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RESEARCH ARTICLE

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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.

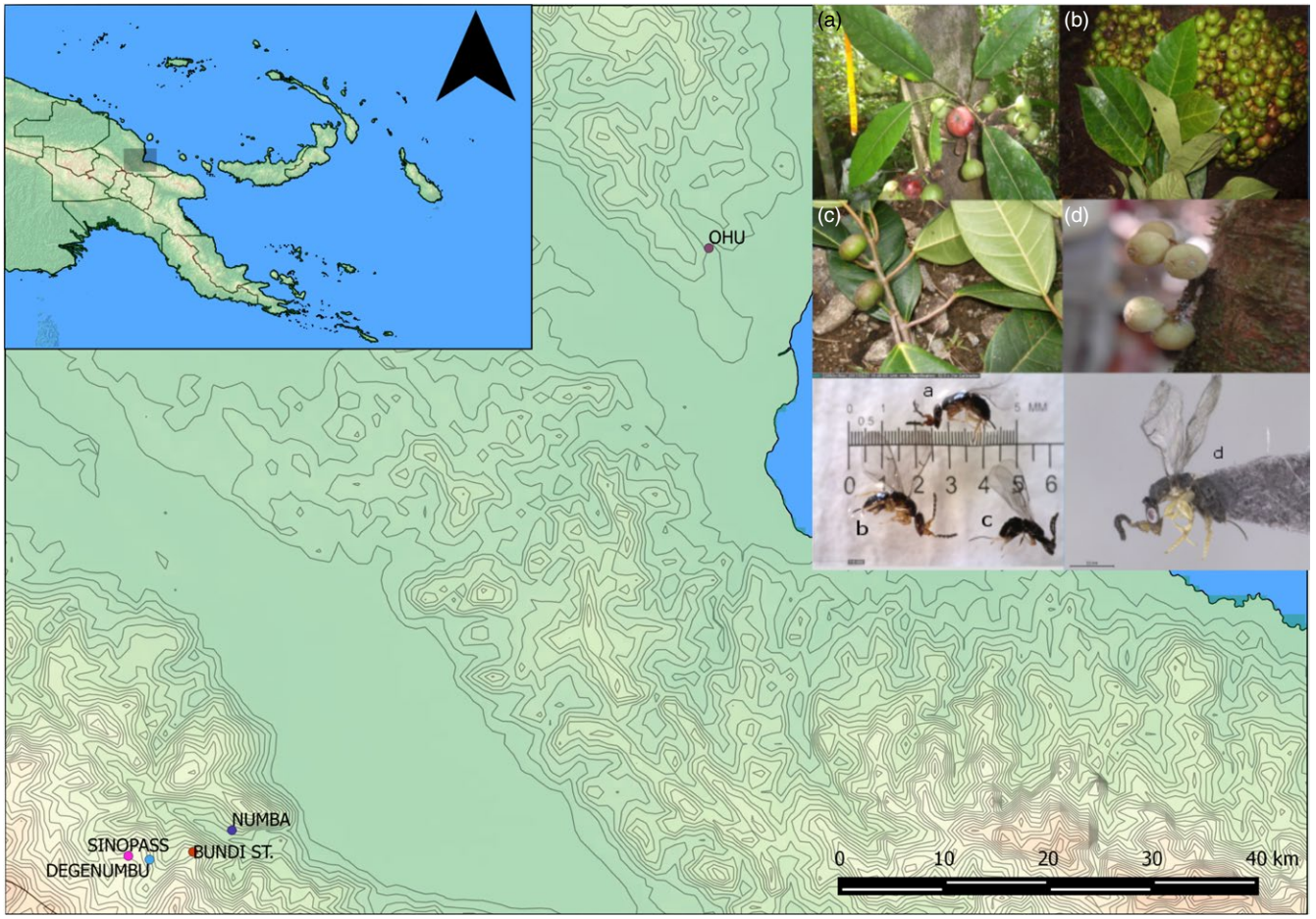

FIGURE 1 Geographical location of field sites along the Mount Wilhelm elevational gradient in Madang province, Papua New Guinea. Contour lines every 100 m. Inset: Focal *Ficus* species used in this study (upper case) and their corresponding pollinating fig wasps (lower case): a: *Ficus itoana*; b: *Ficus*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

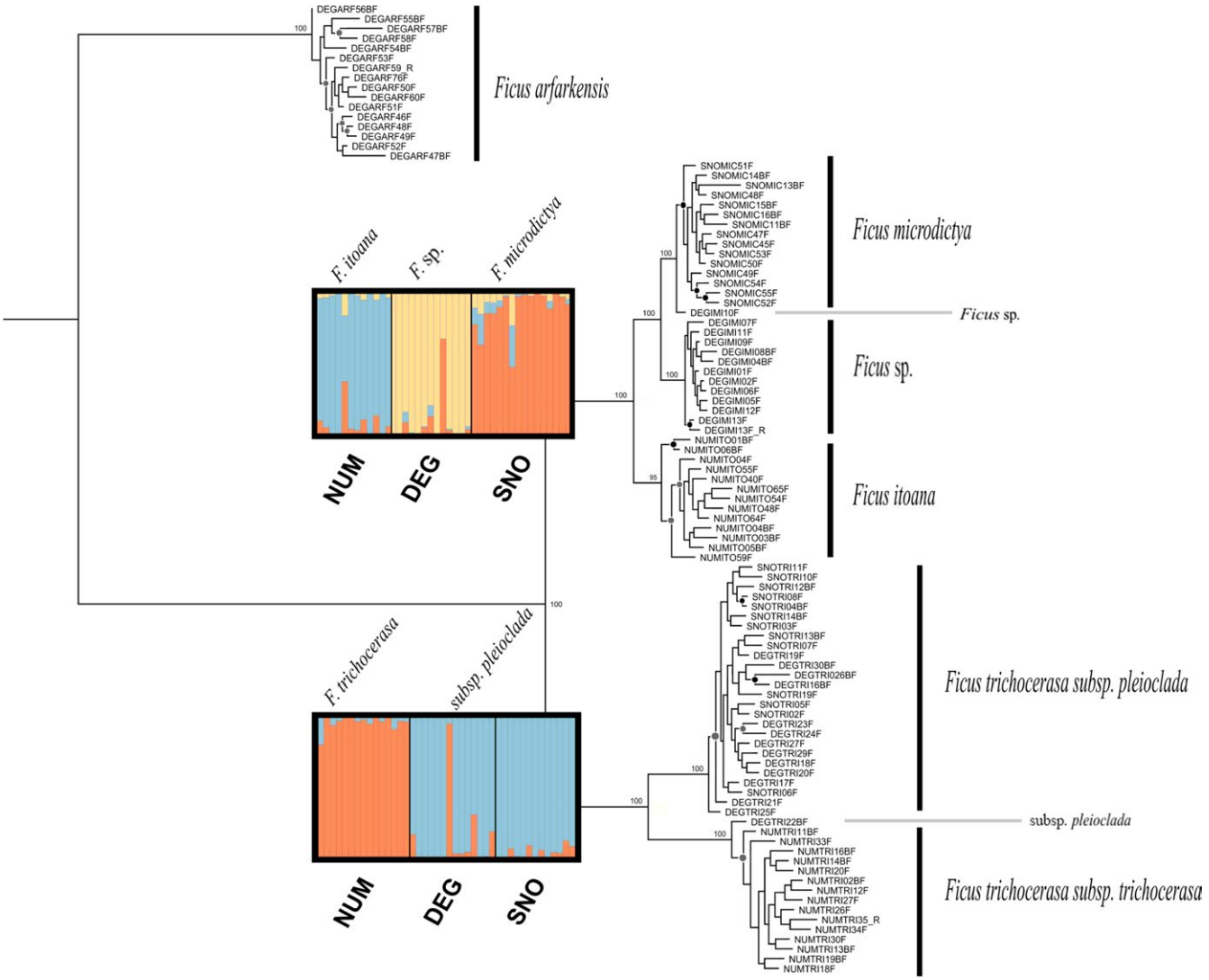

FIGURE 2 RAxML Phylogenetic relationship between analysed *Ficus* species. Values indicate bootstrap support for major branches, black dots indicate internal nodes with bootstrap values >91%; grey dots indicate nodes with bootstrap values between 75% and 90%. Tree rooted to *Ficus arfakensis*. *Collection 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

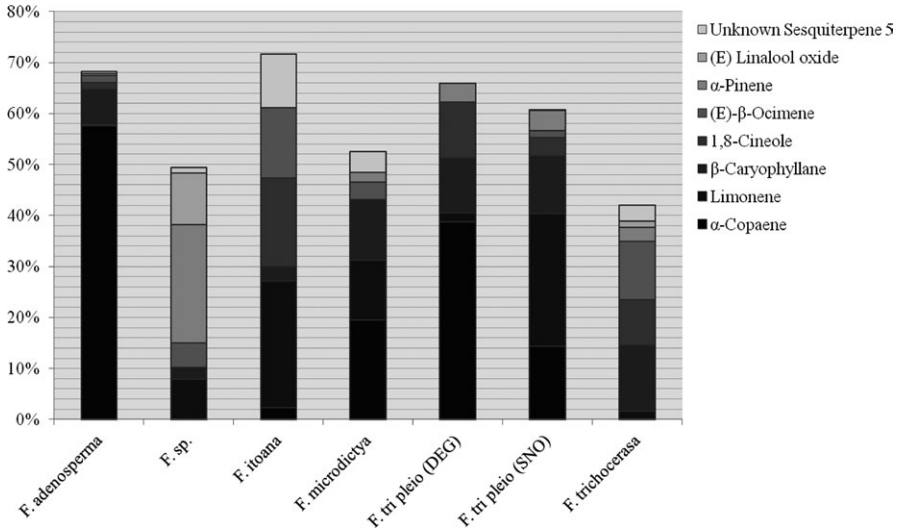

FIGURE 3 Proportions of the main compounds representing more than 40% of total volatile bouquet emitted by receptive figs of the analysed species. F. tri pleio deg and F. tri pleio sno correspond to Ficus trichocerasa subspecies pleioclada individuals collected in Degenumbu and Sinopass, respectively

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**

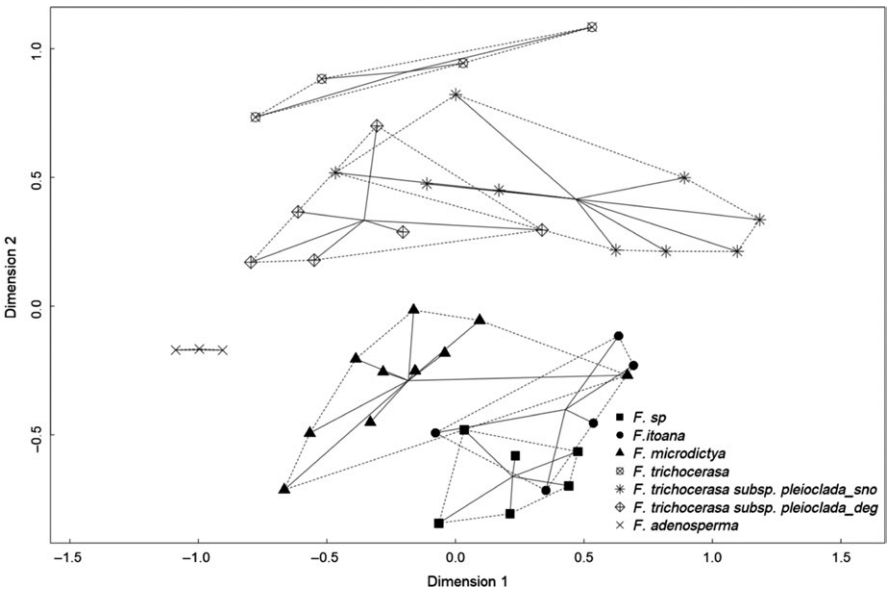

FIGURE 4 Non-metric multidimensional scaling (NMDS) ordination of volatile organic compound composition of studied species at receptive stage, based on Bray–Curtis distance; Two dimensions, stress = 0.22. Dashed lines (generated using ordispider) group samples from the same species; solid lines (generated 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"

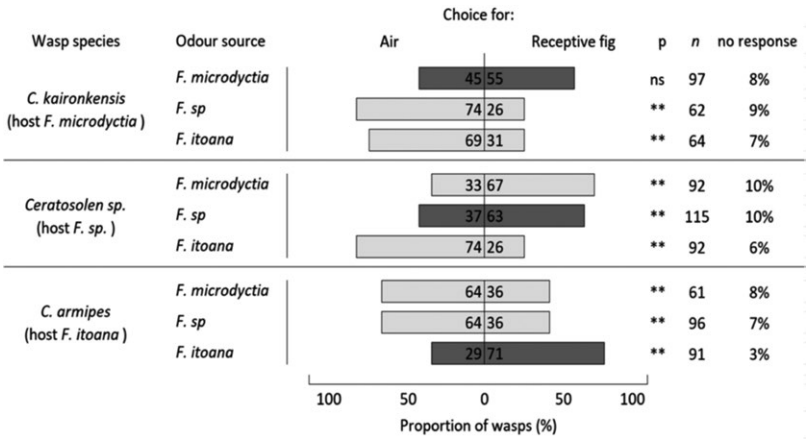

FIGURE 5 Response of different pollinating fig wasps in Y-tube experiments when presented with receptive figs of different species vs. air. Using Fisher's exact test for each series of tests, we compared the proportion of wasps that choose receptive fig odour or "control" air (unresponsive wasps presented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

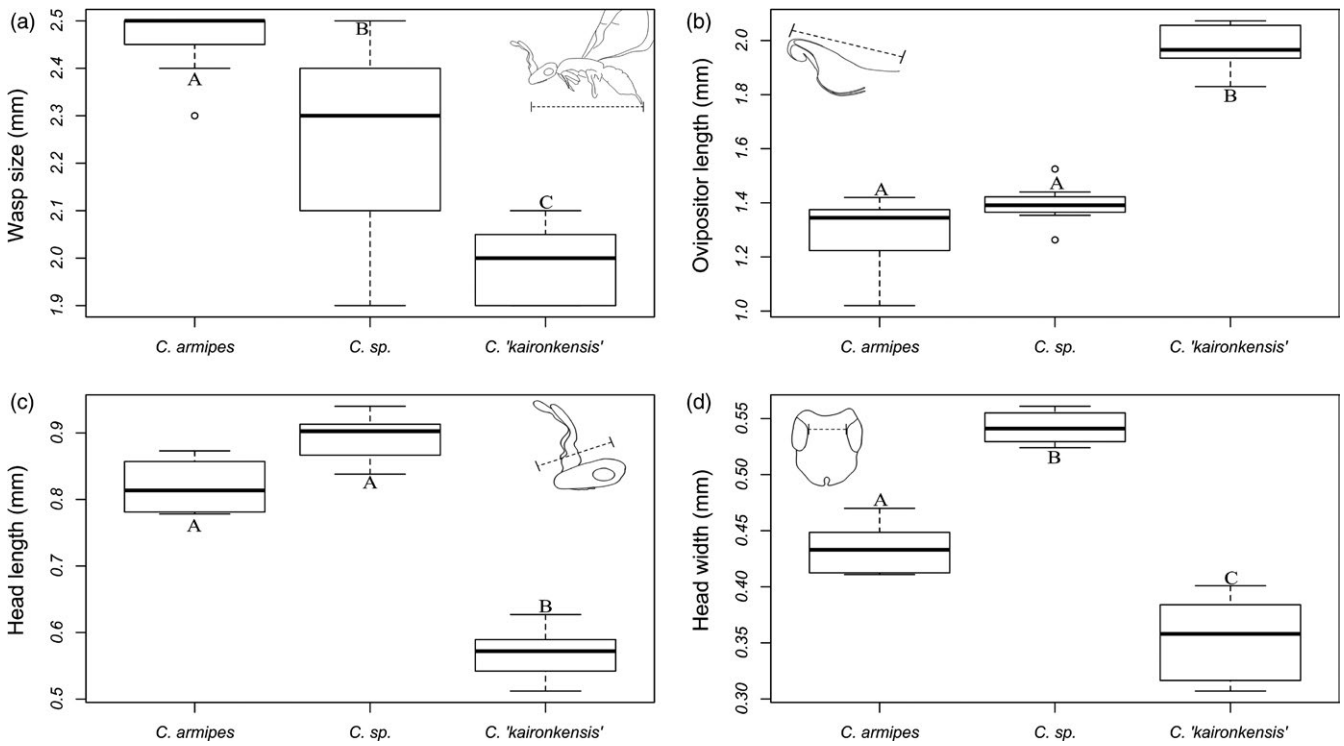

FIGURE 6 Boxplot of fig-wasp measurement of various traits for individuals pollinating figs from section Papuacyse; Ceratosolen armipes, Ceratosolen sp. and Ceratosolen "kaironkensis." a: Wasp size; b: Ovipositor length; c: Head length; d: Head width. Measurements based on eight individuals for each 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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