Systematic, phylogenetic and pollination studies of Specklinia (Orchidaceae)

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Chapter 8

Pollination of *Specklinia* by nectar feeding *Drosophila*: first reported case of a deceptive syndrome employing aggregation pheromones in Orchidaceae

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Background and Aims: The first documented observation of pollination in Pleurothallidinae was that of Augustus Endrés who noticed that the "viscid sepals" of *Specklinia endotrachys* were visited by a "small fly". Mark Chase would later identify the visiting flies as being of the genus *Drosophila*. Here, we document and describe how species of the *Specklinia endotrachys* complex are pollinated by different *Drosophila* species.

Methods: Specimens of *Specklinia* and *Drosophila* were collected in the field in Costa Rica and preserved at JBL and L. Flies were photographed, filmed and observed for several days during a 2-year period and were identified by a combination of noninvasive DNA barcoding and anatomical surveys. Tissue samples of the sepals, petals and labellum of *Specklinia* species were observed and documented with SEM, LM and TEM. EAG experiments were done on *Drosophila hydei*, using the known aggregation pheromones ethyl tiglate, methyl tiglate and isopropyl tiglate. Floral compounds were analysed with GC-MS using those same pheromones as standards.

Key Result: We find that flowers of *Specklinia endotrachys*, *S. pfavii*, S. remotiflora and *S. spectabilis* are visited and pollinated by several different but closely related *Drosophila* species. The flies are arrested by aggregation pheromones, including ethyl tiglate, methyl tiglate and isopropyl tiglate, released by the flowers, and to which at least *D. hydei* is very sensitive. Visible nectar drops on the adaxial surface of sepals are secreted by nectar secreting stomata; encouraging the *Drosophila*, both males and females, to linger on the flowers for several hours at a time. The *Drosophila* frequently show courtship behaviour; occasionally copulating. Several different species of *Drosophila* can be found on a single species of *Specklinia*.

Conclusions: Species of the *Specklinia endotrachys* group share a similar pollination syndrome. There seems to be no species-specific relationships between the orchids and the flies. We do not expect the *Specklinia* species to hybridise naturally as their populations do not overlap geographically. The combination of pheromone attraction and nectar feeding is likely to be a generalised pollination syndrome in Pleurothallidinae.

Keywords: aggregation pheromones, courtship, deceit, *Drosophila repleta* group, nectar secreting stomata, Pleurothallidinae, reward, *Specklinia endotrachys*, *Specklinia pfavii*, *Specklinia spectabilis*, *Specklinia remotiflora*.

Introduction

Epiphytism is likely to be the major contributor to the species richness of the Orchidaceae family, more specifically Epidendroideae (Gravendeel *et al.* 2004). Nonetheless, pollinator adaptation might be the driving force of the remarkable floral diversification in orchids. Jersáková *et al.* (2006) argue that this adaptation is likely to be unilateral, without change in the pollinator (Williams 1982). Co-evolution between orchids and their pollinators is apparently uncommon (Szentesi 2002). Orchids frequently exploit existing plant-pollinator relationships or even sexual systems of insects, exemplified by species that achieve pollination through deception, not offering floral rewards (Ackerman 1986; Jersáková *et al*. 2006; Ramírez *et al.* 2011).

Pollination by deceit is well known among orchids and has been frequently considered another key innovation contributing to the high species richness of the family (van der Pijl & Dodson 1966; Cozzolino & Widmer 2005). Food deception has evolved repeatedly in different angiosperm groups, but is mostly restricted to a few species per family (Renner 2005), while estimates suggest that a third of all orchids might be food deceptive (Ackerman, 1986), where it seems to have arisen many times independently. Sexual deception has been reported in several phylogenetically unrelated orchid clades (van der Pijl & Dodson 1966; Adams & Lawson, 1993; Ayasse *et al.* 2003; Singer 2002; Singer *et al.* 2004; Blanco & Barboza 2005; Ciotek *et al.* 2006; Phillips *et al*. 2009; Peakall *et al.* 2010). If confirmed to be a generalised syndrome in those species rich species' groups, sexual deceit might well represent up to 10% of the pollination syndromes in the Orchidaceae.

Together, those percentages would suggest that deceitful pollination could represent close to half of all pollination syndromes in the orchids. However, considering that only few orchid-pollinator relationships have been studied in detail, and several of those have found "non-obvious" floral rewards being offered to pollinators, including scents, triterpenoid resins, pseudopollen, lipid-rich substances, and low amounts of nectar and oils (Chase *et al*. 2009; Davies and Turner 2004; Mickeliunas *et al*. 2006; Pansarin & Amaral 2006; Whitten *et al.* 2007; Pansarin *et al.* 2008; Stpiczyńska & Davies 2008; Pansarin & Pansarin 2011; Pansarin *et al*. 2013; Papadopulous *et al*. 2013; Davies *et al.* 2014), non-obvious floral rewards might be overestimating the cases in which orchids offer no reward at all. Such a case is that of the *Specklinia endotrachys* (Rchb.f.) Pridgeon & M.W.Chase species complex (Pleurothallidinae).

Pleurothallidinae includes more than 4100 species (Pridgeon 2005), likely making it the largest subtribe among the orchids and one of the largest amongst flowering plants in general. Myophily, or fly pollination, seems to be the general in all the genera of the subtribe, with few exceptions. Myophily is the second largest pollination syndrome in the Orchidaceae, with an estimated 15-25% of the whole family being pollinated by flies (van der Pijl & Dodson 1966; Christensen 1994; Borba & Semir 2001). However, aside from research on *Acianthera* Scheidw. (Borba and Semir 2001; de Melo *et al.* 2010), *Dracula* Luer (Endara *et al.* 2010) *Lepanthes* (Blanco & Barboza 2005), *Octomeria* R.Br. (Barbosa *et al.* 2009), *Pleurothallis* R.Br. (Duque-Buitrago *et al*. 2014) and Stelis Sw. (Albores and Sosa 2006), few pollination syndromes in the Pleurothallidinae have been studied in depth and are yet fully described. Considering the high species and floral morphology diversity it is quite likely that a plethora of different pollination syndromes are present within these fly pollinated orchids.

Endrés, in 1878, noted that flies were attracted to the nectar present in the flowers of *S. endotrachys* (Pupulin *et al.* 2012, Chapter 1). Chase (1985) observed *Drosophila immigrans* visiting and pollinating *Specklinia spectabilis* (Ames & C.Schweinf.) Pupulin & Karremans. He noted that flowers emitted a faint rotten-fruit odor, but did not report the presence of nectar. Nectar production could not be confirmed by Pupulin *et al.* (2012, Chapter 1), but the authors did find that flowers of *Specklinia endotrachys*, *S. pfavii* (Rchb.f.) Pupulin & Karremans, *S. remotiflora* Pupulin & Karremans and *S. spectabilis* were all visited frequently and for long periods of time by Drosophiloid flies at Lankester Botanical Garden in Costa Rica, so they suspected a reward.

Orchidaceae show great adaptability in the rewards offered to potential pollinators, ranging from perfume, to oil, nectar and pollen (Smets *et al*. 2000). Unlike most other Asparagales, and numerous other monocots, Orchidaceae do not possess gynopleural or septal nectaries (Smets & Cresens 1988; Smets *et al*. 2000). Nectar secretion has been observed on the perianth parts (more specifically on the labellum) in some cases but perigonal nectaries are not that common in Orchidaceae as in Liliales where this feature can be considered synapomorphic (Smets *et al.*

2000). Floral fragrances are produced by osmophores (scent glands) occurring in a large group of plants (Vogel 1990; Dressler 1993). In orchids, osmophores may be located on the sepals, petals and labellum (Dressler 1993); the shape seems to vary from unicellular trichomes (Curry *et al*. 1991), pear-shaped or spherical unicellular hairs with irregular cuticle (Stpiczyńska 1993), dome-shaped papillae (Ascensãno *et al.* 2005), papillose cells with smooth cuticle (de Melo *et al.* 2010), to a rugose surface with a sculptured cuticle or wrinkled surface with a smooth cuticle (Antoń *et al*. 2012). The morphology of osmophores in fly-pollinated orchids has been examined only in a few species of Pleurothallidinae. Those studies have shown that osmophores are generally found on the sepals (Vogel 1990; Teixeira *et al*. 2004; de Melo *et al.* 2010).

In this paper we report the outcomes of a multidisciplinary study on the ecology, biology and phylogeny of the *Specklinia endotrachys* species group and allies, and of their pollinators of the *Drosophila repleta* species group. We address two specific questions: (1) how does pollination occur; (2) is pollination of *Specklinia* Lindl. species-specific. To answer these questions we collected plants and flies in the wild, made video documenting pollination and orchid-insect interaction, carried out LM, SEM and TEM observation, used DNA barcoding, and conducted EAG and GC-MS experiments.

Materials and methods

Living material:—Specimens of Specklinia species were field collected in Costa Rica and cultivated at the greenhouses of the Lankester Botanical Garden, University of Costa Rica and the Hortus botanicus of Leiden University (Leiden, The Netherlands), from 2012 till 2014. Voucher specimens of the plants were prepared from cultivated material and deposited at JBL (spirit), L (spirit) and CR.

Flies were photographed, filmed and observed for a total of 30 days during a 2-year period at the open-air greenhouses at Lankester Botanical Garden, in Costa Rica. Observations were mostly made between 06:00 and 18:00 h, with five observations extending that period overnight for all Specklinia species. Flies were identified by a combination of noninvasive (sample rescue after lysis) DNA barcoding of the 660 bp long COI marker by KB and anatomical surveys by DG. Only visitors that would linger on flowers and were highly interactive with sepals and lip (interacting with flower parts for more than 60 min) and/or that carried pollinaria were considered as putative pollinators. Vouchers for the insects were prepared from both field collected and greenhouse collected specimens and are kept at L (spirit) and AMNH.

Phylogenetics:—*Specklinia*. The phylogenetic concept of *Specklinia* follows Pridgeon *et al.* (2001). Those authors found that *Specklinia endotrachys* was closely related to *Specklinia lanceola* (Sw.) Lindl., the type species of the genus *Specklinia*, and a few other mainly orange-flowered species including *S. fulgens* (Rchb.f.) Pridgeon & M.W.Chase, *S. lentiginosa* (F.Lehm. & Kraenzl.) Pridgeon & M.W.Chase and *S. tribuloides* (Sw.) Pridgeon & M.W.Chase (Pupulin *et al.* 2012, Chapter 1). The species belonging to the *S. endotrachys* (*sensu* Pupulin *et al.* 2012, Chapter 1) complex are here treated as a monophyletic group within *Specklinia* based both on morphological similarities and additional unpublished molecular data (Bogarín *et al.* 2013b, Chapter 4; Karremans *et al*. 2013a).

Drosophila. Whole specimens were used for non-destructive extraction, using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. Elution was performed in 150 µl buffer AE. To obtain standard animal DNA barcode fragment of the mitochondrial cytochrome c oxidase subunit COI gene (Hebert *et al*. 2003), PCR was performed using a primer cocktail containing primers LCO1490 and HCO2198 (Folmer *et al*. 1994), and Lep-F1 and Lep-R1 (Hebert *et al.* 2004). PCR reactions contained 18.75 µl mQ water, 2.5 µl 10x PCR buffer CL, 1.0 µl 10mM of each primer, 0.5 µl 2.5 mM dNPTs and 0.25 µl 5 U Qiagen Taq. The PCR protocol consisted of an initial denaturation step of 180 s at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 50 °C and 40 s at 72 °C, with a final extension of 300 s at 72 °C and a pause at 12 °C. Sanger sequencing was performed by Macrogen (http://www.macrogen.com) or BaseClear (http://www.baseclear.com) on an ABI 3730xl (Applied Biosystems).

The Staden *et al.* (2003) package was used for editing of the sequences. Contigs were exported as .fas files and opened in Mesquite v2.72 (Maddison and Maddison 2007), where they were checked for base calling errors, the matrix was aligned manually. *Drosophila melanogaster* was used as outgroup. The trees were produced with an analysis of the COI dataset using BEAST v1.6.0. (Drummond & Rambaut 2007). Parameters were set to preset, except for substitution model GTR with 10 categories, clock model uncorrelated exponential, tree prior Yule process, and number of generations 20,000,000. The resulting trees were combined using TreeAnnotator v1.6.0., using the first 3000 trees as burn-in. FigTree v1.3.1. (Rambaut 2009) was used to edit the resulting tree. Posterior probabilities are given for each node in decimal form. Sequences have been made available through BOLD.

Photo/Video-camera documentation:—*Video Recording*. The videos of the fly visitation were taken with the video option of a Nikon D5100 digital camera and a HD 720p Autofocus Logitech web cam.

Macrophotography. Colour illustrations of flowers and flies were made using a Nikon D5100 digital camera, a DFC295 Leica digital microscope colour camera with Leica FireCam version 3.4.1 software, and a Zeiss SteREO Discover V12 stereomicroscope using the AxioVision stacking software.

Scanning Electron Microscopy (SEM):—Tissue samples of the sepals, petals and labellum were prepared for SEM observation by harvesting tissue from the flowers up to 48 h after the beginning of anthesis, fixing in FAPA (ethanol 50%, acetic acid, formalin at a proportion of 18:1:1 v/v), and dehydration through a series of ethanol steps and critical-point drying using liquid CO_2. Dried samples were mounted and sputter-coated with gold and observed with a JEOL JSM-5300 scanning electron microscope, at an accelerating voltage of 10 kV. All images were processed digitally.

Light Microscopy (LM):—Tissue samples of the sepals of *S. pfavii* were prepared for LM observation by harvesting flowers up to 48 h after the beginning of anthesis, fixing in Ethanol 70%, dehydration through a graded series of ethanol 70%, 96%, 100% and xylene, impregnation with paraffin 60 C, and embedding in paraffin. Sections of 7 µm were cut using a Jung Biocut 2035 rotary microtome. To prepare for staining the samples were de-paraffinated in xylene, rehydrated through a series of ethanol step, and stained by placing in 1% alcian blue for 10 min. The samples were then rinsed in tapwater and demiwater, stained with nuclear fast red for 5 min, rinsed in demiwater, dehydrated through a graded series of ethanol and washed with xylene. Finally a coverslip with Entellan was placed on the sample and photographs were taken with a Zeiss Axioskop connected to a Leica DFC490 camera.

Transmission electron microscopy (TEM):—Freshly collected flowers were fixed for 3 h in a modified Karnovsky fixative (2.5% glutaraldehyde, 2% formaldehyde) and washed in a 0.1 M sodium cacodylate buffer (pH 7.4). After washing in 0.1 M sodium cacodylate buffer the material was postfixed for 2 h in 1% osmium tetroxide and then washed in distilled water. After dehydration in a series of ethanol and propylene oxide, the pieces were infiltrated with Epon by submerging them in a mixture of propylene oxide and Epon (1:1) for 1 hour. After overnight evaporation of the remaining propylene oxide, the material was embedded in fresh Epon and polymerized at 60 ºC for 48 h. Ultrathin sections were cut with an LKB ultratome, mounted on film-coated copper slot grids and poststained with uranyl acetate and lead citrate (Reynolds 1963). The sections were examined with a Jeol 1010 TEM.

Analysis of floral compounds with GC MS:—Floral compounds were extracted by two different methods. The first one consisted of rinsing the flowers in 5 ml heptane for up to 1 min; after removal of the flowers the heptane was concentrated to approximately 0.5 ml using a gentle stream of nitrogen. The second method consisted of trapping odours from open flowers with a volatile collector trap in which air was circulated by a membrane pump in a closed system. After each passage through the membrane-pump, the air was cleaned by a carbon filter. The volatiles were trapped on 50 mg of Porapak Porous Polymer Adsorbent (Sigma Aldrich) in a glass tube. After collecting (typically 3 h) the volatiles were eluted from the adsorbent with 3 ml of pure pentane. The pentane was subsequently concentrated to approximately 0.5 ml using a gentle stream of nitrogen. All samples were stored at -20 °C in anticipation for further analysis.

All extracts were analysed on a Thermo Scientific Trace 1300 gas chromatograph coupled to a Thermo Scientific DSQII mass spectrometer. A Restek Rxi-5ms capillary column (30 m x 0.25 mm, 0.25 mm film thickness) was used.

The initial oven temperature was 80 °C. After 2 min the temperature was increased to 120 °C (10 °C/ min). The final temperature was maintained for 7 min. Helium was used as carrier gas (1.2 ml/min). The split injection mode was used (injection volume 1 ml, inlet temperature 220 °C, split ratio 1:30). Mass spectra were taken in electron ionisation (EI) mode (at 70 eV) in the range of m/z 30-200 (500 amu/s). The ion source temperature and the interface line temperature were set to 250 °C and 200 °C, respectively. Compounds were identified by comparison of their mass spectra and retention times with those of commercially purchased reference samples.

Analysis of the floral droplets:—Drops produced on the adaxial sepal surface were collected with a fine glass pipet point and stored in a glass vial at -20 °C. Fehling's reagent was used to detect sugar presence in the collected drops. Two solutions were prepared and mixed immediately before use, forming a deep blue solution containing a cupric ion. Solution "A" was composed of 17.32 g of hydrated copper sulphate crystals in 250 ml of water, and solution "B" of 86.5 g of sodium potassium tartrate and 35 g of sodium hydroxide in 250 ml of water.

Electrophysiology:—*Fly Culturing and odour stimuli*. *Drosophila hydei* eggs were obtained from a commercial grower and reared at 23 °C, 50% rh and 16:8 L/D cycle. Flies were picked randomly 4-7 days after emergence from the eggs.

Moats *et al.* (1987) and Symonds and Wertheim (2005), reported several aggregation pheromones for D. hydei. Ethyl tiglate, methyl tiglate and isopropyl tiglate (98% purity, Sigma Aldrich) were selected. A volume of 1 µL of hexane diluted pure compounds (10-1, 10-2, 10-3 and 10-4 v/v) was pipetted on 5mm×50mm filter paper. After at least 60 sec to allow the hexane to evaporate, the strip was placed inside a Pasteur pipette. Z-3-Hexen-1-ol (diluted 10-1 v/v) was used as an external standard (positive control) and an empty Pasteur pipette as negative control. Stock solutions were freshly prepared before the experiments and kept at -20 °C in 1.5 ml bottles closed with Teflon lined caps. Pasteur stimulus pipettes were prepared daily.

Insect preparation and Electroantennogram (EAG) recording. Both male and female *D. hydei* were used in the experiments as no behavioural differences have been reported (Bartelt *et al*. 1985; 1986; 1988). A total of 14 animals were tested. Individual flies were cooled, immobilized at 4 °C for approximately 30 minutes and pushed into a plastic pipette just wide enough the catch the head. Recordings were made with a high impedance amplifier (IDAC-4) and EAG2000 software (Syntech, Kirchzarten, Germany), using glass capillaries filled with insect ringer. The recording electrode was inserted at the base of the antenna, the reference electrode only contacted the tip ("surface contact recording", den Otter *et al.* 1980). The preparations lifetime was several hours.

The insect was positioned 1 cm in front of the outlet of a charcoal filtered and humidified airstream (2 l/min). The chemical stimuli from the Pasteur pipettes were injected into the this flow (1 second, 2.5 ml odour pulses) with 60 sec intervals in random order. All EAG responses were expressed relative to the external standard (Z-3-Hexen-1-ol).

Statistics. To evaluate the effects of stimulus compound and concentration on the EAG responses a linear mixed models (Grüber *et al.* 2011) was constructed with Gaussian error function and log link function. The response variable was the standardized EAG amplitude described above. Explanatory variables we the stimulus compound (ethyl tiglate, methyl tiglate and isopropyl tiglate) and the stimulus concentration (dilutions from 10-1 to 10-4 v/v in hexane). To account for the variation caused by differences between individual flies individual was included as a random factor. The input for the models were 162 EAG amplitudes measured in 14 individual insects. To validate the model we visually inspected the fit using a qqplot and also plotted the residuals against the fitted values. No obvious patterns were present. The residuals did not differ from a normal distribution (Shapiro test, $W = 0.9942$, $p = 0.767$) and were homoscedastic (Bartlett test (for compounds), $K2 = 3.094$, $df = 2$, $p = 0.21$). All statistical tests were conducted in R (version 3.0.1; R Core Team 2013).

Results

Plant Biology:—The orchid species studied, *S. endotrachys*, *S. pfavii*, *S. remotiflora* and *S. spectabilis*, belong to a group of species which noticeably share a reddish-orange color of the perianth parts, especially of the sepals (Fig. 72a-d). These species produce long-lived multi-flowered successive inflorescences. Large plants may have 10+ flowers simultaneously opened, however, only one per single inflorescence. The four species have a tendency of flowering all year round in greenhouse conditions, but in field they do have flowering peaks. At least during 6 months all four species were flowering simultaneously in the greenhouses, and capsules were formed eventually. We have not found these species at many different localities in the field; however, when present they are commonly found in large colonies, and we have observed dozens of plants of *S. pfavii* and *S. remotiflora* growing in dense groups (Fig. 72e & f). Fruit-set was observed in both greenhouse and field conditions only in Costa Rica, not in The Netherlands. None of the documented plants of any of the species showed autogamy. In a wild population of *S. pfavii* (Table 13), 40% of the plants had capsules, however only 20% of the inflorescences had a capsule, and 8% of the produced flowers were pollinated. Those plants produced 1 to 7 flowers per inflorescence (can be more +20 under greenhouse conditions), and never more than a single capsule per inflorescence. Capsules are always found on the apex of the inflorescence, suggesting flowering succession is detained after frutification. Drops are produced after anthesis on the rugose areas of the sepals of all four species (Fig. 73). The drops keep growing and accumulate unless removed; they are fed upon by flies, ants and other floral visitors. If not removed they persist even after the flower withers. The drops are transparent and semi-liquid at ambient temperature; they change from liquid and transparent to pasty and opaque with increasing temperatures.

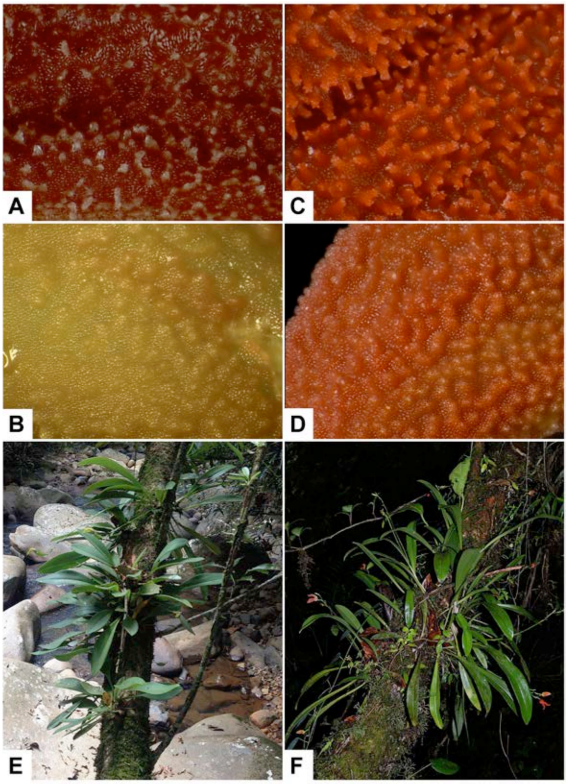


Figure 72. A-D. Adaxial surface of the sepals of diverse members of the orange-flowered *Specklinia* showing the structural and coloration diversity. A. *S. endotrachys* (*Blanco 961*). B. *S. pfavii* (*JBL-11098*). C. *S. remotiflora* (*AK4023*). D. *S. spectabilis* (*JBL-02535*). E-F. *Specklinia* species as found growing in field conditions in Costa Rica. E. *S. pfavii*, growing at a river's edge at 650 m elevation. F. *S. remotiflora*, in the cloud forest at around 2000 m elevation. Vouchers kept at JBL (spirit). Photographs by APK (A-E) and Joszef Geml (F).

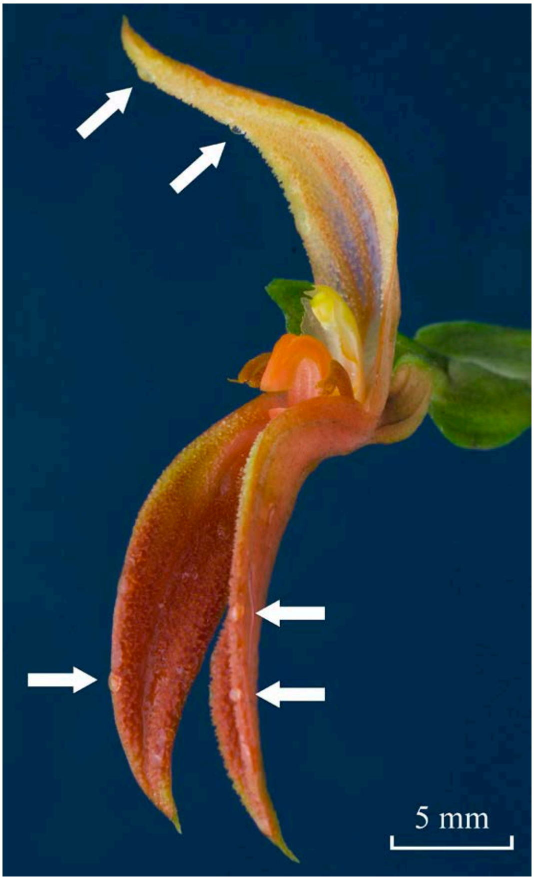


Figure 73. Nectar drops on the sepals of *Specklinia endotrachys* (*Blanco 961*). Photograph by Melania Fernández at Lankester Botanical Garden.

Table 13. Reproductive success relative to flowering in a wild population of *Specklinia pfavii* in Costa Rica.

Population	*Plants*	*Inflorescences*	*Flowers (total)*	*Fruits*
1	16	33	54	7
2	14	27	63	4
3	4	6	21	1
4	5	12	39	3
5	4	5	19	1
Total	43	83	196	16

Interaction	*Value*
Inflorescences per Plant	1.93
Flowers per Plant	4.56
Fruits per Plant	0.37
Fruits per Inflorescence	0.19
Fruits per Flower	0.08
Flowers per Inflorescence	2.36

Figure 74. A-B. *Drosophila* spp. sucking on the nectar secreting stomata on the apex of the papillae on the sepals of *Specklinia remotiflora*. A. Showing several flies at once. B. Shows a single fly and an area of the sepals where the stomata have been depredated by slugs. C. *Drosophila* spp. still attracted to a severed lateral sepal of *S. remotiflora* a few minutes after removal from the flower. D. *Drosophila* sp. on the lateral sepals of *Specklinia pfavii* with a drop on its mouthparts. It is likely to be a nuptial gift, regurgitated after having collected the nectar drops which are also still evident on the sepals. Arrows show the nectar droplets still present on the sepals. Photographs by APK (A-C) and FP (D) at Lankester Botanical Garden.

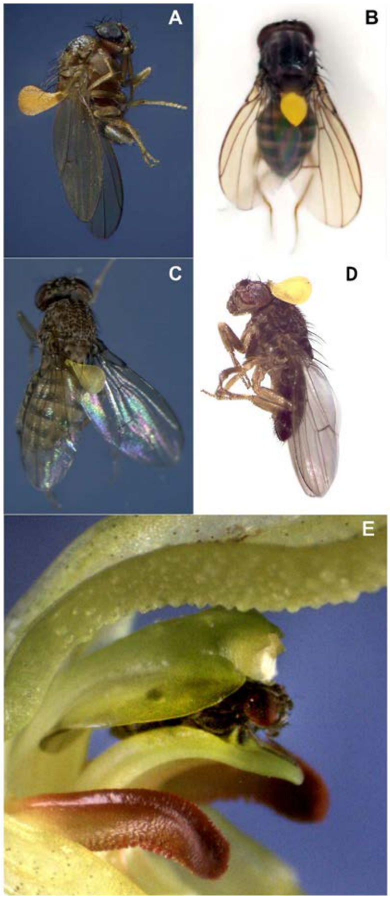


Figure 75. A-D. *Drosophila* flies with the pollinaria of *Specklinia* on the scutellum. A. *Drosophila* sp. with the pollinaria of *S. spectabilis* (*JBL-02643*). B. *Drosophila hydei* with the pollinaria of *S. remotiflora* (*Bogarín 8181*). C. *Drosophila mercatorum* (KB262-02) with the pollinaria of *S. remotiflora*. D. *Drosophila hydei* with the pollinia of *S. pfavii*. E. *Drosophila* sp. trapped between the lip and column of *S. pfavii*. It must be noted that the fly illustrated here is oriented the other way around from what is normally observed; it got the pollinia stuck to the head and not scutellum as would be expected (shown in 13d). Photographs by FP (A, B) and APK (C-E). A and B are copyrighted images reproduced from Phytotaxa 63: 1-20, with permission.

Pollinator Biology:—Flies visit the flowers for up to at least 24 h at a time, during that time they mostly remain on the flowers but occasionally leave for a few minutes and return. Visitation can happen anytime in greenhouse conditions, however it is more frequent in the early morning and late afternoon, possibly when temperatures are lower. With time passing by the visiting flies' motility is greatly reduced, becoming slower and less aware of their surroundings. They can visit singly or in groups of up to 7 individuals (possibly more). The flies move around and "inspect" the entire flower but spend most time (just above 90% of the time spent) on the papillae rich areas in the adaxial surface of the sepals (Fig. 74a & b), on which they suck during the entire time they are there [Supplementary Information - Video 1]. The attraction for the *Specklinia*'s sepals is so strong that even after immediate removal of one of them from the flower the flies still inspect it (Fig. 74c). The removal of all three sepals completely prevents flies from visiting the flowers. Amongst the most observed behaviours were: 1) fencing with the forelegs, occurring once every three minutes; 2) wing flapping and following of other flies, which are done constantly; 3) abdomen bending, about twice in three minutes [Supplementary Information - Video 2]. Two additional events were observed very rarely: 1) copulation, seen twice during the whole study period [Supplementary Information - Video 3]; and a fly with a regurgitated drop in its mouthparts, seen only once during the study period (Fig. 74d). The flies wander from sepal to sepal, frequently stepping on the movable lip. There they explore the conical rugose papillae and when placed in the right position, tilt the lip and are adpressed against the viscid rostellum (Fig. 75). The pollinia (which lack caudicles) are flattened and curved towards the base, and normally grasp the scutellum of the fly whilst the animal tries to leave the column/lip cavity in reverse [Supplementary Information - Video 4]; it can take the fly 20-30 min to liberate itself.

Pollinator Identities:—Fifty-six (56) flies were caught at the greenhouses at Lankester Botanical Garden in Costa Rica, 2 were field collected, and 2 were collected in a private garden (Table 14; Fig. 76). A total of 20 were collected on flowers of *S. remotiflora*, 20 on *S. spectabilis*, 14 on *S. pfavii*, 5 on *S. endotrachys* and 3 on *S*. sp. The flies caught all belonged to the genus *Drosophila* (Drosophilidae), except for one that belonged to genus *Hydrotaea* (Muscidae) and another to the Lauxaniidea. Of the specimens caught, 54 belong to the *Repleta* species group, 2 to the *Coffeata* group, 2 to the *Immigrans* group and 2 to an unknown species group. The *Drosophila* species found were *D. hydei* (35 samples), *D. mercatorum* (7 samples), *D*. aff. *repleta* 1 (2 samples), *D*. aff. *repleta* 2 (4 samples), D. ananassae (2 sample), *D. fuscolineata* (2 samples), *D. immigrans* (2 sample), *D*. aff. *bifurca* (1 sample) *D. nigrohydei* (1 sample), and *D*. spp. (4 different species, a sample each). *Drosophila hydei* was collected on four out of the five species of *Specklinia*, whilst the *D*. aff. *repleta* was found on 3 out of 5. *Drosophila fuscolineata*, *D. immigrans* and *D. mercatorum* were found on two out of the five species of *Specklinia*. All other *Drosophila* species were collected only on one species of *Specklinia*, and the single specimens of *Hydrotaea* (Muscidae) and Lauxaniidae were collected on *S. spectabilis*. Among the flies caught we identified 36 males and 24 females (Table 15).

Floral volatiles and droplets:—A mix of ethyl tiglate, methyl tiglate and isopropyl tiglate was analysed and used as a standard. The signal for the three standards was found back at 3.11 min, 2.48 min and 3.45 min respectively (Fig. 77a). The analysis of individual flowers shows a greater variety of signals, most of which have not been identified. Nonetheless, it is safe to say that ethyl tiglate, methyl tiglate and isopropyl tiglate can be found in both *Specklinia pfavii* (*JBL-11086*) and *S. spectabilis* (*Bogarín 7401*) as strong signals can be found extremely close to the standard times (Fig. 77b & d). In the samples of *S. remotiflora* (*Karremans 4846*) only signals similar to those of the standards of ethyl tiglate and methyl tiglate, and not isopropyl tiglate were found back (Fig. 77c).

The solution of drops collected on the adaxial surface of the sepals turned bright orange with the addition of the Fehling's reagents, evidencing the high sugar content of the drops.

Microstructures:—*SEM*. Lip - The adaxial surface of the lip is completely covered with scale-like epidermal cells. The scales are rounded and flattened towards the apex of the lip, whereas towards the base they are sharply angled and uplifted. The cuticle is somewhat rugose, however without pores or signs of ruptures of any kind. The basal scales are filamentous, and those filaments are capitate (Fig. 78a & b). Petals - Both surfaces of the petals are warty, especially near the apex. The cuticle is smooth, not ornamented, and without pores or signs of ruptures of any kind.

Table 14. Diptera specimens caught on the flowers of the *Specklinia endotrachys* species complex.

Specimen	Sex	Genus	Species	Subgenus	Orchid Species	Origin	BOLD
14003-34	Male	*Drosophila*	*ananassae*	unknown	*S. spectabilis*	JBL	ORCPL050-14
13026-17	Female	*Drosophila*	*ananassae*	unknown	*S. remotiflora*	JBL	ORCPL017-14
14003-14	Male	*Drosophila*	*bifurca aff.*	*repleta*	*S. sp.*	Private	ORCPL031-14
13026-19	Female	*Drosophila*	*fuscolineata*	*coffeata*	*S. pfavii*	JBL	-
13026-10	Male	*Drosophila*	*fuscolineata*	*coffeata*	*S. remotiflora*	Field	ORCPL010-14
13026-01	Female	*Drosophila*	*hydei*	*repleta*	*S. endotrachys*	JBL	ORCPL001-14
14003-17	Male	*Drosophila*	*hydei*	*repleta*	*S. endotrachys*	JBL	ORCPL034-14
14003-18	Male	*Drosophila*	*hydei*	*repleta*	*S. endotrachys*	JBL	ORCPL035-14
14003-26	Male	*Drosophila*	*hydei*	*repleta*	*S. endotrachys*	JBL	ORCPL043-14
14003-35	Male	*Drosophila*	*hydei*	*repleta*	*S. endotrachys*	JBL	ORCPL051-14
13026-11	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL011-14
13026-18	Female	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	-
14003-07	Female	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL024-14
14003-10	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL027-14
14003-11	?	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL028-14
14003-12	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL029-14
14003-13	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL030-14
14003-16	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL033-14
14003-19	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL036-14
14003-20	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL037-14
14003-21	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL038-14
14003-22	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL039-14
14003-23	Male	*Drosophila*	*hydei*	*repleta*	*S. pfavii*	JBL	ORCPL040-14
13026-12	Female	*Drosophila*	*hydei*	*repleta*	*S. remotiflora*	JBL	ORCPL012-14
13026-14	Male	*Drosophila*	*hydei*	*repleta*	*S. remotiflora*	JBL	ORCPL014-14
13026-20	Male	*Drosophila*	*hydei*	*repleta*	*S. remotiflora*	JBL	-
13026-22	Female	*Drosophila*	*hydei*	*repleta*	*S. remotiflora*	JBL	-
13026-23	Male	*Drosophila*	*hydei*	*repleta*	*S. remotiflora*	JBL	-
13026-24	Female	*Drosophila*	*hydei*	*repleta*	*S. remotiflora*	JBL	-
-	Male	*Drosophila*	*hydei*	*repleta*	*S. remotiflora*	JBL	-
13026-03	Male	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	ORCPL003-14
13026-25	Male	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	-
13026-26	Female	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	-
13026-27	Female	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	-
13026-29	Female	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	-
13026-30	Female	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	-
-	Female	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	-
14003-09	Female	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	ORCPL026-14
14003-27	Male	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	ORCPL044-14
14003-39	Male	*Drosophila*	*hydei*	*repleta*	*S. spectabilis*	JBL	ORCPL055-14
14003-15	Female	*Drosophila*	*immigrans*	*immigrans*	*S. sp.*	Private	ORCPL032-14
13026-21	Male	*Drosophila*	*immigrans*	*immigrans*	*S. remotiflora*	JBL	-
13026-13	Male	*Drosophila*	*mercatorum*	*repleta*	*S. remotiflora*	JBL	ORCPL013-14
13026-15	Male	*Drosophila*	*mercatorum*	*repleta*	*S. remotiflora*	JBL	ORCPL015-14
13026-16	Female	*Drosophila*	*mercatorum*	*repleta*	*S. remotiflora*	JBL	ORCPL016-14
14003-25	Male	*Drosophila*	*mercatorum*	*repleta*	*S. spectabilis*	JBL	ORCPL042-14
14003-28	Male	*Drosophila*	*mercatorum*	*repleta*	*S. spectabilis*	JBL	ORCPL045-14
14003-30	Male	*Drosophila*	*mercatorum*	*repleta*	*S. spectabilis*	JBL	ORCPL047-14
14003-38	Male	*Drosophila*	*mercatorum*	*repleta*	*S. spectabilis*	JBL	ORCPL054-14
13026-08	Male	*Drosophila*	*nigrohydei*	*repleta*	*S. remotiflora*	JBL	ORCPL008-14
13026-07	Female	*Drosophila*	*repleta aff. 1*	*repleta*	*S. remotiflora*	JBL	ORCPL007-14
13026-04	Female	*Drosophila*	*repleta aff. 1*	*repleta*	*S. remotiflora*	JBL	ORCPL004-14
14003-24	Male	*Drosophila*	*repleta aff. 2*	*repleta*	*S. sp.*	JBL	ORCPL041-14
13026-09	Female	*Drosophila*	*repleta aff. 2*	*repleta*	*S. remotiflora*	Field	ORCPL009-14
14003-08	?	*Drosophila*	*repleta aff. 2*	*repleta*	*S. spectabilis*	JBL	ORCPL025-14
14003-31	Male	*Drosophila*	*repleta aff. 2*	*repleta*	*S. spectabilis*	JBL	ORCPL048-14
13026-02	Female	*Drosophila*	*sp. 1*	*repleta*	*S. remotiflora*	JBL	ORCPL002-14
13026-05	Male	*Drosophila*	*sp. 2*	*repleta*	*S. remotiflora*	JBL	ORCPL005-14
13026-06	Female	*Drosophila*	*sp. 3*	*repleta*	*S. remotiflora*	JBL	ORCPL006-14
14003-33	Female	*Drosophila*	*sp. 4*	*repleta*	*S. spectabilis*	JBL	ORCPL049-14
13026-28	Female	Lauxaniidae	*unknown*	unknown	*S. spectabilis*	JBL	-
14003-29	Female	Hydrotaea	*unknown*	unknown	*S. spectabilis*	JBL	ORCPL046-14

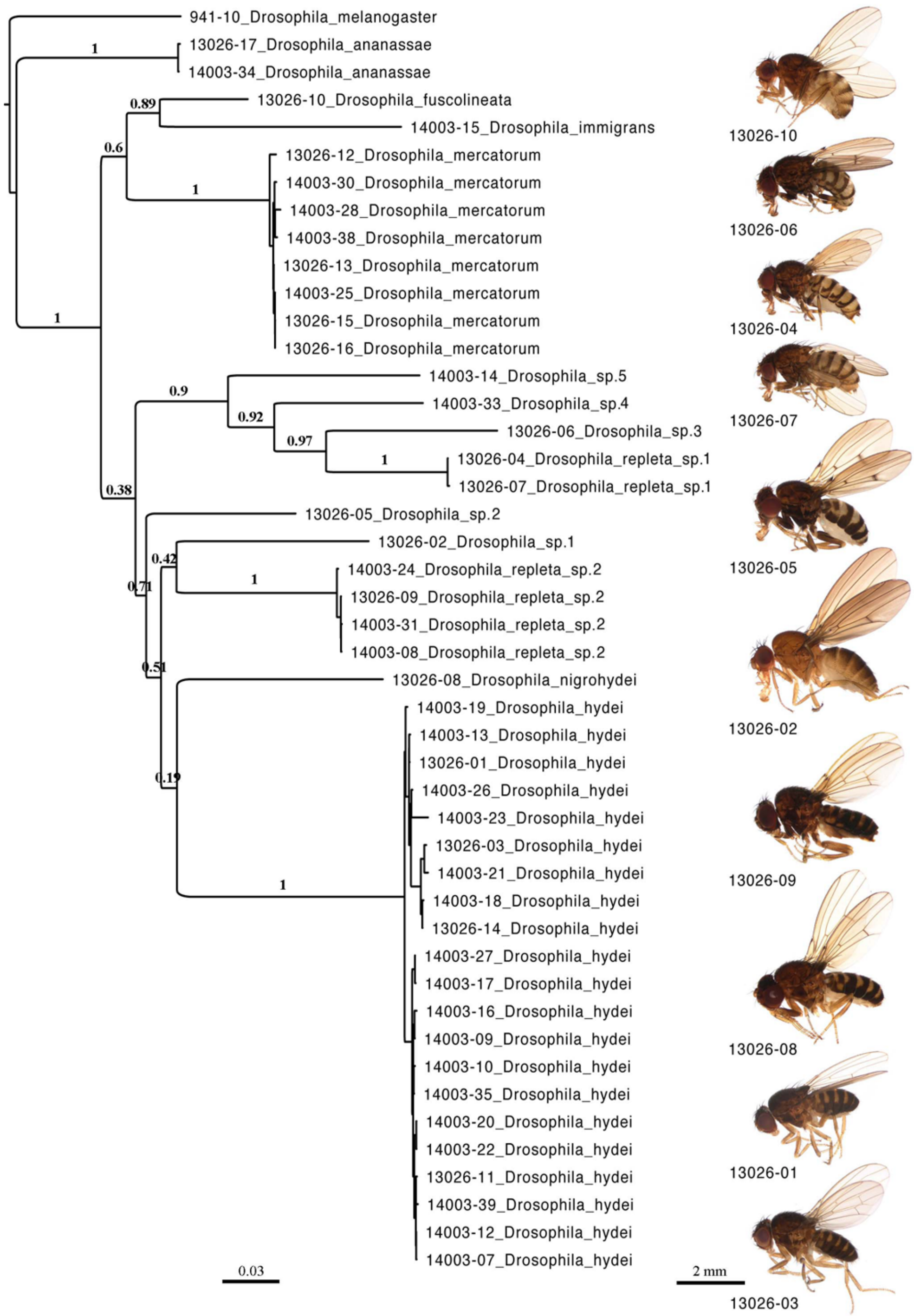


FIGURE 76. Phylogenetic relationship amongst the collected fly specimens. The trees were produced with an analysis of the COI dataset using BEAST v1.6.0. Parameters were set to preset, except for substitution model GTR with 10 categories, clock model uncorrelated exponential, tree prior Yule process, and number of generations 20,000,000. The resulting trees were combined using TreeAnnotator v1.6.0., were the first 3000 trees were used as burn-in. Node values are posterior probabilities. Edited by APK using using FigTree v.1.3.1. Photographs by KB.

Table 15. Diptera species caught summarised per orchids species and sex.

		Orchid Species					
		S. endotrachys	*S. pfavii*	*S. remotiflora*	*S. spectabilis*	*S. sp.*	**Total**
Fly species	*D. ananassae*			1Female	1 Male		1 Male 1 Female
	D. bifurca aff.					1 Male	1 Male
	D. fuscolineata		1 Female	1 Male			1 Male 1 Female
	D. immigrans			1 Male		1 Female	1 Male 1 Female
	D. hydei	4 Male 1 Female	1 + 10 Male 2 Female	4 Male 3Female	4 Male 6 Female		1 + 22 Male 12 Female
	D. mercatorum			2Male 1 Female	4 Male		6 Male 1 Female
	D. nigrohydei			1 Male			1 Male
	D. repleta aff. 1			2 Female			2 Female
	D. repleta aff. 2			1 Female	1 + 1 Male	1 Male	1 + 2 Male 1 Female
	D. sp. 1			1 Female			1 Female
	D. sp. 2			1 Male			1 Male
	D. sp. 3			1Female			1 Female
	D. sp. 4				1Female		1 Female
	unknown				2Female		2 Female

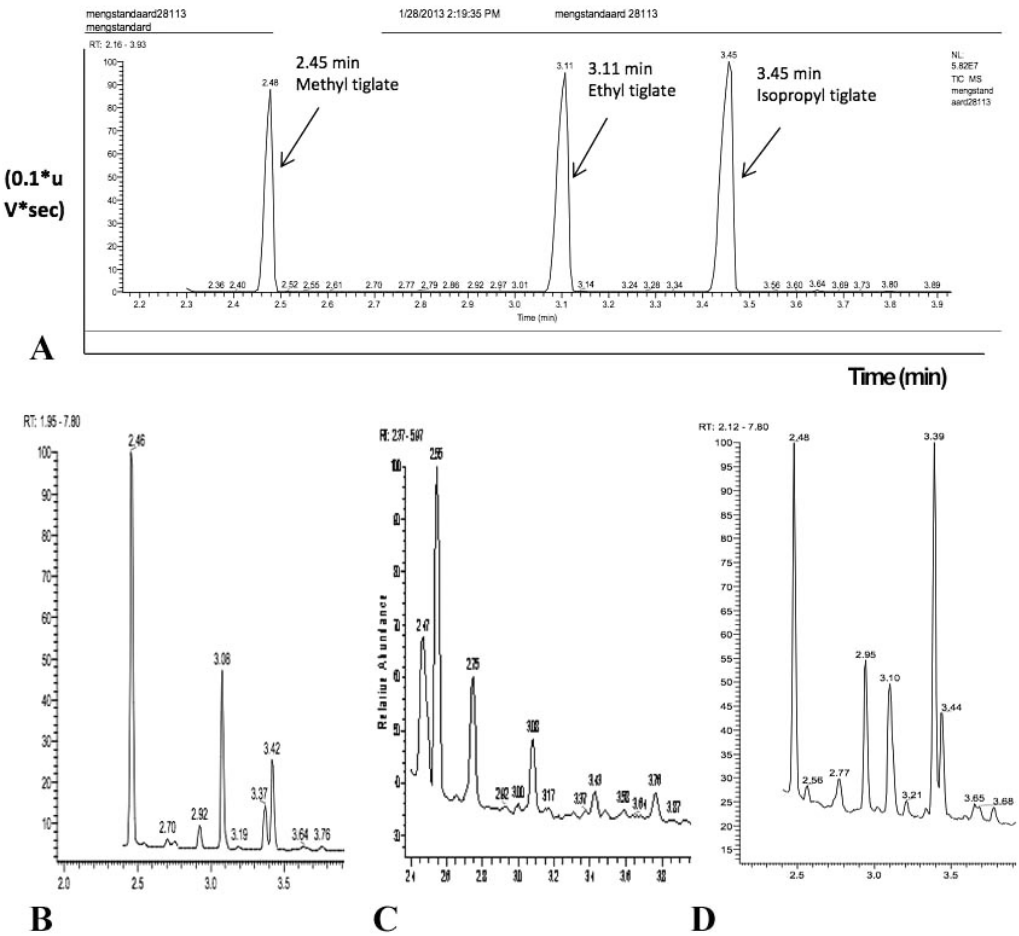


Figure 77. Standard mix of ethyl tiglate, methyl tiglate and isopropyl tiglate measured with GCMS-ITD. A. The graph shows the selected ion monitoring (SIM) signal over time. B. *Specklinia pfavii* (*JBL-11086*) C. *Specklinia remotiflora* (*Karremans 4846*). D. *Specklinia spectabilis* (*Bogarín 7410*). X axis = Time (min); Y axis = Signal (0.1*uV*sec). Figures by Misli Kaya.

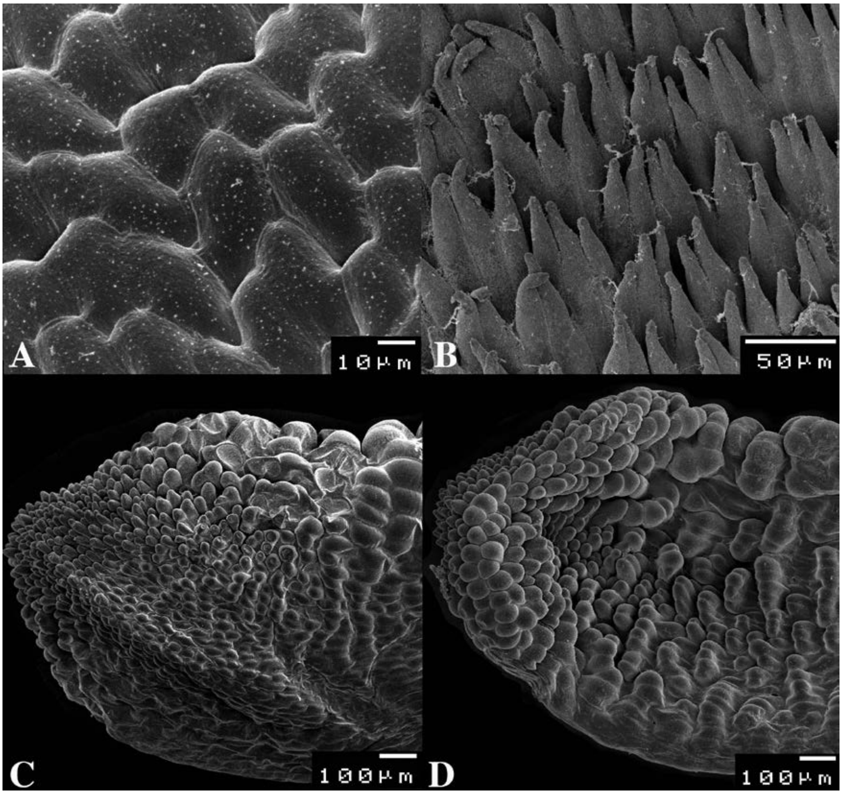


FIGURE 78. Micrographs of *Specklinia pfavii (JBL-11086)*: Scales cover the adaxial surface of the lip; they are flattened and rounded near the apex (A) and elevated and filamentous-capitate closer to the base (B); warts cover the outer (C) and inner (D) surfaces of the petals, especially apically. Photographs by APK.

(Fig. 78c & d). Sepals - The adaxial epidermis of all three sepals is densely rugose and covered with warts, except basally. The apex of each wart carries stomata. The stomata have wide pores and 5-6 somewhat inflated subsidiary cells. The cuticle is somewhat sculptured, not ornamented, and without pores or signs of ruptures of any kind. The stomata were permanently open and no movements were observed (Fig. 79a-d). The abaxial epidermis is smooth and mostly constantly flat, except for rare depressed areas were a sunken trichome is located; this trichome is apically irregular (Fig. 79e & f).

LM. The transversal section of the lip of *S. remotiflora* shows mostly large, rounded parenchyma cells and smaller scaly or pyriform epidermis cells on the adaxial surface (Fig. 80a & b). The petals of *S. pfavii* (Fig. 80c) and S. remotiflora (Fig. 80d) show irregularly, enlarged secretory parenchyma cells, though without apparent openings. The transversal section of the sepal shows two basic cells types, ground parenchyma near the abaxial surface (underpart in fig. 80e), which are larger and sub-rectangular, and secretory parenchyma close to and in the adaxial epidermis (upper part in fig. 80e), which are smaller and rounded. The vascular bundles are visible. The adaxial epidermis is irregular and frequently has stomata, which can be seen in both *S. pfavii* (Fig. 80f) and S. remotiflora (Fig. 80g) as prominent protrusions with an apical opening. The abaxial epidermis in turn is inornate except for the occasional sunken trichomes that can be spotted perforating the surface (Fig. 80h).

TEM. The pores of the stomata are commonly spotted in the transversal section of the adaxial epidermis of the sepals of *S. pfavii*; the subtending guard cells can be distinguished from the subsidiary cells basically by a thicker cell wall. Nevertheless their cytoplasms share the presence of mainly a nucleus with nucleolus, large vacuoles, and a high starch content, with the cells surrounding the subsidiary cells (Fig. 81).

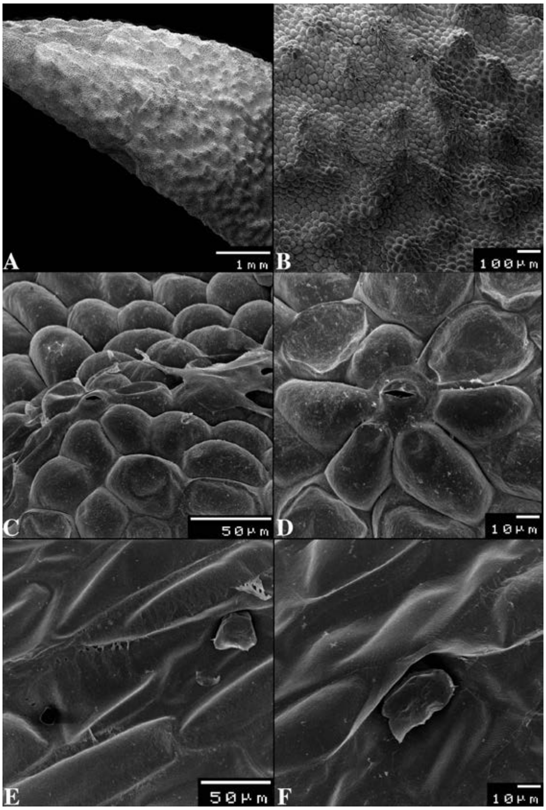


Figure 79. Micrographs of the sepals of *Specklinia pfavii* (*JBL-11086*): A median segment of a lateral sepal showing corrugation of the adaxial surface (A); the elevated cells form papillae, corrugating the adaxial surface (B); the apices of the papillae are formed by nectar secreting actinocytic stomata (C), formed by guard cells and six subsidiary cells (D); the abaxial surface is formed by flattened cells, with occasional depressions that contain a sunken trichome (E & F). Photographs by APK.

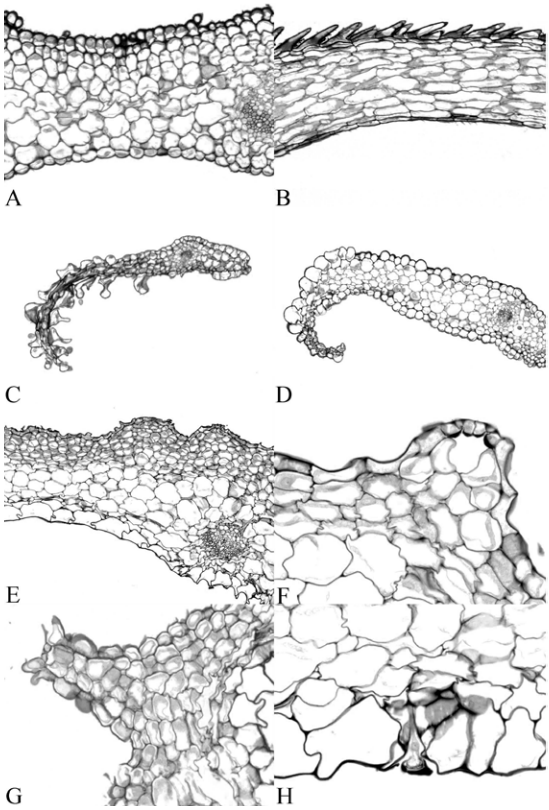
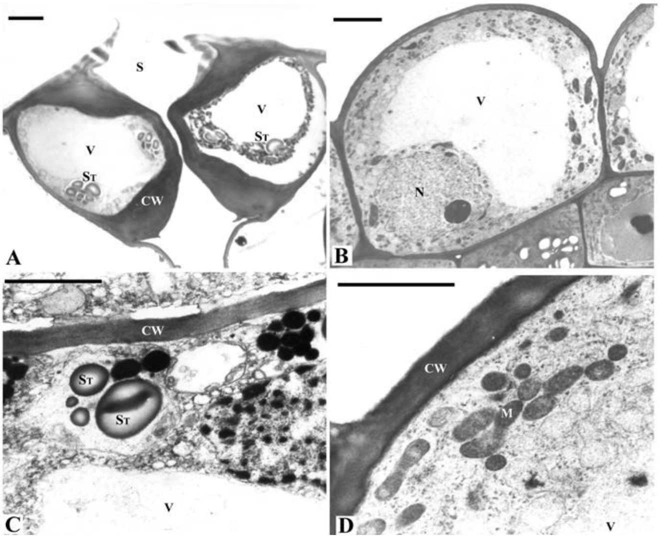


Figure 80. Light Micrographs of *S. pfavii* - *AK4835* (C, E, F, H) and *S. remotiflora* - *AK4798* (A, B, D, G): Transversal section of the lip, showing vascular bundles and keels (A); transversal section of one of the keels, showing the scale-like cells on the adaxial surface (B); large, irregular cells on the transversal section of the petal (C & D); vascular bundles and stomata visible on transversal section of the sepal (E); secretory stomata on the adaxial epidermis (F & G); sunken trichomes on the abaxial epidermis (H). Taken at 200×, 100×, 50×, 100×, 100×, 400×, 200×, 400× respectively.



Left, figure 81. Transmission Electron Micrographs (TEM) of the transversal section of the adaxial epidermis of the sepals of *S. pfavii*, showing the stomata's opened guard cells (A), their subsidiary cells (B), showing the common starch grains (C) and variously sized vesicles (E). Scale bars: A & B = 2 μm, C & D = 1 μm. CW, Cell Wall; M, Mitochondrion; N, Nucleus; S, Stomata; St, Starch; V, vacuole. Photographs by Rob Langelaan.

Electroantennography study:—*Drosophila hydei* is highly sensitive to the stimuli ethyl tiglate, methyl tiglate and isopropyl tiglate and clear dose response relations were found (Fig. 82) The highest response measured was -6.563 mV for the positive control Z-3-Hexen-1-ol (100 % by definition), while the highest values for isopropyl, ethyl and methyl tiglate were -4.462 mV, -4.361 mV and -3.328 mV, respectively. To investigate the effects of concentration, a generalised linear mixed model was used that contained in addition to the random factor 'individual' both the explanatory variables compound and concentration, as well as their interaction (Table 16a). The coefficients for the effects of these factors (Table 16b) showed a highly significant concentration effect, as expected for biological relevant stimuli. Ethyl tiglate (the reference in the linear model) gave a significantly stronger response than methyl tiglate (indicated by the negative coefficient for methyl tiglate), but does not show an interaction, i.e. the slope of the dose response curve was similar for both compounds. In contrast isopropyl tiglate did show an interaction, and the slope of the dose response curve was significantly less steep than that for ethyl tiglate (Fig. 82).

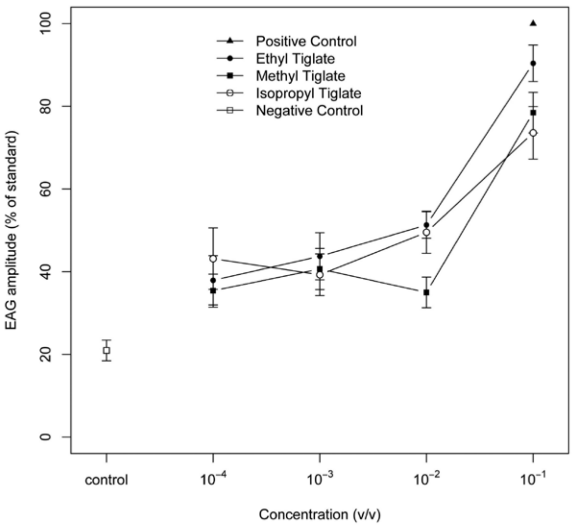


Figure 82. Dose response relationships for the electro antennogram measurements in 14 *Drosophila hydei* flies reacting to ethyl tiglate, methyl tiglate and isopropyl tiglate. All amplitudes are expressed as percentage of the external standard (positive control) Z-3-Hexen-1-ol.

Table 16a. Overview of the tested mixed models ordered by their corrected Aikake information criterium (AICc) values. The best model (top line) contained both concentration and compound as explanatory variables and their interaction.

Model structure	*K*	*AICc*	*Delta_AICc*	*AICcWt*	*Cum.Wt*	*LL*
stimulus * concentration	8	1382.23	0	0.62	0.62	-682.64
stimulus + concentration	6	1383.49	1.26	0.33	0.95	-685.47
concentration	4	1387.36	5.13	0.05	1	-689.55
Null model	3	1503.01	120.78	0	1	-748.43
stimulus + insect	5	1503.59	121.36	0	1	-746.6

Table 16b. Summary of the coefficients in the best model from table 3a. The intercept gives the estimate of the coefficient for ethyl tiglate, the other estimates are indicating the changes in relation to this reference. A highly significant contribution of stimulus concentration was found. Except concentration, all of the coefficients are negative, indicating that ethyl tiglate stimulated the flies significantly better than methyl tiglate The negative coefficient for the interaction of isopropyl tiglate with concentration indicates that the response differed from ethyl tiglate in a concentration dependent way (see also Fig. 82).

Coefficient	*Estimate*	*se*	*Wald z*	*P*	*Sig.*
Intercept	42.55	3.7627	11.3082	0.0000	***
methyl tiglate	-7.2331	3.4729	-2.0828	0.0373	*
Concentration	47.4570	4.9990	9.4932	0.0000	***
isopropyl tiglate : Concentration	-16.5305	7.0646	-2.3399	0.0193	*
methyl tiglate : Concentration	-5.0388	7.0597	-0.7137	0.4754	
Variance (1|insect)	112.48				
Variance (|Residual)	227.31				

Discussion

In Pupulin *et al.* (2012, Chapter 1), we established that under the name *Specklinia endotrachys* at least four similar, yet distinct, recognisable species should be treated. Our findings show that the pollination syndrome of *S. endotrachys*, *S. pfavii*, *S. spectabilis* and *S. remotiflora* is basically the same one. Both male and female flies are arrested by pheromones liberated from the flower sepals. Once on the abaxial surface the pollinators "walk" from sepal to sepal "sucking" on the warty surface of the sepals, where nectar drops have formed on the apex of the stomatal pore. The flies can be seen in groups and spend up to +24 hours, reducing their overall motility, but continuously feeding on the flowers. They display a variety of behaviours including fencing with the forelegs, flapping their wings, following other flies, bending their abdomen, and occasionally copulating. Whilst wandering from sepal to sepal the flies explore the column/lip cavity. When placed in the right position, the fly makes the movable lip tilt and is then adpressed against the viscid rostellum. The pollinia are removed whilst the fly attempts to escape from the cavity.

Biology of the *Specklinia* species studied:—Species of the *S. endotrachys* complex are found in large colonies of dozens of plants. They produce long-lived multi-flowered successive inflorescences, with up to +20 flowers over time. Each plant may be flowering for several months at a time. Overall frutification was found to be low, both in the field and in the greenhouse, making it likely that large colonies and long term flowering are necessary to attain fruitset. *Specklinia endotrachys*, *S. pfavii*, *S. remotiflora* and *S. spectabilis* have all been found growing in Costa Rica, nevertheless never sympatrically (Pupulin *et al*. 2012, Chapter 1; Fig. 83). *Specklinia endotrachys* is a mid-elevation species found only in the north of the country, *S. pfavii* and *S. spectabilis* are lowland species, growing on the pacific and caribbean watersheds respectively, of the Central and Talamanca mountain ranges, whilst *S. remotiflora* is only found in the highland cloud forests close to the continental divide in the south of the Talamanca mountain range. The +2000 m high mountain range serves as a barrier separating the populations of the four species. Allopatry facilitates divergence by both interrupting gene flow and allowing local adaptation without the necessity of high floral divergence or for that matter pollinator shifts (Harder & Johnson 2009).

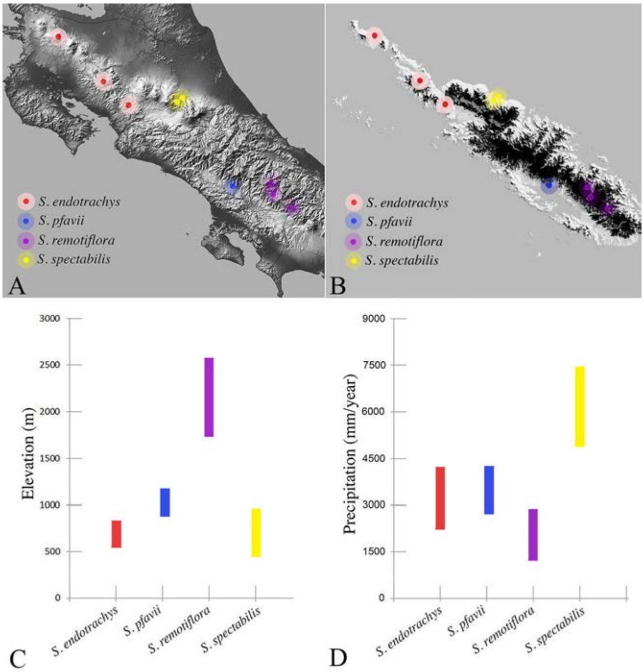


Figure 83. Distribution and ecological preferences of the *Specklinia endotrachys* group in Costa Rica. A. Actual known distribution. B. Distribution with elevations below 400 m converted to sea and elevations above 1500 m blackened. C. Distribution of elevation of found specimens. D. Distribution of precipitation in the areas where the specimens have been found.

Reddish-orange coloured flowers are characteristic of species of the *S. endotrachys* group and close relatives. Although not unique within the Orchidaceae, those colour patterns are uncommon in the family and are notoriously rare in subtribe Pleurothallidinae. Oliveira *et al.* (2012) found that species of the *Drosophila repleta* group, including *D. hydei* and *D. mercatorum*, predominantly use *Opuntia* fruits, which are commonly reddish-orange in colour, for feeding and breeding. It is likely that there is selective pressure on these *Specklinia* species to have and maintain similar colour patterns.

Nectar drops accumulate on the adaxial surface of the sepals of all species of the *S. endotrachys* complex (Fig 73 & 74d). The drops have a pasty consistency, high sugar content, and are persistent unless removed. Practically the entire surface of the sepals is covered with actinocytic stomata which are found elevated on the apex of each one of those warts as can be appreciated in the SEM photographs (Fig. 79). The transversal sections of those stomata, taken with the LM (Fig. 80) and TEM (Fig. 81), evidence high cellular activity in the stomatal guard and subsidiary cells. Starch grains, which are likely to be used as energy source for the production of nectar, are commonly observed. No clear drops nor evidence of nectar secreting stomata are found on the petals and lip, however they are found entirely covered by papillae. Those papillae are morphologically similar to secretory papillae found by Stpiczyńska and Matusiewicz (2001) in the nectary of *Gymnadenia*, and de Melo *et al.* (2010) on the lip of *Acianthera*. The high cellular activity in addition to the presence of nectar residues on the rugose surfaces (Fig. 8 & 9) might well be indicatory of secretory papillae of the lip and petals here as well but this needs further studies.

Using GC-MS we have been able to determine that ethyl tiglate, methyl tiglate and isopropyl tiglate, all of which have been cited as aggregation pheromones for *Drosophila hydei* (Moats *et al.* 1987), are being produced by the flowers of the *Specklinia endotrachys* complex (Fig. 77). The aggregation pheromones, albeit not the only substances produced by the flowers, are likely being released from the sepals, which have been cited to produce and release volatiles (Antoń *et al.* 2012; Kowalkowska *et al.* 2014).

Biology of the *Drosophila* species studied:—Aggregative behaviour in *Drosophila* is mediated by pheromones that can act in concert with odours of the habitat of the flies and indicate a suitable habitat for mating and oviposition (Moats *et al.* 1987; Markow & O'Grady 2005). The pheromones are produced by males and attract flies of both sexes (Bartelt *et al.* 1985, 1986, 1988), as also found here (Table 14 & 15). Aggregation pheromones of *Drosophila* are generally volatile esters, ketones or unsaturated hydrocarbons (Bartelt *et al.* 1985; Hedlund *et al.* 1996). Using EAG experiments we confirmed that *D. hydei* is sensitive to ethyl tiglate, methyl tiglate and isopropyl tiglate, and responds to concentrations as low as 1.0×10^{-5} of the pure substance (Fig. 82; Table 16a, b). The measured concentration of the tiglates in the flowers was about 1µg/L.

Once on the flower the flies wander around feeding on the nectar drops accumulated on the sepals, and displaying courtship behaviours. Following the female, orienting towards her, tapping her with his forelegs, contacting her genitalia with his mouthparts, singing a species-specific courtship song, and bending his abdomen, are commonly cited as courtship behaviour for several *Drosophila* species (Greenspan & Ferveur 2000; Villella & Hall 2008). In *Drosophila subobscura*, nuptial gifts in the sense of males gifting their crop contents in the form of a regurgitated drop have been suggested to play an important role in sexual selection (Steele 1986; Immonen *et al*. 2009). Copulation, albeit rare, was also observed on the *Specklinia* flowers. No oviposition events nor eggs or larvae were ever found. Markow and O'Grady (2005) point out that for any given species mating takes place at particular locations and at specific times of the year and/or day. Markow (1988) found that *Drosophila* species exhibit distinct behaviour patterns on different pieces of fruits. In that study the author found that males of *D. melanogaster* court females on the feeding site (decaying fruit), while females of *D. nigrospiracula* would fly to non-resource-based male territories where the majority of copulations occur; oviposition was found to occur on newly exposed flesh and not elsewhere (Markow 1988).

About 85% of the caught specimens, including samples of both *D. hydei* and *D. repleta*, belong to the *Drosophila repleta* species group. Males of the *Repleta* group have a tendency to court behind the females, suggesting that male visual displays are not the primary form of sexual signalling as in other taxa, which is consistent with having almost no sexual dimorphism in coloration, wing pattern and other morphological traits (Markow & O'Grady, 2005). Adults of most species will feed on a range of food sources, however, ovipositions and larval development are typically more restricted (Carson 1974). The *Repleta* group includes many cosmopolitan species with a neartic and neotropical distribution, which reportedly use both fruits and cacti as breeding sites (Markow 1988; Markow & O'Grady 2005; Markow & O'Grady 2008; Oliveira *et al*. 2012). A particular species of *Drosophila* may feed and breed exclusively in a resource such as flowers (Brncic 1983; Markow & O'Grady 2008). However, together with the lack of observed oviposition events, absence of eggs and larvae, and the short lived flowers, it is safe to say that the flowers of *Specklinia* are a feeding site but not a breeding site for these flies.

Conclusions

We find that *Specklinia endotrachys*, *S. pfavii*, *S. spectabilis* and *S. remotiflora* share not only the same basic pollination syndrome but are also pollinated by the same species of the *Drosophila repleta* group of flies. Species of several unrelated genera of Pleurothallidinae, including *Acianthera*, *Dracula*, *Masdevallia*, *Specklinia* and *Stelis* (*sensu* Pridgeon 2005), share a similar system in which the pollinia removal occurs when a fly is pushed against the column once it walks over the lip; whilst exiting in reverse, the pointed scutellum is smeared with a viscid substance found in the rostellum, and the pollinia are removed by touching their twisted base. In those genera the observed pollen removal is reported to be done mostly by flies of the families Chloropidae, Drosophilidae and/or Phoridae (Chase 1985; Duque 1993; Borba & Semir 2001; Albores & Sosa 2006; Endara *et al*. 2010; de Melo *et al.* 2010). It is thus essentially how the fly is guided to visit the column/lip cavity that differs between these different pleurothallid species' groups.

Pheromones are likely to play an important role in initially aggregating Diptera species to pleurothallid flowers. Blanco and Barboza (2005) supposed that species of *Lepanthes*, which are pollinated by pseudocopulation, attracted male fungus gnats using sexual pheromones. Here we have been able to confirm for the first time that aggregation pheromones are being released from the sepals of *Specklinia* species to attract pollinators. The use of pheromones, be it sexual or aggregation, might be generalised in Pleurothallidinae considering that a wide range of species have secretory structures. Scent is likely to play an important role in specific pollinator attraction thus mediating reproductive isolation (Peakall *et al.* 2010).

Nectar guides are also commonly used by pleurothallids to guide the pollinators to the lip/column cavity. Many studies seem to report no "measurable" or "obvious" rewards, however evidence for nectar guides is frequently found in more detailed pollination studies in the pleurothallids (Borba & Semir 2001; Barbosa *et al*. 2009; de Melo *et al*. 2010; Duque-Buitrago *et al*. 2014). Smith (2010) found that the appearance of nectary glands lead to an increase in reproduction success. Pollination efficiency was found to be significantly lower in food deceptive orchids as compared to rewarding species (Tremblay *et al*. 2005; Scopece *et al.* 2010), and several authors have suggested that deceitful species must be much less frequent than rewarding ones otherwise the evolution of lack of reward is difficult to explain (Darwin 1862; Smithson 2006). In fact we wonder if the cases in which orchids are being considered non-rewarding are not highly over-estimated.