Title

An unusual tricosatriene is crucial for male fungus gnat attraction and exploitation by sexually deceptive *Pterostylis* orchids

Authors

Tobias Hayashi[a,b*], Björn Bohman[a,b,c], Adrian Scaffidi[b], Rod Peakall[a,b] and Gavin R. Flematti[b]

[a]Ecology and Evolution, Research School of Biology, The Australian National University, Canberra ACT, 2600, Australia. [b]School of Molecular Sciences, The University of Western Australia, Crawley WA, 6009, Australia. [c]Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Alnarp, 23053, Sweden

*Lead contact and corresponding author: tobias.hayashi@anu.edu.au
Twitter handle: @TobiasHayashi

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Summary

Cross-kingdom mimicry of female insect sex pheromones by sexually deceptive orchids has evolved multiple times[1]. Fungus gnats (Diptera) are predicted to be pollinators of hundreds of sexually deceptive orchids[2–4], yet unlike orchids that sexually attract bees and wasps (Hymenoptera)[5–11], the chemistry of fungus gnat-pollinated orchids remains unknown. Furthermore, despite the importance of fungus gnats as pollinators, pests and decomposers of organic material, and evidence for sex pheromones since 1971[12–17], no structure of any fungus gnat sex pheromone has to date been reported. In this study, we found a mixture of five hydrocarbons shared between *Pterostylis orbiculata* orchids and female *Mycomya* sp. (Mycetophilidae) fungus gnats, which included three alkanes, a C_{23} diene, and a C_{23} triene. The triene was an undescribed natural product, which we synthesised and confirmed to be (6*Z*,9*Z*)-1,6,9-tricosatriene. Field bioassays with a synthetic blend of the five hydrocarbons elicited attraction and sexual behaviour from male gnats. The triene alone elicited attraction and low levels of sexual behaviour, but the blend without it was unattractive, suggesting that this compound is a key component of orchid pollinator attraction and the female fungus gnat sex pheromone. In two closely related *Pterostylis* species, we found related C_{23} trienes, but not (6*Z*,9*Z*)-1,6,9-tricosatriene. These results suggest that unusual long chain unsaturated hydrocarbons hold the key to sexual deception in *Pterostylis* orchids, and are an important step towards deciphering female fungus gnat sex pheromones.

Keywords

sex pheromone, sexual deception, chemical mimicry, fungus gnat, Mycetophilidae, semiochemical, floral volatile, *Pterostylis*, Orchidaceae, (6*Z*,9*Z*)-1,6,9-tricosatriene

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Results

Male fungus gnats from the highly diverse families Mycetophilidae (4500+ species), Sciaridae (2450+ species) and Keroplatidae (1000+ species)[18] are predicted to be important pollinators of the diverse, putatively sexually deceptive orchid genera *Lepanthes* (1200+ species) and *Pterostylis* (300+ species)[2–4]. *Pterostylis orbiculata* (D.L.Jones & C.J.French) D.J.Jones & C.J.French (recently described as distinct from *P. sanguinea* D.L.Jones & M.A.Clem.) is an Australian orchid which is pollinated by sexually attracted males of an undescribed species of fungus gnat (*Mycomya* sp.; Mycetophilidae). While attempting to copulate with the motile labellum, male fungus gnats are flung upwards and trapped inside the galea, from which they escape via the stigma and brush past the anther to collect pollinia on their thorax (Figure 1A-C)[2].

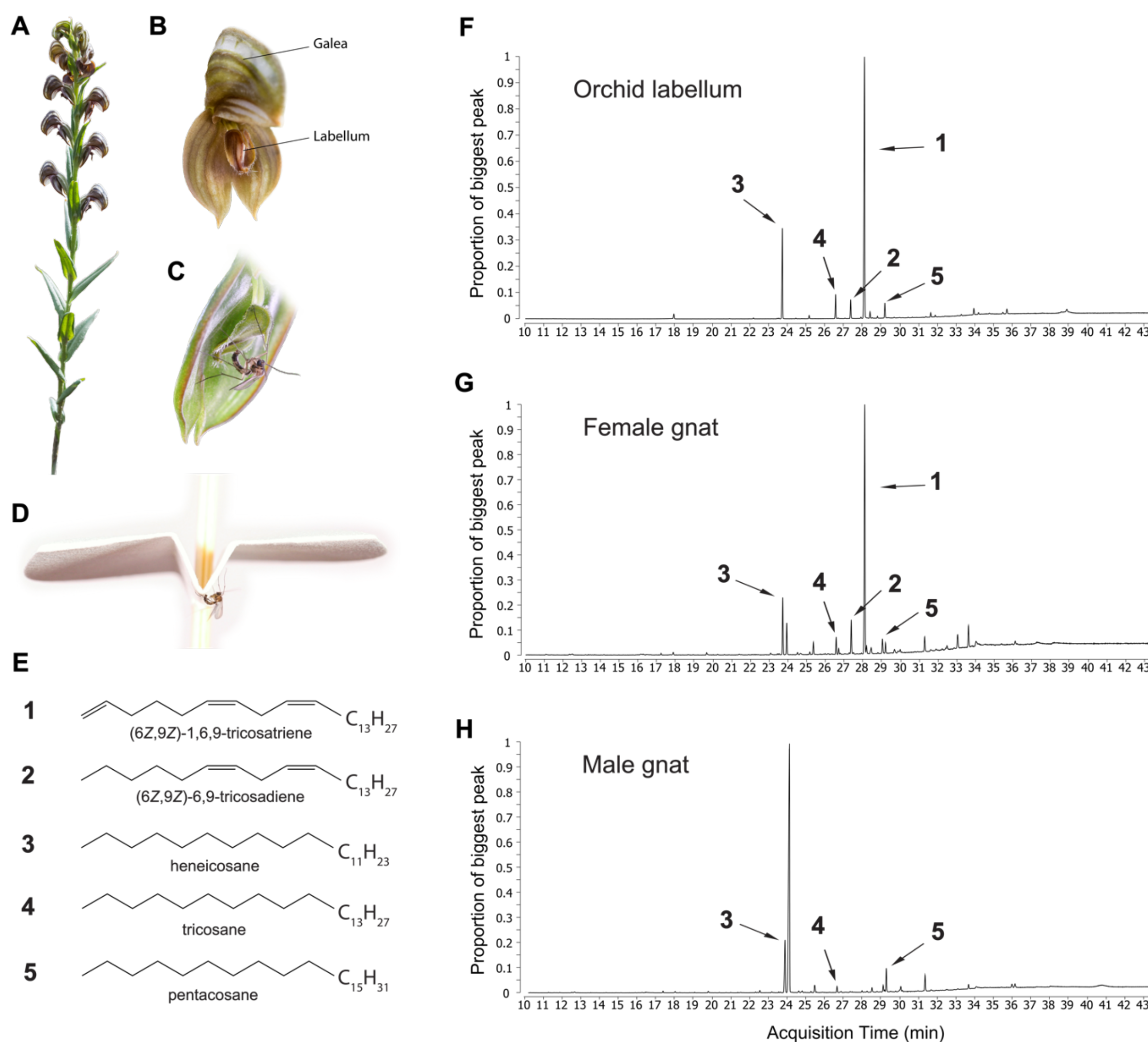

Figure 1. Floral morphology, male gnat copulatory behaviour and compounds in orchid labella, female gnats and male gnats. (**A**) *Pterostylis orbiculata* inflorescence (**B**) details of floral morphology (**C**) male gnat attempting to copulate with *P. orbiculata* labellum (**D**) male gnat attempting to copulate with filter paper in response to synthetic blend (**E**) semiochemicals in synthetic blend: (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**), (6*Z*,9*Z*)-6,9-tricosadiene (**2**), heneicosane (**3**), tricosane (**4**), pentacosane (**5**) (**F**) GC-MS total ion chromatogram (TIC) of orchid labellum extract, (**G**) female gnat extract and (**H**) male gnat extract, with major compounds in common with female gnats and orchid labellum indicated. See also Figure S1 and Table S1.

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In this study, we applied an iterative, bioassay-guided approach[19] to narrow down, isolate and subsequently identify the semiochemicals involved. Given previous dissection experiments have confirmed the labellum is the active floral tissue[2], we first compared the gas chromatography – mass spectrometry (GC-MS) traces of solvent extracts of orchid labella and female gnats with solvent extracts of unattractive orchid tissue and male gnats. Next, we employed preliminary laboratory bioassays with semi-preparative GC fractions containing candidates in common between orchid labella and female gnats. Finally, after identification and synthesis of candidate compounds, we confirmed biological activity with field bioassays.

Unsaturated hydrocarbons are prominent in orchids and female gnats

As identification of female fungus gnats is often difficult, we targeted pairs of fungus gnats *in copula* from which the male could be allocated for DNA barcoding to confirm females were the correct species. Using GC-MS we found five abundant compounds in common between dichloromethane solvent extracts of *P. orbiculata* labella (attractive orchid tissue, extracts comprising five labella from different individuals, replicated across three populations and multiple years) and female fungus gnats (six females from two sites). In order of abundance, they included a C_{23} triene, C_{21} *n*-alkane, a C_{23} diene, and C_{23} and C_{25} *n*-alkanes (Figure 1E-G). These five compounds together accounted for 94% of all detected orchid labellum compounds. Notably, the C_{23} triene alone comprised on average 62% and 60% of compounds detected in orchid labellum and female gnat extracts respectively, but was not detected in floral remains (unattractive orchid tissue comprising petals, sepals and column) or male gnats (Figure 1H, three males from one site). The C_{23} diene was also absent in male gnats, although present in floral remains.

The abundance of the C_{23} triene, present only in attractive orchid tissue and female gnats, as well as the presence of the C_{23} diene in orchid tissue and female gnats, but not in male gnats, indicated these two unsaturated hydrocarbons were candidate semiochemicals.

Laboratory bioassays confirm unsaturated hydrocarbons to be attractive to male gnats

To investigate whether the C_{23} diene and triene are attractive to male gnats, we used semi-preparative GC fractionation in combination with laboratory bioassays. Bioassays in wind tunnels or Y-tubes are commonly used in investigations of insect sex pheromones, including for fungus gnats[20], but so far have not been utilised in sexually deceptive systems. We separated extracts of orchid labella into fractions using semi-preparative GC and presented these fractions to male gnats in laboratory bioassays. Briefly, a single male gnat was placed in a glass tube with gentle airflow, and the stimulus introduced upwind from the gnat. Male gnat behaviour in response to orchid extracts consisted of rapid initiation of upwind flight, and vigorous wing fanning. We targeted the five hydrocarbons in common in extracts of orchid labella and female gnats in creating the following four fractions: fraction one comprised the early eluting part of the extract, fraction two comprised the C_{21} and C_{23} *n*-alkanes, fraction three comprised the candidate C_{23} diene and triene, and fraction four comprised the C_{25} *n*-alkane. Out of a total of 22 bioassays, male gnats presented with fractions one or two did not respond (n=5 and n=4 respectively), while all gnats presented with fraction three responded positively (n=8). Only one gnat responded positively to fraction 4 (n=5). These results indicated that the combination of the C_{23} diene and triene (fraction three) was attractive to male gnats.

Alkenes identified as (6*Z*,9*Z*)-1,6,9-tricosatriene (1) and (6*Z*,9*Z*)-6,9-tricosadiene (2)

High resolution GC-MS confirmed the molecular formulae for the triene ($C_{23}H_{42}$) and diene ($C_{23}H_{44}$) in the active fraction, however the double bond positions remained unknown. A

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comprehensive search of published mass spectral data revealed a possible mass spectrum match of the diene with (6*Z*,9*Z*)-6,9-tricosadiene (**2**), a known pheromone in several species of moth and wasp[21,22]. We could not find any matching literature data for the C_{23} triene, although mass spectral fragments at *m/z* 69 (base peak), 81, 95, 121 and 135 were similar to those of (6*Z*,9*Z*)-1,6,9-heptacosatriene, a compound of unknown function reported in mites[23]. Proton nuclear magnetic resonance (^1H NMR) spectroscopy analysis of the triene isolated from orchid extracts by semi-preparative GC showed the presence of two broad doublets at δ 5.01 and 4.95 ppm, coupled to a multiplet at δ 5.82, which indicated a terminal alkene was present (see Figure S2). Furthermore, an apparent triplet integrating for two protons was present at δ 2.77 ppm, indicating a methylene between two alkenes, and leading us to propose the structure for the triene as (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**).

To confirm these identifications, **1** and **2** were synthesised based on a modified method of Shimizu *et al.*[23] (see Supplemental Information for details). In short, propargyl alcohol was tetrahydropyran (THP)-protected and coupled with 1-bromopentane or 5-bromo-1-pentene respectively, following Giacomina and Alexakis[24]. The THP-protected enynols were brominated with tetrabromomethane, following Wagner *et al.*[25] and formed the required diynes by a copper-promoted coupling with 1-pentadecyne following Tallman *et al.*[26]. Finally, a selective reduction by a titanium catalyst[27] or Lindlar's catalyst provided **1** and **2** respectively, which were purified by $AgNO_3$ modified silica chromatography. Analysis of synthetic **1** and **2** showed identical mass spectra and retention times (confirmed by coinjection on two columns with differing stationary phases) and matching ^1H NMR spectra to the C_{23} triene and diene from orchid extracts. No records of **1** were found in the literature, and to the best of our knowledge this is an undescribed natural product.

Field bioassays confirm biological activity of semiochemicals

In order to confirm the biological activity of our candidate compounds, we conducted field bioassays with synthetic compounds using an established sequential choice design modified from Bohman *et al.*[7,28]. Briefly, the sample was presented alone for 5 min, followed by the addition of an orchid extract control for a further 5 min, providing a comparison and confirming the presence of fungus gnats at each site. Due to the small size of the fungus gnats, we used a filter paper folded to provide an appendage-like tip to which the compound was dispensed (Figure 1D). Gnat behaviour was scored in the following hierarchical categories of increasing sexual response: land only (little or no searching behaviour), land + wing fan (rapid wing movements and searching behaviour), and land + wing fan + sexual behaviour (abdomen curling and/or probing with genitalia). Male gnats at orchid extracts on filter paper demonstrated the full repertoire of sexual behaviour, confirming that our bioassay design is capable of eliciting sexual behaviour.

The blend of all five compounds (**1**-**5**) in common between attractive orchid tissue and female gnats, in similar proportions as in orchid labellum extracts, elicited attraction of male gnats and sexual behaviour in 16% of responses when presented alone in phase 1 ($n=38$, Figure 2A). Alongside the orchid extract control in phase 2, the synthetic blend secured similar proportions of sexual behaviour as the control ($G=0.27$, $p=0.88$), but attracted fewer total responses ($G=19.08$, $p<0.001$, $n=75$). While the similar rates of sexual behaviour indicate that the blend elicits strong sexual activity from male gnats, it is possible that minor additional components, not identified here, may further increase attraction.

Given the abundance of the triene **1** in orchids and female gnats (over 60% of components in extracts), we hypothesised that this compound may play a key role in male gnat attraction. When presented alone, it was attractive and elicited sexual behaviour in 7% of responses in phase 1 ($n=44$, Figure 2B), although in phase 2 the frequency of sexual behaviour was

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significantly lower (G=29.68, p<0.001) and it elicited fewer responses (G=30.07, p<0.001, n=85) than the orchid extract control. Conversely, the synthetic blend without **1** elicited only one response and no sexual behaviour from male gnats in phase 1 (Figure 2C), confirming that this compound is required for male gnat attraction.

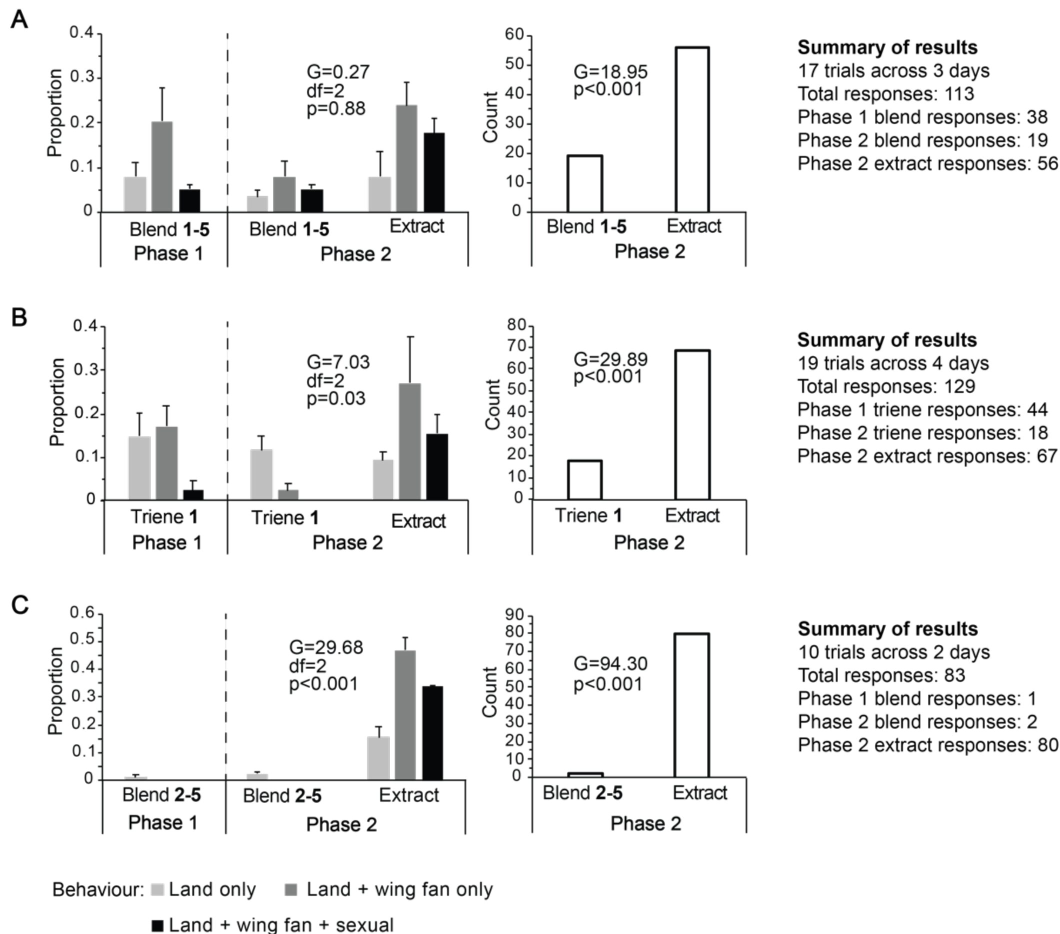

Figure 2. Results from field bioassays with synthetic hydrocarbons. Sequential bioassays with synthetic compound(s) presented alone for 5 min in phase 1, followed by the addition of an orchid extract control for a further 5 min in phase 2 (choice scenario). Male gnat responses were classified into hierarchical categories of increasing sexual behaviour, shown as proportions of the total responses for phase 1 + 2 (± SEM) at left. The total number of responses across experiments in phase 2 is presented separately (middle) and a summary of the baiting effort and total responses is given at right. **(A)** blend of **1-5**; **(B)** only **1**; and **(C)** blend of **2-5**, without **1**.

Closely related *Pterostylis* species have different trienes and tetraenes

Following confirmation that (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**) is a key pollinator attractant in *Pterostylis orbiculata*, we surveyed two closely related species (*P. concava* D.L.Jones & M.A.Clem. and *P. vittata* Lindl.) for the presence of similar compounds (i.e. C_{21}-C_{27} hydrocarbons). We hypothesised that different, but related compounds would be present in floral extracts, as is often the case in sexually deceptive orchids[7,29–31]. We found that odd-chain *n*-alkanes (C_{21}-C_{27}) were common, shared between species and present in both labella

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and floral remains (Figure 3). The diene **2** was also present in extracts of *P. concava* (labella) and *P. vittata* (floral remains), while trienes were species-specific and typically present only in labellum extracts. We failed to detect **1** in *P. concava* and *P. vittata* extracts, although we detected the known moth sex pheromone (3*Z*,6*Z*,9*Z*)-3,6,9-tricosatriene[32] in *P. concava* labellum extracts, confirmed by co-injection with a reference standard. Furthermore, we also detected a potential C_{23} triene (one population only) and a potential C_{23} tetraene in labellum extracts of *P. vittata* (indicated by GC-high resolution MS analysis). The identities of these compounds are currently under investigation.

# carbons: double bonds	Compound name	Orchid labellum (putative active)			Orchid floral remains (putative inactive)		
		P. orbiculata	***P. concava***	***P. vittata***	***P. orbiculata***	***P. concava***	***P. vittata***
21:0	Heneicosane (**3**)	20.8 ± 1.3	38.1 ± 3.8	18.2 ± 3.8	17.3 ± 1.2	14.5 ± 1.1	15.5 ± 1.1
21:1	Heneicosene				5.7 ± 1.2 *		
22:0	Docosane			1.3 ± 0.2	1.1 ± 0.2	1.5 ± 0.2	1.4 ± 0.2
23:0	Tricosane (**4**)	4.0 ± 0.3	4.0 ± 0.5	22.3 ± 5.1	12.5 ± 0.7	9.9 ± 0.9	13.3 ± 1.5
23:1	Tricosene				1.6 ± 0.2	1.5 ± 0.1	2.1 ± 0.4
23:2	(6*Z*,9*Z*)-6,9-Tricosadiene (**2**)	5.6 ± 0.9	2.6 ± 1.3		4.0 ± 0.5		1.0 ± 0.2
23:3	(6*Z*,9*Z*)-1,6,9-Tricosatriene (**1**)	61.6 ± 1.4					
23:3	(3*Z*,6*Z*,9*Z*)-3,6,9-Tricosatriene		27.7 ± 3.8				
23:3	Tricosatriene			43.8 ± 3.1 *			
23:4	Tricosatetraene			11.8 ± 3.6			1.8 ± 0.5 *
25:0	Pentacosane (**5**)	1.9 ± 0.2	6.7 ± 0.9	7.5 ± 1.6	8.8 ± 0.6	7.0 ± 0.6	8.2 ± 0.6
25:1	Pentacosene				3.6 ± 0.3	2.5 ± 0.3	3.5 ± 0.4
27:0	Heptacosane				4.0 ± 0.9	3.9 ± 0.3	4.2 ± 0.6

Figure 3. Results from survey of *Pterostylis* species. Heatmap of compounds detected in survey of *P. orbiculata* and closely related *P. concava* and *P. vittata.* Three individual flowers per population were sampled from three populations (two populations for *P. concava*), and putative active tissue (labellum) and putative inactive tissue (flower without labellum) were sampled separately. Compounds are given as the average proportion (%) of each extract ± SEM. Compounds marked * were only detected in one population and the value given is the average for that population only. See also Figure S1.

Discussion

In this study, we used a combination of techniques within a bioassay guided framework[19] to show that a new natural product, (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**), is the major component of a hydrocarbon blend that acts as a pollinator attractant in the sexually deceptive orchid *Pterostylis orbiculata,* as well as a constituent of the sex pheromone of its fungus gnat pollinator. A synthetic five-compound blend containing **1** elicited attraction and sexual behaviour from male gnats, whereas the blend without this compound was not attractive, pointing to its crucial role in attracting male gnats.

The identification of active semiochemicals involved comparison of orchid and gnat tissue, GC-MS and NMR analysis, semi-preparative chromatography, wind-tunnel bioassays and field bioassays. We show that for insects such as fungus gnats, which are amenable to laboratory work, the combination of wind tunnel type experiments with preparative chromatography is a suitable alternative to electroantennography. Regardless of which tools are employed to assist in the discovery process, it remains imperative to confirm the activity of synthetic blends in bioassays[19]. Here, we were able to conduct replicated field bioassays over multiple days resulting in a total of 325 responses from male gnats, including 122 responses to synthetic compounds and blends.

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The hydrocarbon blend described here represents the first reported semiochemicals for the diverse orchid genus *Pterostylis*, and for fungus-gnat pollinated sexually deceptive orchids more broadly. These semiochemicals from *P. orbiculata* are also the first example of a hydrocarbon-based semiochemical system in Australian sexually deceptive orchids, although there are similarities with pollinator attractants from European orchids. In *Ophrys sphegodes* and *O. exaltata,* blends of alkanes and alkenes are crucial for attracting male solitary bee pollinators, with the double bond position controlling specificity[30,31]. As in the case of *Ophrys*, the double bond position is likely to play an important role in controlling specificity in *Pterostylis orbiculata*, and the finding of similar compounds with different double bond positions in closely related species of *Pterostylis* is in keeping with this hypothesis.

To the best of our knowledge, the hydrocarbon blend of **1**-**5** is the first elucidated sex pheromone blend in fungus gnats. Female-emitted sex pheromones are known to be involved in courtship in *Bradysia*[12,15,33–35] and *Lycoriella* (Sciaridae)[13,14,20], and *Neoempheria* (Mycetophilidae)[16]. Initially, *n*-heptadecane was reported to be a sex attractant in the pest species *Lycoriella ingenua*[14] although this was later questioned[13,20]. Instead, a fraction containing a single unidentified sesquiterpene alcohol has subsequently been reported to be sexually attractive to males of this species[20]. Hydrocarbons are also known to play important roles in sexual attraction and species recognition in other Diptera. The first reported dipteran sex pheromone was (*Z*)-9-tricosene (Muscalure) from female house flies[36]. Blends of alkenes and dienes (C_{23}-C_{33}), but not trienes, are common components of female *Drosophila* sex pheromones[37,38].

Although hydrocarbons are ubiquitous natural products, polyunsaturated hydrocarbons, such as the trienes identified in *P. orbiculata* and *P. concava*, are uncommon. In a review of 1700+ plant volatiles, Knudsen *et al.*[39,40] reported only five different unbranched trienes (C_8 to C_{19}) from the families Araceae, Cactaceae, Orchidaceae and Ranunculaceae. In insects, only 14 unbranched polyenes (three or more double bonds) are reported in Pherobase (accessed July 2020)[41] and all but two are reported from moths[41,42]. Dienes and polyenes in moths are known as Type II sex pheromone components[42,43], comprising two to five double bonds, 17-25 carbons and with double bonds at positions 6 and 9. For example, (3*Z*,6*Z*,9*Z*)-3,6,9-tricosatriene, which was abundant in *Pterostylis concava* extracts, is a major sex pheromone component in some geometrid and oecophorid moths[44,45]. The parallels between *Pterostylis* semiochemicals and moth sex pheromones are particularly interesting as moths have been touted as possible pollinators of sexually deceptive orchids[46], yet so far none have been reported. Our results raise the possibility that some *Pterostylis* orchids may be capable of sexually attracting moths.

The diene and trienes found in *Pterostylis* are unusual plant volatiles as higher plants are generally thought to lack the capability to biosynthesise hydrocarbons with >18 carbons and two or more double bonds[47–49]. However, long chain dienes have previously been reported in maize silk (C_{25}-C_{31})[50] and *Ophrys* flowers (C_{27}-C_{33})[30,51,52]. Perera *et al.*[50] hypothesised that in maize, (6*Z*,9*Z*)-dienes are biosynthesised from linoleic acid, an abundant, di-unsaturated plant metabolite. The double bond configuration of linoleic acid would, following elongation, reduction and decarbonylation, result in the observed odd-chain dienes with a (6*Z*,9*Z*)-configuration[50]. Similarly, in insects, moth biosynthesis of (6*Z*,9*Z*)-diene and (3*Z*,6*Z*,9*Z*)-triene sex pheromone components starts with dietary linoleic and α-linolenic acids respectively, followed by elongation and subsequent reduction and decarbonylation[42]. Given the (6*Z*,9*Z*)- configuration of the diene and trienes observed in *P. orbiculata* and *P. concava*, we speculate that these unusual plant volatiles may also arise from common fatty acid precursors via the action of enzymes capable of elongating polyunsaturated fatty acids.

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In conclusion, (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**) is produced by both *Pterostylis orbiculata* orchids and female *Mycomya* sp. fungus gnats, and sexually attracts male fungus gnats. This compound is crucial for attraction and appears to be the main pheromone component, supported by three alkanes and (6*Z*,9*Z*)-6,9-tricosadiene (**2**), which further increase sexual behaviour. While still to be tested, variation in double bond numbers and position is likely to play a decisive role in maintaining both fungus gnat sex pheromone and orchid semiochemical specificity and thus maintaining strict pollinator specificity in *P. orbiculata*. Our results add yet another variety to the remarkable diversity of sexually deceptive orchid semiochemicals, and are an important step forward in understanding fungus gnat sex pheromones. Furthermore, the similarities between *P. orbiculata* semiochemicals and moth sex pheromones may offer exciting avenues to investigate the possibility of pollination by sexual deception of moths. Finally, evolutionary innovation in the form of unusual enzymes capable of producing long chain polyenes may hold the key to the evolution of sexual deception in *Pterostylis*.

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Author contributions

Conceptualization: T.H., B.B., R.P., G.R.F.; Data curation: T.H.; Formal analysis: T.H., B.B., R.P., G.R.F.; Funding acquisition: T.H., B.B., R.P., G.R.F.; Investigation: T.H., B.B., A.S., R.P., G.R.F.; Resources: Methodology: T.H., B.B., A.S., R.P., G.R.F.; Project administration: T.H.; Resources: B.B., R.P., G.R.F.; Supervision: B.B., R.P., G.R.F.; Visualization: T.H.; Writing – original draft: T.H.; Writing – review and editing: T.H., B.B., R.P., G.R.F.

Declaration of interests

The authors declare no competing interests.

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Methods

RESOURCE AVAILABILITY

Lead contact:

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tobias Hayashi (tobias.hayashi@anu.edu.au).

Materials availability:

There are restrictions to the availability of (6*Z*,9*Z*)-1,6,9-tricosatriene due to the very limited amounts available which are needed for further studies.

Data and code availability:

DNA (CO1 barcoding) sequences of representatives of each fungus gnat taxa are available at GenBank (sequences MW479747 through MW479752).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study system

Pterostylis orbiculata (D.L.Jones & C.J.French) D.L.Jones & C.J.French is a winter-flowering orchid, which is widespread throughout much of south-west Australia and was described as distinct from the widespread *P. sanguinea* D.L.Jones & M.A.Clem. in 2017[53,54]. It is known to be pollinated by sexually deceived males of a single undescribed species of fungus gnat (*Mycomya* sp., Mycetophilidae; Phillips *et al.*[2], as *P. sanguinea*). Like other *Pterostylis* flowers, the variable green or red-brown flowers have an intricate trap system based around a motile, insectiform labellum. While attempting to copulate with the labellum, male fungus gnat pollinators are flung upwards and trapped inside the galea by the movement of the labellum, whereupon they brush past the stigma, escape through a pair of modified column wings and collect pollinia on their thorax. The labellum is the sole source of pollinator attraction[2] so we defined labella as attractive orchid tissue and floral remains (i.e. petals, sepals and column) as unattractive orchid tissue. Specimens of orchids and fungus gnats are held at the Australian National University pending lodgement at the Australian National Herbarium and Australian National Insect Collection respectively. This research was undertaken in accordance with the relevant permits from the Department of Biodiversity, Conservation and Attractions, Western Australia, and Kings Park and Botanic Gardens.

METHOD DETAILS

Orchid sampling

Pterostylis orbiculata flowers were sourced from three main populations in south-west Western Australia at Kings Park, Perth (31.962 °S 115.828 °E), near Mandurah (32.741 °S, 115.724 °E) and near Brookton (32.397 °S 116.756 °E) from June 2017 through August 2019. Flowers from each population were confirmed to attract the same pollinating fungus gnat at a known population in Kings Park (see below). Only fully open flowers were sampled for chemical analysis, as it is not possible to determine whether flowers are recently pollinated without dissection. Five flowers from separate individuals within each population were collected and dissected into labella and floral remains, which were placed into separate vials and covered in dichloromethane for 24 h at ca. 5 °C and then stored at -20 °C. Each population was sampled at least once each year, and all three populations were confirmed to contain the same major compounds. Bulk extracts for fractionation were obtained by excising 50 labella from 1-3 flowers per inflorescence from the same population, and immediately

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extracting in ca. 1 mL dichloromethane for 24 h before concentrating to ca. 200 µL under a gentle stream of nitrogen gas. Preliminary field trials with dichloromethane solvent extracts of orchid labella elicited rapid attraction and sexual behaviour from male gnats, confirming that such extracts of the labella are attractive to pollinators.

In the survey of *P. orbiculata* and two closely related species, *P. concava* D.L.Jones & M.A.Clem. and *P. vittata* Lindl., three flowers from separate individuals were sampled per population per species, and three populations sampled per species (two populations only for *P. concava*) in July and August 2019. *Pterostylis concava* flowers were sourced from populations at Narrogin (32.944 °S 117.162 °E) and near Westdale (32.405 °S 116.649 °E), and *P. vittata* from populations at Narrogin (32.944 °S 117.162 °E), Armadale (32.155 °E 116.041 °E) and near Mandurah (32.757 °S 115.691 °E). Each labellum (putative attractive tissue) and floral remains (putative unattractive tissue) was sampled and analysed separately. Labella were extracted in 100 µL dichloromethane and floral remains in 1 mL dichloromethane for 24 h at ca 5 °C. All extracts were stored at -20 °C and slowly concentrated to 100 µL under a gentle stream of nitrogen gas. Only compounds comprising >1% of extracts were included for analysis.

Fungus gnat sampling

Mycomya sp. (Mycetophilidae) fungus gnats were sourced from populations in south-west Western Australia near Perth (31.956 °S 115.843 °E; 31.911 °S 115.942 °E; 31.982 °S 15.819 °E), including from one site where both the orchids and gnats are known to occur together. To capture female gnats for chemical analysis, we targeted fungus gnat pairs *in copula*, as it is difficult to identify female fungus gnats without reference to male fungus gnat genitalia. Furthermore, the fungus gnat fauna in Australia is poorly described and there is no adequate species-level key to *Mycomya* fungus gnats. To solve this, we captured mating fungus gnat pairs and identified them to genus level using the key in Tonnoir[55]. We then allocated females from mating pairs for chemical analysis and the corresponding males for DNA barcoding analysis (see below) to confirm they were the same species as the orchid pollinator. Although the females captured and analysed were therefore not virgin, observations of males approaching and attempting to mate with already copulating pairs suggested that females continue to be attractive while *in copula*. Several male fungus gnats attracted to orchid flowers were also allocated for extraction.

Gnats were extracted in 25 µL of dichloromethane or a 1:1 mixture of methanol:dichloromethane for 24 h at ca. 5 °C. A total of six females (sourced from two populations, including one population near *P. orbiculata* orchids) and three males were extracted and analysed. All solvent extracts were stored at -20 °C.

Pollinator DNA barcoding

Fungus gnats in Australia are poorly known and many species, including the pollinator of *Pterostylis orbiculata*, remain undescribed. To overcome this, we used DNA barcoding of the mitochondrial CO1 region which has been used previously to establish species-level distinctions in fungus gnats and other insects[56,57], including the pollinator of *P. orbiculata*[2]. We followed the salt extraction method detailed in Phillips *et al.*[2], originally from Bruford *et al.*[58], with a few modifications. Briefly, gnats were placed in 250 µL TNES buffer (12.5 µL 1M Tris-HCl pH 7.5, 83.3 µL 1.2M NaCl, 50 µL 0.5M EDTA, 250 µL 10% SDS with the remainder water) with 30 µL 10 mg mL^{-1} proteinase K, gently mixed and kept at 55 °C for 18+ h. After transferring to a new tube, the gnat was returned to ethanol for storage and 95 µL 4 M ammonium acetate was added to the buffer solution and mixed before being centrifuged at 18,000 x g for 20 min. The supernatant was transferred to a new tube and 1 mL

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cold ethanol added and mixed before being kept at -20 °C for 18+ h. The solution was then centrifuged at 18,000 x g for 20 min to pellet the DNA, then the supernatant was discarded, and the pellet washed with 500 µL cold 70% ethanol/water before being air dried and resuspended in 80 µL Tris-EDTA.

The DNA was amplified and sequenced following the methods of Griffiths *et al.*[56] with minor modifications. Briefly, PCR reactions (35.1 µL) comprised 3 µL DNA template, 4.0 µL 10x PCR buffer (QIAGEN), 2.4 µL of 2 mM dNTPs (Fisher Biotec), 2.4 µL of 25mM $MgCl_2$ (QIAGEN), 0.5 µL of 10 mg μL^{-1} BSA, 2.0 µL of each primer at 2.0 µM, 1.0 U Taq polymerase (QIAGEN) and 22.0 µL Milli-Q water. Amplification was conducted in a Thermo Fisher 2720 thermal cycler using the published touchdown protocol[56] consisting of 3 min at 94 °C followed by 40 cycles, each cycle comprising 30 s at 94 °C, 40 s at 66 °C, and 70 s at 72 °C, with the middle temperature starting at 66 °C and dropping 3 °C every second cycle until 49 °C, before a final 7 min at 72 °C. Purification of PCR products followed the ExoSAP-IT clean-up protocol (USB Corporation) and the sequencing reaction was conducted using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The final product was sequenced in both forward and reverse directions using the original PCR primers on an ABI 3130xl Genetic Analyser.

We developed a new set of primers for the fungus gnat mitochondrial CO1 region (5′-3′ forward GGWGGWTTYGGAAAYTGATTAG and reverse TATWGAWARVACATARTGRAARTGRGC), as the primers of Folmer *et al.*[59], used in Phillips *et al.*[2], were not successful in amplifying the CO1 region in some species of fungus gnats captured as part of a related study. Sequences were edited and aligned using Geneious v9.1.8 (Biomatters Ltd) and phylogenetic analysis was performed in MEGA X 10.1[60] using a maximum likelihood analysis with 1000 bootstrap replicates and a GTR+I+G substitution model.

Other *Mycomya* sp. fungus gnats captured as part of a related study on *Pterostylis* pollinators were included in the phylogenetic analysis, providing a total of more than 60 sequences in our analysis. All fungus gnats from mated pairs, synthetic blends, and *P. orbiculata* flowers in this study had a sequence divergence of <1.7% which is well under the ~4% divergence limit often used to delimit insect species[61]. Furthermore, phylogenetic analysis of the CO1 sequences showed that all 6 males from mated pairs and all 4 males captured at synthetic blends belonged to the same clade as gnats captured at *P. orbiculata* flowers, while other *Mycomya* fungus gnats captured as part of a related study formed distinct, well-supported clades (see Figure S3). Thus, we were able to confirm that the female fungus gnats from mated pairs, bioassays and orchids were the same species as the pollinator of *P. orbiculata*.

General chemical procedures

To obtain GC-EIMS analytical data, solvent extracts of *Pterostylis* orchids and *Mycomya* sp. gnats were analysed on an Agilent 6890 GC equipped with a HP-5MS column [(5% phenyl polysilphenylene-siloxane), 30 m x 0.25 mm x 0.25 µm film thickness, Hewlett-Packard USA] connected to an Agilent 5973 mass selective detector or on an Agilent 8860 GC equipped with a 5977B MSD, and a 7693A autosampler with an Agilent J&W Scientific DB-HeavyWAX column (30m x 0.25 mm x 0.25 µm film thickness). Helium was used as the carrier gas at 1mL / min. Samples (1 µL) were injected splitless (1 min) at 40 °C for 1 min, the temperature then increased at 7 °C/ min to 250 °C (270 °C DB-HeavyWAX) and held for 15 min. Mass spectra were recorded between 50-450 amu and analysed using Agilent ChemStation software or deconvoluted using Agilent MassHunter Quantitative Unknowns software with peaks manually checked. Compounds representing <1% of the total extract are not presented. Compounds were identified based on comparison with mass spectra and

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retention time matching of synthetic standards, or tentatively identified based on comparison with internal libraries and NIST 2011. HR-MS (EI, 70 eV) were recorded on a Waters GCT Premier TOF-MS equipped with a BPX5 column [(5% phenyl polysilphenylene-siloxane), 30 m × 0.25 mm × 0.25 μm film thickness, SGE Australia], using helium as a carrier gas and the same temperature program as above.

All semi-preparative GC fractionations were performed on a HP5890A GC with a Restek RTx-5 column (30 m x 0.53 mm i.d. x 5 μm) or Agilent J&W Scientific DB-Wax column (30 m x 0.53 mm i.d. x 1 μm) equipped with a three- way glass splitter separating the flow post column into the FID and the collector. A manual fraction collector was used, consisting of glass capillaries (100 x 1.55 mm i.d., Hirschmann Laborgeräte, Eberstadt, Germany) held in a custom aluminium holder which was kept cold by partially submerging in a dry ice/acetone bath[28,62]. Fractions were collected from the GC in the cooled glass capillaries, and eluted from the capillaries in dichloromethane (ca. 100 μL) and stored at -20 °C until required.

NMR spectra were acquired on a Bruker Avance 600 MHz (with a 1.7 mm TXI microprobe) spectrometer with $CDCl_3$ as solvent. Chemical shifts were calibrated to resonances attributed to residual $CHCl_3$. The multiplicities of resonances observed in ^{1}H NMR spectra are expressed by the abbreviations: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet), and combinations thereof including br (broad) and app (apparent) for more highly coupled systems.

For laboratory and field bioassays, the amount of compound in fractions, synthetic mixtures and orchid extracts were calculated by co-injection with known amounts of *n*-heneicosane to ensure similar amounts of compounds were presented for each trial. For field bioassays, the synthetic blends were prepared in the laboratory prior to field trials, and the relative abundance of compounds was confirmed by GC-MS to be similar to the orchids. The total amount of (6*Z*,9*Z*)-1,6,9-tricosatriene used in each field bioassay was approx. 2 μg, which was matched by the amount of orchid extract used.

Isolation of semiochemicals from orchid extracts

For the semi-preparative GC fractionation of labellum extracts for laboratory bioassays, extracts were injected (3 μL) splitless (1 min) onto the column (Restek RTx-5) with helium as the carrier gas using the following temperature program: 40 °C for 1 min then increased to 230 °C at 20°C / min, held for 5 min then increased at 2°C / min to 250 °C and held for 15 min. We strategically targeted C_{21}-C_{25} hydrocarbons, as these make up the bulk of the orchid extract. Four fractions were collected: fraction 1 (3 – 24 min, first part of extract); fraction 2 (24 – 28.2 min, containing C_{21} and C_{23} *n*-alkanes); fraction 3 (28.2 – 29.5 min, containing C_{23} diene and triene); fraction 4 (29.5 – 40.5 min, containing C_{25} *n*-alkane).

Semi-preparative GC was also used to isolate the C_{23} triene present in orchid labella for NMR analysis. Concentrated extracts were fractionated as above using the Agilent J&W Scientific DB-Wax column and a shorter method (oven temperature 40 °C held for 1 min then increased at 15 °C/min to 180 °C then increased at 7 °C/min to 230 °C and held for 10 min). Fractions were collected between 25.7 min and 26.5 min, which gave the C_{23} triene in >99% purity for NMR analysis.

^{1}H NMR of (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**) (600 MHz, $CDCl_3$) δ 5.82 (m, 1H), 5.41–5.30 (m, 4H), 5.01 (br d, 1H), 4.95 (br d, 1H), 2.77 (app t, 2H), 2.10–2.02 (m, 6H), 1.49–1.43 (m, 2H), 1.38–1.22 (m, 22H), 0.88 (t, J = 7.0 Hz, 3H).
HRMS: Found 318.3279, $C_{23}H_{42}$ requires 318.3287.

Synthetic procedures

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See below for details of synthetic preparations for (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**) and (6*Z*,9*Z*)-6,9-tricosadiene (**2**), and Figure S1F for the synthetic scheme. As the synthetic procedure for **1** yielded a mixture of geometric isomers, we used semi-preparative GC to purify this compound for bioassays. The synthetic mixture was purified using the same method as for the natural product above, which gave **1** in >99% purity.

2-(Prop-2-ynyl-oxy)tetrahydro-2*H*-pyran: Propargyl alcohol (28.0 g, 0.50 mol) and *p*-toluenesulfonic acid (1.3 g, 7.5 mmol, 1.5%) were dissolved in dichloromethane (250 mL). To this solution, 3,4-dihydro-2*H*-pyran (44.0 g, 0.525 mol) was added slowly at 0 °C over 20 min. The mixture was stirred at RT for 2 h, then basified with NaOH (1 M) and subjected to usual workup (dichloromethane). Concentration *in vacuo* gave 71.6 g crude golden product. Distillation gave the product as 58.0 g clear oil, bp 100-105 °C (house vacuum, ca. 20 Torr). NMR data corresponded with literature data[24].

2-(Octan-2-ynyloxy)tetrahydro-2*H*-pyran and 2-(Oct-7-en-2-ynyloxy)tetrahydro-2*H*-pyran: To a cold solution of protected alcohol (7.3 g, 52 mmol) in dry THF (80 mL) on an ice-bath, was added butyllithium (2.0 M in cyclohexane, 25.0 mL, 50 mmol). The solution was stirred on ice-bath for 15 min, then 1-bromopentane or 5-bromo-1-pentene (6.75 g, 5.6 mL, 45 mmol), followed by DMSO (80 mL) was added (on ice-bath). Each solution was stirred at RT overnight, before being quenched by ice (on ice-bath) and subjected to usual workup (diethyl ether). Concentration *in vacuo*, followed by flash chromatography (hexanes to 30% EtOAc in hexanes) gave the products as 6.96 g of 2-(octan-2-ynyloxy)tetrahydro-2*H*-pyran and 5.44 g of 2-(oct-7-en-2-ynyloxy)tetrahydro-2*H*-pyran, both slightly yellow oils.

8-bromooctan-6-yne: Carbon tetrabromide (4.6 g, 1.4 equiv.) was added to 2-(octan-2-ynyloxy)tetrahydro-2*H*-pyran (2.1 g, 10 mmol, 1 equiv.) in dichloromethane at RT. After 10 min, the mixture was cooled on an ice-bath, triphenylphosphine (7.4 g, 2.8 equiv.) was added, and the mixture was stirred at RT overnight. After 18 h (60% conversion by GC), the mixture was filtered through silica and concentrated *in vacuo* to 2.59 g pale yellow oil. Flash chromatography (hexanes) gave 739 mg clear oil. NMR data corresponded with literature data[25].

8-bromooct-1-en-6-yne: Carbon tetrabromide (3.7 g, 24 mmol) was added to 2-(oct-7-en-2-ynyloxy)tetrahydro-2*H*-pyran (1.7 g, 8.2 mmol) in dichloromethane (50 mL) and the mixture was stirred at RT for 10 min. The mixture was cooled to 0 °C and triphenylphosphene (6.0 g, 23 mmol) was added in one portion. After stirring at room temperature overnight, water was added (2 mL, 111 mmol) and the reaction mixture was stirred for a further 2 h, filtered, dried over $MgSO_4$ and subjected to flash chromatography (0 – 10% EtOAc/hexanes) to return 700 mg of a light-yellow oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ 5.82–5.74 (m, 1H), 5.08–5.02 (m, 1H), 5.01–4.97 (m, 1H), 3.92 (t, *J* = 2.4 Hz, 2H), 2.28–2.23 (m, 2H), 2.18–2.12 (m, 2H), 1.63–1.55 (m, 2H).
^{13}C NMR (150.9 MHz, $CDCl_3$) δ 137.64, 115.33, 87.84, 75.58, 32.69, 27.50, 18.31, 15.68.

Tricosan-6,9-diyne: A suspension of potassium carbonate (552 mg, 4 mmol), copper (I) iodide (760 mg, 4 mmol) and sodium iodide (600 mg, 4 mmol) was stirred in DMF (5 mL). 1-Pentadecyne (832 mg, 4 mmol) and 8-bromooctan-6-yne (534 mg, 2.8 mmol), mixed in DMF (5 mL) were added at RT. The mixture was stirred over night at RT. Diethyl ether (25 mL) was added and the product was subjected to usual workup (diethyl ether). Concentration *in vacuo* to give 1.21 g of a yellow oil. After flash chromatography (hexane) 427 mg pale yellow oil was obtained. NMR data corresponded with literature data[26].

Tricosa-1-en-6,9-diyne: A solution of 8-bromooct-1-en-6-yne (600 mg, 3.2 mmol) and 1-pentadecyne (935 mg, 4.5 mmol) in DMF (5 mL) was added to a suspension of potassium

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carbonate (620 mg, 4.5 mmol), copper (I) iodide (850 mg, 4.5 mmol) and sodium iodide (675 mg, 4.5 mmol) in DMF (5 mL) and the mixture stirred at RT overnight. Diethyl ether (25 mL) was added and the mixture subjected to a usual workup (hexanes) followed by flash chromatography (100% hexanes) to furnish 520 mg of a colourless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ 5.85–5.76 (m, 1H), 5.06–5.04 (m, 1H), 4.99–4.96 (m, 1H), 3.12–3.11 (m, 2H), 2.18–2.13 (m, 6H), 1.62–1.56 (m, 2H), 1.51–1.45 (m, 2H), 1.38–1.22 (m, 20H), 0.88 (t, J = 7.1 Hz, 3H).
^{13}C NMR (150.9 MHz, $CDCl_3$) δ 138.12, 115.17, 80.73, 80.18, 75.02, 74.54, 32.95, 32.07, 29.84, 29.82, 29.80, 29.79, 29.69, 29.51, 29.32, 29.06, 28.92, 28.06, 22.84, 18.89, 18.30, 14.27, 9.88.

(6*Z*,9*Z*)-6,9-Tricosadiene (**2**)**:** Tricosan-6,9-diyne (186 mg) and titanium (IV) isopropoxide (1.34 g, 1.4 mL) were mixed in dry diethyl ether (15 mL) and cooled to – 70 °C. Isopropyl magnesium chloride (10.3 mL, ca 1.1M) was added dropwise and the temperature was increased to – 30 °C and stirred for 2 h, then cooled to – 70 °C and quenched with water (0.5 mL). The mixture was filtered through silica and concentrated *in vacuo*. Flash chromatography (hexane) gave 100 mg white solid product. NMR data corresponded to literature data[27].

(6*Z*,9*Z*)-Tricosa-1,6,9-triene (**1**): Tricosa-1-en-6,9-diyne (1.0 g, 3.2 mmol) was dissolved in hexane (20 mL) followed by the addition of a catalytic amount of Lindlar's catalyst and the mixture was stirred under an atmosphere of hydrogen (2 h). The mixture was filtered and subjected to flash chromatography using silver nitrate doped silica (10% EtOAc/hexanes) to give 320 mg of a colourless oil containing trace amounts of *E/Z* and *Z/E* configured alkenes. Further purification using semi-preparative GC as described for isolating the natural product gave an authentic sample for bioassays of > 99% purity.

^{1}H NMR (600 MHz, $CDCl_3$) δ 5.82 (m, 1H), 5.42–5.30 (m, 4H), 5.01 (m, 1H), 4.95 (m, 1H), 2.77 (m, 2H), 2.11–2.02 (m, 6H), 1.50–1.43 (m, 2H), 1.38–1.22 (m, 22H), 0.88 (t, J = 7.0 Hz, 3H).
^{13}C NMR (150.9 MHz, $CDCl_3$) δ 138.95, 130.43, 129.81, 128.54, 127.99, 114.63, 33.50, 32.08, 29.84, 29.83, 29.81, 29.71, 29.52, 29.49, 29.03, 27.40, 26.82, 25.81, 22.85, 14.27.

Laboratory bioassays

Laboratory bioassays were performed in July and August 2018. Male gnats were collected each morning from known sites using orchid flowers as baits, or by hand using collection vials, and stored at room temperature for < 4 h before use. Gnats were individually placed in a glass tube 255 x 40 mm, which was sealed at either end with organza netting. A Syntech Stimulus controller (Syntech, Kirchzarten, Germany) was used to gently blow charcoal-purified humidified air through the tube to ensure air movement over the gnat. A piece of filter paper was placed upwind approx. 30 mm beyond the gnat tube, onto which the samples were loaded (i.e. the gnat could not reach the sample). Male gnats responded to orchid labellum extracts by rapid initiation of upwind flight (<10 s after commencement of presentation) and performed rapid searching behaviour and/or wing fanning at the netting closest to the sample. Although we did not observe explicit sexual behaviour in laboratory bioassays, the rapid initiation of searching behaviour allowed us to confirm that the male gnats were able to perceive and respond to orchid extracts and certain fractions, thus creating candidates for testing with synthetic compounds in field bioassays (below).

For each bioassay, the gnat was placed at the far end of the tube (i.e. downwind) and allowed to acclimate for 10 min. A solvent blank (negative control) was placed on the filter paper, allowed to evaporate for 1 min, then introduced for 3 min upwind of the gnat tube, and gnat

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behaviour was recorded. After a further 5 min, the sample fraction was placed on filter paper and allowed to evaporate for 1 min, then introduced for 3 min and gnat behaviour recorded. The fraction was determined to be active if the gnat responded < 10 sec with upwind flight and searching behaviour and/or wing fanning at the netting closest to the sample. After a further 5 min, gnats that did not respond positively to the sample fraction were exposed to orchid extract for 3 min and behaviour recorded (positive control). Gnats that did not respond positively to the sample fraction or positive control were not included in analyses. Each gnat was only used once. No gnats responded positively to the dichloromethane solvent (negative) control.

Field bioassays

Field bioassays were performed in July and August 2019 at known populations of *P. orbiculata* pollinators at the University of Western Australia Crawley campus and at Kings Park and Botanic Gardens. Compound(s) and extract were loaded on to the tip of a folded piece of filter paper suspended approx. 25 cm above the ground on a bamboo skewer and placed approx. 30 cm apart during the choice phase (phase 2). Samples were loaded just before commencing each experiment and were kept in an airtight container between trials.

Field bioassays were performed using the established sequential phase bioassay method from Bohman *et al*.[7,19] with minor modifications. Briefly, each trial consisted of two parts: the first part (phase 1) involved the presentation of the candidate compound or blend alone for 5 min. In the second part (phase 2) directly following phase 1, a positive control was added, in this case an orchid extract, for a further 5 min. This bioassay design takes into account the fact that candidate compounds may be partially attractive when presented alone yet may attract few responses in a direct choice scenario with a stronger stimulus (e.g. orchid extract control). The addition of a known attractive control in phase 2 also provides a test for pollinator availability in the event that no responses are observed in phase 1. Trials with no responses in either phase 1 or phase 2 were discarded from further analyses. To take advantage of renewed pollinator responses at novel sites, trials were repeated 4-6 times by moving >3 m between trials. Responses from these trials were summed, resulting in one experiment, and each experiment was replicated 2- 4 times on separate days.

Gnat responses were scored directly in the field into the following hierarchical categories: land only, land and wing fan only, and land and sexual behaviour (abdomen curling and/or attempted copulation). Wing fanning is often considered a pre-copulatory behaviour in fungus gnats and other insects[35,63–65], however we did not have the opportunity to observe pre-mating courtship behaviour in this species. Due to their small size, it was not possible to observe fungus gnats in flight. Similarly, it was difficult to differentiate abdomen curling and brief attempted copulations, so these were combined into one sexual behaviour category.

QUANTIFICATION AND STATISTICAL ANALYSES

Field bioassays

Responses within each field bioassay experiment were converted to proportions relative to the total for phase 1 + 2 (i.e. responses from across phase 1 and 2 summed to 1), and the standard error calculated on the proportional responses across replicated experiments. Gnat behaviour and total responses in phase 2 were compared using *G*-tests applying William's correction, to test for either a difference in behavioural repertoire, or to test the null hypothesis of no differences in the total number of responses regardless of behaviour. *G*-tests were done in GenAlEx 6.5[66] using a significance threshold of 0.05. Because it cannot be ruled out that phase 2 responses are influenced by the control or phase 1, caution may be

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required when comparing phase 1 and phase 2 behaviours. Solvent (dichloromethane) on filter paper was not attractive to male gnats (no responses in phase 1 and only two responses in phase 2 compared with 49 responses to orchid extract; $G=53.8$, $P<0.001$), confirming that the solvent and filter paper set up are not attractive to gnats.

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Supplemental information

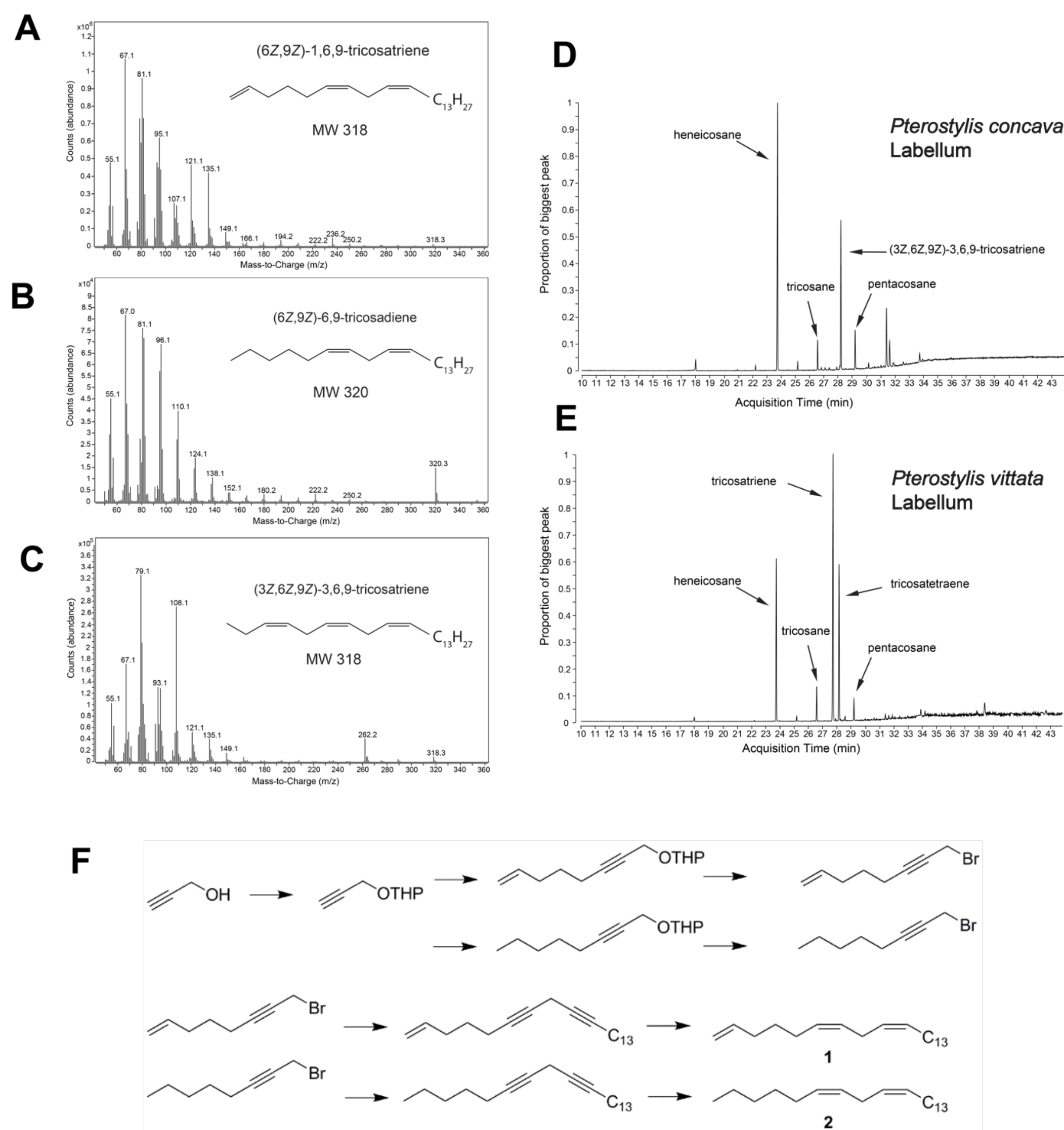

Figure S1. Mass spectra and compounds in *Pterostylis* orchids, and scheme for the synthesis of target compounds. Related to Figures 1 and 3.

Mass spectra of (6Z,9Z)-1,6,9-tricosatriene (**1**, panel **A**) and (6Z,9Z)-6,9-tricosadiene (**2**, panel **B**) obtained from solvent extracts of *Pterostylis orbiculata* labella, and (3Z,6Z,9Z)-3,6,9-tricosatriene obtained from extracts of *P. concava* labella (**C**), showing compound structure and molecular weight (MW). Annotated total ion chromatograms (TIC) from GC-MS analysis of labellum extracts of *Pterostylis concava* (**D**) and *P. vittata* (**E**) with identified compounds labelled. Scheme for the synthesis of 1 and 2 is shown in panel **F**.

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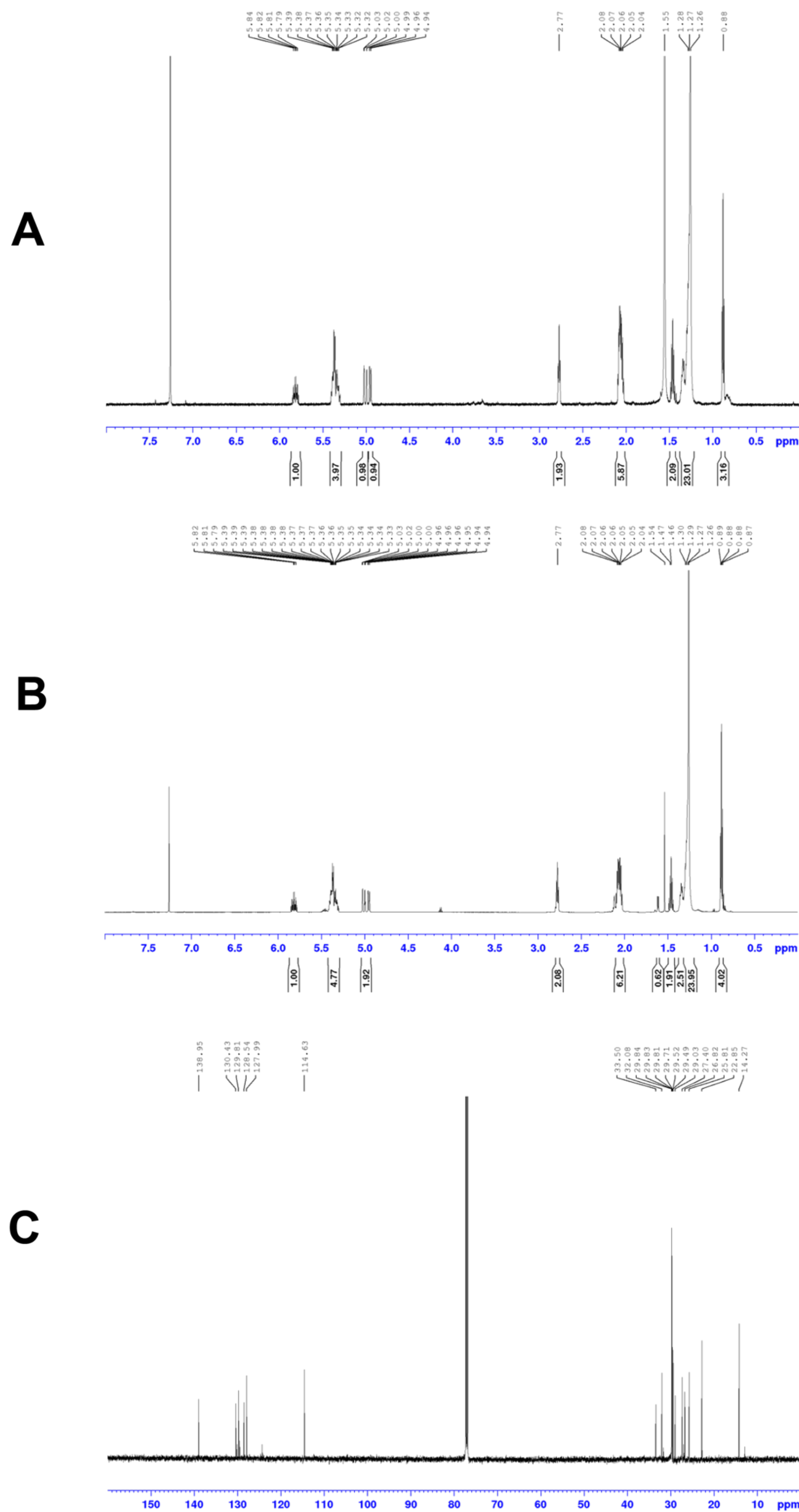

Figure S2. NMR spectra of (6*Z*,9*Z*)-1,6,9-tricosatriene. Related to STAR methods.
^{1}H NMR (600 MHz, $CDCl_3$) spectra of the isolated (**A**) and synthetic (**B** and **C**) (6*Z*,9*Z*)-1,6,9-tricosatriene (**1**).

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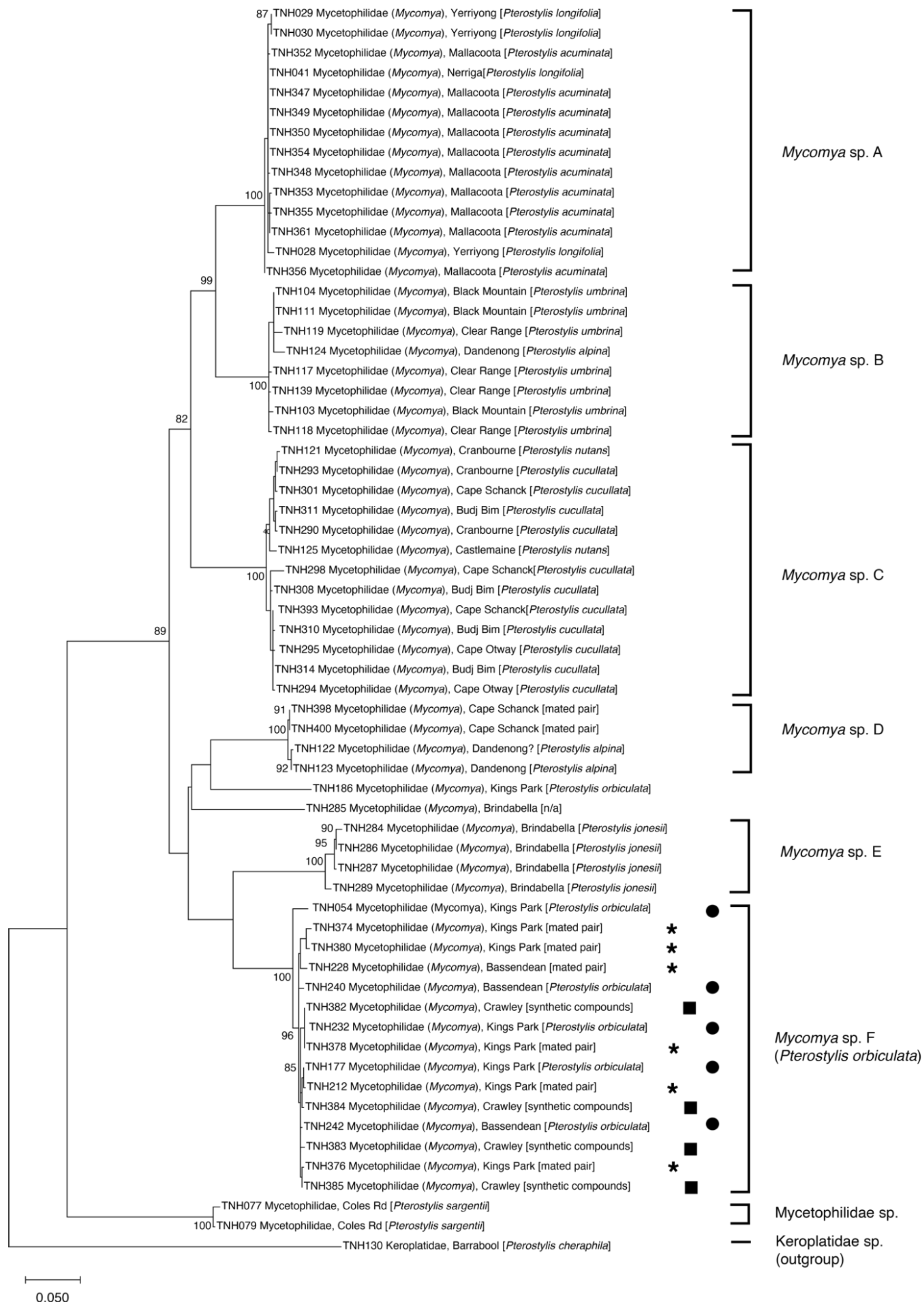

Figure S3. Phylogenetic analysis of CO1 barcoding results. Related to STAR methods. Outcomes of a maximum likelihood analysis of the CO1 region for the pollinator of *Pterostylis orbiculata* (*Mycomya* sp. F), including gnats captured in mated pairs (asterisk), at synthetic blends (squares) and at orchids (circles). Other *Mycomya* species captured as part of a related study are included for comparison. Bootstrap values >85 are shown.

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Table S1. Mean relative percentages ± SEM of the five abundant compounds in common between extracts of orchid labellum and female fungus gnats. Related to Figure 1.

Compound	**Orchid labellum**	**Female fungus gnat**
(6*Z*,9*Z*)-1,6,9-tricosatriene (**1**)	65.5 ± 1.4	74.1 ± 1.4
(6*Z*,9*Z*)-6,9-tricosadiene (**2**)	5.5 ± 0.7	5.9 ± 0.8
Heneicosane (**3**)	22.5 ± 1.4	13.2 ± 0.4
Tricosane (**4**)	4.4 ± 0.3	4.0 ± 0.5
Pentacosane (**5**)	2.1 ± 0.2	2.7 ± 0.2

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