed of 4 μL of Multiplex PCR Master Mix (QIAGEN Inc. Valencia, CA, USA), 0.2 μM of each primer, 1 μL Q-solution (QIAGEN) and approximately 20–50 ng of template DNA and filled with PCR H_2O to the total volume of 10 μL. Conditions for the PCRs were as follows: 15 min of 94 °C, followed by 35 cycles of 94 °C (30 s), 54 °C (90 s) and 72 °C (60 s), with final elongation at 60 °C for 30 min. Genotypes were scored using the software Genemapper 3.7 (Applied Biosystems). We calculated genetic diversity parameters using GenAlEx v 6.5 (Peakall & Smouse, 2006), that is the number of alleles per locus and the observed and expected heterozygosities.

Stepwise analysis of population structure

We used Bayesian inference to determine both the major barriers to gene flow within each species studied (e.g. estimating the minimum k) and the fine-scale relationships between individuals (distance-based clustering to place genotypes across a bifurcating tree). We used methods as implemented in two different software packages and explored several commonly used criteria for defining the number of populations. We then tested for panmixia between our inferred populations using AMOVA before testing the strength and significance of gene flow between elevations and populations using a series of pairwise F_{st} comparisons. For the Bayesian analysis and AMOVA, we used the full data set, including individuals from Ohu. We only calculated pairwise F_{st} values for elevations along the gradient because N was <5 for Ohu.

Bayesian analysis of overall population structure was performed to determine (i) the number of population clusters using both STRUCTURE v2.3.4 (Pritchard *et al.*, 2000) and BAPS v5.4 (Corander *et al.*, 2004) as well as the proportion of the sampled genome of an individual that came from any populations present using STRUCTURE and ii) the fine-scale hierarchical clustering of individuals using BAPS. In STRUCTURE, we used the admixture model with the default settings and a burn-in of 10 000 and 1 000 000 replicates, we did not use sampling location as a *prior* in the analysis. We estimated k (the number of allelic clusters in our data set) using Evanno's ΔK (Evanno *et al.*, 2005), using 10 replicates for each value of k between 1 and 6. We also report the mean of the estimated log probability Ln(K) which is sometimes used to estimate the true value of k and referred to as Ln P(D) in STRUCTURE (Pritchard *et al.*, 2000; Evanno *et al.*, 2005); ΔK gives the minimum level of population genetic structure and can sometimes underestimate the number of clusters present (Waples & Gaggiotti, 2006). We used STRUCTURE Harvester (Earl & von Holdt, 2012) to compare ΔK and CLUMPP v1.1.2 (Jakobsson & Rosenberg, 2007) (using the 'full search' algorithm) and Distruct v1.1 (Rosenberg, 2003) to summarize and plot the output. In BAPS, we grouped individuals using the 'clustering of groups of individuals' and 'clustering of individuals' to assign elevations (but treating Ohu as its own group) and individuals to clusters with k set to 100, the number of clusters (k) was determined using maximum likelihood. The relationships between the clusters of individuals were visualized by plotting neighbour-joining trees using Nei's distance. A species-level neighbour-joining tree was estimated for the *F. hahliana* complex.

To compare genetic variation within and between the major populations, we used analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). We performed AMOVA of pairwise Hamming distances ('diss.dist' function) using the 'poppr.amova' function in the R package 'poppr' (Kamvar *et al.*, 2014). We tested the significance of genetic structure at each level (within individuals, within populations and between populations) using the 'randtest' function in the package 'ade4' (Dray & Dufour, 2007) with 999 permutations. Individuals were grouped to populations using the clusters derived by ΔK as the most conservative estimate of k, and this gave the same conclusions as using the BAPS elevation clusters (results not shown).

Pairwise F_{st} values between elevational sites and their significance levels (10 000 permutations) were calculated using software GenAlEx v 6.5 (Peakall & Smouse,

Table 2 Genetic diversity over 10 microsatellite loci in the two *Ficus* species studied. N = number of alleles; H_o = observed heterozygosity; and H_e = expected heterozygosity. Source studies: A: Garcia *et al.* (2012); B: Moe & Weiblen (2011).

			Ficus arfakensis			*Ficus hahliana*		
Locus name	Multiplex set	Source study	N	H_o	H_e	N	H_o	H_e
Micr2(CA)	1	A	16	0.60	0.89	6	0.23	0.60
Sur1(GA)	1	A	3	0.19	0.21	3	0.13	0.12
Car10(TG)	1	A	5	0.30	0.42	3	0.58	0.53
Sur2(AG)	2	A	1	0.00	0.00	4	0.40	0.36
Car11(CA)	2	A	10	0.53	0.80	9	0.59	0.76
Micr3(CT)	2	A	1	0.00	0.00	4	0.12	0.48
P211(GA)	3	B	5	0.33	0.67	8	0.61	0.72
B83(AG)	3	B	11	0.35	0.77	8	0.83	0.67
B47(GAA)	3	B	14	0.23	0.63	7	0.37	0.46
P215(ATGT)	3	B	13	0.51	0.89	10	0.74	0.79
Mean			9.63	0.38	0.66	6.20	0.46	0.55

2006). In addition, we calculated pairwise F_{st} (and its significance) between the lowland and highland populations revealed via the Bayesian STRUCTURE analysis (as defined by ΔK). We summarized the F_{st} values within and between each population and conducted more detailed analyses of population genetic parameters between the BAPS elevational clusters to describe the finer scale differences along the gradient, given that the mid-elevation populations of each species may represent contact zones which contained a number of private alleles. We used 'poppr' (Kamvar *et al.*, 2014) to calculate the number of private alleles in each population. Differences in pairwise F_{st} values between populations of both species from different elevations were visualized using parametric smoothing as implemented in the R package 'loess', and the smoothing parameter was selected using AIC.

We recognize that using only a limited number of individuals and loci can influence estimates of genetic distance and clustering inferences. We therefore tested the power of our data to detect nonhomogenous population structure using the software 'Powsim' v4.1 (Ryman & Palm, 2006). We stress that our main aim was quite simple, to detect nonhomogeneity and assign major genetic clusters. We tested the power of our data to detect the two major populations inferred using STRUCTURE at an F_{st} threshold of 0.025 for both species. We used the default MCMC chain settings but set N_e to 2000 and t to 100 to give the desired F_{st} threshold of 0.025. We used 100 replicates in each case. Power was assessed as the proportion of significances according to both the chi-square test and Fisher's exact test. We also estimated α (the chance of a type I error) by setting the F_{st} threshold to 0 and sampling directly from the base population.

Results

In our field surveys, we identified 12 880 individuals from 73 species, around 45% of the country's 157 *Ficus* species. The dissimilarity in the *Ficus* communities increased with elevation. Furthermore, there are strong elevational patterns in the distribution of multiple *Ficus* species with unique *Ficus* species found at almost all elevations (Fig. 1). In total, we genotyped 58 individuals of *F. arfakensis* and 49 individuals of *F. hahliana* for 11 microsatellite loci. In *F. arfakensis*, three loci (Car9, Micr3 and Sur2) were either monomorphic or failed to amplify, so that eight polymorphic loci were used for the analysis. In *F. hahliana*, locus Car9 was monomorphic, but the remaining 10 loci were polymorphic and were included in the analyses (Table 2).

We used STRUCTURE to estimate the number of populations and the proportion of each individual genome sampled that came from each population. For *F. arfakensis*, we identified two population clusters using ΔK (mean $L(K) = -1143.8$, $\Delta K = 83.1$) and three population clusters using $L(K)$ (mean $L(K) = -1039.0$, $\Delta K = 6.6$) (Fig. 2). For *F. hahliana*, we identified two clusters of genotypes using both ΔK (mean $L(K) = -896.3$, $\Delta K = 2730.6$) and $L(K)$. For *F. arfakensis* in particular, it was difficult to rule out the existence of more than two clusters of genotypes given the conflicting results of ΔK and $L(K)$. Given the nested structure of our data set and nonhomogenous gene flow (mid-elevation sites represent a mixture of lowland and highland alleles, but the highlands contain a subset of these), we consider the ΔK clusters to represent the major genetic divisions. Despite relatively modest sample sizes at each site, we recovered inferred populations with sizes of between 15 and 43 individuals. We used BAPS to cluster elevations into populations based on their genotypes, and indeed, we recovered four clusters for *F. arfakensis* (cluster 1: Ohu; cluster 2: 200 m and 700 m; cluster 3: 1200 m; and cluster 4: 1700 m) and three clusters for *F. hahliana* (cluster 1: Ohu, 200 m, 700 m, 1200 m; cluster 2: 1700 m; and cluster 3: 2200 m and 2700 m). Our final level of clustering addressed individual genotypes, and we showed a clear contact zone at 1200 m for *F. arfakensis*, with some

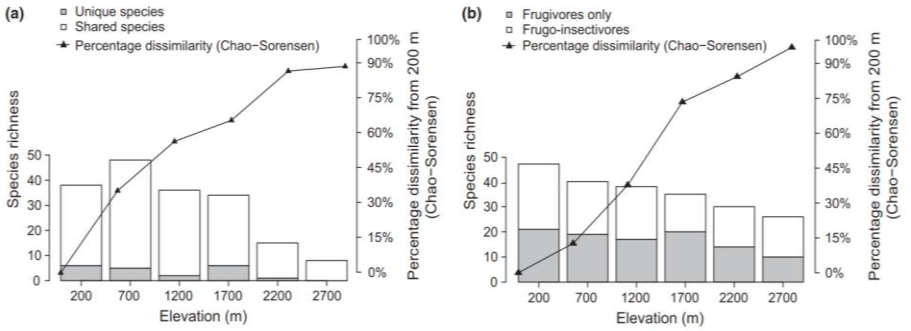

Fig. 1 The species richness of *Ficus* for each of six elevations (bars, left axis) and percentage dissimilarity in comparison with 200 m calculated using the Chao-Sorensen abundance-based distance (line, right axis). Bars are partitioned into species unique only to that elevation (grey) and species shared across more than one elevation (white) (a). The species richness of birds with at least a partially frugivorous diet for each elevation (bars, left axis) and percentage dissimilarity in comparison with 200 m calculated using the Chao-Sorensen abundance-based distance (line, right axis) (b). Bars are partitioned into bird species that are purely frugivorous (grey) and species that also eat insects (white) – data from point count surveys by Sam & Koane (2014).

individuals showing the strongest affinity to the 1700-m cluster, whereas others grouped with genotypes more common at 200 m and 700 m (Fig. 3).

We used AMOVA to test for panmixia across all individuals from each species and to test for hierarchical structure in genetic variation. We found significant between-population genetic structure in our data set and rejected the null hypothesis of panmixia for both species. This is evidenced by the fact that for both *F. arfakensis* and *F. hahliana*, the variance explained at the individual level was significantly less than the value obtained through permutation (Table 3). However, in both cases it explained a considerable amount of genetic variation (45% in *F. arfakensis* and 69% in *F. hahliana*). For both species, genetic variation between populations was much greater than within populations, suggesting that populations represent biologically meaningful groups with some limitations to gene flow between them. In *F. arfakensis*, genetic variation was significantly greater than under null expectations for both between and within populations (Table 3), but genetic variation between populations explained almost twice as much of the total variance (36%) than the variation within populations (19%). For *F. hahliana*, genetic variation between populations was large, whereas genetic variation within populations was very low (Table 3).

Pairwise F_{st} values between elevations along the gradient ranged from 0.03 to 0.36 in *F. arfakensis* and from 0.03 to 0.32 in *F. hahliana*. In *F. hahliana*, all pairwise F_{st} values between elevations were highly significant with the exception of between 200 m and 700 m and 700 m and 1200 m; for *F. arfakensis*, all pairwise F_{st} values between elevations were highly significant, with the exception of between 200 m and 700 m (Table 4). In general, the pairwise F_{st} values between elevations within lowland or highland populations (populations as defined by ΔK) were lower than F_{st} values between elevations from different populations. The mean pairwise F_{st} value within the three lowland elevations was 0.09 for *F. arfakensis*, and this value could not be calculated for the highlands which were represented by only one elevation. The mean pairwise F_{st} value was 0.04 within the three lowland elevations and 0.14 within the three highland elevations for *F. hahliana*. Whereas the F_{st} value between lowland and highland populations was 0.27 in *F. arfakensis* and 0.21 in *F. hahliana*, both between-population F_{st} values were highly significant (Table 4). This distinct reduction in gene flow at mid-elevations is visualized as a sharp increase in pairwise F_{st} values between 1200 m and 1700 m (Fig. 4). This is seen in both species studied here. It is notable that genetic diversity decreases with elevation. Although private alleles could be found for each population, they are more dominant in the lowlands, suggesting a bottleneck effect (Table 5). Our power analysis suggested that we employed a suitable number of loci and individuals to test our simple hypothesis of nonhomogeneity in both species. For *F. arfakensis*, the probability of detecting population differentiation at an F_{st} of 0.025 was 98% using the chi-square test and 97% using Fisher's exact test (α = 4% and 2%), and for *F. hahliana*, it was 93% using the chi-square test and 93% using Fisher's exact test (α = 4% and 4%). We confirmed our observation that the syconia of *F. hahliana* generally increase in size with elevation and form groups that overlap with the genetic clusters recovered, with the largest divide being between 1200 m and 1700 m (Table 1).

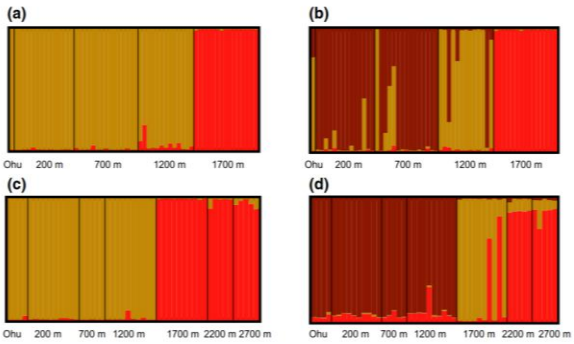

Fig. 2 The proportion of the sampled genome of each individual originating from each population as derived by ΔK using STRUCTURE: *Ficus hahliana* (k = 2) (a), *F. hahliana* (k = 3) (b), *Ficus arfakensis* (k = 2) (c) and *F. arfakensis* (k = 3) (d).

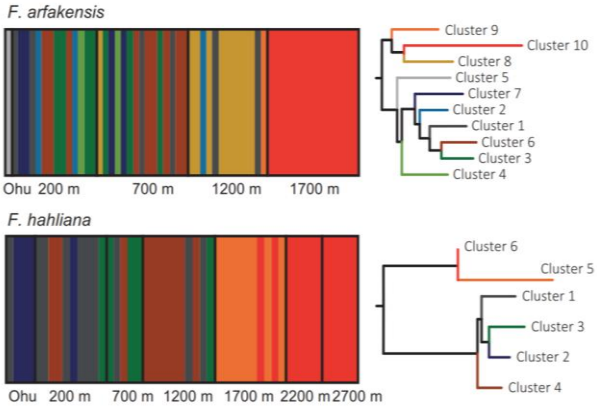

Fig. 3 Clusters resulting from the distribution of alleles amongst individuals for *Ficus arfakensis* (a) and *Ficus hahliana* (left-hand side) (b) and neighbour-joining trees estimated using Nei's distances coloured according to cluster (right-hand side).

Discussion

We demonstrate that strong barriers to gene flow exist between 1200 m and 1700 m for two species of *Ficus*. Our results show that distinct lowland and highland populations exist for *F. arfakensis* and *F. hahliana* growing along a continuously forested elevational gradient in Papua New Guinea. Indeed, most lowland (below 1200 m) individuals of *F. arfakensis* are more similar to those found 70 km away than to those from a population <4 km away but separated by 500 m in elevation. For *F. arfakensis*, at least, these populations are not likely to represent isolated genetic entities (a proportion of alleles are usually shared between peripatric populations along the entire gradient and three loci are invariable in this species). The 1200-m population contains alleles that are otherwise unique to both the lower and higher populations, often in the form of heterozygote individuals suggesting that this population is a contact zone. Genetic diversity also drops considerably at 1700 m, but allele frequency is consistent across two separate 1700-m sites, suggesting genuinely limited gene flow to this elevation has resulted in low genetic diversity. However, there are also a proportion of private lowland and highland alleles for each species and clear genetic and morphological distinctions between lowland and highland *F. hahliana*, which may represent a case of recent divergence into two species (likely to be sisters given our current sampling; Fig. S2). Indeed, we suggest that the highland populations should be referred to as *F.* cf *hahliana* form hereon until further work is conducted to clarify the taxonomic status of these distinct populations. The most obvious limitations of our work are the relatively low numbers of individuals and loci sampled. Furthermore, our sampling strategy includes only one elevational gradient. However, we detected relatively high levels of allelic diversity (see Table 1) amongst a relatively small number of

Table 3 Two-population nested analysis of molecular variance (AMOVA) based on (a) eight polymorphic loci for *Ficus arfakensis* and (b) 10 polymorphic loci for *Ficus hahliana*. *P*-value estimates are based on 999 permutations. d.f. = degrees of freedom; and MS = mean squared deviations. Populations based on ΔK from the STRUCTURE analysis.

AMOVA	d.f.	MS	Variation	% of Total variation	Phi	Direction	*P*-value
(a)							
Between populations	1	54.22	1.16	35.63	0.36	Greater	0.001
Between individuals within populations	56	2.72	0.63	19.39	0.30	Greater	0.001
Within individuals	58	1.46	1.46	44.98	0.55	Less	0.001
Total	115	2.53	3.25	100.00			
(b)							
Between populations	1	52.61	1.07	32.56	0.33	Greater	0.001
Between individuals within populations	47	2.14	−0.07	−1.99	−0.03	NS	0.754
Within individuals	49	2.27	2.27	69.44	0.31	Less	0.001
Total	97	2.73	3.27	100.00			

Table 4 Above: Pairwise F_{st} comparisons between elevational sites for *Ficus arfakensis* (left) and *Ficus hahliana* (right). Below: Pairwise F_{st} comparisons between clusters as derived by ΔK for *F. arfakensis* (left) and *F. hahliana* (right). In all cases, the diagonal is highlighted in bold text, and numbers below the diagonal give Fst values, whereas numbers above give significance.

Elevation	200	700	1200	1700	1700	200	700	1200	1700	2200	2700
200	**0.00**	0.55	<0.001	<0.001	<0.001	**0.00**	0.69	0.03	<0.001	0.001	<0.001
700	0.03	**0.00**	<0.001	<0.001	<0.001	0.03	**0.00**	0.16	0.001	0.008	0.009
1200	0.13	0.11	**0.00**	<0.001	<0.001	0.05	0.05	**0.00**	<0.001	0.001	0.001
1700	0.36	0.34	0.21	**0.00**	<0.001	0.29	0.30	0.32	**0.00**	<0.001	<0.001
2200	NA	NA	NA	NA	NA	0.24	0.24	0.23	0.12	**0.00**	0.014
2700	NA	NA	NA	NA	NA	0.21	0.20	0.18	0.18	0.11	**0.00**

Cluster	Lowlands	Highlands	Lowlands	Highlands
Lowlands	**0.00**	<0.001	**0.00**	<0.001
Highlands	0.27	**0.00**	0.21	**0.00**

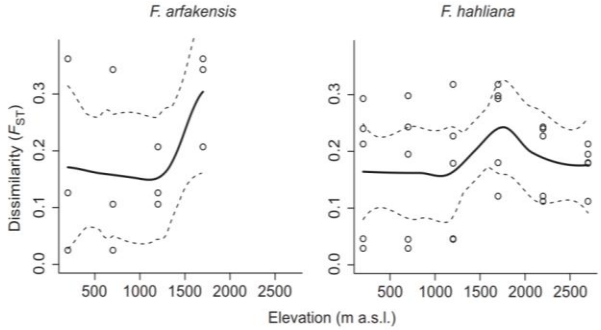

Fig. 4 Pairwise dissimilarity values between groups of individuals form all elevations for *Ficus arfakensis* and *Ficus hahliana*, based on F_{st} with curves and 95% confidence intervals fitted with loess smoothing.

individuals, which allows us to consider the results trustworthy, even with the use of a moderate number of loci (Kalinowski, 2002). This is supported by the results of our power analysis, which suggests that 8–10 polymorphic loci is enough to detect large genetic structure given the number of individuals and variability of the loci used. For more detailed analyses of fine-scale genetic structure and hybridization, we would suggest increasing the number of loci used, because a low number of loci may overestimate genetic distances

Table 5 Genetic diversity of the *Ficus* studied over the three elevational clusters as derived from the BAPS analysis (excluding Ohu). Na, mean number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; Pa, number of private alleles; %Pa, proportion of private alleles. *Ficus hahliana* (cluster 1: 200 m, 700 m, 1200 m; cluster 2: 1700 m; and cluster 3: 2200 m and 2700 m) and three clusters for *Ficus arfakensis* (cluster 1: 200 m and 700 m; cluster 2: 1200 m; and cluster 3: 1700 m).

	F. arfakensis					*F. hahliana*				
	Na	H_o	H_e	Pa	%Pa	Na	H_o	H_e	Pa	%Pa
Cluster 1	6.63	0.35	0.52	19	0.26	4.40	0.46	0.42	24	0.40
Cluster 2	6.13	0.51	0.64	15	0.19	3.10	0.43	0.40	7	0.11
Cluster 3	2.13	0.30	0.27	5	0.06	2.40	0.53	0.43	2	0.03

(Kalinowski, 2002). It would be very useful to include additional elevational gradients, but this would require a considerable amount of extra funding given the costs and practicality of working at one of the world's only fully forested elevational study sites.

Our findings suggest that there are at least two occurrences of limitations to gene flow at our study site. Furthermore, the dissimilarity of *Ficus* communities increases with elevational distance suggesting that elevation may limit the distribution of several other *Ficus* species. This is in contrast to two previous studies on the genetic structure of *Ficus* populations, which have demonstrated high levels of gene flow between sites at the same elevation (Nason *et al.*, 1996; Ahmed *et al.*, 2009). In the latter case, the dispersing wasp is congeneric with the pollinators of *F. arfakensis* (*Ceratosolen solitarius*, Weibes) and *F. hahliana* (*C. hooglandii*, Weibes). At the sites used for both previous studies, dispersing wasps face relatively constant temperatures and are likely to be aided by strong above canopy winds (Compton *et al.*, 2000; Harrison, 2003; Harrison & Rasplus, 2006). Both of these environmental conditions change with elevation. Although there is a predictable decrease in temperature, it is harder to generalize about wind strength and direction, which can vary according to aspect along tropical mountains (Beck *et al.*, 2008). To our knowledge, our study represents the first study of gene flow between *Ficus* populations along an elevational gradient, where environmental conditions change rapidly with vertical distance creating much less homogenous conditions for dispersing wasps than those found in lowland habitats. These apparent barriers to gene flow occur despite continuous forest cover, suggesting a strong abiotic limitation to biotic pollen and/or seed dispersal. It is likely that fig wasp dispersal is important in explaining the observed results. These tiny insects are particularly sensitive to changes in temperature (Jevanandam *et al.*, 2013) and may be unable to cross the 15 °C temperature gradient found between lower and upper elevations. This hypothesis is supported by the occurrence of two species of pollinator associated with *F. sur* Forssk. in West Africa that are also segregated by elevation. Whereas *F. sur* is pollinated by *Ceratosolen capensis* Grandi and *C. silvestrianus* Grandi in the lowlands, the pollinator in the highlands is *C. flabellatus* Grandi (Kerdelhué, 1997). It is possible that vertebrate seed dispersers also have limited ranges, with many endemic birds and mammals having restricted elevational ranges (Winter, 1997). Indeed, we see a strong turnover in bird community structure around 1200–1700 m along our gradient (Fig. 1), with distinct highland and lowland communities potentially limiting the vertical distance that seeds can be dispersed (Sam & Koane, 2014; Marki *et al.*, 2016). However, some degree of limited wasp dispersal is required in both scenarios because long-distance pollen dispersal can mask even highly limited seed dispersal.

Local adaptation in *Ficus* itself may also play a role in reducing gene flow, especially if this is linked to changes in fruit morphology that prevent maladaptation through the exchange of genetic material from higher or lower elevations. Indeed, both *F. arfakensis* and *F. hahliana* exhibit a degree of morphological variation along the gradient with respect to fig size, figs being larger at higher elevations (Table 1). Observations from other *Ficus* species demonstrate even more extreme morphological variation with elevation than species examined in this study. For example, *F. dammaropsis* Diels has cricket ball/baseball-sized fruits in the lowlands which are covered with open bracts; in contrast, highland populations have substantially larger fruits which are generally smoother and have the bracts closed. There are also well-documented instances of highland and lowland varieties or subspecies of *Ficus*; for example, *F. trichocerasa* subsp. *trichocerasa* Diels is found mainly up to elevations of 1400 m but grades slowly into subsp. *pleioclada* (Diels) C.C. Berg in higher elevations up to 2600 m (Berg, 2004). Furthermore, *F. wassa* Roxb. has a similarly large range (up to 3000 m) and is found as var. *nubigena* Diels in the highlands (1300–3000 m) (Berg & Corner, 2005). The main form grows as a tree up to 15 m and has red figs at maturity, and the highland variety has a scadent, scrambling habit, growing up to 3 m and bearing greyish white figs while ripening. Despite these apparently important ecological differences, neither variety can be separated on the mostly vegetative characters listed in Berg & Corner (2005). The extent to which this

variation is genetic or environmental is yet to be established in these species. Indeed, members of the genus *Ficus* can display high levels of phenotypic plasticity (Harrison, 2005). We suggest that additional detailed morphological studies are required across several of the species found at this site to assess the true degree of variation observed and that these should be conducted in conjunction with more detailed and wide-scale population genetic studies of both figs and their pollinating wasps.

It has long been recognized that species turnover (or beta diversity) along elevational gradients is usually high, whereas community level nestedness is low. However, peripatric species are often close relatives, suggesting that speciation is facilitated by local adaptation and decreased gene flow. Although our study addresses gene flow in a specialized pollination mutualism, we suggest that it may have wider implications for less specialized systems because any level of specialization in pollination or seed dispersal may lead to potential isolation. Furthermore, although insect herbivores (Craft *et al.*, 2010) and pollinators (Nason *et al.*, 1996; Ahmed *et al.*, 2009) of *Ficus* show low levels of population structure in lowland habitats, we have little understanding of how pollinator and insect herbivore populations are structured along elevational gradients, but turnover of species within genera appears likely for herbivores (Novotny *et al.*, 2005). *Ficus* species represent one of the key genera in forest communities, supporting extremely species-rich communities of herbivorous insects from several guilds (Novotny *et al.*, 2005). Being one of the most important plant genera for tropical frugivores, *Ficus* also provides an important food source for a broad variety of vertebrates with some of them being dependent on fig consumption (Shanahan *et al.*, 2001). Our data on the composition of *Ficus* communities suggest that multiple *Ficus* species have limited elevational ranges. Elevational barriers to gene flow may therefore be present in additional *Ficus* species not studied here. Divergence in *Ficus* populations and associated variation in their traits, fruit morphology and phenology are thus likely to have pronounced effects on numerous associated organisms. It would certainly be valuable to conduct further studies along tropical elevational gradients to investigate the population genetic structure of additional plant species and its correlation with associated communities of other organisms. We suggest that such an approach would be a useful step in understanding the processes of speciation in some of the world's most biodiverse hotspots.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1 A map of our sampling sites, contour lines are given every 100 m.
Figure S2 A neighbour-joining tree constructed using Nei's distances derived from a 'clustering of groups of individuals' analysis as implemented in BAPS (Corander *et al.*, 2004).
Appendix S1 Plate S1. *Ficus iodotricha* (2200 m a.sl., above) and *Ficus hahliana* (2700 m a.s.l., below).
Plate S2. *Ficus hahliana* 700 m a.s.l. above, 2200 m a.s.l. below.

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Supplementary material for Chapter I. Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus* – Journal of Evolutionary Biology

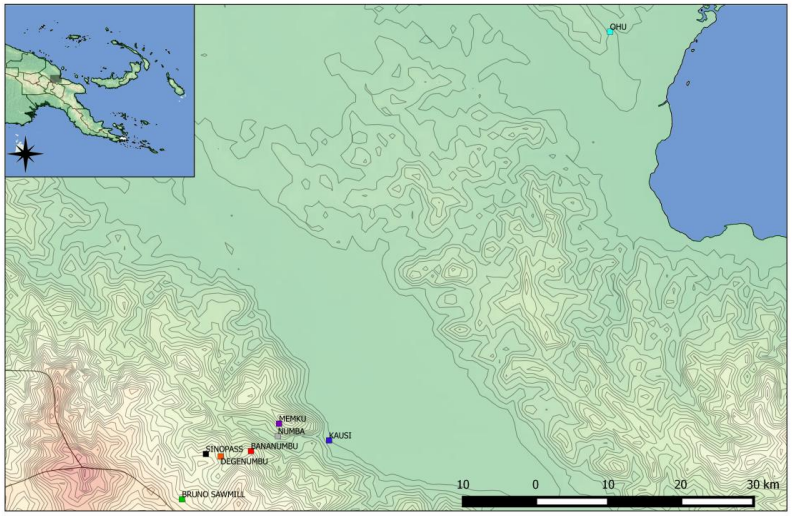


Figure S1. A map of our sampling sites. Contour lines are given for every 100 m. The 1,700m site was spread across two sites (Bananumbu and Degenumbu). The Ohu site does not form part of the Mt. Wilhelm altitudinal gradient but was used in this study to compare lowland population connectivity.

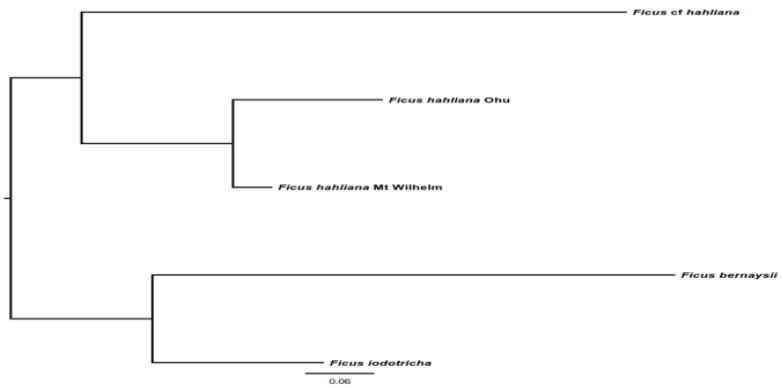


Figure S2. A neighbour-joining tree constructed using Nei's distance derived from a 'clustering of groups of individuals'analysis as implemented in BAPS (Corander et al. 2004).

Appendix S1

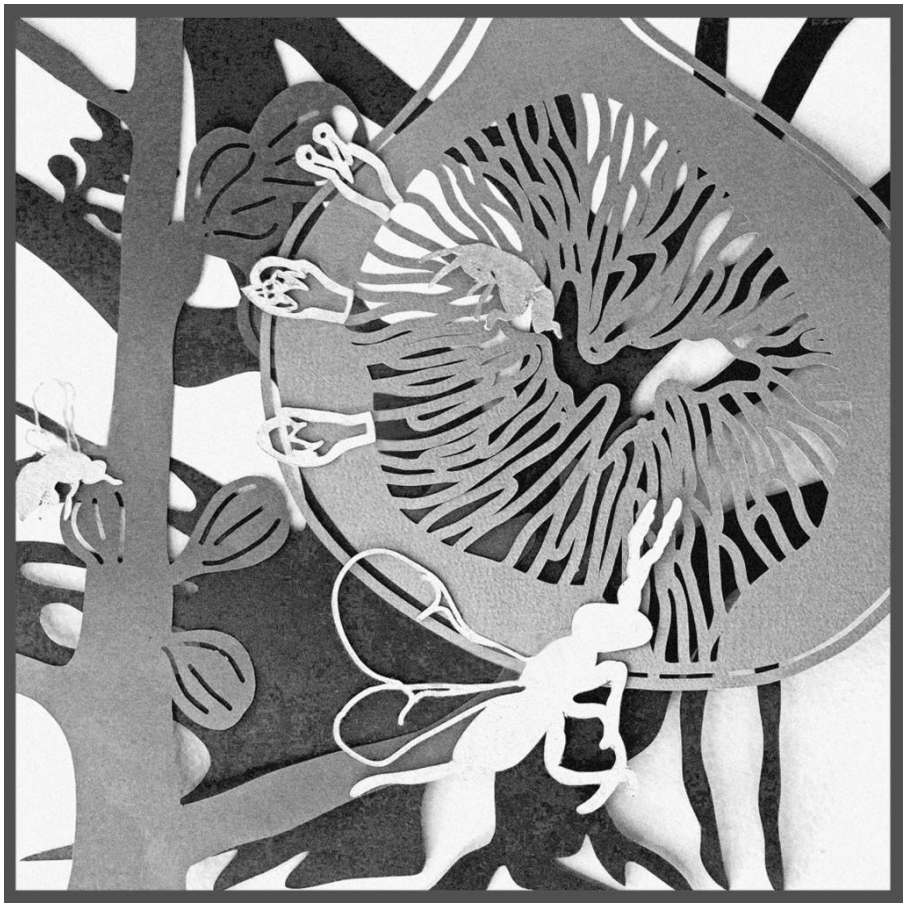
Plate S1. *F. iodotricha* (2,200 m, above) and *F. hahliana* (2,700 m, below). Note the difference in how the petiole joins the leaf, the coloration of the veins and the persistent stipules of *F. hahliana*. *Ficus iodotricha* as described here matches GW2135 from Chimbu Province in PNG (http://ng.atrium-biodiversity.org/).

Plate S2. *Ficus hahliana* 700 m above, 2,200 m below. Note the reduced leaf asymmetry and stem pubescence in high elevation *F. hahliana* but the otherwise similar presentation of the leaves.

Chapter II

Faster speciation of fig wasps than their host figs leads to decoupled speciation dynamics

(manuscript)



Faster speciation of fig-wasps than their host figs leads to decoupled speciation dynamics

Daniel Souto-Vilarós, Antonin Machac, Jan Michalek, Clive T. Darwell, Mentap Sisol, Thomas Kuyaiva, Brus Isua, George D. Weiblen, Vojtech Novotny, Simon T. Segar

ABSTRACT

The genus *Ficus* (Moraceae) is best known for its obligate mutualism with pollinating fig-wasps (Agaonidae), where species are thought to reciprocally trigger genetic differentiation resulting in tight co-speciation. Here, we used nextRAD DNA sequencing to study the population structure of multiple fig species and their corresponding fig-wasps along an elevational gradient in Papua New Guinea. Contrary to the expected one-to-one species specificity in this mutualism we find evidence of multiple pollinating wasp species, which through limited dispersal abilities along the gradient, likely limit pollen flow and influence fig population structure along these slopes. In two cases, where the fig species studied have wide distribution along the mountain, we found between three and four wasp species pollinating closely related populations of fig species. In the case of one fig subspecies complex, we identified two fig-wasp species according to the distribution of their host subspecies. Finally, in a parapatric, three sister species complex, we identified three individual wasp species, each corresponding to its host fig species. Fig-wasps appear to speciate more rapidly through faster generation times, faster rates of local adaptation and/or weak dispersal abilities compared to figs. This in turn restricts pollen movement between fig ecotypes, strengthening reproductive barriers and so facilitating their speciation. Fig speciation along the gradient and wasp lineage extinction may eventually restore the one-to-one rule in this mutualism through split and sort speciation dynamics.

INTRODUCTION

How do new species originate? This has been one of the central questions in biology ever since Darwin (1859). Yet, even after over 150 years, we still have only an incomplete answer, especially when the process involves interactions between multiple species. To help resolve this question, researchers have often turned to interactions between plants and pollinators as the latter are thought to be important agents of selection of many floral traits, while the former similarly influence morphology and behaviour of pollinators. These interactions become particularly interesting when it concerns pollinator species specialized to a single plant species as is the case between orchids and orchid bees, yuccas and yucca moths and figs and fig wasps; all hallmark study systems for studying co-evolution (Kiester, Lande & Schemske 1984; Weiblen 2004; Cruaud *et al.* 2012; Van der Niet, Peakall & Johnson 2014). In the case of the figs and their wasps, it has long been presumed that co-speciation has resulted in a tightly coupled interaction following a one-to-one co-evolutionary dynamic, which make the speciation process easier to study. However, a growing number of exceptions to the one-to-one paradigm have been reported (Cook & Rasplus 2003; Haine, Martin & Cook 2006). These findings have led to the hypothesis that speciation dynamics in figs and their wasps may sometimes be decoupled. Here, we test this hypothesis across multiple fig and wasp species pairs by evaluating their population genetic structure along an elevation gradient in Papua New Guinea (PNG).

Elevation as an obstacle

Elevational gradients have long been regarded as natural laboratories for the study of ecology and evolution (Rahbek & Museum 1995; Körner 2007; McCain & Grytnes 2010). Rapid environmental changes, such as a decrease in temperature or increase of precipitation with elevation, are thought to have shaped biodiversity along mountain slopes sharing the same initial species pool. Tropical mountains harbour a disproportionate amount of the world's biodiversity and likely act as 'species pumps' for adjacent lowlands (Kreft & Jetz 2007). Indeed, the Andes, Mount Cameroon, Mount Kinabalu and New Guinea's central range are among the most species rich places on earth and are frequently the subjects of macroevolutionary studies (Rull 2011; Toussaint *et al.* 2014). Restricted gene flow along such gradients (whether due to environmental heterogeneity and/or non-random mating) likely promotes genetic differentiation, spatial structure in populations, local adaptation and eventual parapatric speciation, explaining the high species turnover along mountains (Kirkpatrick & Barton 1997; Byars, Parsons & Hoffmann 2009; Caro *et al.* 2013; Reis *et al.* 2015).

Pollinators as drivers of speciation

Pollinators may act as reproductive isolating agents between plant populations. This is particularly true for pollinators with limited dispersal since allopatric populations of pollinators may themselves undergo behavioural or physiological changes which influence preference or overall compatibility with the plants they pollinate which may lead to restricted gene flow between plant populations

(Kiester *et al.* 1984; Van der Niet *et al.* 2014; Souto-Vilarós *et al.* 2018). Pollinating fig-wasps are the sole means of pollen transfer between fig trees; these in turn depend exclusively on the fig for their development since flower ovules (enclosed within the 'syconia' or fig) serve as oviposition sites and food resource for the developing wasp larvae (Galil & Eisikowitch 1968). Fig wasps are known for their long distance dispersal as they are transported over wide distances, probably by wind (Ahmed *et al.* 2009; Kobmoo *et al.* 2010; Liu *et al.* 2015), however, recent studies have found that dramatic geographic barriers may limit the dispersal of these tiny, short-lived wasps (Haine *et al.* 2006; Kobmoo *et al.* 2010; Sutton, Riegler & Cook 2016). Additionally, long distance dispersal is most often reported in pollinators of large monoecious trees (approximately half of all *Ficus* species), which are found at low densities in forest habitats. In contrast, pollinating wasps associated with dioecious and under-canopy fig species do not disperse over long distances and dioecious fig trees are clustered into dense local populations (Dev *et al.* 2011; Wachi *et al.* 2016). Such tree clustering and limits to wasp dispersal suggest greater potential for limited gene flow both through pollen and localized seed dispersers, making the study of these interactions along elevational gradients particularly interesting.

Since fig-wasps cycle through many more generations per year than figs, they are expected to undergo local adaptation, and develop barriers to gene flow more rapidly (Cook & Segar 2010; Moe, Clement & Weiblen 2012). Additionally, fig-wasp reproductive success depends exclusively on wasps finding receptive figs shortly

after emergence. This limited time frame results in strong selection against entering the 'wrong' species of fig, since such mistakes represent the ultimate price in terms of wasp fitness (Galil & Eisikowitch 1968; Weiblen 2004; Kjellberg *et al.* 2005). On the other hand, the year-round fruiting of fig trees make them a 'keystone' genus which attracts a wide variety of frugivores responsible for fig seed dispersal, the exact disperser is to some extent determined by fruit syndrome (Shanahan 2000; Thornton *et al.* 2001; Lomáscolo *et al.* 2010). Such seed dispersal may connect otherwise isolated populations allowing some degree of gene flow in figs. For these reasons, we hypothesize that wasps speciate faster than their fig hosts (Figure 1), and subsequently mediate pollen flow between fig ecotypes, eventually resulting in distinct species of both interacting partners.

In this study, we investigate the population genetic structure within carefully selected pairs of fig and wasp species along the Mount Wilhelm elevational gradient in Papua New Guinea. The species and their populations have been selected to encompass various stages of the speciation process: from a continuous species with a wide elevational distribution (Figure 1A) to parapatric sub-species (Figure 1B), and finally parapatric species complexes (Figure 1C), each set representing a step in the 'speciation continuum'. Following standard co-speciation dynamics in the mutualism, we would expect a single pollinating wasp along the entire range of a single host species, regardless of any barriers posed along the gradient. Alternatively, wide ranging fig species may host geographically isolated fig-wasp

species which in turn isolate fig populations, leading to their eventual speciation.

METHODS

The present study was carried out at six sites along Mount Wilhelm. All sites are spaced by approximately 500 m vertical increments, from 200 meters above sea level (m a.s.l.) to 2,700 m a.s.l., ranging from lowland alluvial forest up to lower montane forest. Site names and locations are described in Table 1. Forest types, species composition and climatic conditions have been described in detail elsewhere (McAlpine *et al.* 1983; Toussaint *et al.* 2014; Sam *et al.* 2017).

Focal Species

Previous surveys of *Ficus* diversity carried out at the transect (Segar *et al.* 2017) (L. Sam, unpublished data) reported approximately 70 of the ~ca. 150 *Ficus* species present in PNG. Here we studied five species and one subspecies complex, all of which are endemic to PNG and adjacent islands (Berg & Corner 2005). We selected two well defined species *F. wassa* Roxb. and *F. arfakensis* King, which have wide elevational distributions, their corresponding pollinating wasps are *Kradibia wassae* Wiebes and *Ceratosolen solitarius* Weibes, respectively, however, we identified multiple distinct wasp species responsible for pollinating these species (see results); *F. trichocerasa* Diels is represented by two subspecies that replace each other in parapatry at approximately 1,700 m a.s.l., pollinating wasps are undescribed. Finally, we studied a complex of three species

belonging to the section *Papuacyse* that follow a lowland, mid-elevation, and highland distributions, henceforth referred to as the *F. itoana* species complex which includes *F. itoana* Diels pollinated by *Ceratosolen armipes* Wiebes, *F. microdictya* Diels pollinated by *C.* sp. *"kaironkensis"* (*nom.nud*), and an as of yet undescribed species here referred to as *Ficus* sp. "IMI", pollinating species undescribed. Species names, breeding systems and distributions are detailed in Table 1.

We conducted sampling between August 2015 and November 2016. At each site we geo-referenced and tagged between 10 and 15 individual trees of each locally available focal species. We collected 10 to 15 leaf discs of 2.4 cm in diameter using a cork-borer and stored them in colour changing silica gel for later DNA extraction and molecular analysis. For each male tree tagged, we monitored fig development weekly or daily as necessary, upon ripening to D-stage (Galil & Eisikowitch 1968) we collected 10-15 figs and stored them in plastic pots covered with fine mesh to allow wasps to emerge. Once emerged, we collected ten female and ten male pollinating wasps for storage in 100% ethanol. A second collection of five males and five females was done using tubes filled with colour changing silica gel topped with cotton wool as per (Moe, Rossi & Weiblen 2011). The silica gel collected wasps were used for DNA extraction. Finally, we collected and stored all remaining wasps in 100% ethanol. All samples were stored in a -20˚C freezer and shipped to the Czech Academy of Sciences, Czech Republic. Fig vouchers are stored in the National Herbarium in Lae, PNG, the New Guinea Binatang Research Centre (BRC), PNG, and the Czech Academy of Sciences, Czech

Republic. Wasp vouchers are stored at the BRC and the Czech Academy of Sciences.

DNA extraction and nextRAD sequencing

We performed DNA extractions from leaf discs using the CTAB protocol (Doyle & Doyle 1987) followed by an additional cleaning step using a silica column (Souto-Vilarós *et al.* 2018). From each individual fig, a single female pollinating wasp stored and dried in silica gel was used for DNA extraction. Isolations were performed using DNeasy Blood & Tissue kits (Qiagen) following the manufacturers' protocol with the following modifications: Initial lysis was conducted at 37˚C overnight followed by 30 minute incubation with 1 µL of RNase (Qiagen) per sample. Final yield was further enhanced by using a total of 200 µL of deionised water in two rounds of column washing. For both fig and wasp DNA samples, 1 µL of the final extract was used for QuBit quantification (QuBit 3 ThermoFisher Scientific) and 2 µL were loaded on a GelRed® Nucleic Acid Gel Stain (Biotium) pre-stained 2% agarose gel ran at 120 V for 70 minutes for visual inspection. Finally, fig DNA solutions were diluted to a total of 200 ng in 40 µL of EB buffer (Qiagen), while wasp DNA solutions (due to significantly lower yields) were vacuum dried and resuspended in 35 µL of EB buffer to increase concentration to ~20 ng of DNA per sample.

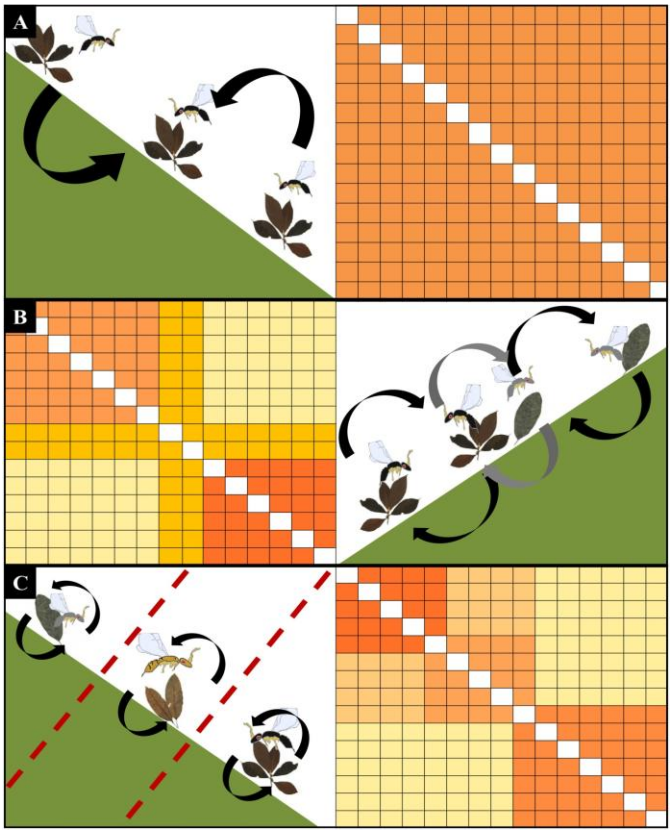


Figure 3. Illustration of how fig-wasp speciation could lead to reproductive isolation of fig ecotypes and eventual speciation along an elevational gradient. Heat maps mimic the co-ancestry matrix; darker colours indicate higher level of relatedness among populations. Black arrows indicate pollen flow, gray arrows indicate occasional pollen flow between fig ecotypes or subspecies; red dashed lines represent clear barriers to geneflow. A: A single fig species is pollinated by a single wasp species along the gradient. Fig-wasps begin to mate non-randomly forming local populations leading to their speciation. B: Separate fig wasps (black and gray wasps) restrict pollen flow between fig ecotypes leading to reproductive isolation with occasional geneflow at the contact-zone. C: Increasing reproductive isolation segregates fig and wasp-populations further, leading to their speciation.

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

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

The genotyping analysis used custom scripts (SNPsaurus, LLC) that trimmed the reads using bbduk (BBMap tools, http://sourceforge.net/projects/bbmap/): bash bbmap/bbduk.sh in=$file out=$outfile ktrim=r k=17 hdist=1 mink=8

ref=bbmap/resources/nextera.fa.gz minlen=100 ow=t qtrim=r trimq=10

Next, a de novo reference was created by collecting 10 million reads in total, evenly from the samples, and excluding reads that had counts fewer than 10 or more than 1000. The remaining loci were then aligned to each other to identify allelic loci and collapse allelic haplotypes to a single representative. All reads were mapped to the reference with an alignment identity threshold of 85% using bbmap (BBMap tools). Genotype calling was done using Samtools and bcftools (samtools mpileup -gu -Q 10 -t DP,DPR -f ref.fasta -b samples.txt | bcftools call -cv - > genotypes.vcf). The vcf was filtered to remove alleles with a population frequency of less than 3%. Loci were removed that were heterozygous in all samples or had more than 2 alleles in a sample (suggesting collapsed paralogs). The absence of artefacts was checked by counting SNPs at each read nucleotide position and determining that SNP number did not increase with reduced base quality at the end of the read.

Additionally, we generated molecular barcodes for wasps using cytochrome oxidase *b* (cyt-*b*), a marker with high specificity and amplification rates in fig wasps which has the ability to delimit closely related fig wasp species (Lopez-Vaamonde *et al.* 2009; Segar *et al.* 2012). Finally, due to unsuccessful amplification of cyt-*b* for individuals of *Ceratosolen* sp. "IMI," we generated COI sequences to compare them against previously published sequences for wasps of the *F. itoana* complex. PCR primers and conditions for both fragments are outlined in (Cruaud *et al.* 2010).

Data analysis

Population genetic structure was inferred using fineRADstructure (Malinsky *et al.* 2018), a pipeline specifically designed for the analysis of RAD data using the fineSTRUCTURE Markov chain Monte Carlo (MCMC) clustering algorithm (Lawson *et al.* 2012). Without previous assumptions of populations, the program generates a "co-ancestry matrix" between all individuals and collection sites which is then clustered with MCMC sampling to identify clusters of individuals which share the highest degree of estimated relatedness. We converted vcf files to the required format using custom python scripts. The SNP matrix, including all individuals for each species, was further filtered to reduce the amount of missing data (<30% and <40% for figs and wasps respectively). The software RADpainter, as implemented in the fineRADstructure package, was then used to calculate the co-ancestry matrix. Individuals were assigned to populations using 100,000 Markov Chain Monte Carlo replications and a burn-in of 10,000. Tree building was conducted using the default parameters and results were visualized using R scripts (available at http://cichlid.gurdon.cam.ac.uk/fineRADstructure.html).

Levels of genetic differentiation between collection sites were estimated by pairwise *Fst* from the vcf files using the Weir and Cockerham method as implemented in VCFtools version 0.1.13 (Danecek *et al.* 2011). For interpretation of *Fst* values, we follow the general classification from Hartl & Clark (1997): *Fst* <0.05 very little, 0.05 – 0.15 moderate, 0.15-0.25 great and > 0.25 very great.

Table 1. Collection sites, focal Ficus species and wasp species collected from each location/Ficus species.

Sampling Locality	GPS Coordinates	Elevation	Species	*n*	Breeding system	Distribution (m a.s.l.)*	Pollinator	*n*
Kausi	5° 44' 33" S, 145° 20' 1" E	200	*F. wassa*	9	Dioecious	0 - 3000	*Kradibia wassae* sp. 1	16
			F. arfakensis	10	Dioecious	0 - 1600	*Ceratosolen solitarius* sp. 1 & 2	20
			F. itoana	3	Dioecious	0 - 1800	*Ceratosolen armipes*	na
Numba	05°44'14" S, 145°16'12" E	700	*F. wassa*	15	Dioecious	0 - 3000	*Kradibia wassae* sp. 2	9
			F. arfakensis	9	Dioecious	0- 1600	*Ceratosolen solitarius* sp. 1 & 2	19
			F. trichocerasa subsp. trichocerasa	15	Dioecious	0 - 1400	*Ceratosolen* sp. "TRI" 1	11
			F. itoana	11	Dioecious	0 - 1800	*Ceratosolen armipes*	15
Memeku	5° 43' 18" S, 145° 16' 17" E	1200	*F. arfakensis*	15	Dioecious	0 - 1600	*Ceratosolen solitarius* sp. 3	12
			F. trichocerasa subsp. trichocerasa	7	Dioecious	0 - 1400	*Ceratosolen* sp. "TRI" 1	3
			F. itoana	5	Dioecious	0 - 1800	*Ceratosolen armipes*	4
Kamugai	5° 44' 49" S, 145° 14' 14" E	1200	*F. wassa*	8	Dioecious	0 - 3000	*Kradibia wassae* sp. 2	4
Degenumbu	05°45'45" S, 145°11'55" E	1700	*F. wassa*	15	Dioecious	0 - 3000	*Kradibia wassae* sp. 3	4
			F. arfakensis	16	Dioecious	0 - 1600	*Ceratosolen solitarius* sp. 4	11
			F. trichocerasa subsp. pleioclada	14	Dioecious	1500 - 2600	*Ceratosolen* sp. "TRI" 2	18
			F. sp. "IMI"	13	Andromonoecious	1700**	*Ceratosolen* sp. "IMI"	30

Sinopass	05°45’34” S, 145°10’49” E	2200	*F. wassa*	14	Dioecious	0 - 3000	*Kradibia wassae* sp. 3	6
			F. trichocerasa subsp. pleioclada	11	Dioecious	1500 - 2600	*Ceratosolen* sp. "TRI" 2	14
			F. microdictya	14	Monoecious	2000 - 2600	*Ceratosolen "kaironkensis"*	18
Bruno Sawmill	5° 48' 57" S, 145° 9' 2" E	2700	*F. wassa*	15	Dioecious	0 - 3000	*Kradibia wassae* sp. 3	3
			F. microdictya	15	Monoecious	2000 - 2600	*Ceratosolen "kaironkensis"*	16

* Species distribution taken from Berg & Corner 20

** personal observation

Further, we obtained molecular operational taxonomic units (MOTUs) for wasps using jMOTU v.1.0.7 (Jones, Ghoorah & Blaxter 2011). This method provides an objective way of defining the cut-off point that should be used to delimit intra- and inter-specific variation, and can be used in combination with coalescence or phylogenetic assignment of MOTUs to provide more robust delimitation of MOTUs. We set the low BLAST identity filter to 97 but otherwise settings were left as default. The 'barcode gap' was visually identified by plotting the number of MOTUs vs. the percentage cut-off and finding the value over which the MOTU numbers plateau following a sharp decrease which results from intra-specific clustering.

RESULTS

Co-ancestry heat maps generated using fineRADstructure provide detailed population structure for both figs and fig-wasps. In general, we were able to detect highland and lowland populations of figs, often with mid-elevation contact zones. Pairwise fixation index (*Fst*) values confirm these to be populations indicating that some of these species do have wide elevational distributions but remain a single species. Contrastingly, our analysis on fig-wasps reveal tightly clustered entities along the gradient which both *Fst* comparisons and genetic barcoding analysis indicate these to be separate pollinating fig-wasp species. For the two widely distributed fig species, our analysis suggests multiple pollinator species along the gradient while for the subspecies and sister species complex, individual wasp species are responsible for pollinating each of the (sub)species maintaining the classic one-to-one species specificity in this mutualism.

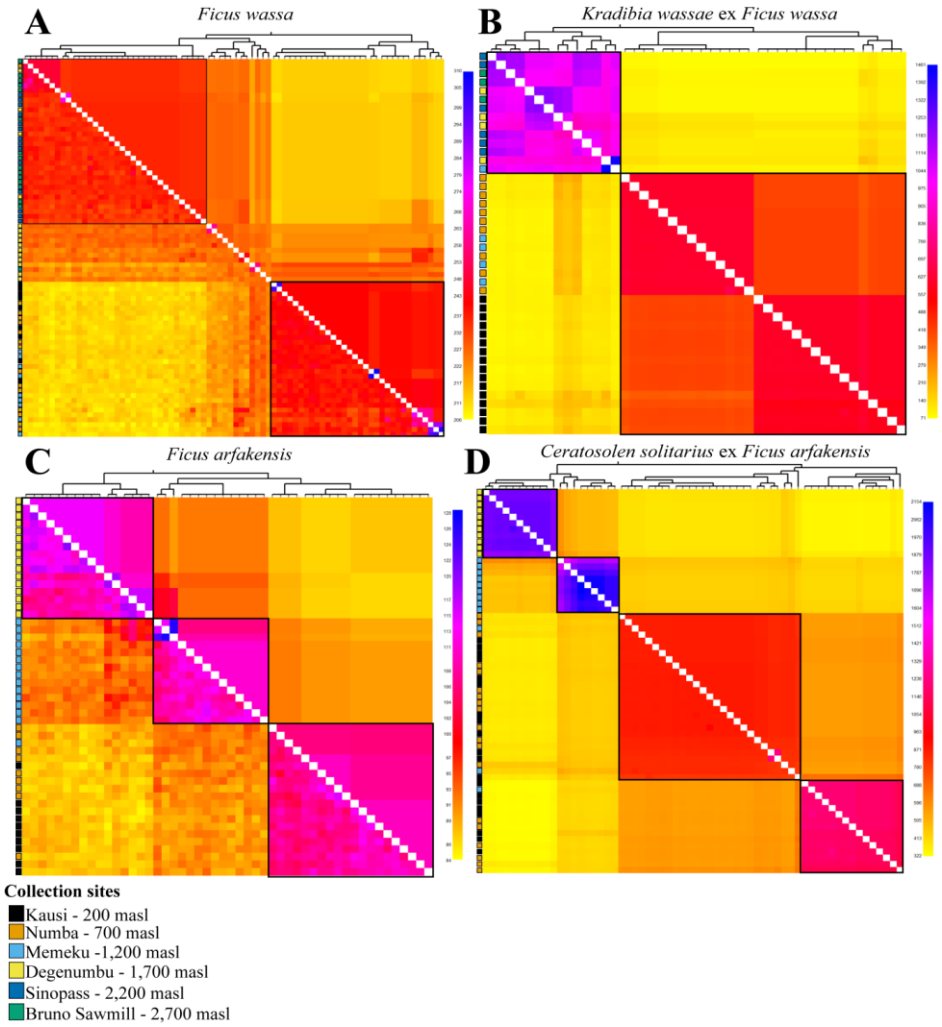


Figure 4. Co-ancestry heat map for F. wassa (above) and F. arfakensis (below) and their corresponding pollinating fig-wasps. FineRADstructure analysis shows substantial population structure among figs while revealing cryptic fig-wasps species responsible for pollination of the different fig populations. Highest levels of co-ancestry are indicated in dark blue. Lowest values of co-ancestry are indicated in yellow. Values above the diagonal are averaged across inferred populations, co-ancestry values for all pairs of individuals are displayed below the diagonal.

Ficus wassa: two populations of figs with a contact zone and three species of wasps

Figure 2 illustrates the level of co-ancestry between *F. wassa* individuals (Figure 2A) and of its corresponding pollinators *Kradibia wassae* spp. 1, 2 and 3 (Figure 2B). *Ficus wassa* has a wide elevational distribution from sea level to 2,700 m a.s.l. (Berg & Corner 2005). At our sites we find distinct highland and lowland populations, while the mid-elevations (here defined as 1,700 m a.s.l.) appear to represent a contact zone (Figure 2A). *Fst* values (Figure 4A) confirm this pattern showing that these populations of *F. wassa* have very little genetic differentiation (*Fst* between sites < 0.01). Wasps, however, display a contrasting pattern with a clear and sub-structured lowland cluster grouping all wasps from the 200 m a.s.l. site and a second group from the 700 and 1,200 m a.s.l. sites. There is further evidence of a deeper split between lowland and highland pollinator groups, while the high co-ancestry levels in highland populations (1,700 – 2,700 m a.s.l.) are suggestive of inbreeding. *Fst* values between wasp populations are correspondingly high (Figure 4B) while *Fst* values within the clusters show moderate genetic diversity (700 m a.s.l. vs. 1,200 m a.s.l. *Fst* < 0.09; between highland populations *Fst* <0.14). Complementary barcoding using cyt-*b* corroborated these results, yielding three MOTUs.

Ficus arfakensis: three populations of figs and four species of wasps

Equivalent plots are given for *F. arfakensis* (Figure 2C) and its corresponding pollinator(s) (*Ceratosolen solitarius*, spp. 1, 2, 3, and 4; Figure 2D). These plots suggest a split between highland (1,700 m a.s.l. and above) and lowland populations (below 1,200 m a.s.l.) and a distinct third entity at mid-elevations (1,200 m a.s.l.). The situation seems comparable to that of *F. wassa* as *Fst* values are moderate (*Fst*<0.16 for all comparisons) suggesting that *F. arfakensis* is a single species with a broad elevational distribution with a low degree of gene-flow between otherwise distinct populations (Figure 4C). The wasp data shows four clear groups: two sympatric wasp species occur in the lowlands (between 200 and 700 m a.s.l.), a third cluster at 1,200 m a.s.l. and a final cluster at 1,700 m a.s.l.. Pollinating wasps display a much more defined grouping with four distinct sets. *Fst* values for wasps retrieve high genetic diversity for each comparison (Figure 4D) and jMOTU analysis retrieved four distinct MOTUs. As with *F. wassa*, these results indicate three distinct *F. arfakensis* populations along the gradient, pollinated by four species of fig-wasps.

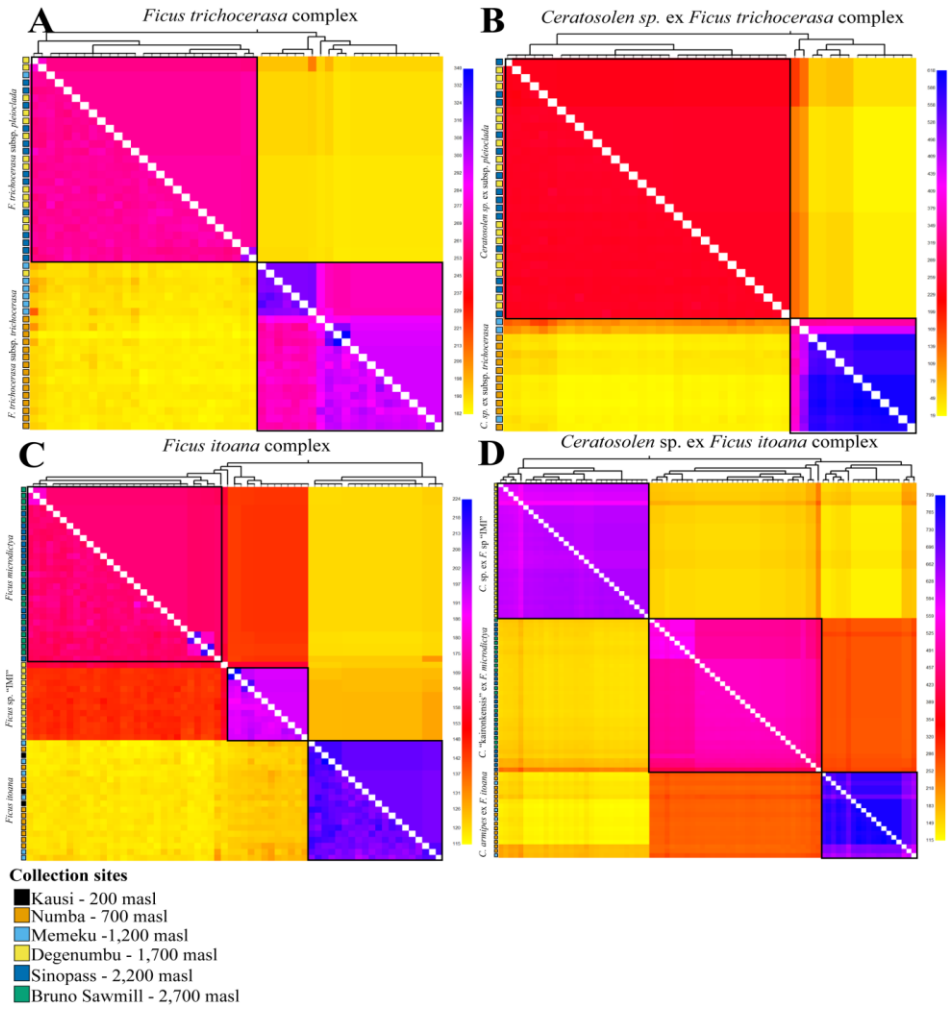


Figure 5. Co-ancestry heat map for F. trichocerasa (above) and the F. itoana complex (below) and their corresponding pollinating fig-wasps. FineRADstructure reveals clear clustering by (sub)species for both fig and pollinating fig wasps except in the case of F. trichocerasa pollinators at mid-elevations (1,200 – 1,700 m a.s.l.) which appear to be a contact zone between both subspecies. Highest levels of co-ancestry are indicated in dark blue. Lowest values of co-ancestry are indicated in yellow. Values above the diagonal are averaged across inferred populations, co-ancestry values for all pairs of individuals are displayed below the diagonal

Ficus trichocerasa: two subspecies of figs and two species of wasps

Figure 3 shows the co-ancestry matrix for *F. trichocerasa* subsp. *trichocerasa* and *F. trichocerasa* subsp. *pleioclada* (Figure 3A) and their corresponding fig-wasps (*Cerastosolen* spp. "TRI"; Figure 3B). *Ficus trichocerasa* has two clear and genetically defined clusters which correspond to the described ranges of both subspecies (Berg & Corner 2005; Souto-Vilarós *et al.* 2018). *Fst* values between subspecies show great genetic dissimilarity (*Fst* >0.20) while there appears to be very little dissimilarity within subspecies at different sites (*Fst* < 0.07; Figure 5A). Similarly, wasp individuals cluster according to the populations of their host subspecies. Unfortunately, due to limited rearing success at 1,200 m a.s.l. we were only able to retrieve information of three individuals which appear to constitute a third cluster or contact zone between subspecies. However, *Fst* values suggest these to be closely related to the 700 m a.s.l. population (*Fst* = 0.07) while they appear to be quite distinct from the highland (1,700-2,200 m a.s.l.) population (*Fst* > 0.80; Figure 5B). Our barcoding analysis recovered one lowland MOTU and one highland MOTU corresponding to host subspecies and a 1,200 m a.s.l. individual as a third MOTU.

Ficus itoana complex: three species of figs and three species of wasps

Finally, Figure 3 shows the co-ancestry matrix for all species in the *F. itoana* complex (*F. itoana*, *F.* sp. "IMI" and *F. microdictya*; Figure 3C) and their corresponding fig-wasps (*Ceratosolen armipes*, *C. sp.*

*"*IMI*"* and *C. "kaironkensis";* Figure 3D). Our analyses recover three distinct clusters corresponding to each of the three species. *Ficus* sp. *"*IMI*"* is closely related to its sister species *F. microdictya* (*Fst* =0.16), however, these two species have distinct breeding systems and pollinating fig-wasps (Souto-Vilarós *et al.* 2018). Interesting to note is that one *F.* sp. *"*IMI*"* individual (DEGIMI10F) appears to have shared ancestry between *F. microdictya* and *F.* sp. *"*IMI*"* suggesting it to be a hybrid between both. Pairwise *Fst* comparisons between clusters show moderate to very high genetic differentiation (*Fst* between 0.16 and 0.34; Figure 5C). In the case of the pollinating fig-wasps of this complex, we identify three clear clusters corresponding to each of the host species and *Fst* values between comparisons show great genetic differentiation between pollinating fig-wasps (Figure 5D). We were unable to amplify cyt-*b* sequences for pollinating wasps of *F.* sp. "IMI," however, the morphological and behavioural differences described by Souto-Vilarós *et al.*(2018) provide compelling evidence that this is indeed a separate species. Similarly, a BLAST (Madden 2003) search for highly similar sequences (megablast) on the NCBI nucleotide collection database of our COI sequences for these individuals found that these sequences are equally dissimilar to those from *C. armipes* and *C. "kaironkensis"*, which are the closest matches.

DISCUSSION

We assessed genetic structure and divergence of several fig species and their corresponding pollinating fig-wasps at different sites along the Mt. Wilhelm elevational gradient. Our analysis suggests that during the early stages of the speciation process, fig-wasps diversify faster than figs, generating locally adapted species along the gradient. These wasp species in turn likely restrict pollen movement and shape fig population structure. If gene flow mediated through seed dispersal remains restricted to these local populations, fig ecotypes remain reproductively isolated from each other, leading to their eventual speciation. Subsequent extinction of co-occurring wasp species, for example due to competition, may then restore the one-to-one ratio. Based on mathematical modelling, Kiester *et al.* (1984) suggested that any small pool of figs which differ enough to promote restricted gene flow, could co-evolve with their pollinating wasps thus promoting cospeciation (Kiester *et al.* 1984). We found that wasps experience far greater limitations to gene flow along the gradient than do figs. Wasp sub-populations may in turn promote character divergence within fig ecotypes (for example through volatile preference) leading to restricted gene flow of figs and eventual speciation. Divergence between these wasp sub-populations, as measured by *Fst* comparisons and jMOTU analyses, suggest that these entities could be considered as distinct wasp species. Our findings held across multiple pairs of interacting species across the different stages of the speciation process, however, further work is required both in terms of taxa and regions. Additional studies within the New Guinean central range would show if this differentiation

occurs in parallel, if this is the case there may be far more pollinating fig-wasps than previously expected.

Elevation as an obstacle

Environmental conditions can change very rapidly over short distances along elevational gradients, which may lead to important reproductive barriers in both plants and animals (Bachman *et al.* 2004; Körner 2007; Rieseberg & Willis 2007; Reis *et al.* 2015). Figs and fig pollinating wasps are known to have coevolved over millions of years (Cruaud *et al.* 2012), however, there remains uncertainty regarding how many wasp speciation events are due to strict cospeciation, host shifts and/or duplications (Cook & Segar 2010). The results presented herein add to the growing list of exceptions regarding the one-to-one species specificity believed to be the norm in the fig and fig-wasp mutualism. It appears that environmental heterogeneity, in this case elevation, plays an important role in the local isolation of wasp populations leading to the formation of new species, without the necessity of a new host fig species. Due to their short life-span and weak dispersal abilities, specifically for below-canopy pollinators (Dev *et al.* 2011; Wachi *et al.* 2016), fig wasps seem to form genetically distinct sub-populations along their host's range. This structuring within wasps may eventually restrict pollen movement, promoting fig population structure and ultimately, host fig speciation (Cook & Segar 2010).

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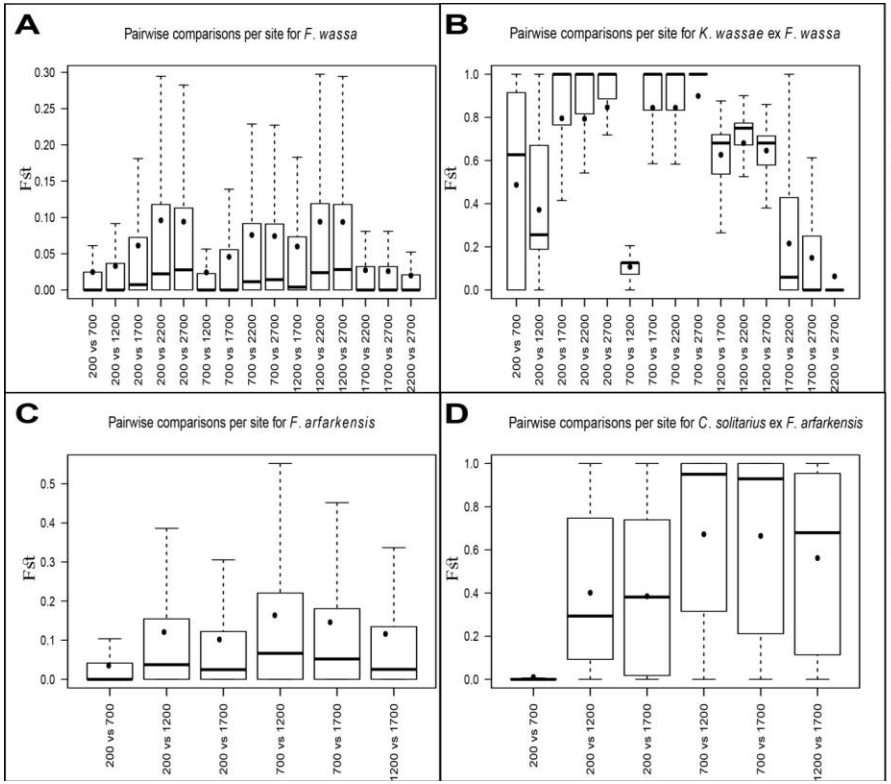
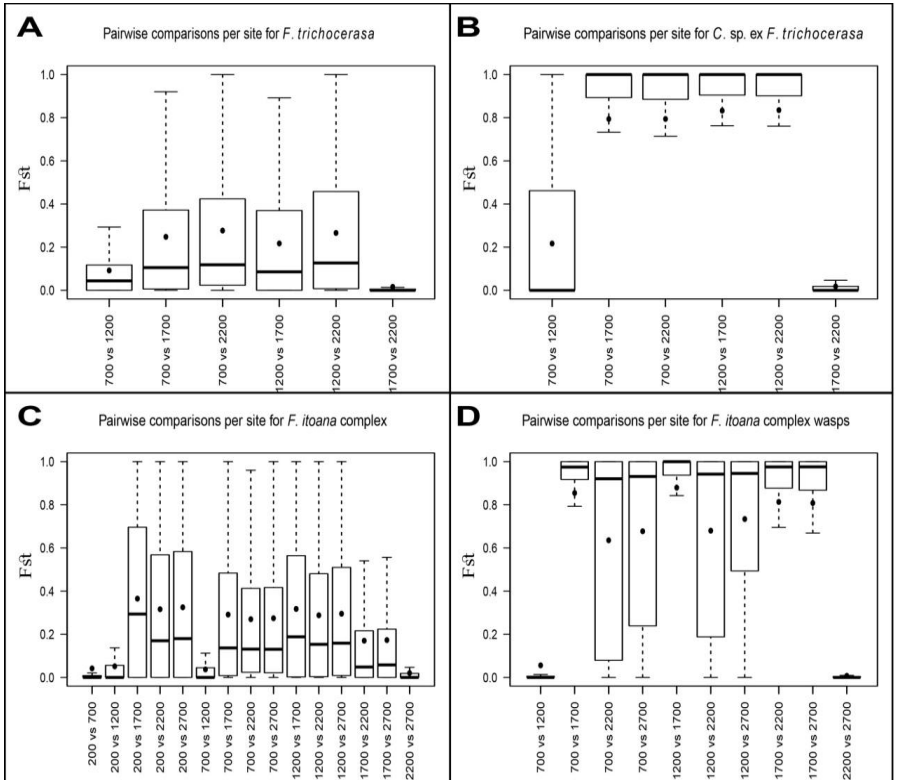


F. wassa	200	700	1,200	1,700	2,200	2,700
200		**0.001**	**0.002**	**0.044**	**0.081**	**0.080**
700	0.479		**0.004**	**0.032**	**0.064**	**0.064**
1,200	0.364	0.095		**0.040**	**0.077**	**0.078**
1,700	0.771	0.830	0.604		**0.012**	**0.011**
2,200	0.774	0.829	0.665	0.142		**0.004**
2,700	0.821	0.893	0.634	0.045	0.117	

F. arfarkensis	200	700	1,200	1,700
200		**0.016**	**0.110**	**0.155**
700	0.013		**0.091**	**0.138**
1,200	0.386	0.368		**0.107**
1,700	0.667	0.659	0.551	

Figure 6. Fst comparisons between sites for *F. wassa* (above) and *F. arfakensis* (below) and their corresponding pollinating fig-wasps. Dots in the box-plots indicate mean Fst values. Fst values above the diagonal in bold indicate mean Fst values for figs while below the diagonal are Fst values for pollinating fig-wasps.



F. trichocerasa complex	700	1,200	1,700	2,200
700		**0.075**	**0.243**	**0.273**
1,200	0.074		**0.206**	**0.256**
1,700	0.770	0.808		**0.001**
2,200	0.767	0.811	0.004	

F. itoana complex	200	700	1,200	1,700	2,200	2,700
200		**0.028**	**0.027**	**0.340**	**0.291**	**0.302**
700	na		**0.003**	**0.286**	**0.265**	**0.270**
1,200	na	0.041		**0.306**	**0.278**	**0.286**
1,700	na	0.853	0.872		**0.163**	**0.167**
2,200	na	0.630	0.652	0.812		**0.005**
2,700	na	0.674	0.714	0.807	0.007	

Figure 7. Fst comparisons between sites for *F. trichocerasa* (above) and the *F. itoana* complex (below) and their corresponding pollinating fig-wasps. Dots in the box-plots indicate mean Fst values. Fst values above the diagonal in bold indicate mean Fst values for figs while below the diagonal are Fst values for pollinating fig-wasp.

Kobmoo *et al.* (2010) found that a single population of *Ceratosolen fusciceps* Mayr pollinates *F. racemosa* Linn. throughout most of South-East Asia, a relatively homogeneous, but large biogeographic region. However, these authors found a genetically distinct entity in mainland Australia pollinating the same fig species. Similarly, studies on *Pleistodontes imperialis* Saunders (the pollinator of *F. rubiginosa* Desf. ex. Vent.) found limited gene flow and possible cryptic species over a wide latitudinal gradient in eastern Australia. Together, these results suggest that pronounced geographic barriers such as large water bodies, or environmental gradients, may lead to wasp population divergence and allopatric speciation (Haine *et al.* 2006; Sutton *et al.* 2016). Our data suggest that even within broadly ranging fig species (i.e. *F. wassa* and *F. arfakensis*), different elevational populations host different species of wasps; pollinator specificity and dispersal abilities in turn restrict gene flow between fig populations. In these cases, a certain degree of gene flow between fig populations maintains fig species integrity, possibly through seed dispersal. It has been reported that immigrant seed gene flow may have evolutionary significant consequences over wide distances, maintaining otherwise isolated entities as a single species (Ellstrand 2014).

The *F. trichocerasa* subspecies complex represents an additional step in the speciation continuum, where these subspecies have developed distinct volatile profiles (Souto-Vilarós *et al.* 2018) and morphological differences (which become less clear at the contact zone, between 1,200 – 1,500 m a.s.l.; (Berg & Corner 2005)). The development of such differences suggests these taxa are in the early stages of ecological speciation, even though they are not yet totally

differentiated (Wu 2001). Since scent is believed to be a major trait in pollinator attraction in the fig-wasp mutualism (Proffit *et al.* 2009; Hossaert-McKey *et al.* 2016), ecological isolation due to pollinator preference for each subspecies may eventually lead to the formation of fully separate fig species.

In the case of the *F. itoana* complex, both highland and lowland fig species have been previously described and shown to be one of the few cases of fig breeding system reversal from dioecy to monoecy (Weiblen 2004; Berg & Corner 2005). It is thought that bisexual flowers are the ancestral state of most, if not all, dioecious angiosperms and that dioecy developed through intermediate steps (monoecy, gynodioecy, distly, etc.) (Weiblen, Oyama & Donoghue 2000; Torices, Méndez & Gómez 2011; Käfer *et al.* 2014). It seems that *Ficus* sp. "IMI" could represent an intermediate step during the transition from dioecy to monoecy in this clade as this seems to be the first described case of an andromonoecious (producing bisexual and male flowers in the same tree) fig species (Souto-Vilarós *et al.* 2018).

Restricted dispersal of both pollen and seeds as promoters of fig speciation

Since fig-wasps are the sole vectors of pollen between figs, multiple species along the gradient certainly restrict pollen flow between fig populations and (sub)species. It is clear that seed dispersal plays an important role in the maintenance of connectivity between fig ecotypes along the gradient. Our focal species have a variety of fruit syndromes which suggests different seed dispersing guilds (Shanahan

2000; Shanahan *et al.* 2001; Lomáscolo *et al.* 2010). *Ficus wassa* and *F. arfakensis* both have small, red berry-like figs, typical of bird dispersed fruits. Despite being pollinated by multiple fig-wasp species, the figs still maintain some degree of genetic connectivity. Seed dispersal by wide ranging birds may be responsible for the maintenance of such population structure. On the other hand, *F. microdictya* has small, green to reddish figs at the top of the canopy, probably eaten by birds. Previous bird surveys on the same transect (Sam & Koane 2014; Marki *et al.* 2016; Sam *et al.* 2017) demonstrated that bird communities differ along the gradient with few species being found throughout its entirety, suggesting that preferential feeding by different types of birds might maintain the observed gene flow patterns. Similarly, *Ficus trichocerasa* and *F. itoana* both display fleshy, green to purple fruits eaten by bats. Frugivorous bats have been shown to be reliable *Ficus* seed dispersers (Shilton *et al.* 1999; Thornton *et al.* 2001), however, the range in which they disperse may be different according to species. Bat abundance decreases sharply with elevation at our site (P. Amick, unpublished data) suggesting that dispersal by bats may be restricted. Finally, *F.* sp. "IMI" has large, brown to purple fleshy fruits clustered at the base of the trunk, eaten by understory animals such as bandicoots (M. S. pers. obs.) and seed dispersal by these animals is strongly linked to their home range. It is clear that seed dispersal plays an integral role in the process of reproductive isolation in figs. In order to make further assumptions on the drivers of fig population structure and speciation, identifying seed dispersers, and the distances at which they are able to disperse seeds seems indispensible.

Figs and wasps present a remarkable system to study speciation, from the early phases of genetic differentiation to the establishment of reproductive barriers. Despite significant research efforts, we still have only insufficient understanding of the mechanisms that generate diversity. Our results demonstrate the cases when simple patterns break down (multiple interacting species of wasps and figs are involved). They also reveal the evolutionary consequences of increased complexity. These findings pave the way toward a more realistic and nuanced understanding of the speciation process when multiple interacting partners are involved. Our results pertain to an understudied region famous for its remarkable diversity, the tropical mountains of New Guinea. They showcase how new diversity is being generated along the mountain slopes in the tropics, and how the origination of species may be unbalanced even within the constraints of a tight mutualism. Our findings further illustrate some of the mechanisms which limit gene flow between species, subspecies, and populations along an elevational gradient and thus promote speciation in the fig and fig-wasp mutualism.

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AUTHOR'S CONTRIBUTIONS

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

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Chapter III

Pollination along an elevational gradient mediated by both floral scent and pollinator compatibility in the fig and fig-wasp mutualism

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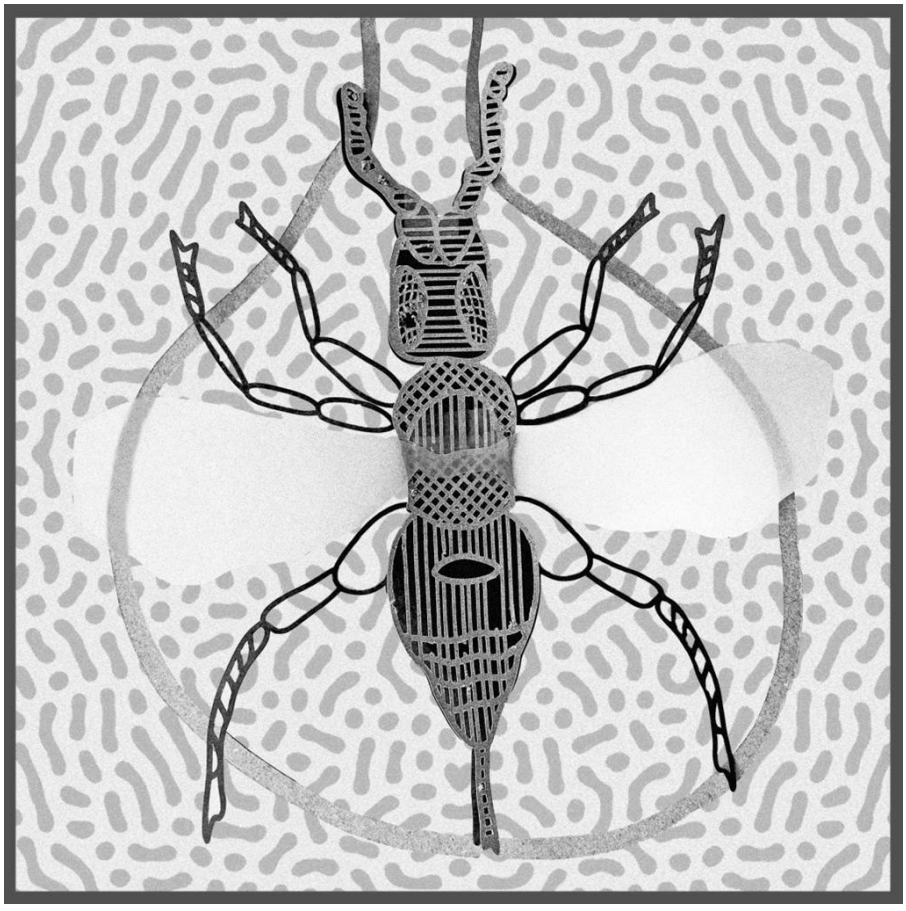

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Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism

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Abstract

1. In the fig (Moraceae) and fig-wasp (Agaonidae) mutualism, scent is believed to be of primary importance in pollinator attraction and maintenance of species specificity. Scent divergence between closely related *Ficus* species seems sufficient in promoting reproductive isolation through pollinator behaviour, starting the process of speciation.
2. We investigated volatile organic compound (VOC) variation from figs in several *Ficus* species endemic to Papua New Guinea. Sister species of section *Papuacyse* and subspecies of *Ficus trichocerasa* substitute each other along the continuously forested Mt. Wilhelm elevational gradient. We placed these species in a phylogenetic context to draw conclusions of scent divergence between close relatives. In addition, pollinator response to VOCs emitted by figs of different species was tested.
3. Volatile profiles differed significantly between focal species, although with a varying degree of overlap between (sub)species and elevations. Pollinators were generally attracted to VOCs emitted only by their hosts except in one case where pollinating fig wasps were also attracted to the sister species of its host. Wasp morphological traits, however, indicate that it is mechanically impossible for this species to oviposit in figs of this atypical encounter.
4. *Synthesis.* This study demonstrates that while scent is an effective signal for partner recognition, there are multiple barriers which help maintain prepollination isolation in fig and pollinating fig-wasp interactions. Speciation along this elevational gradient is reinforced by divergence in key reproductive isolation mechanisms on both sides of the mutualism.

KEYWORDS
character divergence, evolutionary ecology, fig pollination, fig-wasp attraction, reproductive isolation, sister species, speciation, volatile organic compounds (VOCs)

1 | INTRODUCTION

Interactions between plants and insects are a key process shaping species diversity, with over 75% of described species being involved in an insect–plant food web and an estimated 87% of angiosperms being pollinated by animals (Ollerton, Winfree, & Tarrant, 2011; Price, 2002). These two groups exert clear selective pressures on each other, thus reciprocally affecting each other's evolution. In fact, due to ecological relationships between these two groups being tightly linked, it has been proposed that they may codiversify (Ehrlich & Raven, 1964). Whether through herbivory or pollination, reciprocal evolutionary interactions between plants and insects have led to ecologically mediated speciation and the diversification of both parties (Givnish, 2010; Ollerton et al., 2011). For instance, pollinator-mediated selection has often been invoked as a mechanism driving the radiation of angiosperms, since specialization or shifts to different pollinators can, in theory, lead to rapid and effective reproductive isolation (Bischoff, Raguso, Jürgens, & Campbell, 2015; Fenster, Armbruster, Wilson, Dudash, & Thomson, 2004; Grant, 1994; Schemske & Bradshaw, 1999; Sedeek et al., 2014; Whitehead & Peakall, 2014; Van Der Niet, Peakall, & Johnson, 2014). Reproductive isolation, a fundamental step in speciation (Dobzhansky, 1951; Givnish, 2010), is achieved by a series of barriers limiting gene flow between species (Coyne & Orr, 2004; Lowry, Modliszewski, Wright, Wu, & Willis, 2008). In flowering plants, postpollination barriers such as pollen competition, gametic incompatibilities, and hybrid sterility or negative fitness, ensure reproductive isolation in plants (Coyne & Orr, 2004). In addition, prepollination barriers caused by geographical and/or temporal isolation, and barriers mediated through morphological incompatibilities, pollinator attracting signals and pollinator behaviour similarly contribute to reproductive isolation (Sedeek et al., 2014; Whitehead & Peakall, 2014). Reproductive isolation can feasibly lead to local adaptation and selection against the exchange of maladapted genotypes, and thus, we may predict divergent pollinator attracting signals in close relatives along environmental gradients (e.g. with elevation).

Indeed, there is mounting evidence demonstrating how flower colour, odour and morphology can promote reproductive isolation through pollinator preference (Bischoff et al., 2015; Lavi & Sapir, 2015; Peakall & Whitehead, 2014; Schemske & Bradshaw, 1999; Sedeek et al., 2014; Sun, Schlüter, Gross, & Schiestl, 2015), but the general trend is that floral isolation emerges through an interaction of several pre- and postpollination barriers (Sun et al., 2015; Whitehead & Peakall, 2014). Despite some exceptions, pollinators rarely rely on a single cue to differentiate between flowers; rather they depend on a suite of traits. Recent studies in monkeyflowers, some of the classic models for the study of pollinator-mediated evolution, have found that coupled with flower colour, volatile compounds and ecogeographical isolation play an important role in maintaining reproductive isolation between two sister species (Byers, Bradshaw, & Riffell, 2014). *Mimulus lewisii* (Phrymaceae) and *Mimulus cardinalis* have been shown to consistently attract distinct pollinators (bumblebee and hummingbird, respectively) based on flower colour, justifying reproductive isolation through pollinator preference. However, these two species are also ecologically separated by altitude, and only a narrow part of their ranges overlap (Bradshaw & Schemske, 2003). Recently, Byers et al. (2014) found that three monoterpene volatiles present in *M. lewisii* are sufficient to attract bumblebee pollinators, further maintaining reproductive isolation between these two sister species. Similarly, studies in *Ipomopsis* (Polemoniaceae) have found that a single volatile compound (indole) present in flowers of *Ipomopsis tenuituba* but not its close relative *Ipomopsis aggregata* is responsible for attracting hawkmoths to flowers. However, only in the presence of white flowers did the moths feed, and thus pollinate, *I. tenuituba* flowers indicating that hawkmoths require both olfactory and visual cues (Bischoff et al., 2015).

Nevertheless, pollinator specificity is an important isolating mechanism determining the extent of gene flow between taxa, and thus determining species boundaries (Givnish, 2010; Schiestl & Schlüter, 2009; van der Niet & Johnson, 2012). Some of the most species-rich angiosperm groups (e.g. Orchidaceae) often depend on specialized pollinators (Schiestl & Schlüter, 2009), and some studies suggest that divergence in scent between closely related species may be a fundamental mechanism in restricting pollen movement between species, thus promoting floral isolation (Bischoff et al., 2015; Chen et al., 2009; Peakall & Whitehead, 2014; Schiestl, 2015; Sedeek et al., 2014).

Nursery pollination systems are, perhaps, some of the most extreme cases of pollinator specialization, since the reproductive success of both parties often relies on the maintenance of species-specific recognition. Previous studies in nine of the 16 known nursery pollination systems indicate that scent may play a key role in guiding pollinators to find suitable host plants (for a review, see: Hossaert-McKey, Soler, Schatz, & Proffit, 2010 and references therein). In the case of the fig and fig-wasp mutualism, floral scents from many species have been identified, and there are several examples of how these chemical signatures influence pollinator behaviour (Chen et al., 2009; Grison-Pigé, Bessière, & Hossaert-McKey, 2002; Hossaert-McKey et al., 2016; Proffit et al., 2009; Ware, Kaye, Compton, & Van Noort, 1993; Yokoyama, 2003). The pollination ecology of *Ficus* has been extensively described (Galil & Eisikowitch, 1971; Kjellberg, Jousselin, Hossaert-McKey, & Rasplus, 2005), but briefly summarizing, pollen-loaded female agaonid wasps (Chalcidoidea) emerge from the figs (enclosed inflorescences called syconia) in search of trees bearing receptive syconia. Figs emit several common compounds in particular combinations (or bouquets), to attract their obligate pollinating wasps, which upon landing search for the ostiole, a narrow entrance at the apex of the syconia, the only entrance to the flowers enclosed within (Grison-Pigé, Hossaert-McKey, Greeff, & Bessière, 2002; Hossaert-McKey et al., 2016; Soler, Proffit, Bessière, Hossaert-Mckey, & Schatz, 2012; Ware et al., 1993). Once inside the syconia, the wasps oviposit in the ovules of short-styled flowers which generally match the length of the wasp's ovipositor, while simultaneously pollinating long-styled flowers which will produce seeds. Larvae develop

within the syconia and upon reaching maturity, wingless males chew a hole from which fertilized females will exit the fig and repeat the process. In the case of functionally dioecious figs (approximately half of known *Ficus* species), some trees bear only male figs that become nurseries for the next generation of pollinating fig wasps. In synchronous flowering species, female fig trees engage in a type of deceptive pollination where through mimicry of male fig volatile emissions they lure fig wasps to entering the female figs which will house no wasps and produce only seeds (Hossaert-McKey et al., 2016).

Similar to sexually deceptive orchids, speciation of figs could potentially arise from changes in the composition of the plant's attractive volatiles (Rodriguez et al. 2017; Sedeek et al., 2014; Ware et al., 1993). On the other hand, there is increasing evidence suggesting that there may be pollinator sharing between certain species of figs and in some cases, being explicitly attracted to volatile emissions of sympatric species (Moe, Rossi, & Weiblen, 2011; Wang, Cannon, & Chen, 2016). This has some important implications to the species delimitation and evolutionary history of this mutualism, and although Moe et al. (2011) and Moe and Weiblen (2012) report a low frequency of natural hybrid trees, Wang et al. (2016) report pollinator sharing, a significant number of hybrids and high levels of geneflow between five sympatric fig species, likely due to pollinators being attracted to atypical host species.

In addition to unique volatile profiles, it seems that non-volatile cuticular cues, ostiole size and shape, and floral arrangement within the syconia act together as prepollination barriers which help maintain the stability of this mutualism (Borges, 2016; Galil & Eisikowitch, 1971; Ganeshaiah, Kathuria, Shaanker, & Vasudeva, 1995; Gibernau, Hossaert-mckey, Frey, & Kjellberg, 1998; Grison-Pigé, Hossaert-McKey, et al., 2002; Hossaert-McKey et al., 2016; Wang, Compton, & Chen, 2013). Moe and Weiblen (2012) developed a method to coerce pollinating wasps to enter non-natal fig species and found that seed viability resulting from these crosses was only marginally affected, while wasps could lay their eggs and develop galls, but offspring failed to reach maturity. In other cases, due to a mismatch between ovipositor and style length of flowers, wasps are mechanically unable to lay eggs altogether, resulting in zero reproductive success for wasps entering the wrong host (Borges, 2016; Kjellberg et al., 2005; Weiblen, 2004).

Efficient attraction by the host fig, coupled with recognition and morphological compatibility of these tiny (1–2 mm), short-lived wasps (24–48 hr, but estimates vary depending on species) is crucial for ensuring the reproductive success of both parties. Although the link between fig volatile profiles and pollinator attraction has been well established (Chen et al., 2009; Grison-Pigé, Bessière, et al., 2002), few studies have focused on volatile profiles of closely related species, and how these affect pollinator behaviour (Wang et al., 2016). In tropical forests, many closely related *Ficus* species occur in sympatry (Berg & Corner, 2005; Cornille et al., 2012; Moe & Weiblen, 2012; Soler et al., 2011; Wang et al., 2016), making such encounters are especially interesting. Focusing on species pairs which replace each other with altitude allows us to study the multiple barriers acting to promote specificity and speciation in such systems.

This study combines molecular data and volatile profile analysis of one *Ficus* species complex and a single species (with two recognized subspecies) along an elevational gradient. Together with pollinating wasp morphology and behaviour, we attempt to reveal the prepollination barriers which help maintain species specificity in such a tightly linked system. Overall, we predict that parapatric sister species and populations along an altitudinal transect will diverge in their volatile signals to avoid gene flow between maladapted genotypes and species. These differences should also be reflected in the behaviour of their highly co-evolved pollinators. Furthermore, fig and wasp morphology can also serve as an additional "lock and key" mechanism to ensure compatibility in cases where volatile signals appear too ambiguous for wasps.

More specifically, our expectations concerning pairs of (sub) species replacing each other along the transect are that: (1) volatile profiles should strongly diverge in order to avoid attracting the wrong pollinators, since VOCs are of primary importance during the identification of receptive figs (Gibernau et al., 1998; Grison-Pigé, Bessière, et al., 2002). (2) Pollinator behaviour will reflect preference to receptive figs of its host species rather than to close relatives, as behaviour alone could be an effective isolating mechanism explaining the rarity of natural *Ficus* hybrids (Moe & Weiblen, 2012). Expectations (1) and (2) are, therefore, directly linked. Finally, (3) wasp morphology must also be compatible with fig host morphology, since wasps must be able to crawl through the ostiole, and oviposit in the ovules of flowers with compatible lengths, serving as a final barrier for wasps entering an atypical host (Kjellberg et al., 2005; Weiblen, 2004).

2 | MATERIALS AND METHODS

2.1 | Study system and collection sites

There are at least 150 *Ficus* (Moraceae) species recorded from the island of New Guinea (Berg & Corner, 2005), some of these have wide elevational ranges (from 200 to 2,700 metres above sea level [masl]) and are key-stone species in forest communities (Novotny et al., 2005; Segar et al., 2017). Along the Mount Wilhelm elevational gradient in the central range of Papua New Guinea (PNG) almost half of these species are found. Here, we focus on an island endemic species complex in *Ficus* section *Papuacyse* including *Ficus itoana* Diels and *Ficus microdictya* Diels, sister species according to Weiblen (2004). A third entity, here referred to as *Ficus* sp., is a yet to be named species discovered by morphological and genomic analyses (see Section 3). *Ficus itoana* is pollinated by *Ceratosolen armipes* Wiebes and is distributed in hill forests up to 1,200 m a.s.l., while *F. microdictya*, pollinated by *C.* sp. "*kaironkensis*" (*nom.nud*; Weiblen, 2001) occurs at higher altitudes, ranging from 1,500 to 2,000 m a.s.l. At Mt. Wilhelm, *Ficus* sp. is most commonly encountered in a contact zone around 1,700 m a.s.l. (pers. obser.). Its pollinating fig wasp has not been described, and is here on referred to as *Ceratosolen* sp. *Ficus itoana* is functionally dioecious, while *F. microdictya* is monoecious, representing one of the few known cases of evolutionary reversal to

the monoecious condition of the genus (Weiblen, 2004). However, Berg and Corner (2005) noted that some specimens attributed to *F. itoana* bear monoecious figs. Here, we report on dissections of figs from the mid-elevation contact zone between *F. itoana* and *F. microdictya* that suggests a third sexual system in *Ficus* sp. that is neither strictly dioecious nor monoecious.

A second species complex we examined includes both subspecies of *F. trichocerasa* Diels, a documented example of lowland and highland subspecies (Berg & Corner, 2005). Subspecies *trichocerasa* is most commonly found between 700, and 1,200 m a.s.l. although there are some collections made at altitudes between 1,400 and 1,700 m a.s.l. (and up to 2,150 m a.s.l.; Berg & Corner, 2005), while subspecies *pleioclada* is found at altitudes between 1,500 and 2,600 m a.s.l. The morphological differences between the two are easily recognizable but become less clear in the zone of contact (Berg & Corner, 2005). Both are dioecious species pollinated by *Ceratosolen* wasps (species undescribed). Focal species and their corresponding pollinating wasps, along with their localities, are summarized in Table 1. Figure 1 shows the geographical distribution of the collection sites. Voucher photographs for both figs and wasps are presented as supporting information (Figures S1–S6). Vouchers of figs are deposited at the National Herbarium in Lae, PNG, and at the New Guinea Binatang Research Centre, PNG, as well as at the Czech Academy of Sciences, Czech Republic. Wasp vouchers are stored at the Czech Academy of Sciences, Czech Republic.

All collections were performed at the three sites along the elevational transect where these species were most abundant, site details are summarized in Table 1. *Ficus itoana* and *Ficus* subspecies *trichocerasa* were collected at Numba (700 m a.s.l.); *Ficus* sp. and subspecies *pleioclada* at Degenumbu (1,200 m a.s.l.) and *F. microdictya* plus a second collection of subspecies *pleioclada* were collected at Sinopass (2,200 m a.s.l.). During the study period (October to November 2016), it was possible to find several individual trees bearing figs at different developmental stages. This allowed us to collect both receptive figs for volatile collection and figs ready for hatching out wasps to use in Y-tube assays. In addition, during a previous field season (September to December 2015) and as part of a wider population genomic study (Souto-Vilarós et al., in prep.), using a cork borer (2.4 cm diameter), we collected 15 leaf discs from 10 individual trees into colour indicating silica gel and subsequently stored them at −20°C before DNA extraction and next-generation sequencing analysis.

2.2 | DNA extraction and sequencing

DNA was isolated from one leaf disc (*c.* 2 mg dry tissue) using CTAB protocol (Doyle & Doyle, 1987) followed by an extra cleaning step through a silica column (as per Segar et al., 2017). This step removed all traces of polyphenols and secondary metabolites yielding highly concentrated and pure DNA. Samples were diluted to a total of 200 ng (quantified in a Qubit 3 Fluorometer; ThermoFisher Scientific) in 40 μL of EB buffer (Qiagen) and sent to SNPsaurus, LLC for genotyping-by-sequencing using Nextera-tagmented reductively amplified DNA sequencing (NextRAD; as per Russello, Waterhouse, Etter, & Johnson, 2015). Genomic DNA is first fragmented with Nextera reagent (Illumina, Inc.) which also adds short adapter sequences to the end of the fragments. The Nextera reaction was scaled for fragmenting 7 ng of genomic DNA, although 17.5 ng of genomic DNA was used for input to compensate for degraded DNA in the samples. Fragmented DNA was then amplified for 26 cycles at 73°C, with one of the primers matching the adapter and extending nine nucleotides into the genomic DNA with the selective sequence GTGTAGAGC. Thus, only fragments starting with this sequence can be efficiently amplified. The nextRAD libraries were sequenced single end on a HiSeq 4000 with two lanes of 150 bp reads, single individual per lane (University of Oregon, USA). Because not all trees sampled for DNA analysis were found at receptive stage during the volatile collection, not every tree matches both molecular and volatile analyses (Table S2).

2.3 | Volatile organic compounds (VOCs) collection

VOCs were collected in situ using an adsorption-desorption headspace technique (Cornille et al., 2012; Hossaert-McKey et al., 2016; Soler et al., 2011). For each species (Table 1), between three and 10 individual trees were sampled for volatile collection. For each collection, an average of 35 receptive figs per tree were enclosed in polyethylene terephtalate (Nalophane®, Kalle Nalo GmbH, Wursthüllen, Germany) bags and shut tightly with cotton string. ChromatoProbe® quartz microvials of Varian Inc. (length: 15 mm; inner diameter: 2 mm), previously cut closed-end and filled with 3 mg of a 1:1 mix of Tenax-TA and Carbotrap® (60–80 and 20–40 mesh, respectively; Sigma Aldrich, Munich, Germany), were used as adsorbent traps. One microlitre of a solution of internal standards (n-Nonane and n-Dodecane, 110 ng/μl of each) was added to each trap before scent extraction, to ensure that samples did not suffer loss during storage and transport so that our analysis could run properly. Traps were attached to silicone tubing within the collection bags and connected on the other end to flowmeters and a standard 12-V air pump. Fig collections were left in the shade for 30 min and 200 ml/min air flow was drawn out of the bag and over the trap for 5 min. In parallel, blank extractions were performed using empty bags, to control for ambient contaminant compounds; we collected one blank sample per site per collection day. Collections were done under natural light and ambient temperature, which ranged from 15°C in the highland sites to 30°C in the lowlands, between 10:00 and 17:00 hr. All samples were kept in clean glass vials and stored in the dark, in a portable cooler, until transport to a −20°C freezer where samples remained until analysis. Due to varied field conditions, the time before samples reached the freezer was between 3 and 10 days from collection. Chemical analyses were conducted within 1 month of collection. One additional volatile collection of three individuals of *Ficus adenosperma* (subgenus *Sycomorus*, section *Adenosperma*) was conducted at Ohu village, and this species was used as an "outgroup."

TABLE 1 Sampled species, reproductive system of each species, corresponding pollinating wasp and name of sampling locality and GPS coordinates

Ficus species	Sexual system	Pollinating wasp	Sampling locality	Elevation (masl)	GPS coordinates
Ficus itoana	Dioecious	*Ceratosolen armipes*	Numba	700	05°44′14″S, 145°16′12″E
Ficus sp.	Andromonoecious	*Ceratosolen* sp.	Degenumbu	1,700	05°45′45″S, 145°11′55″E
Ficus microdictya	Monoecious	*Ceratosolen "kaironkensis"*	Sinopass	2,200	05°45′34″S, 145°10′49″E
Ficus adenosperma[a]	Dioecious	*Ceratosolen cf adenospermae*	Ohu	200	05°14′00″S, 145°41′00″E
Ficus arfakensis[a]	Dioecious	*Ceratosolen solitarius*	Degenumbu	1,700	05°45′45″S, 145°11′55″E
Ficus trichocerasa subsp. *trichocerasa*	Dioecious	*Ceratosolen* sp. 1	Numba	700	05°44′14″S, 145°16′12″E
Ficus trichocerasa subsp. *pleioclada*	Dioecious	*Ceratosolen* sp. 2	Degenumbu and Sinopass	1,700 and 2,200	05°45′45″S, 145°11′55″E and 05°45′34″S, 145°10′49″E

[a]These species were used as outgroups for the volatile (*Ficus adenosperma*) and phylogenetic (*Ficus arfakensis*) analyses.

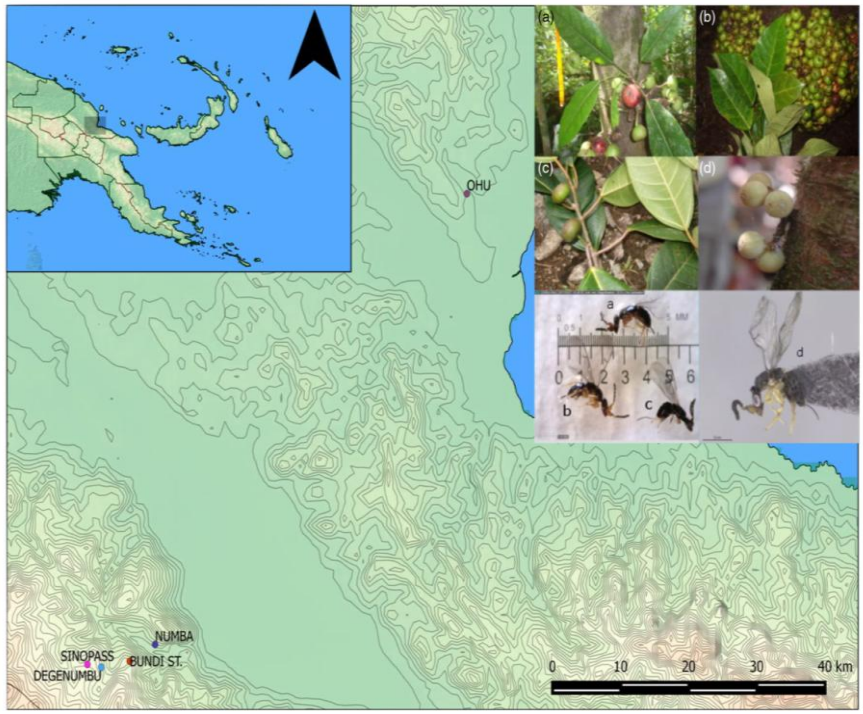

FIGURE 1 Geographical location of field sites along the Mount Wilhelm elevational gradient in Madang province, Papua New Guinea. Contour lines every 100 m. Inset: Focal *Ficus* species used in this study (upper case) and their corresponding pollinating fig wasps (lower case): a: *Ficus itoana*; b: *Ficus* sp.; c: *Ficus microdictya*; d: *Ficus trichocerasa*

2.4 | VOC analysis

Samples were analysed at the "Platform for Chemical Analyses in Ecology" (PACE), technical facilities of the LabEx CeMEB (Centre Méditerranéen pour l'Environnement et la Biodiversité, Montpellier, France), using a gas chromatograph (GC, Trace™ 1310, Thermo Scientific™ Milan, Italy) coupled to a mass spectrometer (ISQ™ QD Single Quadrupole, Thermo Scientific™ Milan, Italy). The

column used was an Optima 5-MS capillary column (30 m, 0.25-mm internal diameter, 0.25-μm film thickness, Machery-Nagel, Düren, Germany). Absorbent traps were handled with a Multi Purpose Sampler (Gerstell, Mülheim, Germany) and desorbed with a double stage desorption system, composed of a Thermal Desorption Unit (TDU) and a Cold Injection System (CIS) (Gerstell, Mülheim, Germany). First, the filters were splitless with a temperature of 250°C on the CIS trap cooled at −80°C by liquid nitrogen. Then, the CIS trap was heated to 250°C with a 1:4 split ratio to inject the compounds in the column. The carrier gas used was helium at 1 ml/min. Oven temperature was held at 40°C for 3 min, increased from 40 to 220°C at a rate of 5°C/min and from 220 to 250°C at 10°C/min, and finally held for 2 min. The temperature of the transfer line and the ion source of the mass spectrometer were 250 and 200°C, respectively. The acquisition was from 38 to 350 *m*/*z*, at a 70-eV ionization energy. Xcalibur™ software (Thermo Scientific™, Milan, Italy) was used for data processing. Retention times of a series of *n*-alkanes (Alkanes standard solution, 04070, Sigma Aldrich®) were used to convert retention times into a retention index. Compound identification was based on computer matching of mass spectra with a database (NIST 2007 MS library, Wiley 9th edition), on retention indices reported in the literature (Adams, 2007), and finally whenever available, by comparison with reference compounds. By comparing samples to the controls collected on the corresponding days of collection, potential contaminant compounds were subtracted from the samples prior to statistical analysis.

2.5 | Y-tube assays

Pollinator choice experiments were only conducted for species belonging to the *Papuacyse* complex. All experiments were performed at Bundi Station (*c.* 1,700 m a.s.l.; 05°45′21″S, 145°14′11″E), a central site along the transect which allowed us to transport figs from the lowland and highland sites; the walk between the collection sites to Bundi Station takes 3–4 hr. For each species, fig trees were previously identified and monitored for the duration of the experiments (between the 10th and 29th of October 2016). Between 5 and 10 unhatched figs were collected and left overnight in plastic boxes with a mesh lid to allow fig wasps to emerge. Every other day, as many receptive figs from as many possible individuals were collected and brought to the experimental site. Receptive figs were used on the day of collection and were kept in a closed Nalophan® bag in a cool box before use, only receptive figs collected on that day were used for the experiments and were discarded 4 hr after arrival at Bundi Station. A glass Y-tube (dimensions: base = 6 cm; arms = 2.5 cm; internal diameter = 0.5 cm, as per Tooker, Crumrin, & Hanks, 2005) was used to test pollinator response to receptive figs from each of the selected species. Each arm of the olfactometer was connected to Nalophan ® bags containing 10–20 receptive figs or air as a "control." Airflow was maintained at 200 ml/min by flow metres connected to each bag and fed through a standard air pump powered by a 12-volt battery. The experiments were performed between 11:00 and 15:00 hr in a darkened room. All doors were shut and covered with black fabric, one window was completely covered to avoid light coming into the room, while the second window was left as the only source of light. The olfactometer was placed on a flat surface with the arms of the Y-tube facing the uncovered window, thus avoiding any other light source which would distract the wasps.

Emerged female fig wasps were individually introduced to the base of the olfactometer and were given 3 min to make a choice between the arm containing an odour source or an empty Nalophan® bag. The choice was recorded only after the wasp crossed more than 1 cm past the Y junction, and wasps which did not make a choice after the allocated time was over were recorded as unresponsive and removed from the analysis. After 10 trials, the Y-tube was rinsed with 100% ethanol and left to air dry. In addition, the odour arm was swapped to avoid any directional bias. Each wasp was only tested once and the experiment was repeated until a minimum of 60 wasps had made a choice. Wasps were kept in 70% ethanol for later species confirmation, dissection and measurement of morphological traits.

2.6 | Wasp morphology

Dissections were made under an Olympus light dissecting microscope using a graded eyepiece to take basic wasp measurements (hind tibia length and total body length to the nearest 0.1 mm). Head length and width as well as ovipositors were measured (to the nearest 0.001 mm) using a Dino-Lite® USB microscope. Voucher photographs were made with a Leica DFC 450 camera (lens Leica Planapo 1,0× WD 97 mm).

2.7 | Data analysis

Genotyping analysis used custom scripts (SNPSaurus, LLC) that trimmed the reads using bbduk (BBMap tools, http://sourceforge.net/projects/bbmap/; ktrim = r, k = 17, hdist = 1, mink = 8, ref = bbmap/resources/nextera.fa.gz, minlen = 100, ow = t, qtrim = r, trimq = 10) followed by a de novo reference created by collecting 10 million reads in total, evenly from the samples (excluding reads with counts fewer than 30 or greater than 3,000). Remaining loci were aligned to each other to identify allelic loci and collapse allelic haplotypes to a single representative. All reads were mapped to the de novo reference with an alignment similarity threshold of 88% using bbmap (BBMap tools). Genotype calling was done using SAMtools and bcftools (SAMtools, https://sourceforge.net/projects/samtools/files/samtools/;mpileup−gu,-Q10,-tDP,DPR,|bcftoolscall-cv->genotypes.vcf). The vcf was filtered to remove alleles with a population frequency of less than 5%. Heterozygous loci in all samples or those which had more than two alleles per sample were removed. Absence of artefacts was checked by counting SNPs at each read nucleotide position and determining that SNP number did not increase with reduced base quality at the end of the read. The vcf file was converted to a phylip format variant file using PGDSpider v2.1.1.3 (Lischer & Excoffier, 2012). The phylogenetic tree was generated using RAxML version 7.2.7 (Stamatakis, 2014) using GTRCAT model of rate heterogeneity.

For population genomic analyses, alternative vcf files were generated for both focal groups using the *denovo_map* program (*M* = 2, *N* = 4, *n* = 1) in Stacks v. 1.45 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) and analysed for missing data using the *populations* program (*r* = .5, max_obs_het = 0.5, min_maf = 1/[2 × *n*]). Next, we used VCFtools v 0.1.15 (Danecek et al., 2011) to identify and remove individuals with too much missing data and calculated Weir and Cockerman's F_{ST} values between populations/species. Finally, we used sNMF v. 1.2 (Frichot, Mathieu, Trouillon, Bouchard, & Francois, 2014) to estimate the number of ancestral populations (*K*) to run on the STRUCTURE software v.2.3.4 (Pritchard, Stephens, & Donnelly, 2000) using the *distruct* program.

To compare scent composition between different species, we performed non-metric multidimensional scaling (NMDS) using the function meta MDS in the R package "VEGAN" (Oksanen et al., 2013). We used the relative proportions of all compounds emitted by the six species (semiquantitative data). To prevent NMDS from being influenced by the most abundant compounds, before analysis, data were square root transformed and standardized using a Wisconsin double standardization. A pairwise between sample distance matrix was calculated using the Bray–Curtis distance index, which ranges between 0 and 1. NMDS was used to find the best n-dimensional representation of the distance matrix (our analysis retrieved a two-dimensional representation with a stress level of 0.22). Volatile profile differences were tested for significance using permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) using a customized script based on the "adonis" function in Vegan. Pairwise PERMANOVAs were run on the Bray–Curtis distance matrix with 999 permutations per analysis; *p*-values were adjusted for multiple comparisons using the FDR method (Benjamini & Hochberg, 1995). In all PERMANOVA models, the response variable was the distance matrix derived from volatile composition of each individual, while the explanatory variable was the categorical variable (sub)species. No interaction terms were included in the models. In addition, similarity percentage (SIMPER) analysis was used to identify the compounds which explain up to 30% of the differences between the species analysed (presented as Table S1). Wasp choice comparisons were analysed using two-tailed Fisher's exact test. Wasp morphological comparisons were analysed using the nonparametric Kruskal–Wallis test in R followed by a post hoc pairwise comparison using Dunn's multiple comparison test and the FDR *p* value adjustment method using the PMCMR package in R (Pohlert, 2014).

3 | RESULTS

3.1 | Phylogenetic relationships and fig morphology

According to our phylogenetic hypothesis (Figure 2), section *Papuacyse* forms a well-supported clade including *Ficus* sp. as sister to *F. microdictya* and *F. itoana*. The phylogenetic relationships between *F. microdictya* and *F. itoana* and their pollinating fig wasps have been previously reported as a case of cospeciation (Weiblen, 2004). The relationship between the pollinating fig wasps falls beyond the scope of this study but, from Weiblen (2004), we predict *Ceratosolen* sp. to belong to the same clade as *C. armipes* and C. sp. "kaironkensis."

Dissections of *F. itoana* herbarium specimens (collection numbers GW619, GW622, GW2088, GW1236, B200 and B201) supported the dioecious condition, with trees bearing either male figs (containing short-styled florets, staminate florets and *Ceratosolen* galls) or female figs (containing only long-styled florets). Specimens of *F. microdictya* (collection numbers GW954 and GW2127) had monoecious figs with a unimodal style length distribution, staminate florets, *Ceratosolen* galls and seeds. Material from *Ficus* sp. included both monoecious figs (collection numbers DEGIMI008 and GW406) and male figs (DEGIMI010 & GW421). These observations together with those of Berg and Corner (2005) suggest that *Ficus* sp. is functionally andromonoecious.

Ficus trichocerasa subspecies *trichocerasa* and *F. trichocerasa* subspecies *pleioclada* form well-supported clades agreeing with previous taxonomic descriptions of two distinct subspecies (Berg & Corner, 2005). Similarly, both populations sampled for subspecies *pleioclada* form a well-supported clade suggesting that these populations to be well connected.

Interestingly, our phylogeny recovers one *Ficus* sp. (DEGIMI010) individual which falls within the *F. microdictya* clade and a single *F. trichocerasa* subspecies *pleioclada* (DEGTRI022) within the subspecies *trichocerasa* clade. In both these cases, voucher collections have been revisited and we can rule out misidentification in the field. Important to note, however, is that these two individuals were not used for volatile collection.

3.2 | Population genomic summary

Detailed population genomic relationships and the evolutionary history between these and other *Ficus* species along the transect are being analysed and prepared as separate manuscripts (Souto-Vilarós et al., in prep.); however, preliminary analysis suggests some genetic structure between these groups supporting these as individual, closely related entities. Weir and Cockerman's weighted F_{ST} values between (sub)species were relatively high (*Ficus itoana* vs. *Fius* sp. = 0.604; *F. itoana* vs. *F. microdictya* = 0.518; *Ficus* sp. vs. *F. microdictya* = 0.394; *F. trichocerasa* vs. subspecies *pleioclada* = 0.52 and 0.58 for both DEG and SNO populations, respectively, while both populations of subspecies *pleioclada* reveal very little genetic structure between these populations F_{ST} = 0.022). In addition, STRUCTURE analysis (Figure 2) supports this pattern representing the major genetic divisions with a certain degree of SNP sharing between the different (sub)species. For the *Papuacyse* complex, we identified three separate clusters (*K* = 3) matching species level relationship with *F. itoana* in the lowlands, *F. microdictya* in the highlands and a third entity at the mid-elevation. Similarly, for *F. Trichocerasa*, we recovered two distinct clusters (*K* = 2) matching the subspecies distribution proposed by Berg and Corner (2005), with one individual clearly showing closer relationship to *F. trichocerasa* than to the subspecies *pleioclada*.

3.3 | Variation in scent profiles

We detected a total of 47 VOCs produced by receptive figs from these five species, mainly composed of fatty acid derivatives, monoterpenes and sesquiterpenes (Table 2). It was possible to identify most compounds and these have been found in other angiosperm families (Knudsen, Eriksson, Gershenzon, & Ståhl, 2006). Only a few compounds were responsible for approximately 40% of the total blend, but this differed among species (Figure 3). For instance, the *F. adenosperma* bouquet was mostly dominated by α-copaene (*c.* 57% of total scent), while other species displayed more varied profiles with up to seven compounds adding up to 40% of total scent for subspecies *trichocerasa*. Pairwise PERMANOVA analysis between the distance matrix confirmed significant differences in VOC composition between all species (volatile composition–(sub)species identity; $F_{6,36} = 4.67$, $p = .001$; Table 3). As expected, the NMDS plot (Figure 4) indicated that the differences between *F. trichocerasa* and figs from section *Papuacyse* are larger than the differences within these groups. There is some overlap in the scent composition of figs from section *Papuacyse*. On the other hand, the odour bouquet from subspecies *trichocerasa* differs considerably from subspecies *pleioclada*, but the latter also displays a different (though overlapping) profile depending on collection site. *Ficus adenosperma*, which belongs to the same section as *F. trichocerasa*, displays a distinct odour profile. Despite there being certain overlap between species in the ordination plot, the positions of the centroids (indicated by the solid lines connecting each point) of the groups are significantly different (Table 3, in all cases $p < .01$). One-way SIMPER analysis revealed that up to 30% of the difference between scents is explained by a suite of between five and six compounds, each contributing individually to a small proportion

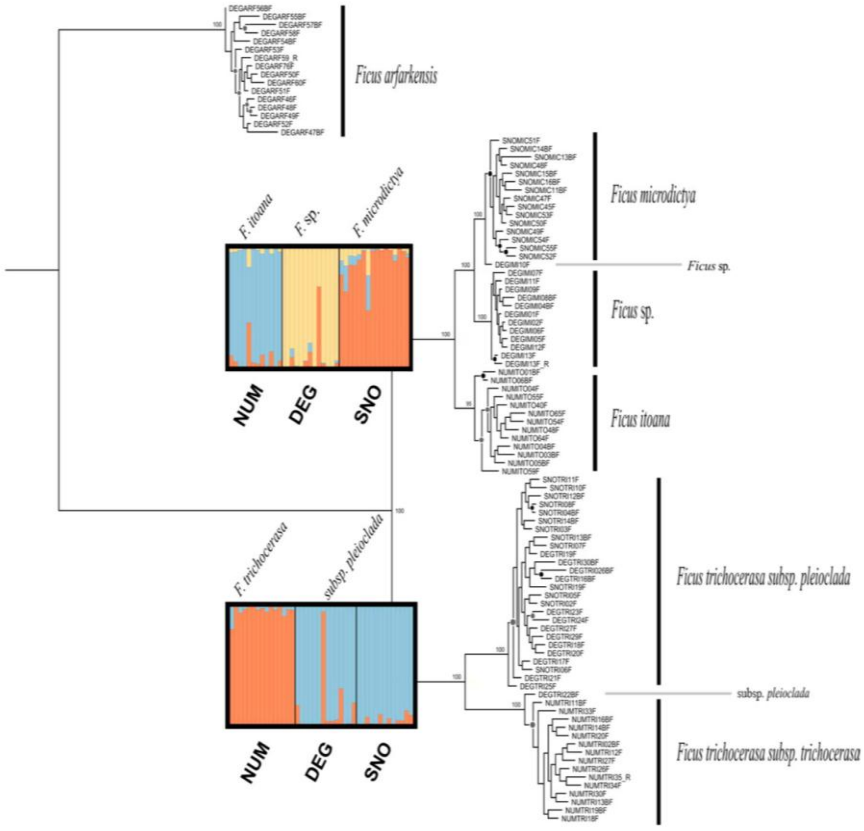

FIGURE 2 RAxML Phylogenetic relationship between analysed *Ficus* species. Values indicate bootstrap support for major branches, black dots indicate internal nodes with bootstrap values >91%; grey dots indicate nodes with bootstrap values between 75% and 90%. Tree rooted to *Ficus arfakensis*. *Collection sites:* NUM = Numba (700 masl); DEG = Degenumbu (1,700 masl); SNO = Sinopass (2,200 masl). Structure plots based on SNPs for individuals of each (sub)species. Top: *K* = 3; Bottom: *K* = 2 as derived through sNMF software for identifying ancestral populations

of the dissimilarity (*c.* 3%–7%; supporting information Table S1). For example, within section *Papuacyse*, an unidentified monoterpene derivative present in *Ficus* sp. but not in the two other sister species explained approximately 6% of the variation between species. Similarly, the presence of (*E*)-4,8-dimethyl-1,3,7-nonatriene in subspecies *trichocerasa* explains approximately 7% of the variation between this and subspecies *pleioclada* from both collection sites.

3.4 | Y-tube assays

Behavioural results are summarized in Figure 5. Female wasps of *C. armipes* presented with a choice between air and receptive figs from different fig species showed a significant preference for figs of their host (*F. itoana*, $n = 91$; Fisher's exact test $p < .0001$), but were not attracted to receptive figs from either *Ficus* sp. or *F. microdictya*, preferring air over receptive figs ($n = 96$, $p < .0001$; $n = 61$, $p < .0001$, respectively). Similarly, *C. "kaironkensis"* clearly avoided figs from *Ficus* sp. and *F. itoana* ($n = 62$, $p < .0001$; $n = 64$, $p < .0001$, respectively) consistently choosing air instead of figs, but when presented with receptive figs from their host species (*F. microdictya*), no significant preference for its host species was detected; however, they were not significantly avoiding these figs either ($n = 97$, $p = .25$). Finally, *Ceratosolen* sp. showed a significant preference for both its host and receptive figs from *F. microdictya* ($n = 115$, $p < .0001$; $n = 92$, $p < .0001$, respectively), while they avoided figs from *F. itoana* ($n = 92$, $p < .0001$). Unfortunately, due to the rapid mortality of *F. trichocerasa* pollinating wasps, we were unable to perform choice experiments on these insects. During the time of experiments, *F. trichocerasa* pollinating wasps died approximately 6 hr after hatching (D. Souto, pers. obs.), while wasps from the other species lasted considerably longer (up to 3 days for *C. armipes*), allowing us to perform these experiments.

3.5 | Wasp morphology

Wasp morphology of pollinators is summarized in Figure 6. The differences in ovipositor length between *C. armipes* and *C. "kaironkensis"* have been previously discussed by Weiblen (2004); however, it is worth noting that Kruskal–Wallis test confirmed significant differences in ovipositor length ($\chi^2 = 16.812$, $df = 2$, $p = .0002$). Post hoc tests show that there is no significant difference in ovipositor length between *C. armipes* and *Ceratosolen* sp. ($p = .229$), while the ovipositor length of *C. "kaironkensis"* is significantly longer when compared with *C. armipes* and *Ceratosolen* sp. ($p = .0001$ and $p = .007$, respectively). Similarly, head length between *C. armipes* and *Ceratosolen* sp. is comparable ($p = .066$), but it is significantly longer when compared with *C. "kaironkensis"* ($p < .0001$ for *C. armipes* and $p = .02$ for *Ceratosolen* sp.). Head width varied significantly between all three species (*C. armipes* vs. C. sp. and *C. "kaironkensis"*, $p = .024$ in both cases, and *C. "kaironkensis"* vs. *C.* sp., $p < .0001$). Finally, overall body size differed significantly between *C. armipes* and *C. "kaironkensis"* ($p = .0002$), while the size of *Ceratosolen* sp. was marginally different to the two other species ($p = .056$ in both comparisons).

4 | DISCUSSION

Volatile profiles between species in the *Papuacyse* complex varied significantly, supporting the hypothesis that closely related species should clearly differ in traits responsible for attracting their specific pollinators. Volatile profiles are also divergent within *F. trichocerasa* subspecies occupying different elevations, in concordance with known morphological and (newly demonstrated) molecular differences. Similarly, our behavioural experiments revealed a general trend of pollinators avoiding non-natal figs, except in one case where the pollinator was also attracted to its host's sister species. It appears that in this case, volatile signals are equally attractive to these pollinators, suggesting further barriers are necessary to maintain reproductive isolation between these two fig species. We demonstrate that wasp morphology can enforce prepollination barriers and suggest that limited pollinator dispersal may further reinforce reproductive isolation.

Pollinator specificity in the fig–fig wasp mutualism has been widely studied, and despite examples of pollinator sharing in some *Ficus* species (Cook & Rasplus, 2003; Cornille et al., 2012; Wang et al., 2016), hybridization in natural populations appears to be low (<1% of individuals; Moe & Weiblen, 2012; but see Wang et al., 2016) indicating limited introgression, explained by the tight specificity of this mutualism. Our study reveals that a combination of character divergence in both figs and pollinating wasps are important prepollination barriers between these species.

Examples from a similarly tight-knit mutualism, the *Yucca* (Agavaceae) and its pollinating Yucca-moths, have shown eastern and western species (*Yucca filamentosa* and *Yucca elata*) having nearly identical volatile signatures, indicating that the maintenance of specificity is due mainly to geographical distribution rather than volatile signals (Svensson, Pellmyr, & Raguso, 2006). Contrastingly, in this study, fig volatile blends are found to be significantly different from each other, but there is certain overlap between figs from section *Papuacyse*. These three species are parapatrically separated by elevation, and together with volatile signatures, pollinating fig-wasp dispersal range and morphology may be important for the effective isolation of these species. Morphologically, pollinating wasps of *Ficus* sp. are more similar to the pollinators of *F. itoana* (Figure 6); most importantly they have a very similar ovipositor length. Our behavioural tests show that these two species show no reciprocal host attraction, suggesting that in an encounter with non-natal figs, volatile cues are enough to deter wasps from entering figs in which egg deposition may otherwise be possible.

Contrastingly, *Ceratosolen* sp. wasps showed significant attraction to receptive figs from *F. microdictya*, which according to our phylogenetic hypothesis, is the sister species of *Ficus* sp. This indicates that in the event of a pollinating *Ficus* sp. wasp drifting uphill, it may be potentially attracted to figs from *F. microdictya*. In this case, morphological barriers may prevent oviposition at different stages. Fig size at receptivity is known to be correlated with wasp head morphology, indicating that head dimensions play an important role when the wasp is entering through the ostiole (van Noort &

TABLE 2 Percentage (*M* ± *SE*) of volatile organic compounds found in bouquets emitted by receptive figs from Section *Papuacyse* and both subspecies of *Ficus trichocerasa*

Part one									
		Ficus adenosperma (*n* = 3 trees)		*Ficus itoana* (*n* = 5 trees)		*Ficus* sp. (*n* = 6 trees)		*Ficus microdictya* (*n* = 10 trees)	
Compounds	RI:	% ±*SE*	O	% ±*SE*	O	% ±*SE*	O	% ±*SE*	O
Aliphatic compounds									
(*Z*)-3-Hexenol*	857	6.45 ± 2.3	3	n.d.	0	n.d.	0	n.d.	0
2-Heptanone*	896	n.d.	0	18.012 ± 9.326	3	23.016 ± 6.98	6	5.881 ± 1.582	10
Unknown ramified alkane 1	983	0.018 ± 0.018	1	5.732 ± 1.616	5	1.319 ± 0.619	3	3.348 ± 1.384	8
2-Heptyl acetate	1038	n.d.	0	n.d.	0	n.d.	0	4.568 ± 4.135	2
Nonanoic acid*	1264	0.024 ± 0.024	1	n.d.	0	0.56 ± 0.52	2	0.916 ± 0.79	2
Unknown ramified alkane 2	1273	0.114 ± 0.086	3	0.984 ± 0.984	1	0.551 ± 0.543	2	3.405 ± 1.675	9
Monoterpenic compounds									
α-Pinene*	937	0.342 ± 0.128	3	n.d.	0	23.113 ± 9.284	4	1.907 ± 0.576	6
Myrcene*	991	0.031 ± 0.031	1	n.d.	0	4.924 ± 3.136	2	0.772 ± 0.568	2
(*E*,*E*)-Cosmene	1011	n.d.	0	n.d.	0	n.d.	0	n.d.	0
Limonene*	1035	0.314 ± 0.124	3	24.891 ± 16.657	3	7.805 ± 3.093	5	11.744 ± 4.072	10
(*Z*)-β-Ocimene*	1038	n.d.	0	0.734 ± 0.734	1	n.d.	0	0.371 ± 0.249	2
1,8-Cineole*	1038	1.153 ± 0.385	3	17.315 ± 8.046	3	n.d.	0	n.d.	0
(*E*)-β-Ocimene*	1048	1.4 ± 0.218	3	13.662 ± 13.662	1	4.923 ± 2.95	3	3.419 ± 1.343	6
(*E*)-Linalool oxide*	1091	n.d.	0	n.d.	0	10.062 ± 4.705	4	n.d.	0
Linalool*	1102	0.791 ± 0.195	3	0.275 ± 0.275	1	6.244 ± 3.761	5	0.547 ± 0.198	8
Unknown Monoterpene derivative	1110	n.d.	0	n.d.	0	10.263 ± 2.285	6	n.d.	0
E)-4,8-Dimethyl-1,3,7-nonatriene	1114	1.47 ± 0.394	3	n.d.	0	n.d.	0	1.647 ± 1.332	3
Sesquiterpenic compounds									
δ-Elemene	1341	0.073 ± 0.073	1	n.d.	0	0.099 ± 0.099	1	5.165 ± 2.735	4
α-Cubebene*	1356	4.277 ± 0.164	3	n.d.	0	n.d.	0	0.057 ± 0.057	1
Cyclosativene*	1383	0.329 ± 0.109	3	n.d.	0	n.d.	0	4.574 ± 0.736	9
α-Copaene*	1388	57.629 ± 1.971	3	2.308 ± 2.308	1	0.235 ± 0.235	1	19.64 ± 5.24	9
β-Elemene*	1398	3.632 ± 0.647	3	n.d.	0	0.477 ± 0.477	1	1.491 ± 0.627	4
(*Z*)-α-Bergamotene	1422	0.494 ± 0.494	1	0.104 ± 0.051	3	0.046 ± 0.046	1	0.665 ± 0.349	4
α-Gurjunene	1421	1.275 ± 0.363	3	n.d.	0	0.321 ± 0.321	1	1.572 ± 0.55	8
β-Ylangene	1432	0.275 ± 0.275	1	n.d.	0	n.d.	0	n.d.	0
β-Caryophyllene*	1435	6.997 ± 2.591	3	2.884 ± 1.926	2	2.24 ± 1.051	4	11.95 ± 3.259	9
(E)-α-Bergamotene	1441	0.036 ± 0.036	1	n.d.	0	0.119 ± 0.119	1	2.891 ± 0.935	8
Unknown Sesquiterpene 1	1444	1.078 ± 0.575	3	n.d.	0	n.d.	0	0.198 ± 0.198	1
α-Guaiene	1446	0.957 ± 0.658	2	n.d.	0	n.d.	0	0.042 ± 0.03	2
Geranyl acetone*	1450	n.d.	0	n.d.	0	0.343 ± 0.343	1	0.049 ± 0.049	1
Aromadendrene	1454	n.d.	0	n.d.	0	n.d.	0	0.386 ± 0.342	2
Unknown Sesquiterpene 2	1459	0.112 ± 0.083	2	n.d.	0	n.d.	0	0.221 ± 0.147	3

(Continues)

TABLE 2 (Continued)

Part one

Compounds	RI:	*Ficus adenosperma* (*n* = 3 trees) % ±*SE*	O	*Ficus itoana* (*n* = 5 trees) % ±*SE*	O	*Ficus* sp. (*n* = 6 trees) % ±*SE*	O	*Ficus microdictya* (*n* = 10 trees) % ±*SE*	O
Unknown Sesquiterpene 3	1466	0.087 ± 0.087	1	n.d.	0	n.d.	0	n.d.	0
α-Humulene*	1471	1.621 ± 0.695	3	0.37 ± 0.37	1	1.738 ± 1.206	4	2.514 ± 0.623	9
Allo-Aromadendrene	1476	2.986 ± 0.33	3	n.d.	0	n.d.	0	0.479 ± 0.342	2
γ-Muurolene	1486	0.381 ± 0.223	2	n.d.	0	0.091 ± 0.091	1	0.171 ± 0.171	1
Unknown Sesquiterpene 4	1490	0.018 ± 0.018	1	n.d.	0	n.d.	0	0.439 ± 0.393	2
Germacrene-D*	1496	0.463 ± 0.272	3	n.d.	0	n.d.	0	0.227 ± 0.227	1
Unknown Sesquiterpene 5	1505	0.407 ± 0.051	3	10.564 ± 6.488	2	1.068 ± 1.068	1	4.126 ± 1.023	8
α-Muurolene	1509	1.28 ± 0.056	3	n.d.	0	n.d.	0	n.d.	0
Bicyclogermacrene	1510	n.d.	0	n.d.	0	n.d.	0	2.61 ± 1.686	3
α-Bulnesene	1514	0.439 ± 0.399	2	n.d.	0	n.d.	0	0.721 ± 0.358	4
δ-Cadinene	1529	1.625 ± 0.135	3	n.d.	0	n.d.	0	0.064 ± 0.064	1
(Z)-Calamenene	1534	0.71 ± 0.427	2	0.07 ± 0.07	1	0.029 ± 0.029	1	0.144 ± 0.07	4
(*E*)-Cadina-1,4-diene	1546	0.194 ± 0.004	3	0.557 ± 0.341	2	0.185 ± 0.051	6	0.12 ± 0.036	7
α-Calacorene	1556	0.384 ± 0.12	3	0.055 ± 0.055	1	n.d.	0	0.132 ± 0.085	4
β-Calacorene	1577	0.135 ± 0.035	3	1.481 ± 0.931	3	0.231 ± 0.19	3	0.829 ± 0.322	9

Part two

Compounds	RI:	*Ficus trichocerasa* (*n* = 4 trees) % ±*SE*	O	*Ficus trichocerasa* subsp. *pleioclada* (DEG) (*n* = 6 trees) % ±*SE*	O	*Ficus trichocerasa* subsp. *pleioclada* (SNO) (*n* = 9 trees) % ±*SE*	O
Aliphatic compounds							
(Z)-3-Hexenol*	857	1.841 ± 1.841	1	n.d.	0	n.d.	0
2-Heptanone*	896	n.d.	0	2.418 ± 1.64	2	0.623 ± 0.623	1
Unknown ramified alkane 1	983	1.029 ± 0.81	2	1.988 ± 1.294	2	8.207 ± 2.082	7
2-Heptyl acetate	1038	n.d.	0	n.d.	0	n.d.	0
Nonanoic acid *	1264	1.465 ± 0.891	3	3.511 ± 2.354	2	n.d.	0
Unknown ramified alkane 2	1273	0.484 ± 0.484	1	2.059 ± 1.901	3	11.097 ± 3.215	8
Monoterpenic compounds							
α-Pinene*	937	2.684 ± 2.205	2	3.693 ± 1.947	6	4.012 ± 1.491	6
Myrcene*	991	1.219 ± 0.728	2	n.d.	0	0.351 ± 0.351	1
(*E*,*E*)-Cosmene	1011	0.687 ± 0.687	1	n.d.	0	n.d.	0
Limonene*	1035	1.55 ± 1.355	2	1.739 ± 1.642	2	26.01 ± 7.075	7
(Z)-β-Ocimene*	1038	0.8 ± 0.8	1	0.506 ± 0.506	1	4.684 ± 2.825	1
1,8-Cineole*	1038	8.926 ± 8.498	2	10.984 ± 6.364	5	3.469 ± 3.469	1
(*E*)-β-Ocimene*	1048	11.524 ± 5.531	4	n.d.	0	1.329 ± 1.329	1

(Continues)

TABLE 2 (Continued)

Part two							
		Ficus trichocerasa (*n* = 4 trees)		*Ficus trichocerasa* subsp. *pleioclada* (DEG) (*n* = 6 trees)		*Ficus trichocerasa* subsp. *pleioclada* (SNO) (*n* = 9 trees)	
Compounds	**RI:**	**% ±SE**	**O**	**% ±SE**	**O**	**% ±SE**	**O**
(*E*)-Linalool oxide*	1091	1.193 ± 0.697	2	n.d.	0	n.d.	0
Linalool*	1102	2.545 ± 2.129	3	1.094 ± 0.509	3	2.035 ± 1.185	4
Unknown Monoterpene derivative	1110	5.196 ± 3.234	2	0.52 ± 0.52	1	n.d.	0
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	1114	23.089 ± 7.324	4	n.d.	0	n.d.	0
Sesquiterpenic compounds							
δ-Elemene	1341	n.d.	0	0.638 ± 0.638	1	n.d.	0
α-Cubebene*	1356	n.d.	0	0.422 ± 0.284	2	0.017 ± 0.017	1
Cyclosativene*	1383	n.d.	0	n.d.	0	n.d.	0
α-Copaene*	1388	n.d.	0	38.773 ± 12.968	4	14.438 ± 6.537	5
β-Elemene*	1398	n.d.	0	n.d.	0	n.d.	0
(Z)-α-Bergamotene	1422	2.265 ± 1.577	4	1.022 ± 0.354	6	0.702 ± 0.394	3
α-Gurjunene	1421	2.532 ± 1.803	3	1.127 ± 0.59	4	1.031 ± 0.543	4
β-Ylangene	1432	n.d.	0	n.d.	0	5.797 ± 4.923	2
β-Caryophyllene*	1435	13.048 ± 8.766	2	10.773 ± 6.382	5	11.403 ± 6.202	4
(E)-α-Bergamotene	1441	1.534 ± 0.508	4	4.914 ± 1.41	6	1.284 ± 0.77	3
Unknown Sesquiterpene 1	1444	3.96 ± 3.96	1	n.d.	0	n.d.	0
α-Guaiene	1446	4.24 ± 3.876	2	n.d.	0	n.d.	0
Geranyl acetone*	1450	n.d.	0	10.401 ± 4.095	5	0.279 ± 0.204	2
Aromadendrene	1454	n.d.	0	n.d.	0	n.d.	0
Unknown Sesquiterpene 2	1459	n.d.	0	n.d.	0	n.d.	0
Unknown Sesquiterpene 3	1466	n.d.	0	n.d.	0	n.d.	0
α-Humulene*	1471	0.598 ± 0.368	2	1.618 ± 1.034	2	0.915 ± 0.402	4
Allo-Aromadendrene	1476	n.d.	0	n.d.	0	n.d.	0
γ-Muurolene	1486	n.d.	0	n.d.	0	n.d.	0
Unknown Sesquiterpene 4	1490	0.243 ± 0.243	1	n.d.	0	n.d.	0
Germacrene-D *	1496	n.d.	0	n.d.	0	n.d.	0
Unknown Sesquiterpene 5	1505	3.06 ± 1.935	2	n.d.	0	0.161 ± 0.107	2
α-Muurolene	1509	n.d.	0	n.d.	0	n.d.	0
Bicyclogermacrene	1510	n.d.	0	n.d.	0	n.d.	0
α-Bulnesene	1514	4.225 ± 3.737	2	n.d.	0	0.332 ± 0.332	1
δ-Cadinene	1529	n.d.	0	0.844 ± 0.844	1	0.445 ± 0.445	1
(Z)-Calamenene	1534	n.d.	0	0.349 ± 0.228	2	0.402 ± 0.228	4
(*E*)-Cadina-1,4-diene	1546	0.064 ± 0.054	2	0.249 ± 0.135	3	0.265 ± 0.1	5
α-Calacorene	1556	n.d.	0	0.119 ± 0.119	1	0.057 ± 0.04	2
β-Calacorene	1577	n.d.	0	0.239 ± 0.081	4	0.656 ± 0.289	4

O = occurrence of number of individuals where that compounds was found. RI = retention index. n.d. = compound not detected. * = compounds identified using chemical standards.

Compton, 1996). Body size of *Ceratosolen* sp. wasps is comparable to *C. "kaironkensis"* wasps; however, head morphology between these species differed substantially, and may act as a barrier for entering the fig. In order for wasps to reach the enclosed inflorescences, fig wasps crawl through the tightly closed ostiole and often have head morphologies equipped for travelling through this narrow passage. Foundress wasps often lose their wings and parts of the antennae through the process, and once reaching the cavity within, a further barrier preventing oviposition might present itself. Individuals of *Ceratosolen "kaironkensis"* have ovipositors that are nearly twice the

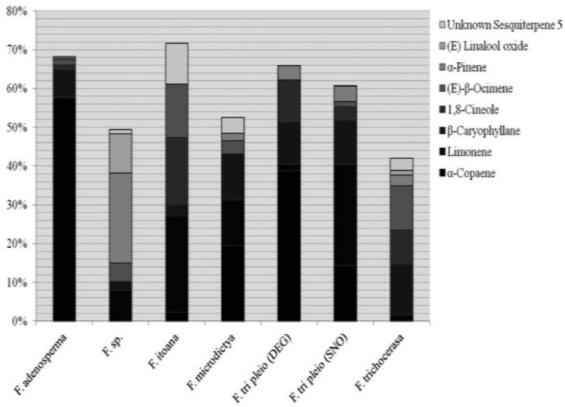

FIGURE 3 Proportions of the main compounds representing more than 40% of total volatile bouquet emitted by receptive figs of the analysed species. F. tri pleio deg and F. tri pleio sno correspond to Ficus trichocerasa subspecies pleioclada individuals collected in Degenumbu and Sinopass, respectively

TABLE 3 Results of the permutational analysis of variance (PERMANOVA) performed on volatile compound proportions (data transformed using squared root and Wisconsin double standardization). *p*-values adjusted using FDR method. Significant *p*-values ($p < .05$) indicated in bold

	df	*F*	R^2	*p* (adjusted)
Interspecies variation (all species)	6,36	4.67	.437	**.001**
Pairwise Comparisons:				
Section Papuacyse				
Ficus itoana vs. *Ficus* sp.	1,10	3.880	.279	**.004**
F. itoana vs. *Ficus microdictya*	1,14	5.310	.290	**.004**
Ficus sp. vs. *F. microdictya*	1,15	5.299	.261	**.003**
Ficus trichocerasa				
Ficus trichocerasa vs. subsp. *pleioclada* DEG	1,9	4.903	.352	**.004**
F. trichocerasa vs. subsp. *pleioclada* SNO	1,12	4.824	.286	**.009**
subsp. *pleioclada* DEG vs. subsp. *pleioclada* SNO	1,14	3.879	.229	**.005**

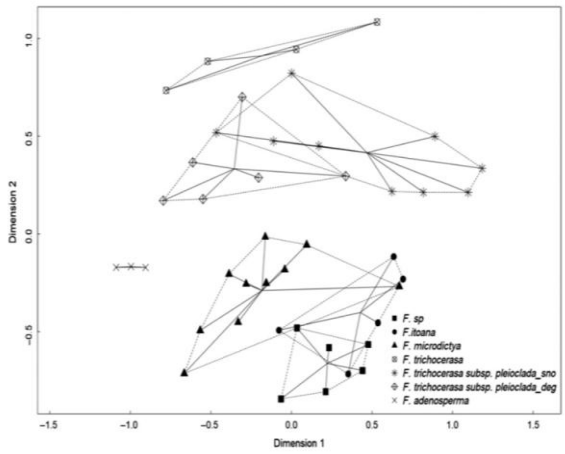

FIGURE 4 Non-metric multidimensional scaling (NMDS) ordination of volatile organic compound composition of studied species at receptive stage, based on Bray–Curtis distance; Two dimensions, stress = 0.22. Dashed lines (generated using ordispider) group samples from the same species; solid lines (generated with ordihull) connect each point to a centroid which is significantly different between species. Samples corresponding to Ficus trichocerasa subspecies pleioclada are written as pleioclada_deg and pleioclada_sno and correspond to Degenumbu and Sinopass collection sites, respectively. Ficus adenosperma (in blue) was used as an "outgroup"

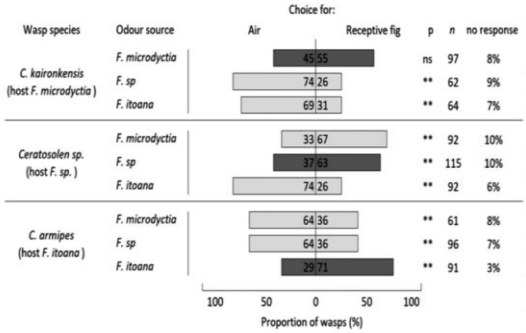

FIGURE 5 Response of different pollinating fig wasps in Y-tube experiments when presented with receptive figs of different species vs. air. Using Fisher's exact test for each series of tests, we compared the proportion of wasps that choose receptive fig odour or "control" air (unresponsive wasps presented as % of total wasps tested for each species). Darker shaded bars indicate host fig species. [ns = non-significant difference ($p > .05$); **$p < .0001$]

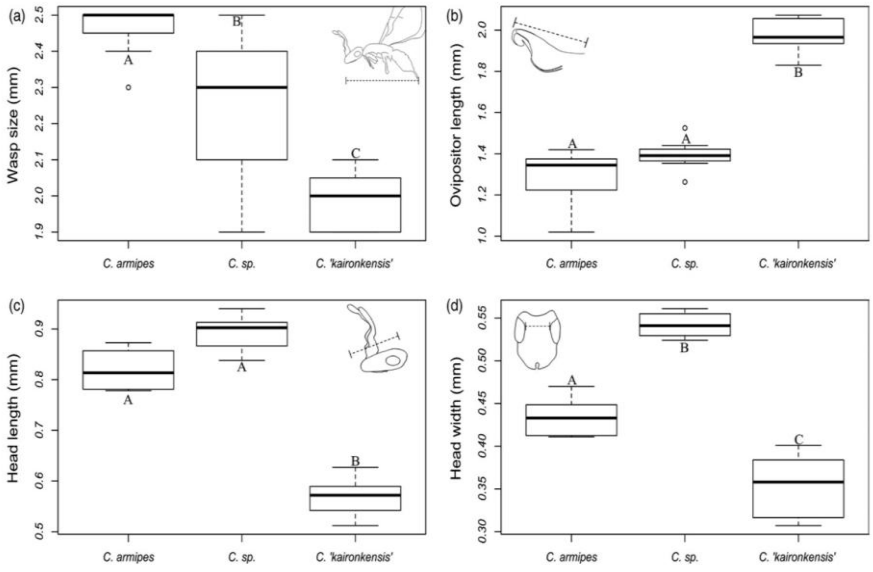

FIGURE 6 Boxplot of fig-wasp measurement of various traits for individuals pollinating figs from section Papuacyse; Ceratosolen armipes, Ceratosolen sp. and Ceratosolen "kaironkensis." a: Wasp size; b: Ovipositor length; c: Head length; d: Head width. Measurements based on eight individuals for each species. Letters indicate significant differences between comparisons, bars indicate one *SE*

length than those of *C. armipes* and *Ceratosolen* sp., which is compatible for oviposition in the long-styled flowers from the monoecious species of the section (Weiblen, 2004). The inability of *Ceratosolen* sp. wasps to penetrate and successfully oviposit in *F. microdictya* figs represents the ultimate fitness cost from the wasps' perspective, suggesting strong selection against making such a choice. Nevertheless, the measurements herein serve as indirect evidence for the inability of *Ceratosolen* sp. of ovipositing within the figs from *F. microdictya* as this was not explicitly tested in this study. From the tree's perspective, evidence from hybrid seed viability in other Papua New Guinean *Ficus* species indicates that postpollination barriers are, perhaps, less defined; however, seedling survival was lower in hybrids than non-hybrids, suggesting negative selection as a further step towards maintenance of species specificity (Moe & Weiblen, 2012).

Finally, *Ceratosolen "kaironkensis"* did not display any significant host recognition, but this species of wasp clearly avoids the other two fig species, suggesting that volatile signal alone may prevent these wasps from entering these figs, while a lack of avoidance from its host species might be enough to maintain this relationship. Fig wasps pollinating monoecious figs are known to disperse further than their dioecious relatives, since the density of monoecious trees bearing receptive figs is often low (Borges, 2016; Harrison & Rasplus, 2006). This suggests that these wasps must be well adapted

to distinguish between the different *Ficus* species present throughout their range.

In the case of *F. trichocerasa*, the marked differences in scent composition, with no overlap between subspecies, suggest that volatile signatures may be an important component in limiting geneflow between them. Unfortunately, this study failed to conduct choice experiments due to rapid wasp mortality (<6 hr after emergence, D. Souto, pers. obs.). The short life-span of these wasps, however, highlights the need for them to rapidly find a suitable host, effectively limiting their dispersal ability. Indeed, limited wasp dispersal along a steep environmental gradient may be an important contributing factor limiting gene flow in this system.

Ficus trichocerasa displays highland (*F. trichocerasa* subspecies *pleioclada*) and lowland (*F. trichocerasa* subspecies *trichocerasa*) morphological differences which become less evident at mid elevations, where their ranges overlap (1,200–1,500 m a.s.l.; Berg & Corner, 2005). Among the clearest trait differences between them is the densely hairy syconia in subspecies *pleioclada* (supplementary material); divergent traits linked to pollinator attraction and behaviour could play a role in reducing gene flow, which may result in reproductive isolation between these two subspecies. Wang et al. (2016) found that the pollinators of *F. semicordata* were attracted to volatile signatures produced by a sympatric fig variety, but avoided entering atypical hosts after physically contacting the surface of the fig, suggesting a secondary mechanism for host recognition. Gibernau et al. (1998) suggest that visual or physical cues (e.g. hairs) are of minor importance, but that tactile chemical cues (as cuticular waxes in the fig surface) may act as stimuli to enter the fig. The densely hairy figs from subspecies *pleioclada* may, perhaps, provide an additional tactile cue as a complementary prepollination barrier.

Previous studies on interpopulation scent variation in figs, and other nursery pollinator systems, have found that scent can be constant over wide ranges, but may vary in the presence of geographical barriers (Ibanez et al., 2010; Rodriguez et al., 2017; Svensson et al., 2006; Soler et al., 2012). Elevational differences, coupled with scent variation could lead to speciation, so long as pollinators remain faithful visitors to their local hosts, and seed dispersal remains localized. Our population genomic analysis was not able to separate subspecies *pleioclada* between different collection sites, but the volatile composition between figs originating in Degenumbu (1,700 masl) or Sinopass (2,200 masl) is different. It is possible that these subtle differences in scent may eventually lead to even more localized preferences in pollinating wasps. The influence of elevational differences in volatile compositions deserves to be studied in more detail. Other *Ficus* volatile studies have also found within species scent differences and suggest that differences may be due to variation in compounds not necessary for mediating host species recognition (Rodriguez et al., 2017; Soler et al., 2011). Population-level relationships between both figs and pollinators in this case would help elucidate the level of isolation between these subspecies, as well as within-site, allowing us to estimate the relative importance of interpopulation scent variation in maintaining species specificity.

This study reveals the complexity of pollination barriers at play even in highly specific, obligate mutualisms. Odour has often appealed as one of the most important mechanisms for pollinator isolation in *Ficus* (Gibernau et al., 1998; Grison-Pigé, Bessière, et al., 2002); however, this signal has been shown to vary across wide geographical ranges, and this study found contrasting responses from pollinators to scents from related species. Contact stimuli were not tested in this study, but Wang et al. (2013) suggest that it plays a complementary role in host recognition. A further constraint is the apparent physical inability of these pollinators to oviposit in atypical hosts, as this should suffice as a major deterrent to avoid such encounters. Divergent volatile signals between figs could represent an initial isolating mechanism between subspecies which is later reinforced by pollinator behaviour and morphological adaptation. Plant genera which have specialized pollination systems seem to have greater diversity than those with more generalized interactions. In *Ficus*, if volatile and morphological cues are enough to maintain pollinator isolation, coupled with geographical barriers and limited wasp range, these mechanisms could contribute to speciation in this large plant genus. Also interest for further investigation is the evolution of the *Ficus* sexual system. *Ficus* section *Papuasyce* along the Mt. Wilhelm transect in PNG presents a zone of contact between closely related dioecious and monecious species where a third, unnamed species at mid-elevation has sexual characteristics of both relatives and appears to represent the first case of functional andromonoecy in the genus.

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AUTHORS' CONTRIBUTIONS

V.N., S.T.S. and D.S.V. designed the research and provided input at all stages. G.D.W. suggested suitable species for the study. D.S.V. and M.R. designed the Y-tube experiment and photographed the specimens. D.S.V., M.S., B.I. and T.K. conducted fieldwork, with initial assistance from S.T.S. M.P., B.B. and M.H.-M. assisted with the GCMS

and volatile analysis and all aspects of sample collection in the field. C.T.D. and J.M. assisted with NGS data management and phylogenetic analysis. D.S.V. analysed the data and wrote the manuscript with substantial input from all authors. All authors contributed and approved the final version of the manuscript.

DATA ACCESSIBILITY

Data used for this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.hm83f7t (Souto-Vilarós et al., 2018). Demultiplexed sequence data are deposited in the Short Read Archive (https://www.ncbi.nlm.nih.gov/sra/SRP136650).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary material for Chapter III. Pollination along an elevational gradient mediated by both floral scent and pollinator compatibility in the fig and fig-wasp mutualism – Journal of Ecology

The following Supporting Information is available for this article:

Figure S1 Voucher photographs for each focal *Ficus* species used in this study.

Figure S2 Voucher photographs of *Ceratosolen armipes*, pollinator of *F. itoana*.

Figure S3 Voucher photographs of *Ceratosolen* sp., pollinator of *Ficus*. sp.

Figure S4 Voucher photographs of *Ceratosolen "kaironkensis"*, pollinator of *F. microdyctia*.

Figure S5 Voucher photographs of *Ceratosolen* sp. 1, pollinator of *F. trichocerasa* subsp. *trichocerasa* from the Numba collection site.

Figure S6 Voucher photographs of *Ceratosolen* sp. 2, pollinator of *F. trichocerasa* subsp. *pleioclada* from the Sinopass collection site.

Table S1 Volatile compounds which contribute ~30% of variation between analysed species.

Table S2 Samples used for genomic and volatile analysis.

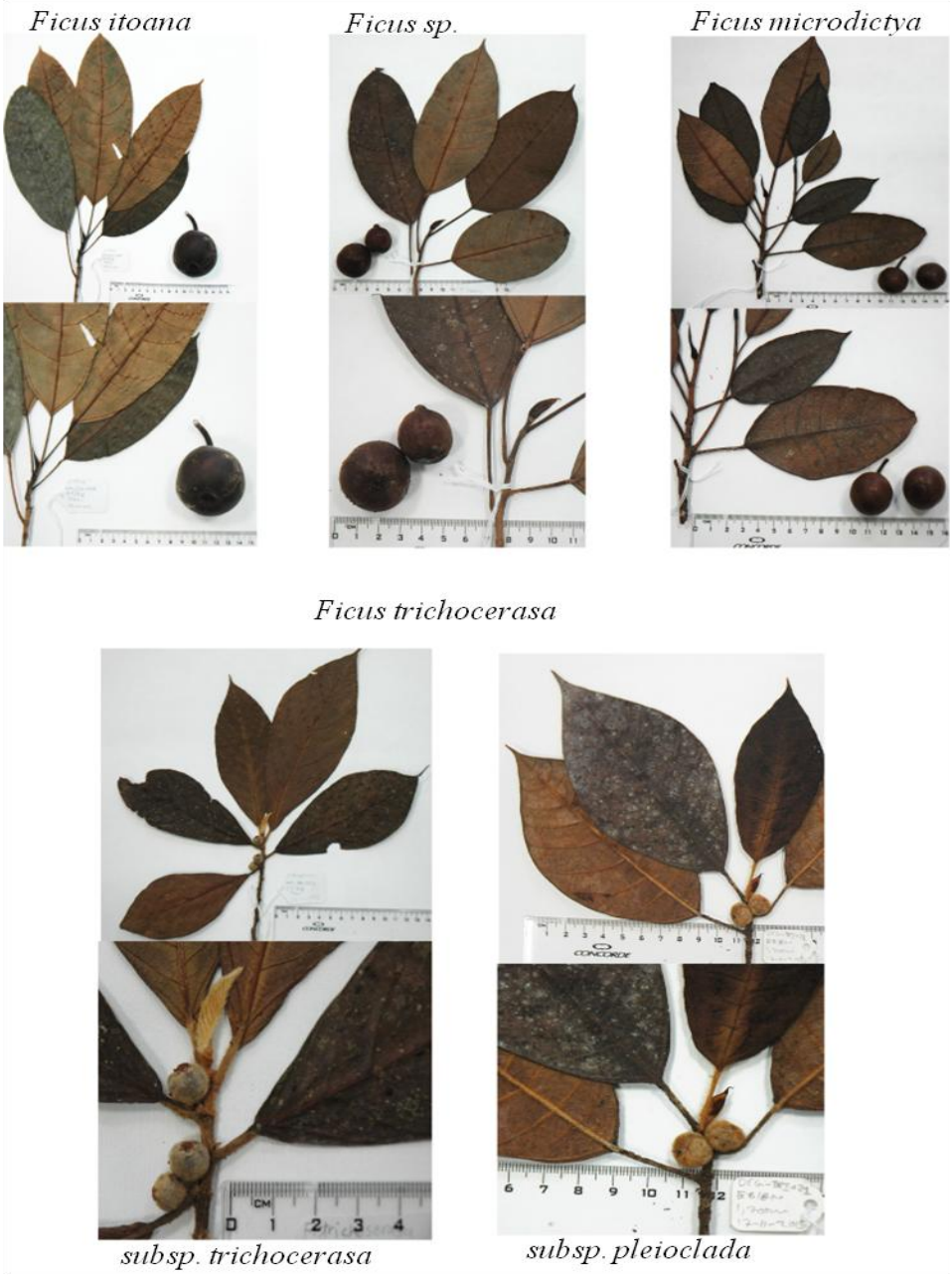


Figure. S1 Voucher photographs of focal *Ficus* species used for this study. Top three photographs correspond to species in the Papuacyse section while bottom pictures are both subspecies of *Ficus trichocerasa*.

Figure S2 Voucher photographs of *Ceratosolen armipes*, pollinating wasp of *F. itoana*.

Figure S3 Voucher photographs of *Ceratosolen* sp., pollinating wasp of *Ficus* sp.

Figure S4 Voucher photographs of *Ceratosolen "kaironkensis",* pollinating wasp of *F. microdictya.*

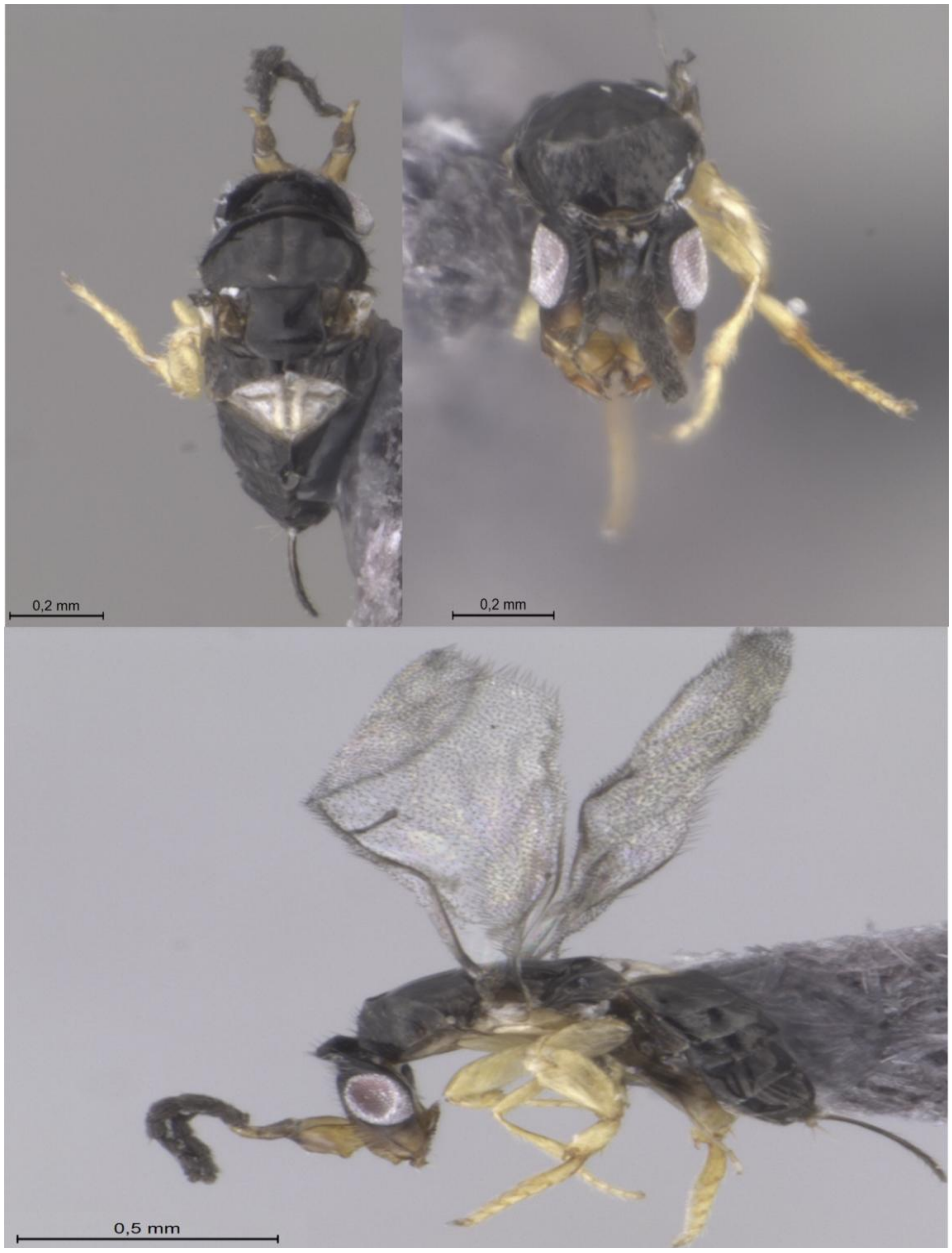


Figure S5 Voucher photographs of *Ceratosolen* sp. 1, pollinating wasp of *F. trichocerasa* subsp. *trichocerasa* from Numba collection site.

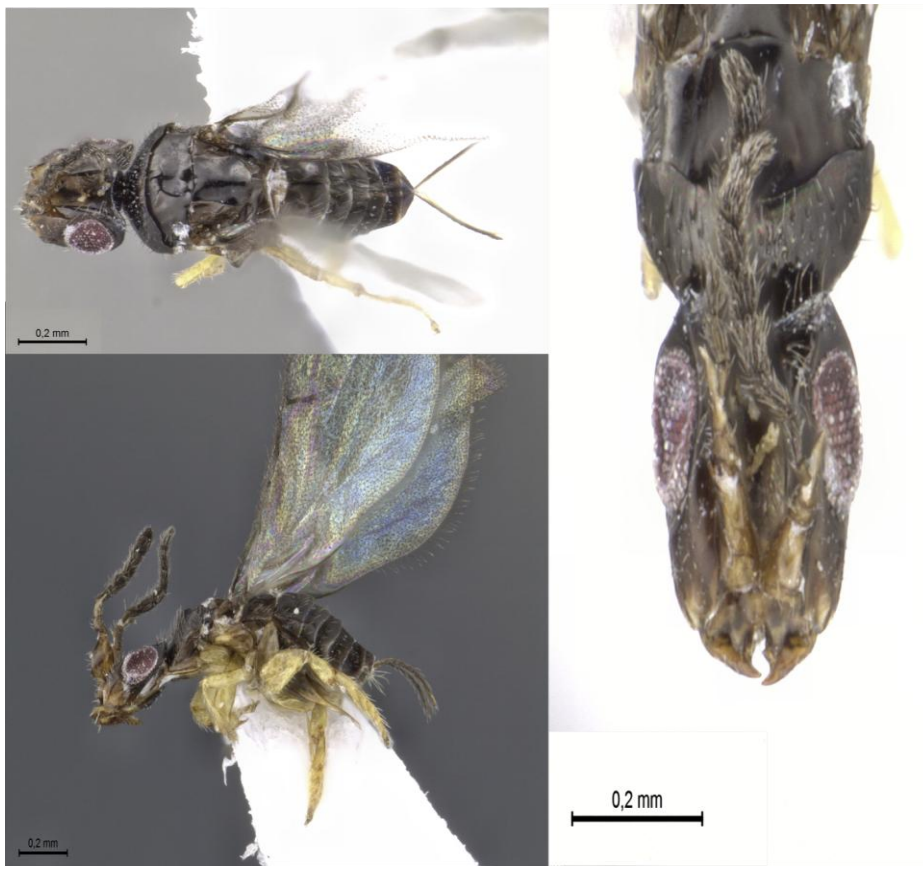


Figure S6 Voucher photographs of *Ceratosolen sp*. 2, pollinating wasp of *Ficus trichocerasa* subsp. *pleioclada* from Sinopass collection site.

Table S1 Volatile compounds contributing ~30% of the variation between pairwise comparisons as recovered from Similarity Percentage Analysis (SIMPER); figures in % of total scent:

Section *Papuacyse*

F. itoana* vs. *F. microdictya

Compounds	%
1,8-Cineole	7.52
Limonene	7.05
Unknown Sesquiterpene 5	6.97
Unknown ramified alkane 1	6.60
2-Heptanone	6.36
Total:	34.50

***F. itoana vs. Ficus* sp.**

Compounds	%
Unknown Monoterpene derivative	8.71
1,8-Cineole	7.41
Limonene	6.99
α-Pinene	6.76
Unknown ramified alkane 1	6.53
Total:	36.40

***F. microdictya vs. Ficus* sp.**

Compounds	%
Unknown Monoterpene derivative	9.26
α-Pinene	6.76
Cyclosativene	5.75
2-Heptanone	5.61
(*E*)-Linalool oxide	5.32
Total:	32.70

Ficus trichocerasa

***F. trichocerasa* vs. *pleioclada* DEG**

Compounds	%
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	7.52
α-Copaene	7.21
Geranyl acetone	6.36
1,8-Cineole	4.42
α-Humulene	4.19
β-Caryophyllene	3.89
Total:	33.59

***F. trichocerasa* vs. *pleioclada* SNO**

Compounds	%
Unknown ramified alkane 1	7.03
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	6.95
Unknown ramified alkane 2	6.51
Limonene	5.99
(*E*)-Cadina-1,4-diene	4.06
Total:	30.54

***pleioclada* DEG vs. *pleioclada* SNO**

Compounds	%
Unknown ramified alkane 2	7.69
Unknown ramified alkane 1	7.00
Limonene	6.86
α-Copaene	6.52
Geranyl acetone	6.40
Total:	34.47

Table S2: Samples used for volatile and genomic analysis. Due to collections performed in two different field seasons, not every tree used for DNA analysis bared fruit a the receptive stage in order to collect volatiles and so additional trees were used for volatile collection.

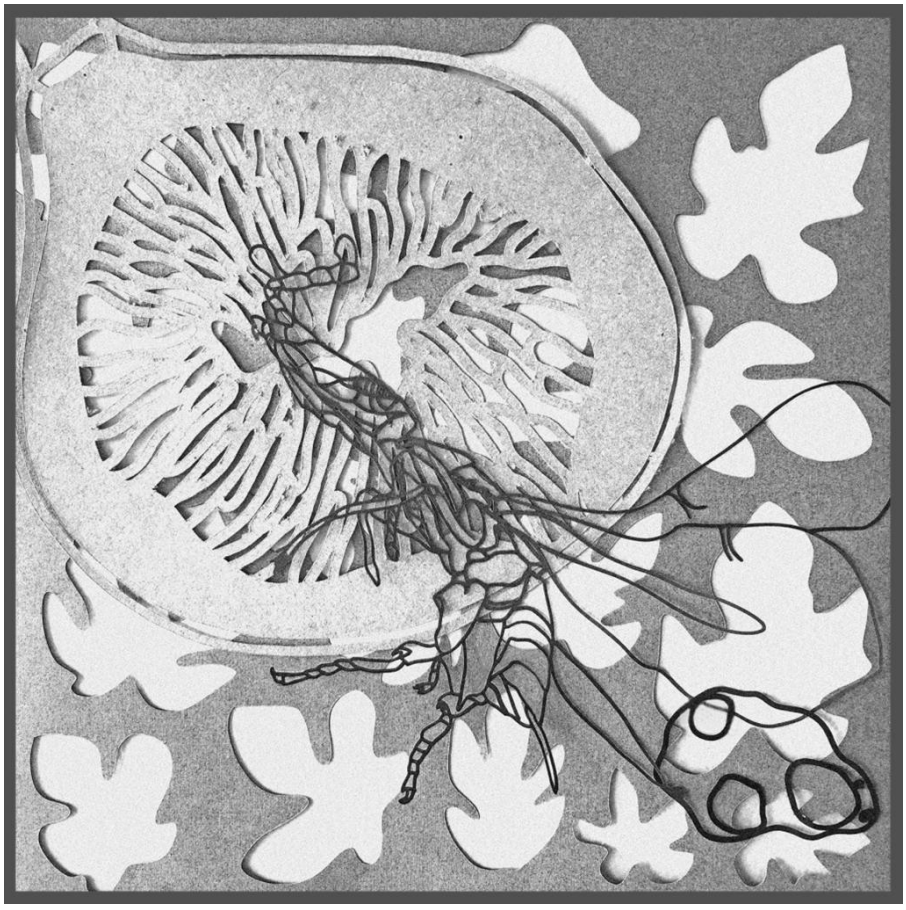
Sample	Species	Site	Volatile	DNA
sno_mic_004	microdictya	sinopass	x	x
sno_mic_005	microdictya	sinopass	x	x
sno_mic_047	microdictya	sinopass	x	x
sno_mic_048	microdictya	sinopass	x	x
sno_mic_050	microdictya	sinopass	x	x
sno_mic_052	microdictya	sinopass	x	x
sno_mic_v01_bis	microdictya	sinopass	x	
sno_mic_v02	microdictya	sinopass	x	
sno_mic_v03_bis	microdictya	sinopass	x	
sno_mic_v06	microdictya	sinopass	x	
sno_tri_001	pleioclada	sinopass	x	x
sno_tri_002_bis	pleioclada	sinopass	x	x
sno_tri_004	pleioclada	sinopass	x	x
sno_tri_007	pleioclada	sinopass	x	x
sno_tri_009	pleioclada	sinopass	x	x
sno_tri_011	pleioclada	sinopass	x	x
sno_tri_v01	pleioclada	sinopass	x	
sno_tri_v02	pleioclada	sinopass	x	
sno_tri_v03	pleioclada	sinopass	x	
deg_imi_003	F sp	degenumbu	x	x
deg_imi_004	F sp	degenumbu	x	x
deg_imi_008	F sp	degenumbu	x	x
deg_imi_011_bis	F sp	degenumbu	x	x
deg_imi_012_bis	F sp	degenumbu	x	x
deg_imi_v01	F sp	degenumbu	x	
deg_tri_v01	pleioclada	degenumbu	x	
deg_tri_v02	pleioclada	degenumbu	x	
deg_tri_v03	pleioclada	degenumbu	x	
deg_tri_v04	pleioclada	degenumbu	x	
deg_tri_v05	pleioclada	degenumbu	x	
deg_tri_v06	pleioclada	degenumbu	x	

mem_ito_006	itoana	numba	x	
mem_ito_007	itoana	numba	x	
num_ito_007	itoana	numba	x	x
num_ito_008	itoana	numba	x	x
num_ito_006_bis	itoana	numba	x	x
num_tri_016	trichocerasa	numba	x	x
num_tri_025	trichocerasa	numba	x	x
num_tri_v01	trichocerasa	numba	x	
num_tri_v02	trichocerasa	numba	x	
ohu_ade_001	adenosperma	ohu	x	
ohu_ade_002	adenosperma	ohu	x	
ohu_ade_003	adenosperma	ohu	x	

Chapter IV

Non-random and predictable distribution of *Wolbachia* strains along an elevational gradient in Papua New Guinea

(Manuscript)



Non-random and predictable distribution of *Wolbachia* strains along an elevational gradient in Papua New Guinea

Daniel Souto-Vilarós, Jan Michalek, Clive T. Darwell, Mentap Sisol, Brus Isua, Thomas Kuyaiva, George D. Weiblen, Vojtech Novotny, Simon T. Segar.

Abstract

Wolbachia is a maternally inherited endobacterium known to have negative effects on its host such as male killing, distortion of sex-ratio and inducing cytoplasmic incompatibility (CI). Through CI, *Wolbachia* has also been thought of as a speciation agent or at least, responsible for the maintenance of reproductive isolation between incipient species. Fig wasps are among the insects with the highest known incidences of *Wolbachia* and as such may promote their speciation. We screened 284 pollinating fig wasps (Agaonidae) associated with six different New Guinean *Ficus* (Moraceae) species for presence and strain identity of *Wolbachia*. Thirty-six percent of screened wasps were positive for *Wolbachia* and their distribution across our generated wasp phylogeny was not different than that expected by change. Strain typing of infected individuals revealed six different strains and their distribution deviated significantly from random across the phylogeny. Additional LDA analysis revealed a high predictability of infection status (70%) using host identity and collection site (as elevation) as only input. In general, strain type and infection status of wasps correspond to collection site and species suggesting a potential for CI induced reproductive isolation between wasps pollinating the same species but at different elevations.

INTRODUCTION

The endobacterium *Wolbachia* is widespread among insects, with an estimated 40% of species being infected (Shoemaker *et al.* 2002; Yang *et al.* 2012). One of the most widespread and important effects of *Wolbachia* infections is imposing cytoplasmic incompatibility (CI) on their hosts, and this has been detected in almost every major insect order (Stouthamer, Breeuwer & Hurst 1999; Zabalou *et al.* 2004; Engelstädter & Telschow 2009). CI renders progeny between individuals with different *Wolbachia* infection status unviable. It can be unidirectional, where infected males cannot fertilize eggs from uninfected females, or bidirectional, where matings between infected individuals with different strains of *Wolbachia* result in unviable offspring. Since *Wolbachia* is predominantly maternally inherited (in the cytoplasm of eggs along with mitochondria rather than in sperm), males are exploited by the infection to kill the offspring of uninfected females. Infection spreads through host reproductive manipulation such as distorting sex-ratio in favour of females, male killing and feminization (Shoemaker *et al.* 2002; Engelstädter & Telschow 2009; Yang *et al.* 2012). These strategies to increase transmission through female hosts give infected females a selective advantage over non-infected ones (since they can successfully reproduce with all males of a population) and thus *Wolbachia* can strongly influence host evolutionary history, gene-flow patterns and can even lead to rapid speciation (Engelstädter & Telschow 2009).

Pollinating fig wasps (Agaonidae) appear to be a prime candidate for exploring CI because many closely related and often cryptic

pollinating species can share an enclosed space and must regularly come into contact (Shoemaker *et al.* 2002; Molbo *et al.* 2003; McLeish & Van Noort 2012; Yang *et al.* 2012). Matings between brothers and sisters are also common and under certain population structure scenarios, high inbreeding may decrease the likelihood of incompatible matings as well as decreasing the overall invasion speed and/or persistence of CI-inducing microbes (Engelstädter & Telschow 2009). Incipient species must split quickly and maintain post-speciation barriers, implying that any pre- or post-zygotic barriers would be advantageous. Indeed, evidence suggesting higher rates of diversification in wasps compared to figs is accumulating (Cook & Rasplus 2003; Machado *et al.* 2005). However, most studies to date have shown a random distribution of *Wolbachia* strains across sympatric wasp populations, with strong indications for multiple infections and horizontal transmission (Shoemaker *et al.* 2002; Yang *et al.* 2012). It has also been estimated that *Wolbachia* can be purged over the course of five to nine million years (Bailly-Bechet *et al.* 2017). *Wolbachia* studies on fig wasps have generally targeted pollinator and non pollinating fig wasp communities associated with well differentiated host species in homogenous rainforest environments and find a 'chaos of *Wolbachia* sequences' within fig syconia, often including multiple infections and high incidences of horizontal transfer (Shoemaker *et al.* 2002; Yang *et al.* 2012). We argue here that focusing on pollinating wasps alone reveals contrasting *Wolbachia* infections found in incipient species, acting as facilitators of speciation. Our hypothesis is that sister populations of

wasps associated with diverging hosts should be infected with different strains or exist as infected/non-infected pairs.

Study system

Our field collection site is located along an elevational gradient in the central mountain range in northern Papua New Guinea. There is a steep turnover of *Ficus* species along this gradient (Segar *et al.* 2017) and several monophyletic species complexes comprising lowland/highland sister species can be found growing alongside morphologically homogenous species with wide elevational ranges (Souto-Vilarós *et al.* 2018; Souto-Vilarós *et al.* submitted). We focused on collecting fig wasps from one species complex (*F. itoana* species complex) two sub-species (*F. trichocerasa*) and two species with wide elevational ranges (*F. wassa* and *F. arfakensis*). Population genomic analyses of these confirm them to be *Ficus* species subpopulations along the gradient, while the same analysis on their corresponding pollinating wasps revealed that these subpopulations are pollinated by genetically distinct wasp species along the gradient (Souto-Vilarós *et al.* submitted). In order to identify sister-species *Wolbachia* infection status, we screened 284 fig pollinating wasp individuals. Positive individuals were then sequenced for the *Wolbachia* surface protein gene (*wsp*). Following strain identity of these sequences, we sequenced multiple individuals per *wsp* clade for the five Multi Locus Strain Typing (MLST) genes to identify infection strain type.

METHODS

Field collection

Sampling was done between August 2015 and December 2016 along the Mt. Wilhelm elevational gradient in Papua New Guinea. Focal trees at each of our six collection sites were monitored regularly for ripening crops. We collected individual near-ripe syconia (enclosed inflorescences) from 10-15 fig individuals of each species at each site and placed these into breathable rearing pots to allow for the emergence of fig wasps within. A selection of five male and five female pollinating fig wasps were then stored in 2ml tubes three-quarters filled with silica gel and a small piece of cotton wool before being transferred to a -20°C freezer for long term storage and later manipulation (Moe & Weiblen 2012). Remaining wasps were collected in 100% ethanol and used for a wider population genomic and fig-wasp community studies (Souto-Vilarós *et al.* submitted).

DNA extraction and PCR

Wasp samples in open tubes were submerged in liquid nitrogen and manually homogenized with a sterile plastic pestle. Subsequent DNA extractions were performed using DNeasy Blood & Tissue kits (Qiagen) following a several modifications of the manufacturer's protocol. The initial lysis step was done gently at 37°C overnight and finished by 30 minutes incubation with 1 µL of RNase A (Quiagen) per sample. To enhance the yield the final elution step was done with a total of 200 µL of deionised water separated in two rounds of column washing. The resulting DNA solution was then dried using

vacuum concentrator and resuspended in 38 µL of buffer EB in order to increase the concentration. One microliter was used for Qubit quantification, 2 µL were loaded on 2% agarose gel pre-stained with GelRed® Nucleic Acid Gel Stain (Biotium) and ran at 120V for 70 min. to assess the quality of extracted DNA. Samples were quantified using a Qubit 3 Fluorometer (ThermoFisher Scientific) and diluted to a total of ~20 ng in 35 µL of EB buffer (Qiagen). We used the primers and protocols of Baldo et al. (2006) to amplify the *Wolbachia* surface protein gene (*wsp*) and the five Multi Locus Strain Typing (MLST) genes used for accurate strain typing and better detection of recombination. All PCR products were sequenced using Sanger sequencing, chromatograms were checked for multiple peaks and edited before alignment using MAFFT v.7 (Katoh & Standley 2013). While the rest of the wasp DNA was sent to SNPsaurus (LLC) for NextRAD genotyping-by-sequencing as per (Russello *et al.* 2015). Genotyping analysis used custom scripts (SNPsaurus, LLC) which trimmed reads using bbduk (BBMap tools, http://sourceforge.net/projects/bbmap/). A de novo reference was created using 10 million reads in total, selected evenly form all samples and excluding reads that had counts less than 10 or more than 1000. Remaining loci were then aligned to each other to identify allelic loci and collapse allelic haplotypes to single representatives. All reads were mapped to the reference with alignment identity threshold of 85% using bbmap (BBMap tools). Genotype calling was done using Samtools and bcftools. Vcf files were filtered to remove alleles with a population frequency of less than 3%. Heterozygous loci in all samples or samples which had more than two alleles

(suggesting collapsed paralogs) were removed and absence of artefacts were checked by counting SNPs at each site and determining that SNP number did not increase with reduced base quality at the end of the read. Detailed NextRAD sequencing protocol and bioinformatic pipeline is provided in the supplementary material.

Phylogenetic reconstruction

Wasp phylogenetic tree was generated by converting the vcf files to a phylip format variant using PGDSpider v2.1.1.3 (Lischer & Excoffier 2012) and generated the tree using RAxML version 7.2.7 (Stamatakis 2014) using the GTRCAT model rate of heterogeneity. For the *Wolbachia* sequences, separate phylogenetic trees were generated in the same way for each gene to further assess the consistency of strain groupings. Further phylogenetic manipulations were performed in R using the 'ape' package (Paradis & Schliep 2018). Tree visualization and annotations were done using the web-based interactive tree of life (iTOL) tool (Letunic & Bork 2016). All *wsp* and MLST sequences were compared to those in the MLST data base (Baldo *et al.* 2006) to assess strain similarity. Sequences were assigned to the nearest matching allele to derive consistent MLST defined strains allowing us to identify 6 strains (see results).

Statistical analyses

We assessed the distribution of *Wolbachia* infection status (infected or non-infected) across the entire 284 wasp phylogeny by comparing the observed mean phylogenetic distance between all infected individuals to a distribution of values generated by shuffling the tip

labels across the phylogeny and assessing the deviation of observed and null values. Null models were implemented using functions in the R package 'picante' (Kembel *et al.* 2010).

In order to evaluate whether the distribution of the identified *Wolbachia* strain types along the wasp species and populations are non-random, we analysed these data under an assembly rule framework (Gotelli 2000). Wasp phylogenetic tree was pruned to include only infected wasps and binary characters indicating the presence or absence of each of the 6 identified strains. We compared the observed data to multiple randomized datasets which serve as a null hypothesis to test the data against. We randomised an association matrix of 103 rows (wasp species/population) and 6 columns (*Wolbachia* strains) such that row incidences were retained (one infection per wasp) while column incidences (strain identity per wasp) were shuffled across a series of permutations, showing a random distribution of strain types along the wasp phylogeny. Model construction was done using the 'permatfull' function in the R package 'vegan' (Oksanen *et al.* 2016). We fixed row margins, set shuffle type to 'both' and set model type to 'prab'. Therefore, overall matrix fill and sums were maintained. Permutations were restricted to take place in strata delimiting wasp species. Statistical significance was evaluated by comparing the chi square value for the original matrix to the distribution of values generated through 999 permuted matrices.

Additionally, to test whether or not *Wolbachia* strains were associated to different fig wasp species, we used linear discriminant analyses

(LDA) as implemented in the 'MASS' R package (Venables & Ripley 2002). This analysis attempts to predict *wsp* strain based on wasp (sub)species and site collection (as elevation). First, a random 70% subset of the host species and strain type association data is used to train a *wsp* strain type assignment model which is then used to predict strain type of the remaining 30% of the data. This is then repeated for 1000 permutations, each with a randomly selected training dataset and a mean prediction success rate is calculated.

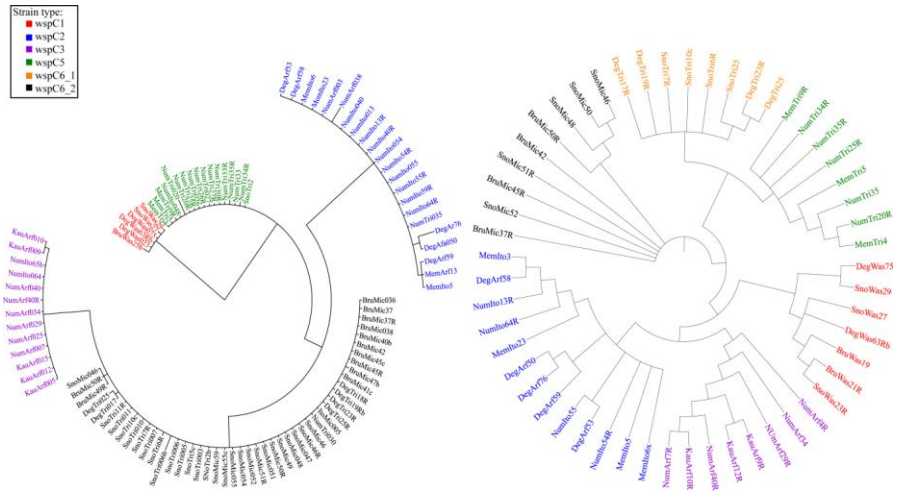


Figure 8. Phylogenetic trees generated with the obtained wsp (left) and MLST (right) sequences. Under both scenarios, we retrieved the same number of different strains except for the MLST tree, where the wspC6 clade is divided in two.

RESULTS

Out of the 284 screened wasps, 36% (103 individuals) were infected with *Wolbachia*. We found no evidence for multiple infections in the sampled wasps as no chromatograms contained multiple peaks and strain identifications were consistent across MLST loci. We used individual phylogenies using *wsp* and MLST markers to confirm the monophyly of the strains identified; the *wsp* tree contained five major clades (Figure 1a) while the MLST tree contained six (Figure 1b). *Wsp* clade assignment corresponded to that using MLST for the most part, with the exception of the clade associated with *F. trichocerasa* subsp. *pleioclada* and *F. microdyctia* which were contained within the same *wsp* clade. We therefore followed *wsp* clade designation in all cases apart from this one, in which we split *wsp* clade six into two (wspC6_1 and wspC6_2).

Based on null model expectation, the mean phylogenetic distance between infected individuals is no different than expected by chance (z=-0.314, p=0.346), suggesting a random distribution of *Wolbachia* across the phylogeny. However, it is notable that while 77% of all *Ceratosolen armipes* sampled (the pollinator of *F. itoana*) were infected with *Wolbachia* only one (out of 34) of the mid-elevation *C.* sp (the pollinator of *Ficus* sp. "IMI") was infected. Similarly, ~63% of *C. "kaironkensis"* (the pollinator of *F. microdyctia*) were infected. Parapatric pollinators of *F. arfakensis* also showed disjunct infection status, with only 26% of infected pollinators overall, but with different infection rates at different elevations (Table 1). In the case of *F. trichocerasa*, infection rates differed between subspecies with

subsp. *trichocerasa* having an 84% infection rate and subsp. *pleioclada* had a 54% infection rate. Finally, in the case of *F. wassa* only 0.08% of samples were infected; all individuals from highland populations (between 1,700 and 2,700m). Thus, parapatric populations of pollinating wasps often have contrasting infection statuses.

Under random expectations we would not expect any clustering of our six *Wolbachia* strains across the fig wasp populations that we sampled. Our analyses demonstrated that the association of strain types with wasp species ($z=-5.793$, $p=0.001$) was not randomly distributed, with strain type being clustered with wasp population and species. Shuffling of strain types across the wasp phylogenetic tree demonstrated that strains clustered in wasp clades, such that they occupied less branch length than expected under a random distribution across the tree in these strains. Sister species/populations usually had different *Wolbachia* infection status or strain type (Figure 2b). Similarly, LDA analysis was able to predict strain type ~70% of the times based exclusively on wasp species and collection site.

It is interesting to note that there appears to be wsp strains that are restricted to the lowlands and highlands. For instance, wsp clades 1, 6_1 and 6_2 are present in wasps from elevations above 2,200m while the rest occur in the lowlands (below 1,200m). A notable exception is for wasps originating from the mid-elevation site (here considered as 1,700m) "Degenumbu" where both lowland and highland strains occur in wasps from *F. wassa* (wsp clade 1) and *F. arfakensis* (wsp clade 2). Similarly, bar a few exceptions, strain type

segregates by (sub)species while infection status seems to be influenced elevation.

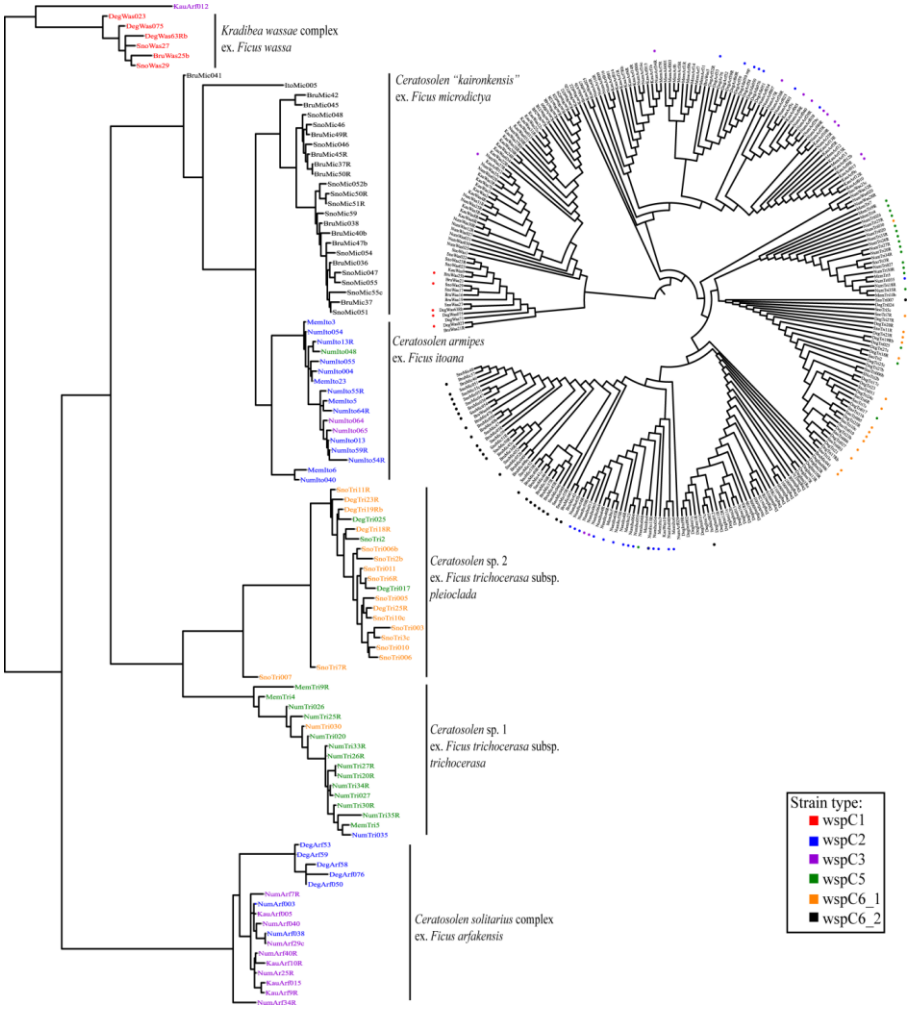


Figure 9. Wolbachia strains mapped along the pollinating wasp phylogeny. Circular phylogeny (right) includes all sampled individuals, Wolbachia infection status and strain indicated by a coloured dot. Left phylogeny is pruned only to include infected wasps. Strain type is indicated by the different colours.

DISCUSSION

We were able to estimate the proportion of *Wolbachia* infected pollinating wasps associated with six (sub)species of figs along an altitudinal gradient in Papua New Guinea. This proportion (36%) is lower than previous estimates for fig-wasps in general (Shoemaker *et al.* 2002; Yang *et al.* 2012), however, comparable to studies done in pollinating wasps exclusively (Sun *et al.* 2011). Future estimates including non-pollinating fig wasps will certainly reveal a higher proportion of infection rates along our transect. This study is, to our knowledge, the first to include pollinating wasps from the same host fig species along a steep environmental gradient. Previous studies on these fig species along the same gradient (Souto-Vilarós *et al.* submitted) revealed fig sub-populations pollinated by genetically distinct wasp species, suggesting multiple cryptic wasp species pollinating populations of the same fig host. The analysis presented herein reveals complex infection patterns that seem to depend on both, host-species identity and elevation. We find no evidence of strict cospeciation between wasp species and their *Wolbachia*; however, we do find different strain types associated with different wasp species. These findings are consistent with the idea that different *Wolbachia* strains could reinforce, and even speed up, the speciation process through cyotplasmic incompatibility. For instance, pollinating wasps from *Ficus trichocerasa* subsp. *trichocerasa* are mostly infected with *Wolbachia wsp* strain type 5 while wasps pollinating *F. trichocerasa* subsp. *pleioclada* are mostly infected by strain type 6_1. The population structure and trait divergence of these *Ficus* subspecies indicate these to be in the process of speciation

(Souto-Vilarós *et al.* 2018), coupled with evidence suggesting these subspecies are pollinated by two separate wasp species, *Wolbachia* strain type could be responsible for the maintenance of reproductive isolation between wasp species and by consequence, fig subspecies. Similarly, in the case of the *F. itoana* complex, pollinating wasps from *F. itoana* are mostly infected by strain type 2, while the sister species is mostly infected by the distantly related strain type 6_2; pollinators of the third species in the complex, *F. sp.* "IMI", seem to be *Wolbachia* free (only one out of 34 individuals screened was infected) again supporting strain type and infection status as a mechanism for maintaining reproductive isolation, even in cases where pollinators are attracted to the scent of a different fig species (Souto-Vilarós *et al.* 2018). In the case of both *F. arfakensis* and *F. wassa,* pollinating wasps showed an overall low infection rate. It is interesting to note that according to Souto-Vilarós *et al*. (submitted) these two fig species are pollinated by more than one wasp species. In the case of *F. wassa,* Souto-Vilarós *et al.* (submitted) identified three pollinating wasp species, two in the lowlands (between 200 to 1,200m) and one in the highlands (1,700m and above). We were unable to detect *Wolbachia* infections in the two lowland wasp species, while 35% of the screened highland wasps were infected with *Wolbachia* strain type 1. Similarly, of the four pollinating wasp species identified by Souto-Vilarós *et al.* (submitted), only two of these were positive for *Wolbachia* infections, and these presented different strain types according to species (strain type 3 and 2 in the lowlands and highlands respectively).

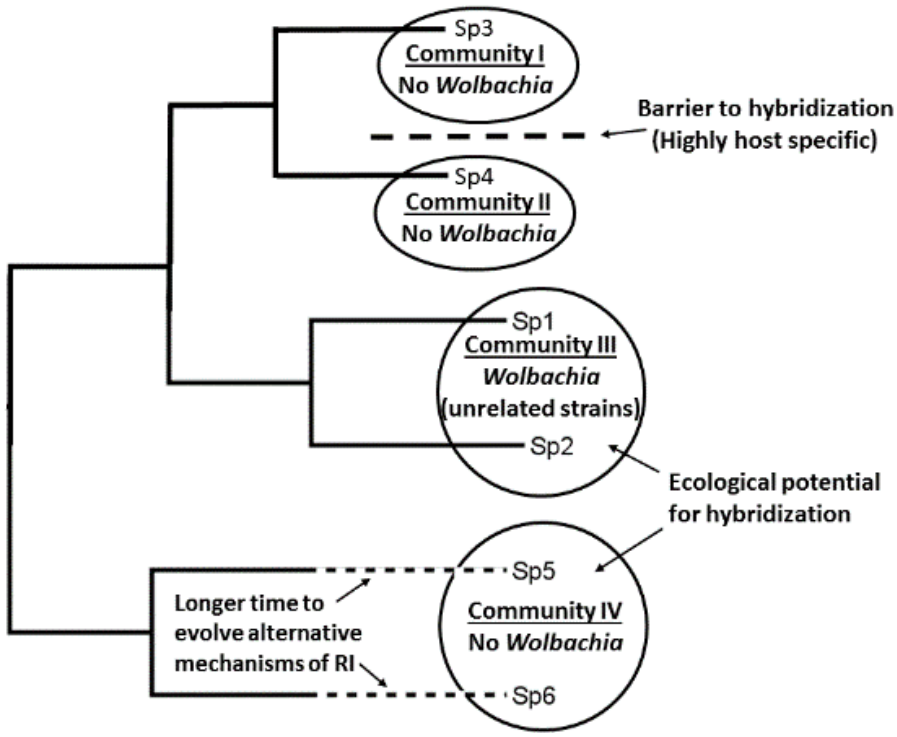


Figure 3. Hypothetical fig wasp relationships and predicted status of RI inducing *Wolbachia* according to variation in ecological contact and evolutionary time since speciation. We predict *Wolbachia* infection to occur only in community III where, critically, species 1 & 2 should harbour alternate strains. Sister-species in communities I & II are not in ecological contact, while sister-species in community IV, despite ecological contact, have had sufficient evolutionary time for alternative (less costly) RI mechanisms to evolve.

Whilst our approach cannot test for specific mechanisms that determine *Wolbachia* distributions across our study system, we propose a verbal model for CI-inducing strains reliant on key predictions consistent with our findings and the *Wolbachia* literature. It should be stressed that our proposed model is particularly suited to

testing in fig wasps due to the high degree of host-specificity exhibited when wasps are breeding. For most arthropods we have limited knowledge about ecological contact which provides direct opportunity for horizontal exchange of microbes or genetic material between species. Such data have not been previously incorporated into a predictive phylogenetic model of *Wolbachia* distributions. Nevertheless, we expect that our proposed model is relevant to other arthropod groups among which patterns may appear random due to difficulties in assessing inter-specific ecological contact.

First, we predict that recently evolved sister-species that diversified within the same host communities will have different *Wolbachia* infection status to overcome the initial stages of speciation. This is because we expect that co-diverging *Wolbachia* strains would not confer reproductive isolation between diverging host species. Thus, we argue that co-diversification between *Wolbachia* and wasp (or other arthropod) hosts should not be an expected outcome. Second, because *Wolbachia* infection itself appears to impose a fitness cost (Zug & Hammerstein 2015), we predict that these patterns should not be evident among sister-species that are not in regular ecological contact (i.e. fig wasps in alternative fig hosts) and may therefore be prone to preferentially purge *Wolbachia* infection due to the absence of any RI-enforcing benefit. Similarly, as mentioned before, high levels of inbreeding can render infection and prevalence of *Wolbachia* impossible (Engelstädter & Telschow 2009). Interestingly, non-pollinating fig wasps may offer a testable counterpoint as some species breed externally from the syconia and may therefore not benefit from RI-enforcement even if they breed within the same fig

host as a sister-species (Yang *et al.* 2012). In general, selection should favour tolerance of *Wolbachia* by newly-forming species if the fitness costs of potential hybridization are high and species are in contact. Finally, as *Wolbachia* typically remains within host lineages for approximately 7 million years (±5.2-9.6; Bailly-Bechet *et al.* 2017), and because alternative mechanisms of RI that require cytogenetic or morphological modification may take longer to evolve (Bordenstein & Werren 2007), we predict that closely-related species that have not recently diverged should be free from *Wolbachia* infection. This would reflect a hypothesis that observed lineage dropout (Bailly-Bechet *et al.* 2017) results from temporal changes in the adaptive benefits of *Wolbachia*, which may subsequently become redundant and be eventually eradicated.

Following these inter-connected lines of reasoning, we propose that predicable phylogenetic structure should be apparent among study systems where suitably detailed ecological data have been recorded (Figure 3). A key feature of our model is that it predicts alternate *Wolbachia* infection statuses for species that have otherwise equitable ecological circumstances. However, the key discriminant for our model is the shared community in which the focal species occur. For example, two wasp species may occupy fig hosts with similar syconia size, seed dispersal vectors and habitat and may also hail from the same wasp lineage. However, only by considering whether each species' constituent community contains a recently diverged congener do we predict relative *Wolbachia* infection status. We encourage method development and mathematical modelling strategies to test these proposed mechanisms.

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AUTHOR'S CONTRIBUTIONS
V.N., S.T.S., and D.S.V. planned the research and provided input at all stages. G.D.W. and B.I. suggested suitable species for the study. D.S.V., M.S. and T.K. conducted and managed all fieldwork aspects with initial assistance of S.T.S. J.M. coordinated all molecular lab manipulations. C.T.D. and S.T.S. analysed the data and interpreted the results. D.S.V. wrote the manuscript with substantial help from all authors. All authors contributed and approved the final version of the manuscript.

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Chapter V

Contrasting patterns of fig wasp communities along Mt. Wilhelm, Papua New Guinea

(Manuscript)

Contrasting patterns of fig wasp communities along Mt. Wilhelm, Papua New Guinea

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ABSTRACT

The fig (Moraceae) and fig wasp (Agaonidae) mutualism is best known as a model system for the study of coevolution in plant-pollinator interactions and its central role in underpinning vertebrate species diversity in tropical forests. Figs also host myriad antagonistic non-pollinating fig wasps which impose costs on both partners threatening its stability. Spatio-temporal variation in parasitic wasp abundance is a key factor in mitigating these effects. Because fig wasps are temperature sensitive and likely vary in their ability to traverse environmental gradients, we expect community assemblages and abundance of both pollinating and non-pollinating fig wasps to respond to changes along an elevational gradient. In the present study, we compare the fig wasp communities and abundance of three fig species growing along the slopes of the Mount Wilhelm altitudinal gradient in Papua New Guinea. We quantified wasps from over 100 male fig trees and calculated seed set for 55 female trees along their distribution on the transect. Our results show that the abundance of both pollinating and non-pollinating follows a mid-elevation peak, consistent with fig species richness found in the same transect. The patterns, however, are different according to the host's species distribution. Seed set remained relatively constant along the gradient for all species with some decrease along higher elevations potentially affecting connectivity along the gradient. As suggested for insects in general, temperature and habitat diversity appear to play a fundamental role in the species richness and abundance of fig wasps.

INTRODUCTION

Insect species richness and composition along altitudinal gradients is known to change with elevation, these patterns, however, are different among taxonomic groups (Warren, Harper & Booth 1988; McCoy 1990; Peck *et al.* 2008; Maunsell *et al.* 2015). Endothermic insects are particularly sensitive to temperature (Arroyo, Primack & Armesto 1982; García-Robledo *et al.* 2016) and correspondingly less diverse at higher elevations and lower latitudes. The vast majority of angiosperms are pollinated by insects which inextricably links plant and insect fitness (Lowry *et al.* 2008; Ollerton, Winfree & Tarrant 2011; Ellstrand 2014). Species richness of hymenopterans and coleopterans tends to decrease with elevation as they are replaced by dipteran communities. This implies a shift in pollinator composition which is likely to influence plant reproductive strategies and success (Warren *et al.* 1988). Similarly, there is evidence of parasitoid wasp abundance and species richness being affected by elevation, with greater abundance and richness at the mid elevation, due in part to abundance of potential hosts influencing structure and function of food webs by affecting plant herbivore and/or pollinator interactions (Peck *et al.* 2008; Maunsell *et al.* 2015).

Obligate pollination mutualisms offer a tractable and relatively simple model for measuring fitness related traits along environmental gradients (Souto-Vilarós *et al.* 2018) because species specificity is high and traits can be easily quantified. Parasites and parasitoids of mutualisms add an extra layer of complexity because direct and indirect costs on mutualistic partners can influence the stability of

such mutualisms (Bronstein 2001), and in some cases, abiotic factors may even shift mutualists into parasites and vice versa (Kawakita, Mochizuki & Kato 2015). Studies focusing on the response of trophic interactions to elevation have found that while there is a general trend for insect predation and parasitism rates to decline, the players involved do not necessarily respond in the same manner and in many cases depend on host distribution, and its density and temporal overlap during key life cycles (Péré, Jactel & Kenis 2013; Maunsell *et al.* 2015; Corcos *et al.* 2018). Thus, network structure varies with elevation (Plowman *et al.* 2017).

For this study, we focus on the fig (Moraceae) and fig-wasp (Agaonidae) mutualism, one of the most specialized nursery pollination systems (Cook & Rasplus 2003), where the reproductive success of both parties depends on species specific encounters. Briefly summarizing, female wasps emerge from the figs (called syconia) and are guided by volatile signals as they search for a receptive fig of the same host species. Upon landing, the gravid and pollen-loaded wasps enter the floral cavity through a narrow passage (ostiole) and pollinate the flowers within becoming foundress wasps. While approximately half of described fig species are monoecious having both male and female functions within the same fig, the remainder are gyno(dioecious) meaning that sexual function is partitioned across individual trees (Bronstein 1988; Corlett, Boudville & Seet 1990). Monoecious figs contain both long-styled flowers (which frequently develop as seeds) and a subset of short-styled flowers that are more accessible for wasps to oviposit, thus housing

the next generation of wasps. In functionally dioecious figs, male syconia become nurseries for wasps while female fig trees deceive the wasps to enter and pollinate, but wasps are unable to oviposit since they encounter only long-styled flowers inside and so female fig trees produce only seeds (Galil & Eisikowitch 1968; Kjellberg *et al.* 2005). Some fig-wasps are known for long distance pollen dispersal as these minute insects (1-2 mm) appear to be transported by wind over wide distances of up to 160km (Ahmed *et al.* 2009; Kobmoo *et al.* 2010; Liu *et al.* 2015). Thus far, these findings have been restricted mostly to large monoecious trees which occur at naturally low densities. But there is evidence that dioecious and under-canopy fig trees are clustered into dense local populations and so pollinating fig-wasps do not disperse over such long distances (Dev *et al.* 2011). In addition to this, figs house a large number of non-pollinating fig wasps (NPFW) which also develop within the syconia inflicting a cost through predation of pollinator larva or competition for seed resources (Weiblen, Yu & West 2001; Weiblen 2002). NPFW have a fascinating ecology of their own: ranging from primary gallers, entering the syconia alongside pollinators, secondary gallers who oviposit in previously galled flowers; to kleptoparasites which oviposit into pre-existing galls or parasitoids, which oviposit from the outside of the fig, the ovipositor length correlated with the syconia developmental stage at which these wasps oviposit (Weiblen 2002; Cook & Segar 2010; Borges 2015).

Some authors have suggested that the negative effect of parasitism is stabilized through temporal and spatial heterogeneity in non-

pollinator occurrence and abundance, as well as location of figs at the right developmental stage for them to invade. Breeding system of figs has also been suggested to mitigate the effect of parasitism as NPFW waste time searching for sites in female figs, thus reducing parasitism of male figs without a cost to seed set (Weiblen & Bush 2002), although less frequently, however, some non-pollinating gallers which sustain kleptoparasites and parasitoids can also occur in female figs (Wu *et al.* 2013; Borges 2015).

To date the distribution and fitness costs non-pollinating wasps along environmental gradients has not been well studied. We suggest that elevational gradients, which to some extent control for species pool effects, make excellent systems to study environmentally mediated variation in fitness traits and parasite loads. Such gradients provide natural spatial structure and environmental clines which mimic more wide scale variation across the range of a given resource species.
Specifically, we test the hypotheses that (1) pollinating wasp abundance decreases with elevation due to increased constraints on dispersal, (2) non-pollinator diversity and abundance will show a mid-elevation peak due to mixing between lowland and highland communities but correspond to trends in pollinator abundance, finally (3) seed set will remain constant along the gradient since dioecy confers an advantage on mitigating the overall effects of parasitism without reducing seed set. Because fig size is bound limit the amount of seeds or developing wasps that can fit within them, we also test whether or not syconia size (as volume) varies along the gradient.

METHODS

The study was conducted along the Mount Wilhelm elevational transect in the central range of Papua New Guinea (PNG). Approximately half of the 150 *Ficus* (Moraceae) species recorded for the island occur there (Berg & Corner 2005). Previous surveys of the transect reported that some of these species have wide elevational ranges and provide fundamental resources to forest communities year round, making these key-stone species (Novotny *et al.* 2005; Segar *et al.* 2017). The continuously forested gradient ranges from lowland alluvial forest up to lower montane forest and has been previously described in detail elsewhere (Toussaint *et al.* 2014; Marki *et al.* 2016; Robillard *et al.* 2016). We focus on three dioecious species endemic to PNG and adjacent islands selected on the basis of their distribution above sea level along the transect: *Ficus wassa* Roxb., is a well-defined species that is abundant throughout the gradient with a wide distributional range occurring between 200m and 2,700m. This species is pollinated by the fig-wasp *Kradibia wassae*, however, recent genomic analyses reveal highland and lowland genetically distinct pollinating wasps (Souto-Vilarós *et al.* in prep). The second focal species is *Ficus arfakensis* King, distributed between 200m and 700m pollinated by *Ceratosolen solitarius*; similarly, genomic analysis reveals at least four distinct pollinating wasps throughout the transect (Souto-Vilarós *et al.* in prep). Finally, *Ficus trichocerasa* Diels is species complex of a lowland and a highland subspecies with *F. trichocerasa* distributed between 700 and 1,200m and the highland *F. trichocerasa* subsp. *pleioclada* (1,200 to 2,600m). These species appear to be pollinated by two distinct *Ceratosolen* wasps (species

undescribed; Souto-Vilarós *et al.* 2018) and for these reasons, all analyses carried out were performed between subspecies rather than treating them as a single species.

Sampling was conducted between August 2015 and November 2016. At each of the six sites along the transect, we tagged several male and female trees of each locally available focal species and monitored them during the duration of the sampling efforts. For each of the focal trees, we collected up to five ripe syconia for each female tagged tree, stored them in plastic pots with a 70% ethanol solution and exported to the University of South Bohemia, Czech Republic for later dissection. Using a microscope, up to two syconia selected haphazardly from each pot were dissected and all fully developed seeds and all available ovules were counted, seed set was calculated as:

$$seed\ set = \frac{number\ of\ seeds}{number\ of\ available\ ovules}$$

For each male tree, figs were sampled either through emergence or dissection methods (Segar *et al.* 2014). For the emergence method, we collected up to five D-stage syconia (Galil & Eisikowitch 1968), the stage when wasps are already hatched from the galls and ready to emerge, and stored them in individual plastic pots covered with fine mesh. Wasps were allowed to emerge naturally from the figs and were immediately collected and stored in 100% ethanol. For the dissection method, a second collection of D-stage syconia were directly stored in 70% ethanol solution. All collections were sent to

the University of South Bohemia for later dissection and sorting. Up to two syconia were selected haphazardly and dissected under a microscope and recorded the total number of wasps. For the emerged wasps, individuals were sorted to genus and morpho-species. For all syconia stored in 70% ethanol (male (n = 113) and female (n = 112)), width and height were measured to the nearest 0.01mm using vernier callipers. Syconial volume was calculated using the standard cone volume formula (as per Segar *et al.* 2017):

$$V = \pi r^2 \frac{h}{3}$$

Generalized linear models (GLMs) were performed separately using collection site (as elevation) and fig species as explanatory variables. Minimal models were retained using standard backward selection by removing non-significant higher-level interactions. As there was no significant difference between fig volume and sex (see results), volume analyses combined both sexes. Analyses on seed set and wasp load were conducted separately for female and male figs. The full models run were: i) fig volume as a response to elevation and species, ii) seed set as a response to elevation and species, iii) total wasps as a response to elevation and species; further, we separated analyses on wasps to include -iv) total pollinating wasps as a response to elevation and species and - v)- parasitic wasps as a response to elevation and species. For all models we fitted a quasipoisson error structure, except for seed set for which we used a Gaussian distribution. Finally, pairwise comparisons between species and elevation were tested for significance using Tukey's range test

(TukeyHSD package). All analyses were conducted in R version 3.5.1 (R Core team 2015).

RESULTS

Collection details are summarized in Table 1 including sampling sites, syconia volume, seed set, percentage of infested figs, pollinator and parasitic wasp per elevation. Model summaries and analysis of variance are presented in Table 2.

Syconia volume variation between species and elevation. - We initially tested syconia volume separately according to tree sex but found no significant difference between them (t = -1.048, df = 266.7, p = 0.295) and so we combined these data to analyze the effect elevation has on fig volume. The effect of elevation varied between species (Fig 1): there was a positive effect of altitude for of *F. arfakensis* while fig volume remains relatively constant for *F. wassa* with declines at the 1,700m and the 2,700m sites. In the case of *F. trichocerasa*, volume increases with elevation for both subspecies, but subspecies *pleioclada* has smaller figs than the lowland subspecies. Alternative plots showing the effect of elevation on fig volume are presented in the supplementary material (Fig. S1).

TABLE 1 cont. Summary of collections per species including name of collection sites of collections (male figs only)

***Ficus* species**	**Collection Site**	**Elevation (m)**	**Male figs[a] (Trees = 103)**	**Infested syconia (%)**	**Number of pollinators (±SE)**	**Number of parasitoids (±SE)**	**Percentage of parasitism (±SE)**
F. arfakensis	Kausi	200	35(8)	96	116.571 ± 8.152	22.677 ± 3.489	0.173 ± 0.031
	Numba	700	9(7)	88	247.222 ± 34.29	23.222 ± 9.212	0.098 ± 0.037
	Memeku	1200	12(8)	100	475.833 ± 63.768	98.833 ± 16.692	0.172 ± 0.036
	Degenumbu	1700	9(7)	25	526.556 ± 82.802	0.75 ± 0.496	0.002 ± 0.001
F. trichocerasa	Numba	700	8(8)	100	136.875 ± 23.394	33.125 ± 9.48	0.225 ± 0.074
	Memeku	1200	10(10)	100	89 ± 5.55	14.889 ± 2.939	0.138 ± 0.027
F. pleioclada	Degenumbu	1700	27(13)	95	65.296 ± 8.552	14.792 ± 1.689	0.245 ± 0.018
	Snowpass	2200	12(11)	91	64.667 ± 18.915	20.917 ± 3.487	0.377 ± 0.074
F. wassa	Kausi	200	15(6)	75	126.2 ± 20.018	10.417 ± 3.306	0.082 ± 0.03
	Numba	700	27(10)	95	176.556 ± 36.166	29.792 ± 5.08	0.31 ± 0.071
	Memeku	1200	15(4)	73	344.467 ± 42.177	8.8 ± 4.018	0.037 ± 0.022
	Degenumbu	1700	10(6)	75	77.9 ± 17.805	7.625 ± 2.656	0.073 ± 0.026
	Snowpass	2200	27(13)	75	31.074 ± 6.403	17.826 ± 3.258	0.323 ± 0.059
	Bruno Sawmill	2700	7(2)	50	63.571 ± 31.742	3.333 ± 1.846	0.083 ± 0.039

[a]Numbers within parenthesis indicate the number of figs which were sorted from emerged wasps. Total numbers include sorted and dissected fig samples

TABLE 2. Summary of generalized linear model results and Analysis of Variance for each model tested. Values in bold indicate significant effect of the predictive term on the response variable.

Response	Interaction	χ^2	*df*	*p*
Volume				
	Elevation	253.01	5	**<0.001**
	species	941.88	3	**<0.001**
	Elevation:species	104.90	5	**<0.001**
Seed Set				
	Elevation	9.393	4	**0.051**
	species	24.580	3	**<0.001**
Total Wasps				
	Elevation	185.835	5	**<0.001**
	species	128.351	3	**<0.001**
	Elevation: species	66.865	5	**<0.001**
Pollinator abundance				
	Elevation	163.735	5	**<0.001**
	species	105.359	3	**<0.001**
	Elevation: species	59.101	5	**<0.001**
Parasitoid abundance				
	Elevation	62.987	5	**<0.001**
	species	36.070	2	**<0.001**
	Elevation: species	65.677	6	**<0.001**

TABLE 3. Summary of collections per species including name of collection sites (female figs only)

***Ficus* species**	**Collection Site**	**Elevation (m)**	**Female figs (dissected) (Total trees = 55)**	**Syconia volume (±SE)**	**Seed set (±SE)**
F. arfakensis	Kausi	200	6	1.13 ± 0.049	0.739 ± 0.058
	Numba	700	6	1.565 ± 0.063	0.758 ± 0.028
	Memeku	1200	4	2.599 ± 0.311	0.91 ± 0.031
	Degenumbu	1700	6	3.408 ± 0.114	0.794 ± 0.039
F. trichocerasa	Numba	700	9	0.781 ± 0.051	0.98 ± 0.008
	Memeku	1200	10	1.5 ± 0.219	0.975 ± 0.007
F. pleioclada	Degenumbu	1700	9	0.479 ± 0.017	0.7 ± 0.087
	Snowpass	2200	8	0.627 ± 0.064	0.796 ± 0.092
F. wassa	Kausi	200	12	0.599 ± 0.059	0.748 ± 0.053
	Numba	700	12	0.569 ± 0.03	0.786 ± 0.035
	Memeku	1200	10	0.653 ± 0.054	0.79 ± 0.047
	Degenumbu	1700	12	0.464 ± 0.037	0.546 ± 0.071
	Snowpass	2200	8	0.512 ± 0.05	0.795 ± 0.112
	Bruno Sawmill	2700	na	0.351 ± 0.026	na

Seed set variation between species and elevation - The effect of fig volume and seed set were analyzed using data for female figs only (Fig. 2A) and shows that in general, larger figs produce more seeds. Overall, seed set remains constant for all species along transect (Fig. 2B; Fig. S2) with the exception of a significant decrease of seed set for *F. wassa* at the 1,700m site. Important to note is that for this species, all mature female syconia found at this site were infested by maggots or decaying on the tree and so we were unable to calculate seed set.

Wasp abundance and variation between species and elevation - All analysis pertaining to wasp loads (both pollinators and parasites) were conducted on data from male figs only. Elevation played a significant role on total wasp load forming a distinct mid-elevation peak (Fig 3) where all parasitic wasp communities and pollinator abundance occur in tandem. Pollinator abundance steadily increases with elevation in *F. arfakensis* and *F. wassa* until the 1,200m site where the former appears to plateau at its range limit (1,700m) and the latter sharply decreases beyond this point (Fig S3). In the case of *F. trichocerasa*, there is a significant decrease of total wasp abundance in subsp. *trichocerasa* (Fig 3).

Pollinator abundance varies in response to elevation with a sharp increase at the mid-elevation peak (between 1,200m and 1,700m) followed by a decrease of pollinators in the highlands (Fig 4). The NPFW community associated with the three focal species is summarized in Table 2. The most diverse community was found in *F. wassa* with as many as nine different genera found within a single fig, while for *F. arfakensis* a single but very abundant genus, *Apocrypta*,

was found in nearly all of the samples collected. The NPFW community of *F. trichocerasa* is richer in subspecies *pleioclada* with up to seven different genera within a single fig.

Percentage of parasitism, taken as the total number of parasitic wasps divided by the total number of wasps per syconia is presented in Table 1. Generalized linear models revealed a significant effect of both host species identity and a correlation between NPFW abundance and elevation for all three species (Fig 4). As was the case for pollinators, the interaction between parasitic wasp abundance and elevation for *F. arfakensis* was positive but with a sharp decline at the species range limit (1,700m) where very few NPFW were found (mean = 0.75 ± 0.49; Table 1; Fig S5). For *F. trichocerasa*, subspecies *trichocerasa* shows a sharp decrease in NPFW numbers per fig with elevation (from 33.12 ± 9.48 at 700m to 14.88 ± 2.93 at 1,200m) while there appears to be no effect for *pleioclada.*

Finally, NPFW wasp abundance in *F. wassa* remains constant through the gradient albeit with sharp increases at 700m and 2,200m asl (Fig 4). These increases are negatively correlated with pollinating wasp abundance, particularly at the 2,200m site where we recorded the lowest number of *F. wassa* pollinators and the highest abundance of parasitic wasps (Table 1). Similarly, the increase of parasitic wasp loads at the 700m asl site is due to a considerable increase in parasitic wasp richness, rather than exclusively numbers, as at this site we found most syconia with between three and six different morphospecies of NPFW.

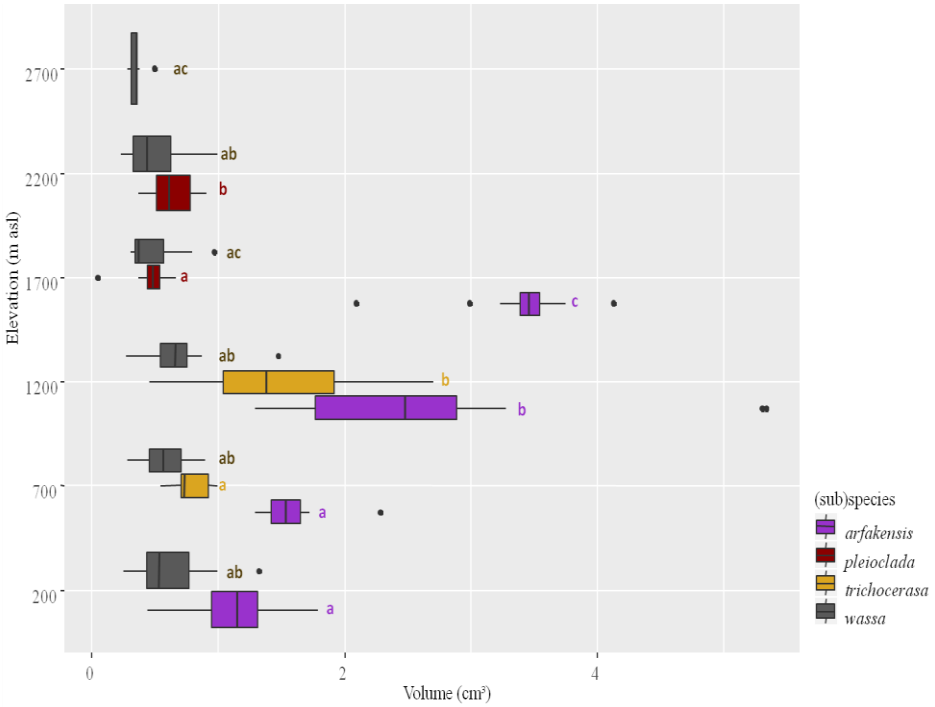

FIGURE 1 Effect of elevation on fig size per species. Pairwise differences between elevations were tested using Tukey HSD post-hoc test. Different letters indicate significant difference between comparisons ($P < 0.05$).

DISCUSSION

The present study is, to our knowledge, the first to offer insight on fig seed set and wasp load variation along an elevational gradient. We found that elevation plays an important role in all the parameters studied here (fig size, seed set and wasp production), however, the direction of the effect varies between species. As with other taxa (García-Robledo *et al.* 2016; Peters *et al.* 2016; Robillard *et al.* 2016), altitude plays an important role in species richness and abundance of both pollinating and NPFWs. Indeed, predictable climatic changes that occur with increasing elevation are some of the major factors

affecting the distribution and survival of many insect species (Jevanandam, Goh & Corlett 2013; García-Robledo *et al.* 2016). Both temperature and rainfall decrease with elevation directly affecting insect development and survival while the same factors influence the surrounding vegetation, similarly affecting links along the trophic chain (i.e. herbivores and parasitoids). The results presented herein follow the *Ficus*-wide species trends presented by Segar *et al.* (2017) where fig species richness decreases with increasing elevation in the presence of a clear mid-elevation peak. Not surprisingly, perhaps, we find wasp abundance to follow this trend with a clear increase with elevation up to between 1,200m and 1,700m followed by a sharp decrease at higher elevations.

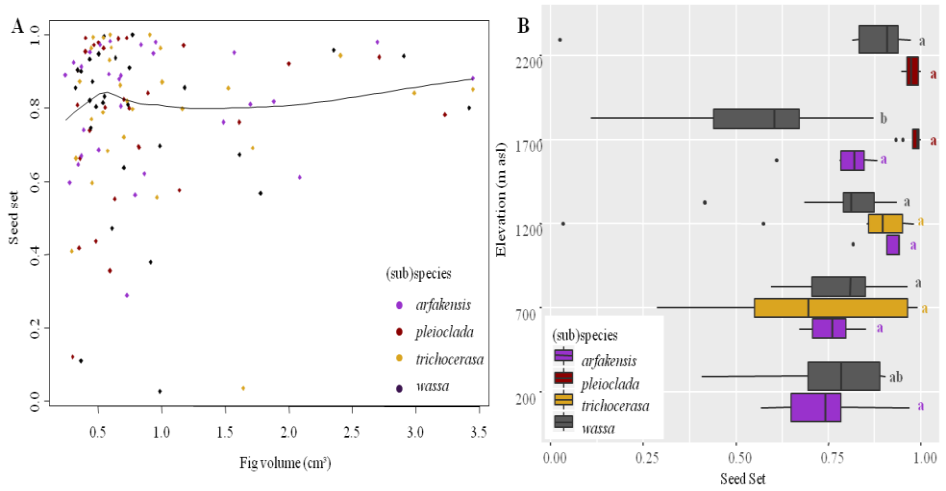

FIGURE 10. Scatter plot displaying the effect of fig size on seed set for all species (A) and boxplots showing seed set per species and elevation (B). Pairwise differences between elevations were tested using Tukey HSD post-hoc test. Different letters indicate significant difference between comparisons ($P < 0.05$).

Fig female fitness, measured as seed set, remains relatively stable for all species (Fig 2) throughout the transect, similar to findings by Weiblen, Flick & Spencer (1995) in *F. variegata* (69% seed set). There are clear exceptions of high seed set for subspecies *pleioclada* at both collection sites and a low seed set for *F. wassa* at the 1,700m population (Table 1). It is known that reduced seed set in *Ficus* is explained almost exclusively by the number of pollen loaded wasps entering syconia at receptivity (Corlett *et al.* 1990). Hence, total production of pollinating fig wasps does not have a direct effect on seed set, since only a fraction of emerged wasps eventually become foundress wasps (Jandér, Charlotte & Allen Herre 2010). Our results show that even at the range limits of *F. trichocerasa* subsp. *pleioclada* (2,200m asl), nearly every available ovule in female syconia produced seeds suggesting little pollen limitation occurring at this site. There is evidence suggesting that seed set increases with foundress wasps entering receptive figs (Nefdt & Compton 1996; Moore & Greeff 2003), as well as more wasps entering bigger figs (Anstett, Bronstein & Hossaert-McKey 1996). We did find size variation at some of the sites along the gradient, interestingly, *F. arfakensis* size steadily increases with elevation, and seed set seems to remain constant throughout the gradient. We did not record the number of foundress wasps entering the figs analyzed and the occurrence of foundress wasps found in the dissected syconia was hard to assess and so we are unable to relate seed set to the number of wasps entering receptive figs.

In dioecious species, female figs serve as a time sink in which NPFW waste time in search for oviposition sites, thus reducing overall parasitism without a decrease in seed production (Weiblen *et al.* 2001). The percentage of parasitic wasps per fig in the species studied varied widely with as many as 38% of wasp production of figs from subsp. *pleioclada* represented by parasitic wasps at the 2,200m site (Table 1). Amongst other possible explanations, ant predation has been shown to play an important role in regulating both pollinators and parasitic wasp numbers (Weiblen *et al.* 2001; Jandér 2015), but overall the interaction appears to be beneficial to pollinating wasps. Ant communities and structure along the same transect have been shown to decrease with elevation (Plowman *et al.* 2017; Orivel *et al.* 2018) and this could contribute to the observed changes in both parasitic and pollinating wasps along the gradient. Similarly, the sharp decline in parasitic wasps in *F. arfakensis* can be simply explained by the non-overlapping ranges of the single NPFW genus (*Apocrypta*) found within this species. Ecology and life history stages of these NPFWs is beyond the scope of this study, however, placing these wasps along the various trophic levels would greatly contribute to our as of yet limited understanding of NPFW communities in Papua New Guinea. Species richness and abundance of galling wasps influences the diversity of parasitoids and hyperparasitoids. Larger figs have greater number of flowers, which in turn offer more opportunities for wasp colonization (Borges 2015). Indeed, the largest figs in this study, *F. arfakensis* support the largest number of pollinating and non-pollinating fig wasps, but not the most diverse communities. Contrastingly, the decline of pollinating wasp numbers

in *F. wassa* at the 2,200m site could be explained by the increase of NPFW, perhaps due to fewer predatory ants at this elevation.

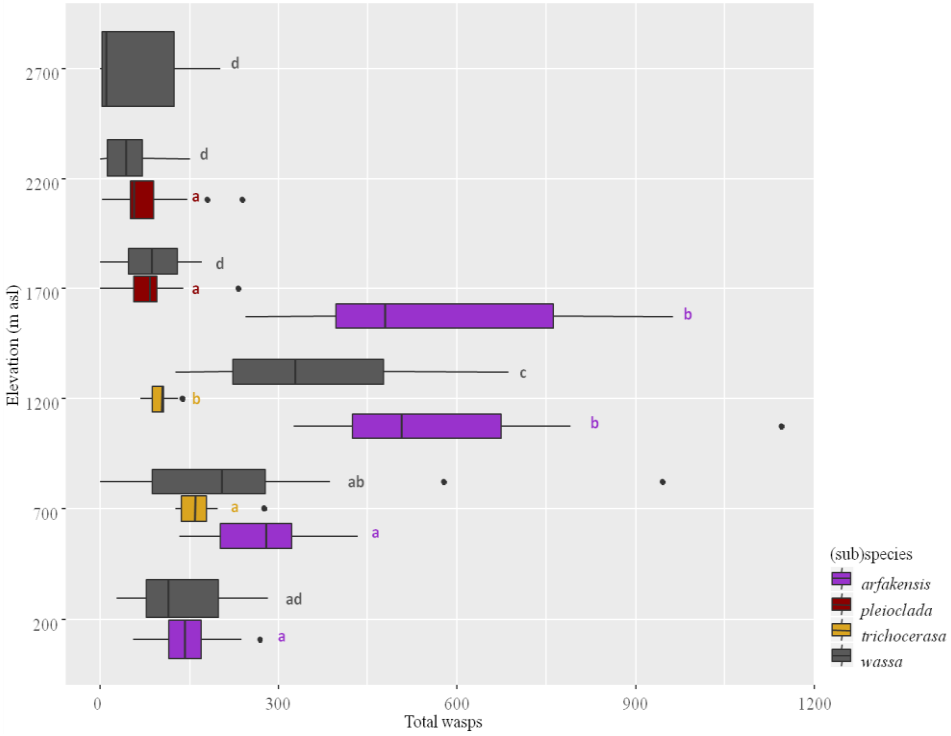

FIGURE 11. Effect of elevation on total wasp load for all species combined. Pairwise differences between elevations were tested using Tukey HSD post-hoc test. Different letters indicate significant difference between comparisons ($P < 0.05$).

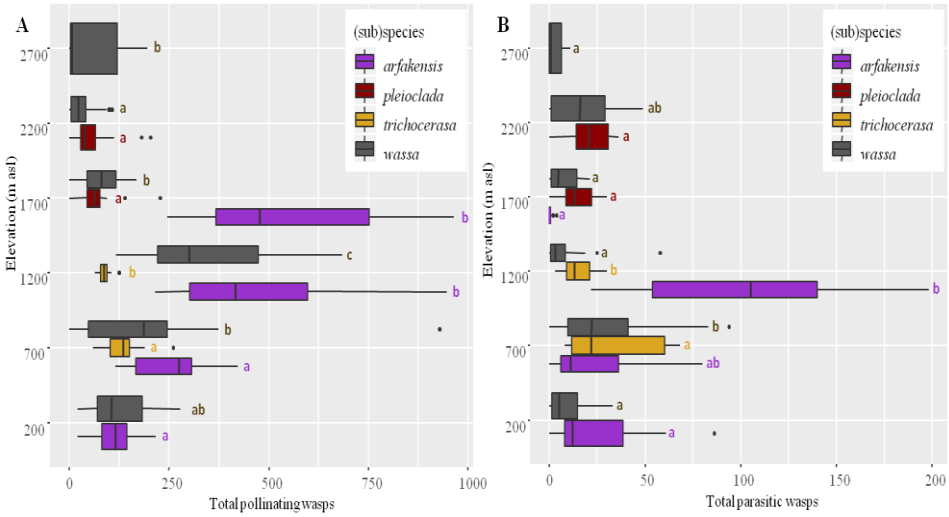

FIGURE 12. Effect of elevation on pollinating wasp abundance (A) and parasitic wasps (B) separated by species. Pairwise differences between elevations were tested using Tukey HSD post-hoc test. Different letters indicate significant difference between comparisons ($P < 0.05$).

TABLE 4. Summary of non-pollinating fig wasp community found at each elevation and Ficus species. Individuals were reared from individual syconia and sorted to morphospecies.

Ficus species	Collection Site	Elevation (m asl)	*Philotrypesis black 1*	*Philotrypesis black 2*	*Philotrypesis orange*	*Otitesella 1*	*Otitesella 2*	*Apocrypta 1*	*Apocrypta 2*	*Apocrypta 3*	*Apocrpytophagus*	*Arachonia*	*Megastigmus 1*	*Megastigmus 2*	*cf. Ficobracon*	*Ficobracon*	*Epichrysomallinae 1*	*Epichrysomallinae 2*	*Epichrysomallinae 3*	*Eurytomidae 1*	*Eurytomidae 2*	*Sycophila*
F. arfakensis	Kausi	200						87														
	Numba	700						158														
	Memeku	1200						692														
	Degenumbu	1700						5														
F. trichocerasa	Numba	700	6		14						185							2		1		
	Memeku	1200							2		132											
F. pleioclada	Degenumbu	1700									109		11					9		1		
	Snowpass	2200	91			8					99		5		1			3				
F. wassa	Kausi	200					42															
	Numba	700		6			41					34		26		3	51		37		8	3
	Memeku	1200		6																		
	Degenumbu	1700		11			7			1												
	Snowpass	2200		90			59															
	Bruno Sawmill	2700		11						8												

Due to our limited taxonomic identification, we cannot rule out the ability of some of these NPFWs to use multiple host species. Although host specificity for NPFW may be less constrained than that of pollinators, it has been suggested that some degree of specificity is still necessary (Jousselin *et al.* 2008; McLeish *et al.* 2012). Ecological and/or morphological requirements for NPFW development such as synchrony with syconia development, volatile cues for host recognition, fig wall thickness and/or the presence of other wasps either as hosts, competitors or parasitoids may promote species specificity (Weiblen *et al.* 2001; Marussich & Machado 2007; McLeish *et al.* 2012; Borges 2015). The co-occurrence of specific genera in different fig species at the same elevation may be of great interest from a community network perspective. Similarly, under-sampling individual trees may be a constrain in our results since it is known that NPFWs do not colonize all available figs within a patch due to asynchrony of developing syconia as well as the available species pool which is likely to vary over time and space, hence, wasp communities in a given fig crop depend on a multitude of factors (McLeish *et al.* 2012). Molecular approaches would help greatly in determining population genetic and species turnover within this system, which we suggest is an excellent system for studying speciation in parapatry.

As mentioned before, factors affecting vegetation distribution and insect development, abundance and survival are evident along altitudinal gradients. Due to the strong and predictable abiotic changes within small geographical distances, elevational gradients are

increasingly used as natural laboratories for the study of how species respond to climate change (Maunsell *et al.* 2015; García-Robledo *et al.* 2016). Through both herbivory and pollination, plants and insects are intrinsically linked, and indirectly serve as fundamental links up the trophic cascade. Climate warming predictions over the next decades spell dire consequences for species unable to track their climatic niche or locally adapt to a changing environment (IPCC 2014). For these reasons, the study of organisms along elevational gradients is increasingly relevant. In the case of *Ficus* along the Mt. Wilhelm gradient, we found that the abundance of both pollinators and parasitic wasps is clearly affected by elevation, and in turn intrinsically linked to the hosts range and distribution.

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AUTHORS' CONTRIBUTIONS

V.N., S.T.S., and D.S.V. planned the research and provided input at all stages. G.D.W. and B.I. suggested suitable species for the study. D.S.V., M.S. and T.K. conducted and managed all fieldwork aspects with initial assistance of S.T.S. J.M. assisted with data analysis and management of fig dissections. D.S.V. and M.H. analysed the data and interpreted the results. D.S.V. wrote the manuscript with substantial help from all authors. All authors contributed and approved the final version of the manuscript.

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Supplementary material for Chapter IV. Contrasting patterns of fig wasp communities along Mt. Wilhelm, Papua New Guinea.

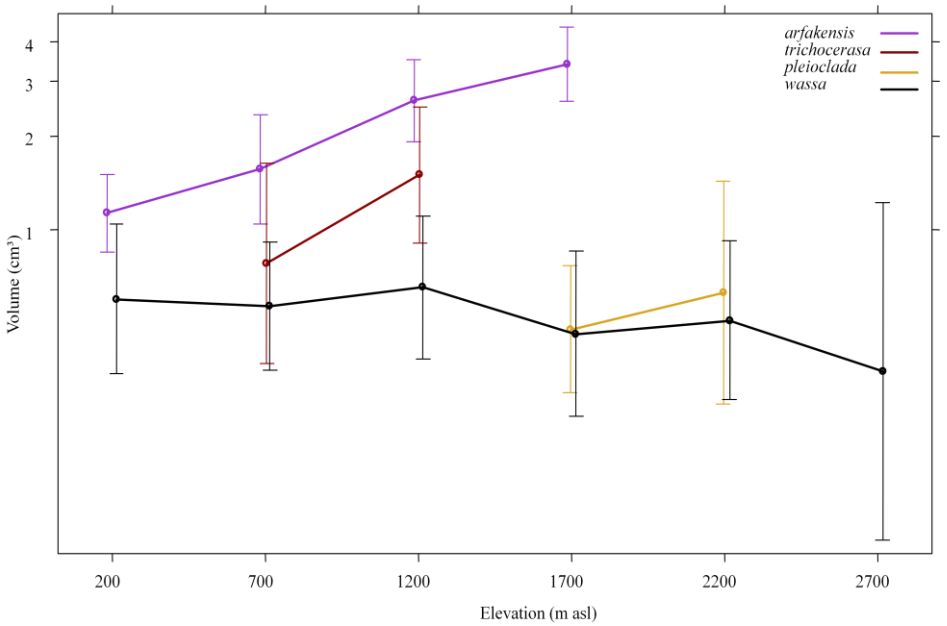

FIGURE S13. Effect of elevation on fig volume for all (sub)species. Effect was calculated using generalized linear model with volume as the response variable to elevation and (sub)species interaction. The interaction of elevation and(sub)species identity is highly significant ($\chi^2 = 104.90$, df = 5, $p < 0.001$).

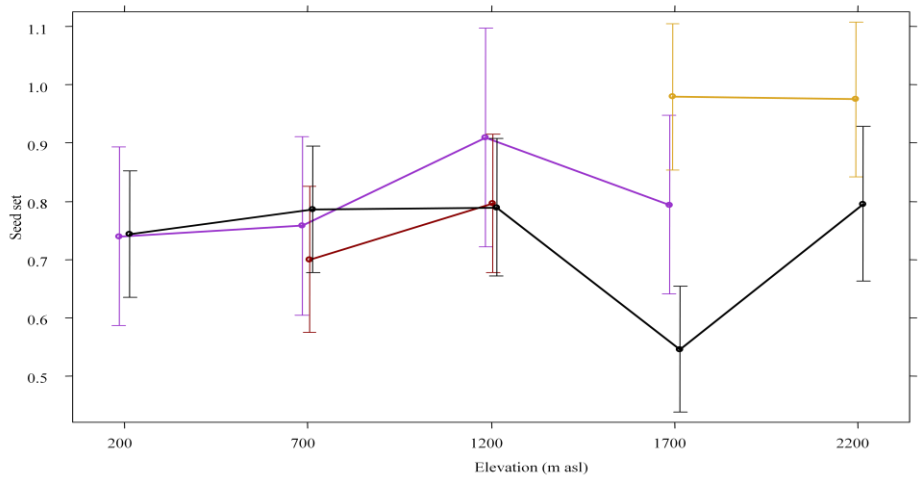

FIGURE S2. Effect of elevation on seed set for all (sub)species. Effect was calculated using generalized linear model with seed set as the response variable to elevation and (sub)species interaction. The interaction of elevation and(sub)species identity is not significant and so removed from the model through backwards elimination. Elevation and subspecies are significant (Elevation $\chi^2 = 9.393$, df = 4, $p < 0.051$; (sub)species $\chi^2 = 24.580$, df = 3, $p < 0.001$).

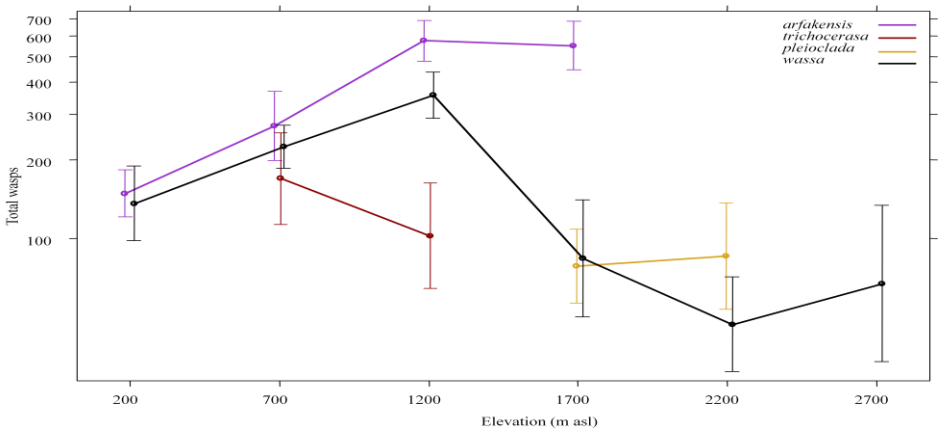

FIGURE S3. Effect of elevation on total wasp production for all (sub)species. Effect was calculated using generalized linear model with total wasp as the response variable to elevation and (sub)species interaction. The interaction of elevation and(sub)species identity is highly significant ($\chi^2 = 66.865$, df = 5, $p < 0.001$).

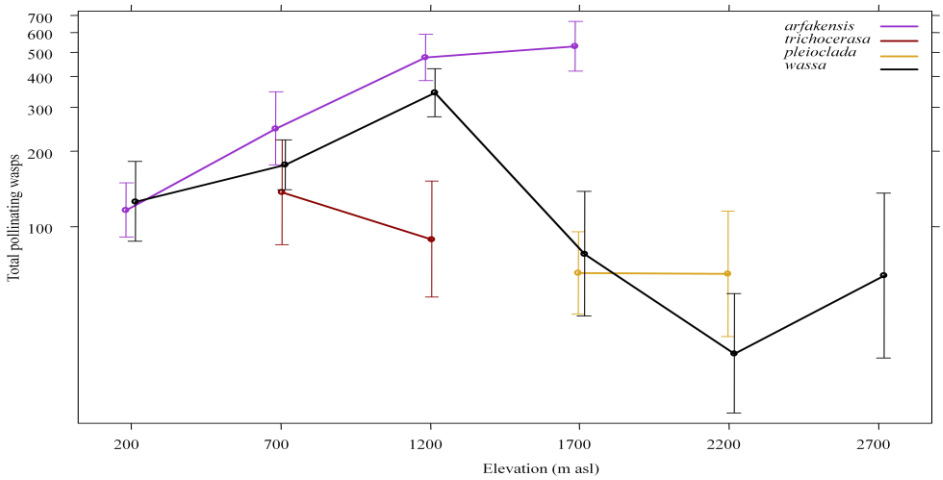

FIGURE S4. Effect of elevation on pollinating wasp production for all (sub)species. Effect was calculated using generalized linear model with total pollinating wasps as the response variable to elevation and (sub)species interaction. The interaction of elevation and (sub)species identity is highly significant ($\chi^2 = 59.101$, df = 5, $p < 0.001$).

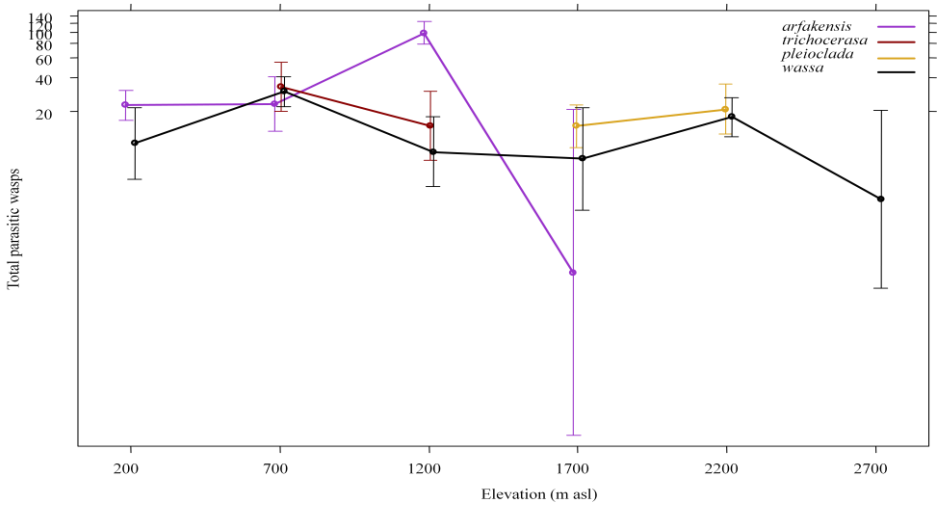

FIGURE S5. Effect of elevation on parasitic wasp production for all (sub)species. Effect was calculated using generalized linear model with total parasitic wasps as the response variable to elevation and (sub)species interaction. The interaction of elevation and (sub)species identity is highly significant ($\chi^2 = 65.677$, df = 6, $p < 0.001$).

Summary

Thesis Summary

The aim of this thesis was to explore how pollinating insects might influence plant population structure and lead to their speciation along elevational gradients. We focused on the obligate nursery mutualism between figs and pollinating fig wasps; a ~75 million year old plant-pollinator relationship which has given rise to a diverse and ecologically important genus in tropical forests (Shanahan *et al.* 2001; Novotny *et al.* 2005; Cruaud *et al.* 2012). This long standing relationship has often been considered to be the product of multiple cospeciation events, where single species of pollinating wasps breed within, and pollinate a single species of figs. Evidence against this pattern continues to accumulate with cases of multiple pollinating wasps per *Ficus* species, or single wasp species visiting multiple *Ficus* species (Cook & Rasplus 2003).

As the exclusive means of pollen transport between figs, wasps are intrinsically linked to the fig's reproductive success. Likewise, flowers within the fig cavity are the only suitable site for these wasps to lay their eggs meaning they are also dependent on figs for their own reproduction (Galil & Eisikowitch 1971). Given these reasons, fig and wasp ranges should overlap. Fig wasps have been shown to disperse over long distances (up to 160 km) and serve as effective pollinators throughout their host's range (Ahmed *et al.* 2009), while other studies have found isolated wasp populations and restricted gene flow between fig populations (Liu *et al.* 2013; Rodriguez *et al.* 2017).

In chapter I we demonstrate population structure of figs along an elevational gradient (Segar *et al.* 2017). The species studied therein have relatively wide elevational distributions, yet, we reveal restricted gene flow

between highland and lowland populations. Fig wasps have been shown to be unable to disperse over marked geographic barriers such as large expanses of sea (Kobmoo *et al.* 2010; Rodriguez *et al.* 2017). We argue that fig structuring may be due to the wasp's inability to disperse along the gradient given the rapidly changing conditions along mountain slopes.

In chapter II we expand this idea to include a variety of species complexes along the 'speciation continuum,' with varying degrees of range distribution along the mountain. Following the generalization of one-to-one species rule our expectation was to find single wasp species responsible for the pollination of each fig species throughout its range. Our unique genomic dataset of both figs and corresponding fig wasps allowed us to study fine-scale ancestry between individuals at each of our collection sites. As in chapter I, we recovered population structure among figs with distinct highland and lowland populations often with a mid-elevation contact zones. Surprisingly, in the case of the pollinating wasps, the clusters retrieved were genetically distinct enough to be considered as separate species. We found that wide ranging fig species had several parapatric pollinating wasp species distributed along the gradient. Incipient species, such as the *F. trichocerasa* subspecies complex had separate lowland and highland pollinating wasp species, restoring the one to one rule.

First, we argue that due to their short life span, dispersal abilities and urge to find receptive figs in which to lay their eggs, pollinating wasps form localized and isolated populations. Due to their shorter generation time, wasps speciate faster than figs and thus restrict gene flow among fig populations (Cook & Segar 2010). We suggest that as long as these

clustered fig populations remain isolated enough to continue to diverge, wasp species may be responsible for the parapatric speciation of figs, thus restoring the species-specific paradigm. Seed dispersal plays a fundamental role in maintaining fig population connectivity so future research must consider paternal (pollen) and maternal (seed) reproductive success and dispersal.

Chapters III and IV further investigate some of the possible mechanisms maintaining reproductive isolation between closely related fig species and diverging subspecies as well as the possible role of *Wolbachia* as an agent of speciation in fig wasps. Scent is thought to be one of the most relevant traits for attracting pollinators to receptive figs (Grison-Pigé *et al.* 2002). Scent profiles for all studied species differed significantly, and interestingly in some cases it varied according to collection site (Souto-Vilarós *et al.* 2018).

In order to test whether these differences in scent could also be perceived by pollinating wasps, we employed commonly used 'Y-tube' choice tests on wasps form the Papuacyse section. In general, wasps are able to distinguish between their host fig and the other species, except in one case where mid-elevation wasps are equally attracted to their host fig as well as to its sister species (*F. microdictya*). We demonstrate that although scent plays a fundamental role in the attraction of pollinators, multiple barriers act as a 'lock and key' mechanism preventing crosses between close relatives.

Faster wasp speciation could be attributed to short generation times relative to figs, within fig assortative mating, routine inbreeding, and/or *Wolbachia* induced reproductive isolation. Fig wasps have a high prevalence of

Wolbachia infection which is known to cause cytoplasmic incompatibility (Bordenstein & Werren 2007; Engelstädter & Telschow 2009). It has been shown that differences in *Wolbachia* infection status and/or strain type can have negative effects in the offspring of otherwise compatible crosses. In chapter IV we explored *Wolbachia* infection status and strain type for pollinating wasps along the gradient. We discovered a non-random distribution of *Wolbachia* strain types along the pollinating wasp phylogeny and in many cases, we find sister clades with contrasting infection status (as infected or noninfected or infected with different strains). These clades broadly agree with the species clusters recovered with genomic data (chapter II) which allows us to speculate on the role *Wolbachia* may have in the speciation of pollinating fig wasps, or at least in maintaining reproductive isolation between incipient species.

Besides pollinating wasps, figs house a large number of non-pollinating fig wasps (NPFW) which often have detrimental effects on the mutualism through predation of pollinator larvae and competition for seed resources (Weiblen 2002). Some genera have similar ecology to pollinating wasps, galling flowers through entering the ostiole alongside pollinators, while others behave as kleptoparasites and parasitoids which oviposit from the outside of the fig (Cook & Segar 2010; Borges 2015) and have been suggested as a mechanism for introducing *Wolbachia* into an otherwise enclosed system (Yang *et al.* 2012). The final chapter of this thesis establishes the relationship between elevation and NPFW community and pollinator abundance. Spatio-temporal heterogeneity has been suggested as a mitigating factor for the negative effects of parasitism in mutualisms since there needs to be an overlap during key life cycles of both host and parasite. We expected elevation to play a major role in shaping community

diversity as well as pollinator abundance, thus the cost of parasitism on both figs and pollinating wasps would differ along the gradient. A comprehensive quantification of costs to reproductive fitness among parapatric populations will surely contribute to our understanding of local selective pressures on both partners in the mutualism. Nevertheless, limited taxonomic identification and life history information lacking for these NPFWs prevent us from placing these wasps along the various trophic levels as well as defining range distribution and their degree of host specificity.

Future directions

This thesis employed several methods for studying the role of pollinating fig wasps as promoters of fig diversification. With the costs of high-throughput sequencing technologies continuously decreasing (Levy & Myers 2016), it is tempting to suggest the use of whole genome sequencing (WGS) to answer some of these questions. Undoubtedly, having fully annotated genomes for figs and wasps would allow us to explore many exciting questions such as correlated evolution of traits (i.e. floral style length and ovipositor length), changes in breeding systems (from dioecy to andromonoecy to monoecy), or even recovering mapped and more densely sampled SNP datasets with which to study population genomics in far greater detail. Equally interesting would be to expand similar studies to other environmental gradients. The rates of wasp speciation found along our study gradient suggest that populations of pollinating wasps might be localized throughout their host's range. This suggests that there could be potentially many more pollinating wasp species across different gradients, mountain ranges and other isolated populations. Papua New Guinea offers

an ideal arena for this type of study as not only does it include a large and rugged mainland terrain, but multiple islands along the archipelago share similar flora and fauna. It would be interesting to note if similar fig species occurring in such islands or different mountain ranges have different pollinating wasp species.

Temperature is thought to be one of the most important factors affecting insect abundance, distribution and survival (Andrewartha & Birch 1954). We argue that fig wasp speciation may be due to the inability of pollinating wasps to disperse along the elevation gradient because of dramatic temperature changes along its slope. Fig wasps appear to be sensitive to temperature (Jevanandam *et al.* 2013) supporting the idea of local adaptation facilitating speciation of cold adapted populations as has been seen in other tropical systems (García-Robledo *et al.* 2016). To date, data on thermal tolerance of pollinators is rather scarce, despite the general awareness of the consequence's climate change could have on obligate mutualisms. It would be of great value to test thermal tolerance of fig wasps along the elevational gradient to evaluate whether these species are adapted to local conditions along the mountain.

Although this thesis specifically investigates the fig and fig wasp mutualism, plants with a certain degree of pollinator specialization are likely to be subject to similar mechanisms driving population structure along mountains. Elevational gradients serve as a proxy to investigate how evolution has shaped biodiversity under different climatic conditions. Plant-pollinator interactions in the tropics have rarely been studied under this light, even though current projections of climate warming suggest a scenario that will be highly disruptive to intimate mutualisms.

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Curriculum vitae

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