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doi:10.1093/aob/mcv086, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

PART OF A HIGHLIGHT ON ORCHID BIOLOGY

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

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Received: 17 October 2014 Returned for revision: 12 February 2015 Accepted: 5 May 2015

• **Background and Aims** The first documented observation of pollination in Pleurothallidinae was that of Endrés, who noticed that the 'viscid sepals' of *Specklinia endotrachys* were visited by a 'small fly'. Chase would later identify the visiting flies as being members of the genus *Drosophila*. This study documents and describes how species of the *S. endotrachys* complex are pollinated by different *Drosophila* species.
• **Methods** Specimens of *Specklinia* and *Drosophila* were collected in the field in Costa Rica and preserved in the JBL and L herbaria. Flies were photographed, filmed and observed for several days during a 2-year period and were identified by a combination of non-invasive DNA barcoding and anatomical surveys. Tissue samples of the sepals, petals and labellum of *Specklinia* species were observed and documented by SEM, LM and TEM. Electroantennogram experiments were carried out on *Drosophila hydei* using the known aggregation pheromones ethyl tiglate, methyl tiglate and isopropyl tiglate. Floral compounds were analysed by gas chromatography–mass spectometry using those same pheromones as standards.
• **Key Results** Flowers of *S. 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 male and female *Drosophila* to linger on the flowers for several hours at a time. The flies frequently show courtship behaviour, occasionally copulating. Several different *Drosophila* species can be found on a single *Specklinia* species.
• **Conclusions** Species of the *S. endotrachys* group share a similar pollination syndrome. There seem to be no species-specific relationships between the orchids and the flies. It is not expected that *Specklinia* species will hybridize naturally as their populations do not overlap geographically. The combination of pheromone attraction and nectar feeding is likely to be a generalized pollination syndrome in Pleurothallidinae.

Key words: Aggregation pheromones, courtship, deceit, deceptive pollination syndrome, *Drosophila repleta* group, nectar-secreting stomata, Orchidaceae, Pleurothallidinae, pollination biology, *Specklinia endotrachys*, *Specklinia pfavii*, *Specklinia spectabilis*, *Specklinia remotiflora*.

INTRODUCTION

Epiphytism is likely to be the major contributor to the species richness in Orchidaceae, 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) argued that this adaptation is probably unilateral, without change in the pollinator (Williams, 1982), and 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 and Dodson, 1966; Cozzolino and Widmer, 2005). Food deception has evolved repeatedly in different angiosperm groups, but is mostly restricted to a few species per family (Renner, 2005), whereas estimates suggest that a third of all orchids might be food-deceptive (Ackerman, 1986), and it seems to have arisen

many times independently in Orchidaceae. Sexual deception has been reported in several phylogenetically unrelated orchid clades (van der Pijl and Dodson, 1966; Adams and Lawson, 1993; Singer, 2002; Ayasse *et al*., 2003; Singer *et al*., 2004; Blanco and Barboza, 2005; Ciotek *et al*., 2006; Phillips *et al*., 2009; Peakall *et al*., 2010). If confirmed to be a generalized syndrome in these species-rich groups, sexual deceit might well represent up to 10 % of the pollination syndromes in Orchidaceae.

Together, those percentages would suggest that deceitful pollination could represent close to half of all pollination syndromes in orchids. However, considering that only a few orchid–pollinator relationships have been studied in detail, and several of these have found non-obvious floral rewards being offered to pollinators, including scents, triterpenoid resins, pseudopollen, lipid-rich substances and small amounts of nectar and oils (Davies and Turner, 2004; Mickeliunas *et al*., 2006; Pansarin and Amaral, 2006; Whitten *et al*., 2007; Pansarin *et al*., 2008, 2013; Stpiczyńska and Davies, 2008; Chase *et al*., 2009; Pansarin and Pansarin, 2011; Papadopulos *et al*., 2013; Davies *et al*., 2014), non-obvious floral rewards might lead to an over-estimation of the number of cases in which orchids offer no reward at all. One such example is the *Specklinia endotrachys* species complex (Pleurothallidinae).

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

Endrés, in 1878, noted that flies were attracted to the nectar present in flowers of *S. endotrachys* (quoted by Pupulin *et al*., 2012). Chase (1985) observed *Drosophila immigrans* visiting and pollinating *Specklinia spectabilis*. He noted that flowers emitted a faint rotten-fruit odour, but did not report the presence of nectar. Nectar production could not be confirmed by Pupulin *et al*. (2012), but the authors did find that flowers of *S. endotrachys*, *Specklinia pfavii*, *Specklinia remotiflora* 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, including perfume, 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 and 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 as common in Orchidaceae as in Liliales, in which this feature can be considered synapomorphic (Smets *et al*., 2000). Floral fragrances are produced by osmophores (scent glands), which occur 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 varies and types include unicellular trichomes (Curry *et al*., 1991), pear-shaped or spherical unicellular hairs with an irregular cuticle (Stpiczyńska, 1993), dome-shaped papillae (Ascensão *et al*., 2005), papillose cells with a smooth cuticle (de Melo *et al*., 2010) and a rugose surface with a sculptured cuticle or a 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. These 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 phylogenetics of the *S. 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? and (2) is pollination of *Specklinia* species-specific? To answer these questions, we collected plants and flies in the wild, made videos documenting pollination and orchid-insect interaction, carried out light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM), used DNA barcoding and conducted electroantennogram (EAG) and gas chromatography–mass spectometry (GC–MS) experiments.

MATERIALS AND METHODS

Living material

Specimens of *Specklinia* spp. were collected in the field in Costa Rica and cultivated in the greenhouses of the Lankester Botanical Garden, University of Costa Rica, and the Hortus botanicus, Leiden University (The Netherlands), from 2012 to 2014. Voucher specimens of the plants were prepared from cultivated material and deposited in the JBL (spirit), L (spirit) and CR herbaria.

Flies were photographed, filmed and observed for a total of 30 d during a 2-year period in the open-air greenhouses at Lankester Botanical Garden. Observations were mostly made between 0600 and 1800 h, with five observations extending this period overnight for all *Specklinia* species. Flies were identified by a combination of non-invasive (sample rescue after lysis) DNA barcoding of the 660-bp COI (mitochondrial cytochrome *c* oxidase subunit COI marker) by K.B. and anatomical surveys by D.G. Only visitors that lingered on flowers and were highly interactive with sepals and the lip (interacting with flower parts for >60 min) and/or that carried pollinia were considered as putative pollinators. Vouchers for the insects were prepared from both field-collected and greenhouse-collected specimens and are kept in the L (spirit) and AMNH herbaria.

Phylogenetics

Specklinia. The phylogenetic concept of *Specklinia* Lindl. follows Pridgeon *et al*. (2001). Those authors found that *S.*

endotrachys (Rchb.f.) Pridgeon & M.W.Chase was closely related to *Specklinia lanceola* (Sw.) Lindl., the type species of *Specklinia*, and a few other mainly orange-flowered species, including *Specklinia fulgens* (Rchb.f.) Pridgeon & M.W.Chase, *Specklinia lentiginosa* (F.Lehm. & Kraenzl.) Pridgeon & M.W.Chase and *Specklinia tribuloides* (Sw.) Pridgeon & M.W.Chase (Pupulin *et al*., 2012). The species belonging to the *S. endotrachys* (*sensu* Pupulin *et al*., 2012) complex are here treated as a monophyletic group in *Specklinia* based on morphological similarities and additional unpublished molecular data (Bogarín *et al*., 2013; Karremans *et al*., 2013).

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 the standard animal DNA barcode fragment of the 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). The PCR reactions contained 18·75 µl MQ water, 2·5 µl 10× PCR buffer CL, 1·0 µl of 10 mM each primer, 0·5 µl of 2·5 mM dNPTs and 0·25 µl of 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 genetic analyser (Applied Biosystems).

The Staden *et al*. (2003) package was used to edit 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 the outgroup. The trees were produced with an analysis of the COI dataset using BEAST v1.6.0. (Drummond and Rambaut, 2007). Parameters were set to preset, except for substitution model GTR with ten 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 (www.boldsystems.org).

Photographic/video-camera documentation

Video recording. The videos of fly visitation were taken with the video option of a Nikon D5100 digital camera and a HD 720 p 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

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, formaldehyde; 18:1:1 v/v), 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

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 70 % ethanol, dehydration through a graded series of ethanol (70 %, 96 %, 100 %) and xylene, impregnation with paraffin 60 °C, and embedding in paraffin. Sections (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 steps and stained by placing in 1 % Alcian blue for 10 min. The samples were then rinsed in tap water and demineralized water, stained with nuclear fast red for 5 min, rinsed in demineralized water, dehydrated through a graded ethanol series and washed with xylene. Finally, a coverslip with Entellan mounting medium was placed on the sample and photographs were taken with a Zeiss Axioskop connected to a Leica DFC490 camera.

Transmission electron microscopy

Freshly collected flowers were fixed for 3 h in modified Karnovsky fixative (2·5 % glutaraldehyde, 2 % formaldehyde) and washed in 0·1 M sodium cacodylate buffer (pH 7·4). After washing, the material was post-fixed for 2 h in 1 % osmium tetroxide and then washed in distilled water. The pieces were dehydrated in an ethanol series and propylene oxide, then infiltrated with Epon by submerging them in a mixture of propylene oxide and Epon (1:1) for 1 h. After overnight evaporation of the remaining propylene oxide, the material was embedded in fresh Epon and polymerized at 60 °C for 48 h. Ultrathin sections were cut with an LKB ultratome, mounted on film-coated copper slot grids and post-stained with uranyl acetate and lead citrate (Reynolds, 1963). The sections were examined with a Jeol 1010 transmission electron microscope.

Analysis of floral compounds by 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 ∼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 with a carbon filter. The volatiles were trapped on 50 mg of Porapak porous polymer adsorbent (Sigma Aldrich) in a glass tube. After

collection (typically 3 h), the volatiles were eluted from the adsorbent with 3 ml of pure pentane. The pentane was subsequently concentrated to ~0·5 ml using a gentle stream of nitrogen. All samples were stored at –20 °C to await 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-5 ms capillary column (30 m × 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 the carrier gas (1·2 ml min^{-1}). The split injection mode was used (injection volume 1 ml, inlet temperature 220 °C, split ratio 1:30). Mass spectra were taken in electron ionization 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 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 floral droplets

Drops produced on the adaxial sepal surface were collected with a fine glass pipette point and stored in a glass vial at –20 °C. Fehling's reagent was used to detect sugar 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 water, and solution B of 86·5 g of sodium potassium tartrate and 35 g sodium hydroxide in 250 ml water.

Electrophysiology

Fly culture and odour stimuli. *Drosophila hydei* eggs were obtained from a commercial grower and reared at 23 °C, 50 % relative humidity and a 16:8 light/dark cycle. Flies were picked randomly 4–7 d 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 onto 5 mm × 50 mm filter paper. After at least 60 s 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 EAG recording. Male and female *D*. *hydei* were used in the experiments as no behavioural differences have been reported (Bartelt *et al*., 1985, 1986, 1988). Fourteen animals were tested. Individual flies were cooled, immobilized at 4 °C for ~30 min 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 solution. 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 lifetime of the preparations was several hours.

The insect was positioned 1 cm in front of the outlet of a charcoal-filtered and humidified airstream (2 L min^{-1}). The chemical stimuli from the Pasteur pipettes were injected into this flow (1 s, 2·5 ml odour pulses) at 60-s 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 model (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 were 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 comprised 162 EAG amplitudes measured for 14 individual insects. To validate the model we visually inspected the fit using a quantile–quantile plot (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, $W = 0{\cdot}9942$, $P = 0{\cdot}767$) and were homoscedastic [Bartlett test (for compounds), $K^2 = 3{\cdot}094$, d.f. = 2, $P = 0{\cdot}21$]. 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 that share a notable reddish-orange colour of the perianth parts, especially of the sepals (Fig. 1A–D). These species produce long-lived multi-flowered successive inflorescences. Large plants may have ten or more flowers open simultaneously, but only one per single inflorescence. The four species have a tendency to flower all year round in greenhouse conditions, but in the field they do have flowering peaks. For at least 6 months all four species were flowering simultaneously in the greenhouses, and capsules were eventually formed. We have not found these species at many 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. 1E, F). Fruit-set was observed in 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 1), 40 % of the plants had capsules, but only 20 % of the inflorescences had a capsule and 8 % of the flowers produced were pollinated. These plants produced one to seven flowers per inflorescence (20 or more under greenhouse conditions), and never more than a single capsule per inflorescence. Capsules are always found

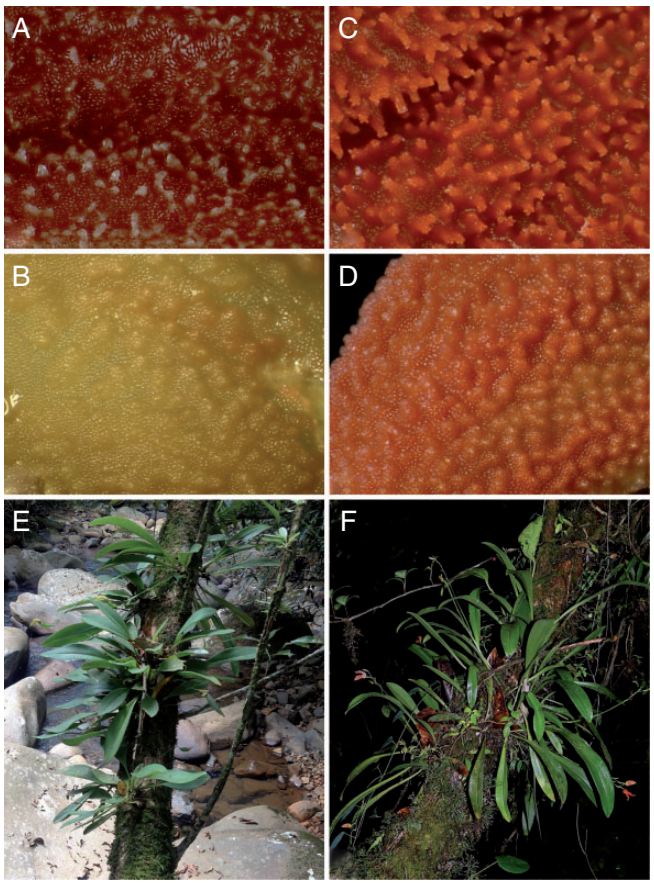

FIG. 1. (A–D) Adaxial surface of the sepals of diverse members of the orange-flowered *Specklinia* showing the structural and colour diversity. (A) *S. endotrachys* (Blanco 961). (B) *S. pfavii* (JBL-11098). (C) *S. remotiflora* (Karremans 4023). (D) *Specklinia* spectabilis (JBL-02535). (E–F) *Specklinia* spp. as found growing in field conditions in Costa Rica. (E) *S. pfavii* growing by the edge of a river at 650 m elevation. (F) *S. remotiflora* in the cloud forest at ~2000 m elevation. Vouchers kept at JBL (spirit). Photographs by A.P.K. (A–E) and Joszef Geml (F).

TABLE 1. *Reproductive success relative to flowering in a wild population of* S. 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

Parameter	Ratio
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

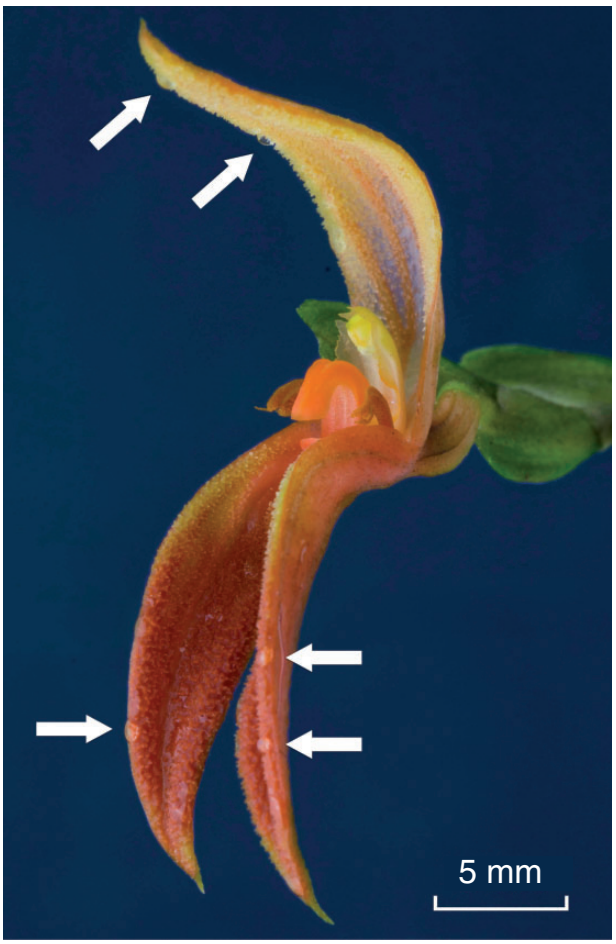

FIG. 2. Nectar drops on the sepals of *S. endotrachys* (Blanco 961). Photograph by Melania Fernández at Lankester Botanical Garden.

on the apex of the inflorescence, suggesting flowering succession is arrested after fruit formation. Drops are produced after anthesis on the rugose areas of the sepals of all four species (Fig. 2). The drops keep increasing in size 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 temperature.

Pollinator biology

Flies visit the flowers for up to +24 h at a time; during this time they mostly remain on the flowers but occasionally leave for a few minutes and return. Visitation can happen any time in greenhouse conditions, but it is more frequent in the early morning and late afternoon, possibly when temperatures are lower. With increasing time the mobility of the visiting flies is greatly reduced and they become slower and less aware of their surroundings. They can visit singly or in groups of up to seven individuals (possibly more). The flies move around and 'inspect' the entire flower, but spend most time (just over 90 % of

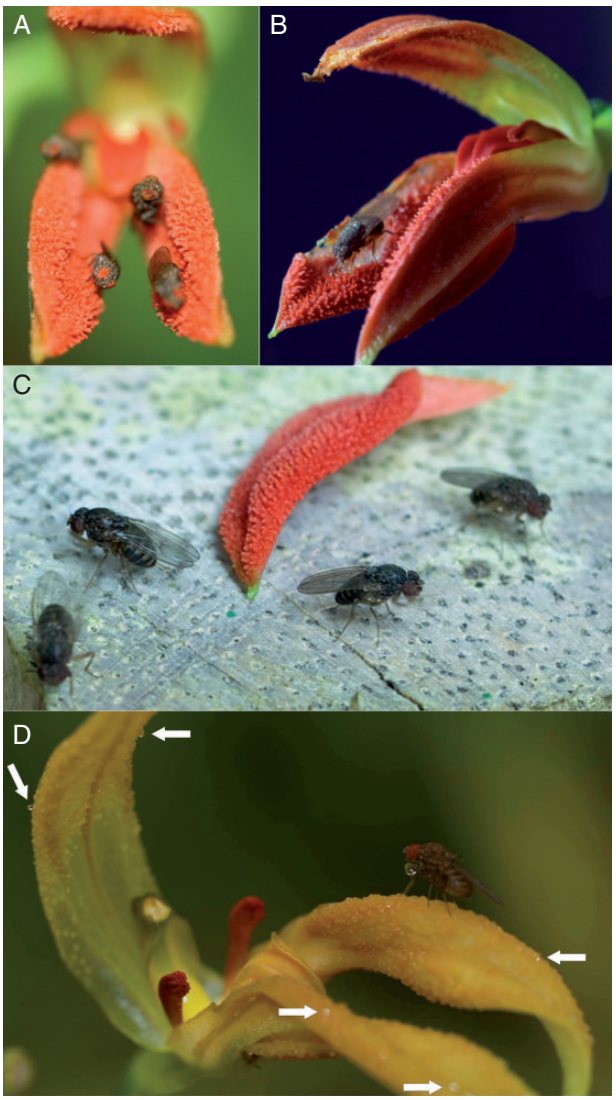

FIG. 3. (A, B) *Drosophila* spp. sucking on nectar-secreting stomata on the apex of the papillae of the sepals of *S. remotiflora*. (A) Showing several flies at once. (B) A single fly and an area of the sepals where the stomata have been depredated by slugs. (C) *Drosophila* spp. still attracted to a severed lateral sepal of S. *remotiflora* a few minutes after removal from the flower. (D) *Drosophila* sp. on the lateral sepals of *S. 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 A.P.K. (A–C) and F.P. (D) at Lankester Botanical Garden.

the time spent) on the papillae-rich areas in the adaxial surface of the sepals (Fig. 3A, B), on which they suck during the entire time they are there (Supplementary Data Video 1). The attraction to sepals of *Specklinia* is so strong that even after immediate removal of one of them from the flower the flies still inspect it (Fig. 3C). The removal of all three sepals completely prevents flies from visiting the flowers. Among the most observed behaviours were: (1) fencing with the forelegs, occurring once every 3 min; (2) wing flapping and following of other flies, which are done constantly; and (3) abdomen bending, about twice in 3 min (Supplementary Data Video 2). Two additional events were observed rarely: (1) copulation, seen twice during the whole study period (Supplementary Data Video 3); and (2) a fly with a regurgitated drop in its mouthparts, seen only once during the study period (Fig. 3D). 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. 4). The pollinia (which lack caudicles) are flattened and curved towards the base, and normally grasp the scutellum of the fly while the animal tries to leave the column/lip cavity in reverse (Supplementary Data Video 4); it can take the fly 20–30 min to liberate itself.

Pollinator identities

Fifty-six flies were caught at the greenhouses at Lankester Botanical Garden in Costa Rica, two were field-collected and two were collected in a private garden (Table 2; Fig. 5). Twenty were collected on flowers of *S. remotiflora*, 20 on *S. spectabilis*, 14 on *S. pfavii*, five on *S. endotrachys* and three on *Specklinia* sp. The flies caught all belonged to the genus *Drosophila* (Drosophilidae), except for one that belonged to *Hydrotaea* (Muscidae) and another to the Lauxaniidea. Of the specimens caught, 54 belonged to the *repleta* species group, two to the *coffeata* group, two to the *immigrans* group and two to an unknown species group. The *Drosophila* species found were *D. hydei* (35 samples), *D. mercatorum* (seven samples), *D.* aff. *repleta* 1 (two samples), *D.* aff. *repleta* 2 (four samples), *D. ananassae* (two samples), *D. fuscolineata* (two samples), *D. immigrans* (two samples), *D.* aff. *bifurca* (one sample) *D. nigrohydei* (one sample) and *Drosophila* spp. (four species, one sample each). *Drosophila hydei* was collected on four of the five *Specklinia* species and the *D.* aff. *repleta* was found on three *Specklinia* species. *Drosophila fuscolineata*, *D. immigrans* and *D. mercatorum* were found on two out of the five species of *Specklinia*. All other *Drosophila* species were collected on only one *Specklinia* species, 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 3).

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 retrieved at 3·11, 2·48 and 3·45 min, respectively (Fig. 6A). The analysis of individual flowers showed 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 *S. pfavii* (JBL-11086) and *S. spectabilis* (Bogarín 7401), as strong signals were found extremely close to the standard times (Fig. 6B, D). In the samples of *S. remotiflora* (Karremans 4846) only signals similar to those of the standards of ethyl tiglate and methyl tiglate, not isopropyl tiglate, were retrieved (Fig. 6C).

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

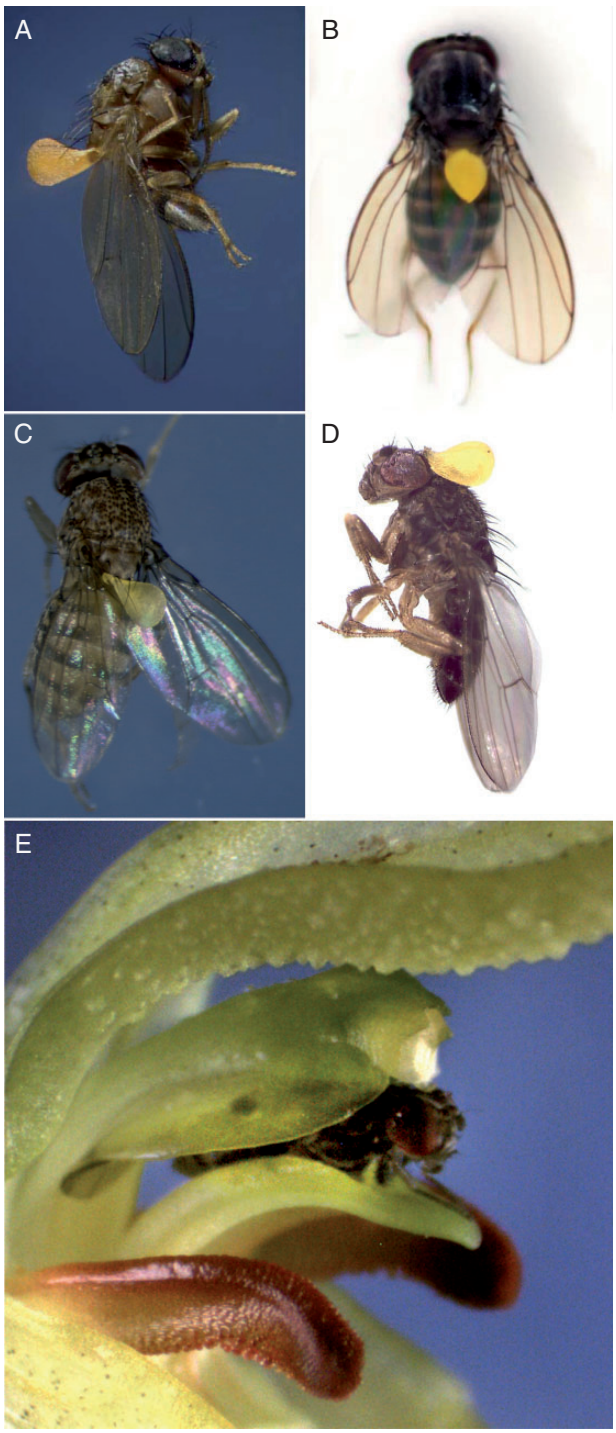

FIG. 4. (A–D) *Drosophila* flies with pollinia of *Specklinia* on the scutellum. (A) *Drosophila* sp. with pollinia of *S. spectabilis* (JBL-02643). (B) *Drosophila hydei* with pollinia of *S. remotiflora* (Bogarín 8181). (C) *Drosophila mercatorum* (KB262-02) with pollinia of *S. remotiflora*. (D) *Drosophila hydei* with the pollinia of *S. pfavii*. (E) *Drosophila* sp. trapped between the lip and column of *S. pfavii*. Note that the fly illustrated here is oriented the other way around from what is normally observed; the pollinia were stuck to the head and not the scutellum, as would be expected (Fig. 4D). Photographs are by F.P. (A, B) and A.P.K. (C–E). Panels (A) and (B) are copyrighted images reproduced from *Phytotaxa* **63**: 1–20, with permission.

Microstructures

SEM The adaxial surface of the lip was completely covered with scale-like epidermal cells. The scales were rounded and flattened towards the apex of the lip, whereas towards the base they were sharply angled and uplifted. The cuticle was somewhat rugose, but without pores or signs of rupture of any kind. The basal scales were filamentous, and these filaments were capitate (Fig. 7A, B). Both surfaces of the petals were warty, especially near the apex. The cuticle was smooth, not ornamented, and without pores or signs of rupture of any kind. (Fig. 7C, D). The adaxial epidermis of all three sepals was densely rugose and covered with warts, except basally. The apex of each wart carried stomata. The stomata had wide pores and five or six somewhat inflated subsidiary cells. The cuticle was somewhat sculptured, not ornamented, and without pores or signs of rupture of any kind. The stomata were permanently open and no movements were observed (Fig. 8A, D). The abaxial epidermis was smooth and mostly constantly flat, except for rare depressed areas where a sunken trichome was located; this trichome was apically irregular (Fig. 8E, F).

LM. The transverse section of the lip of *S. remotiflora* showed mostly large, rounded parenchyma cells and smaller scaly or pyriform epidermis cells on the adaxial surface (Fig. 9A, B). The petals of *S. pfavii* (Fig. 9C) and *S. remotiflora* (Fig. 9D) showed irregular, enlarged secretory parenchyma cells, though without apparent openings. The transverse section of the sepal showed two basic cells types: ground parenchyma near the abaxial surface (underpart in Fig. 9E), which were larger and subrectangular; and secretory parenchyma close to and in the adaxial epidermis (upper part in Fig. 9E), which were smaller and rounded. The vascular bundles were visible. The adaxial epidermis was irregular and frequently had stomata, which could be seen in both *S. pfavii* (Fig. 9F) and *S. remotiflora* (Fig. 9G) as prominent protrusions with an apical opening. The abaxial epidermis was inornate except for the occasional sunken trichomes that could be seen perforating the surface (Fig. 9H).

TEM The pores of the stomata were commonly seen in the transverse sections of the adaxial epidermis of the sepals of *S. pfavii*; the subtending guard cells could be distinguished from the subsidiary cells basically by a thicker cell wall. Nevertheless, their cytoplasm showed the presence of mainly a nucleus with nucleolus, large vacuoles and a high starch content, with the cells surrounding the subsidiary cells (Fig. 10).

EAG study

Drosophila hydei is highly sensitive to the stimuli ethyl tiglate, methyl tiglate and isopropyl tiglate and clear concentration–response relations were found (Fig. 11). The highest response measured was –6·563 mV for the positive control *Z*-3-hexen-1-ol (100 % by definition), whereas the highest values for isopropyl, ethyl and methyl tiglate were –4·462, –4·361 and –3·328 mV, respectively. To investigate the effects of concentration, a generalized linear mixed model was used that contained, in addition to the random factor 'individual', the explanatory variables 'compound' and 'concentration' and their interaction. The coefficients for the effects of these factors (Table 4) showed a highly significant concentration effect, as

TABLE 2. *Diptera specimens caught on flowers of the* S. endotrachys *species complex*

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

*www.boldsystems.org.

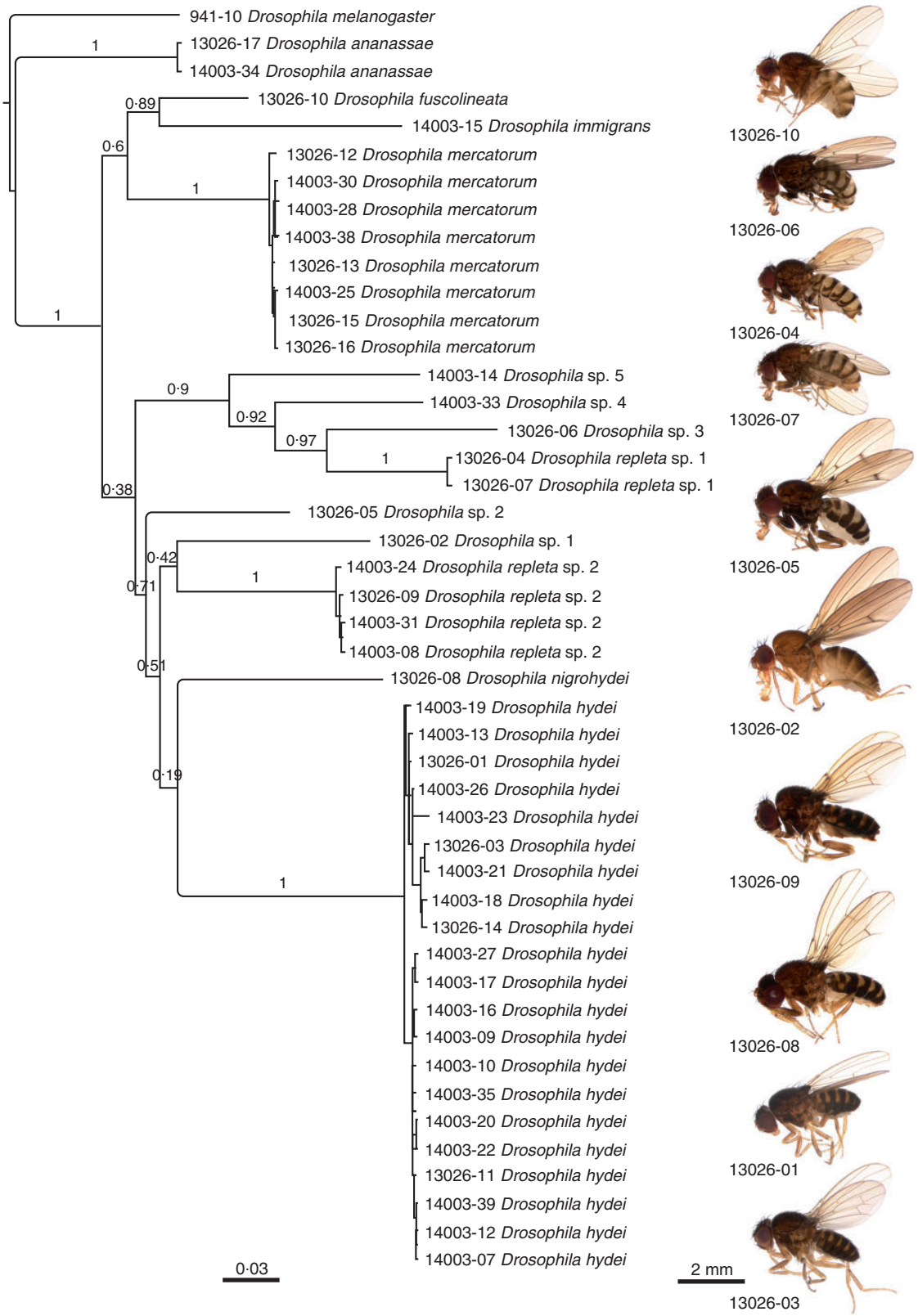

FIG. 5. Phylogenetic relationship amongst the collected fly specimens. The trees were produced by analysis of the COI dataset using BEAST v1.6.0. Parameters were set to preset, except for substitution model GTR with ten categories, clock model uncorrelated exponential, tree prior Yule process, and number of generations 20 000 000. The resulting trees were combined using TreeAnnotator v1.6.0.; the first 3000 trees were used as burn-in. Node values are posterior probabilities. Edited by A.P.K. using using FigTree v.1.3.1. Photographs by K.B.

TABLE 3. *Diptera species caught summarized per orchid species and sex*

Fly species	Orchid species					
	S. endotrachys	*S. pfavii*	*S. remotiflora*	*S. spectabilis*	*Specklinia* sp.	Total
D. ananassae			1 ♀	1 ♂		1 ♂, 1 ♀
D. bifurca aff.					1 ♂	1 ♂
D. fuscolineata		1 ♀	1 ♂			1 ♂, 1 ♀
D. immigrans			1 ♂		1 ♀	1 ♂, 1 ♀
D. hydei	4 ♂, 1 ♀	1 + 10 ♂, 2 ♀	4 ♂, 3 ♀	4 ♂, 6 ♀		1 + 22 ♂, 12 ♀
D. mercatorum			2 ♂, 1 ♀	4 ♂		6 ♂, 1 ♀
D. nigrohydei			1 ♂			1 ♂
D. repleta aff. 1			2 ♀			2 ♀
D. repleta aff. 2			1 ♀	1 + 1 ♂	1 ♂	1 + 2 ♂, 1 ♀
D. sp. 1			1 ♀			1 ♀
D. sp. 2			1 ♂			1 ♂
D. sp. 3			1 ♀			1 ♀
D. sp. 4				1 ♀		1 ♀
Unknown				2 ♀		2 ♀
Total	4 ♂, 1 ♀	1 + 10 ♂, 3 ♀	10 ♂, 10 ♀	1 + 10♂ 9♀	2♂ 1♀	2 + 36♂ 24♀

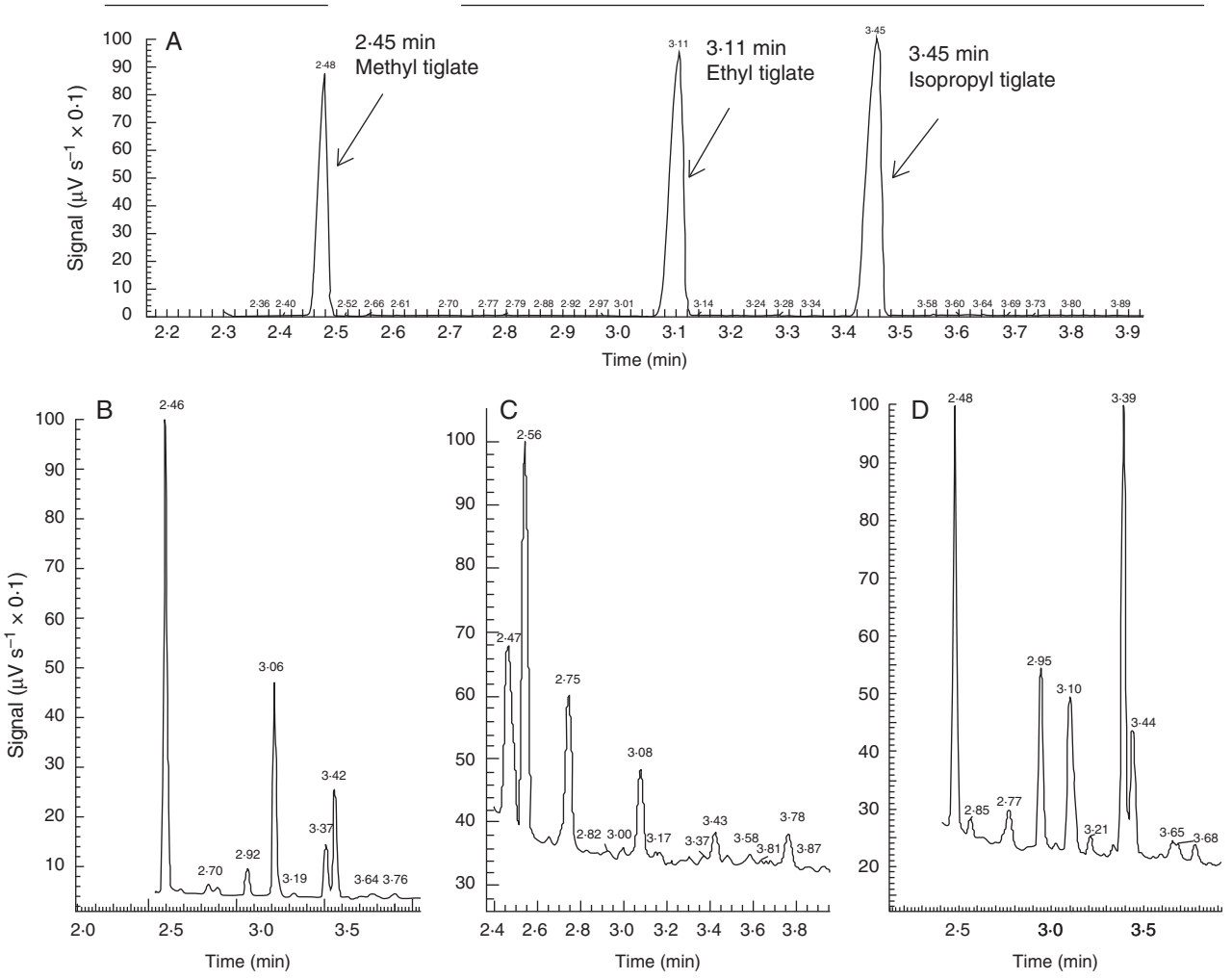

FIG. 6. Standard mix of ethyl tiglate, methyl tiglate and isopropyl tiglate measured with GCMS-ITD. (A) Selected-ion monitoring signal over time. (B) *S. pfavii* (JBL-11086). (C) *S. remotiflora* (Karremans 4846). (D) *S. spectabilis* (Bogarín 7410). The *x* axis shows time (min) and the *y* axis shows the signal (0·1 μV s^{-1}). Figures by Misli Kaya.

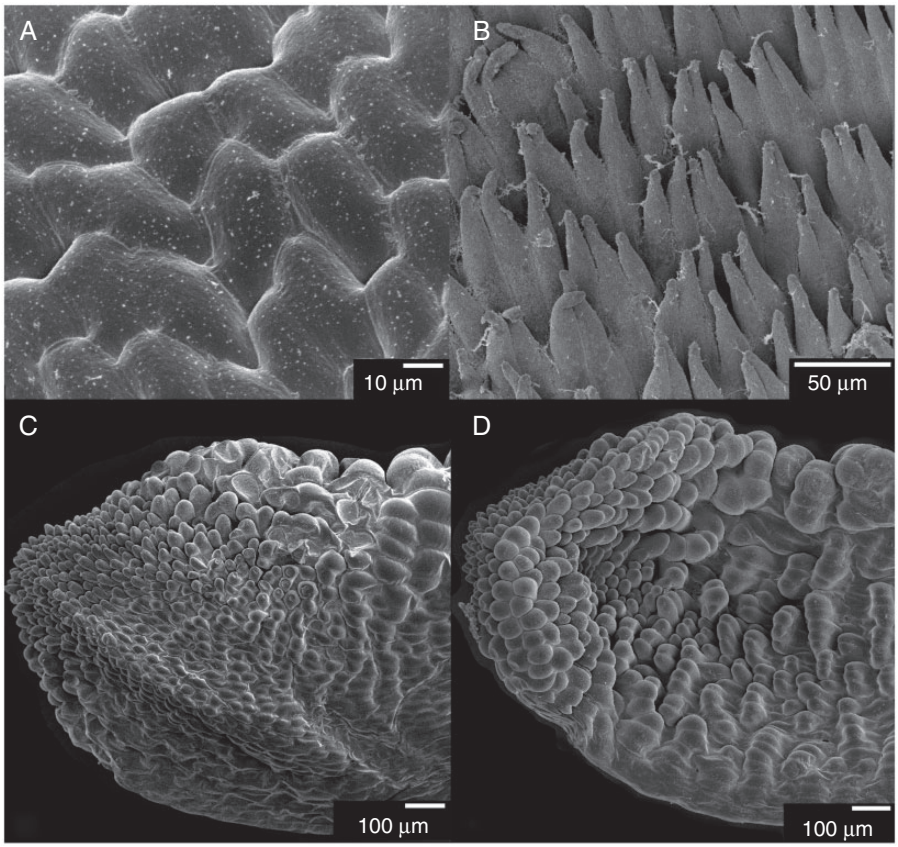

Fig. 7. Micrographs of *S. pfavii* (JBL-11086). Scales cover the adaxial surface of the lip; they are flattened and rounded near the apex (A) and elevated and filamentous–capitate closer to the base (B). Warts cover the outer (C) and inner (D) surfaces of the petals, especially apically. Photographs by A.P.K.

expected for biologically 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 did not show an interaction, i.e. the slope of the concentration–response curve was similar for the two compounds. In contrast, isopropyl tiglate showed an interaction, and the slope of the concentration–response curve was significantly less steep than that for ethyl tiglate (Fig. 11).

DISCUSSION

In Pupulin *et al*. (2012), we established that at least four similar, yet distinct, recognizable species should be treated under the name *S. endotrachys*. Our findings show that the pollination syndrome of *S. endotrachys*, *S. pfavii*, *S. spectabilis* and *S. remotiflora* is basically the same. Both male and female flies are arrested by pheromones liberated from the 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 h continuously feeding on the flowers, and with reduced mobility over time. They display a variety of behaviours, including fencing with the forelegs, flapping their wings, following other flies, bending their abdomen and occasionally copulating. While 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 when 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, multiflowered, successive inflorescences, being able to produce up to +20 flowers over time. Each plant may flower for several months at a time. Overall fruit production was found to be low, in the field and in the greenhouse, making it likely that large colonies and long-term flowering are necessary to attain fruit set. *Specklinia endotrachys*, *S. pfavii*, *S. remotiflora* and *S. spectabilis* have all been found growing in Costa Rica, but never sympatrically (Pupulin *et al*., 2012) (Fig. 12). *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, and *S. remotiflora* is only found in the highland cloud forests close to the continental divide in the south of the Talamanca mountain range. The mountain range (>2000 m high) serves as a barrier separating the populations of the four species. Allopatry facilitates divergence by interrupting gene flow and allowing local

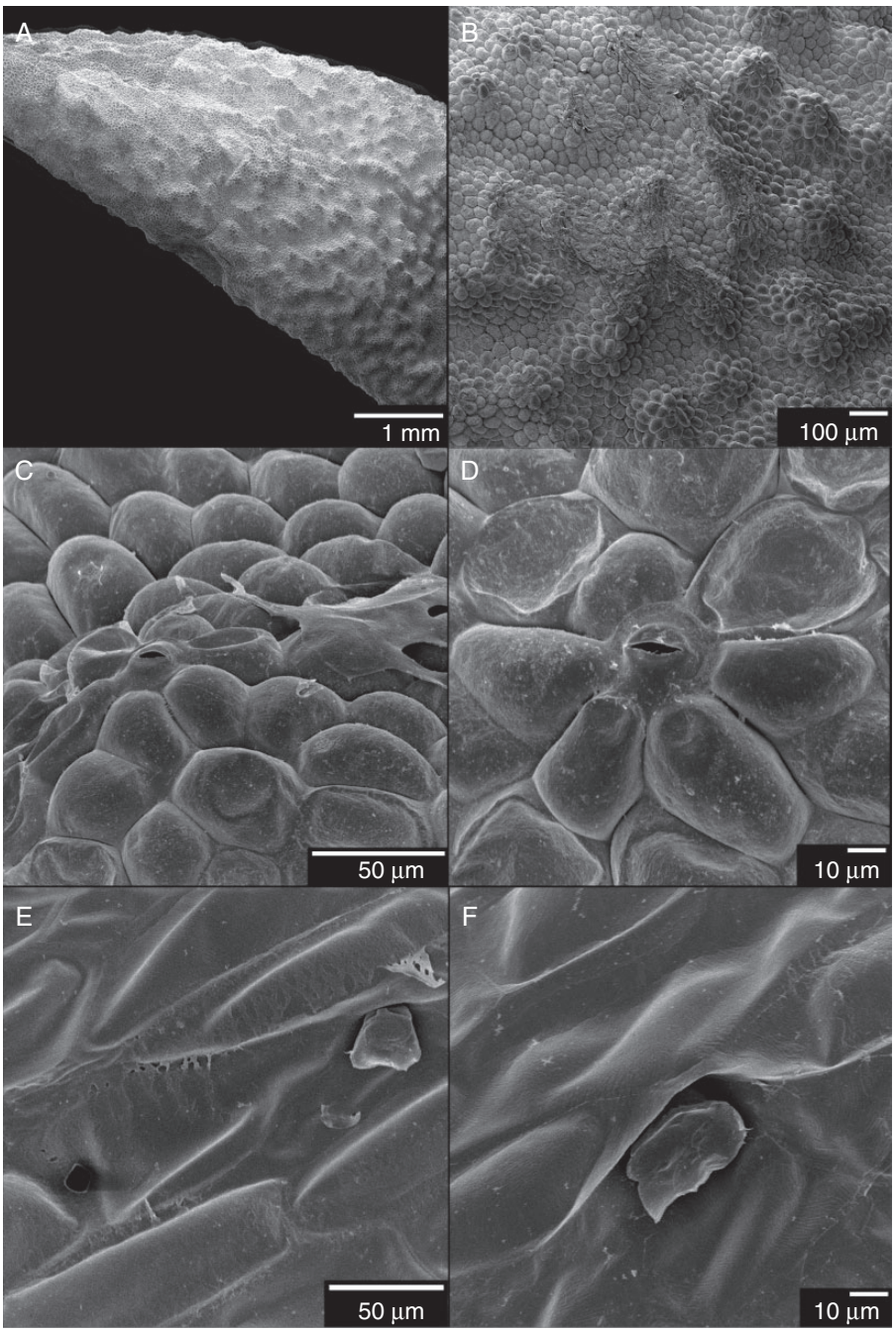

FIG. 8. Micrographs of sepals of *S. pfavii* (JBL-11086). (A) Median segment of a lateral sepal showing corrugation of the adaxial surface. (B) Elevated cells form papillae, corrugating the adaxial surface. (C) Apices of the papillae are formed by nectar-secreting actinocytic stomata, formed by guard cells and six subsidiary cells (D). (E, F) The abaxial surface is formed by flattened cells, with occasional depressions that contain a sunken trichome. Photographs by A.P.K.

adaptation without the necessity of high floral divergence or, for that matter, pollinator shifts (Harder and Johnson, 2009).

Reddish-orange flowers are characteristic of species of the *S. endotrachys* group and close relatives. Although not unique in Orchidaceae, these colour patterns are uncommon in the family and are notably rare in subtribe Pleurothallidinae. Oliveira *et al.* (2012) found that species of the *D. 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 (Figs 2 and 3D). The drops have a pasty consistency and 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 of the warts, as can be seen in the SEM photographs (Fig. 8). The transverse sections of those

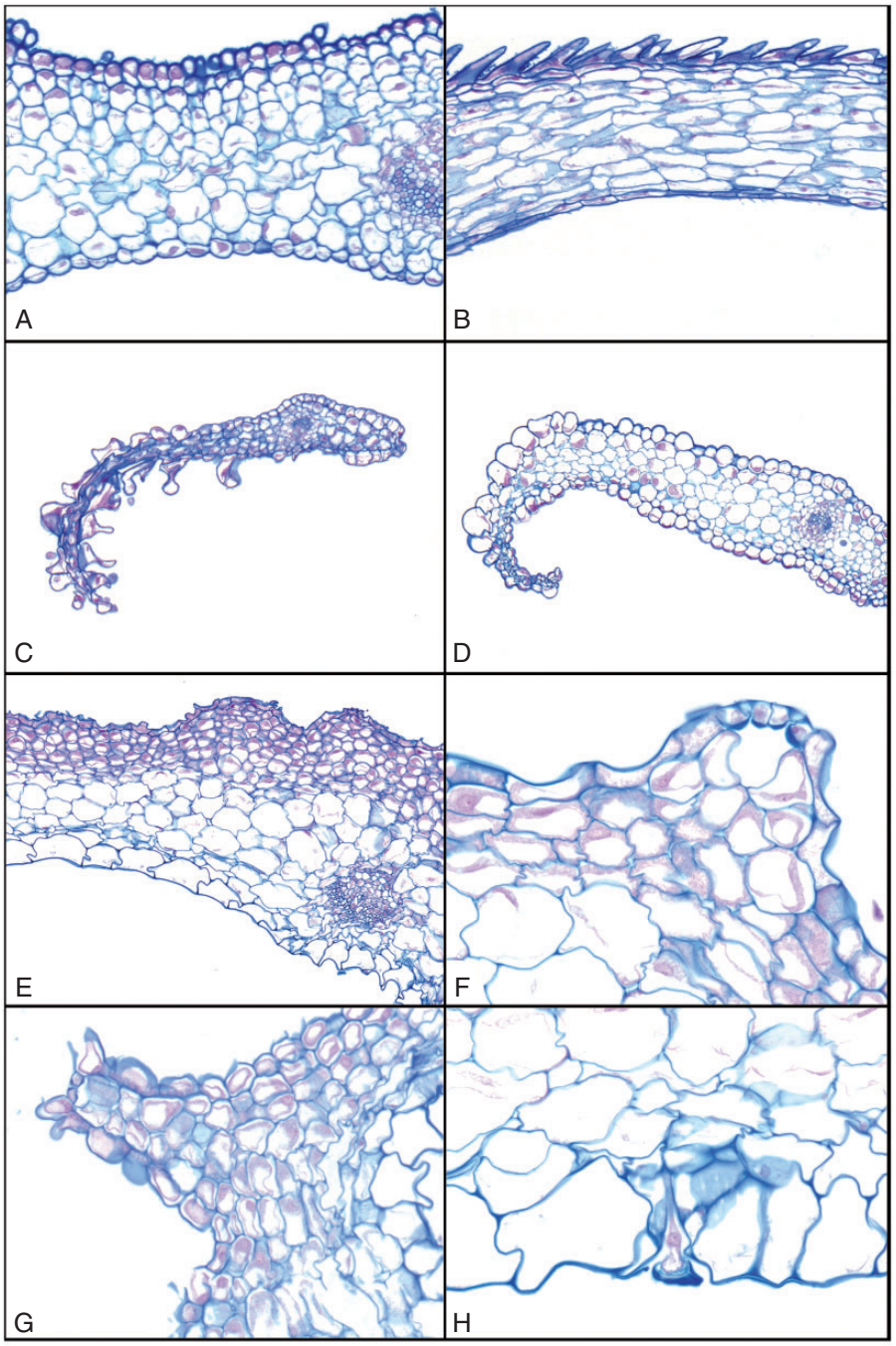

FIG. 9. Light micrographs of *S. pfavii* (AK4835) (C, E, F, H) and *S. remotiflora* (AK4798) (A, B, D, G). (A) Transverse section of the lip, showing vascular bundles and keels. (B) Transverse section of one of the keels, showing the scale-like cells on the adaxial surface. (C, D) Large, irregular cells on transverse section of the petal; vascular bundles and stomata are visible on transverse section of the sepal (E). (F, G) Secretory stomata on the adaxial epidermis. (H) Sunken trichome on the abaxial epidermis. Magnification (A–H respectively): 200×, 100×, 50×, 100×, 100×, 400×, 200×, 400×.

stomata, taken by LM (Fig. 9) and TEM (Fig. 10), evidence high cellular activity in the stomatal guard and subsidiary cells. Starch grains, which are likely to be used as an energy source for the production of nectar, are commonly observed. No clear drops or evidence of nectar-secreting stomata are found on the petals and lip, but these structures are entirely covered by papillae. These papillae are morphologically similar to the secretory papillae found by Stpiczyńska and Matusiewicz (2001) on the nectary of *Gymnadenia* and by 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 (Figs 8 and 9) might also indicate the presence of secretory papillae on the lip and petals here, but this needs further studies.

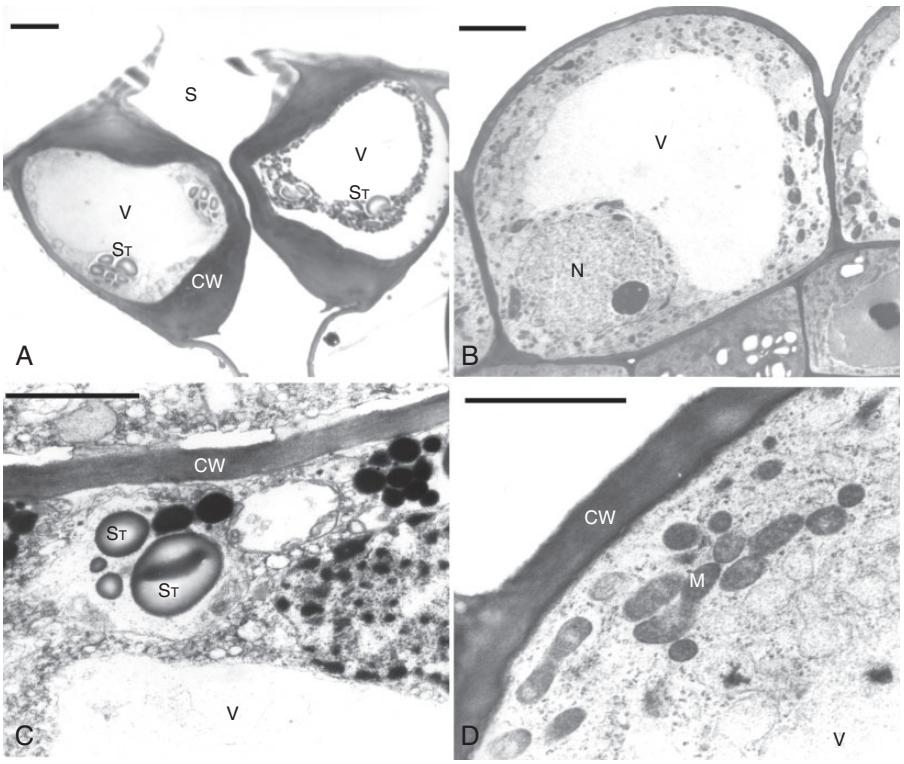

FIG. 10. Transmisison electron micrographs of transverse section of the adaxial epidermis of the sepals of *S. pfavii*, showing opened guard cells of stomata (A), their subsidiary cells (B), with frequent starch grains (C), and variously sized vesicles (D). Scale bars: (A, B) = 2 µm; (C, D) = 1 µm. CW, cell wall; M, mitochondrion; N, nucleus; S, stomata; St, starch; V, vacuole. Photographs by Rob Langelaan.

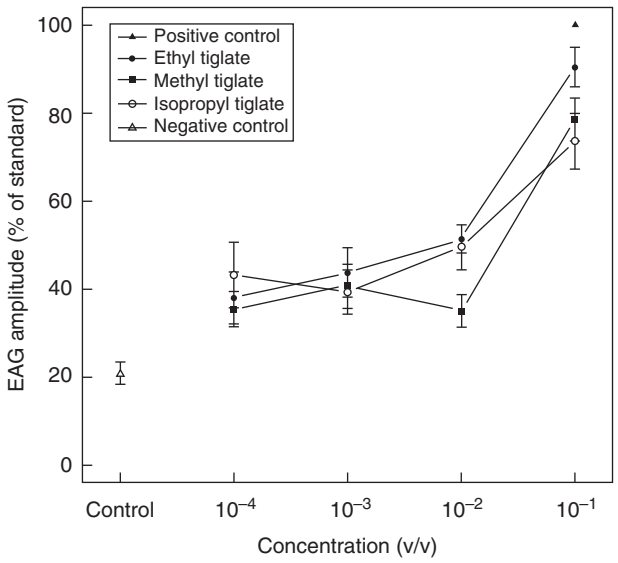

FIG. 11. Concentration–response relationships for EAG measurements in 14 *D. hydei* flies reacting to ethyl tiglate, methyl tiglate and isopropyl tiglate. All amplitudes are expressed as percentage of the external standard (positive control) *Z*-3-hexen-1-ol.

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 *D. hydei* (Moats *et al.*, 1987), are produced by flowers of the *S. endotrachys* complex (Fig. 6). The aggregation pheromones, although not the only substances produced by the flowers, are probably 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 and 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 (Tables 2 and 3). 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 $1{\cdot}0 \times 10^{-5}$ v/v of the pure substance (Fig. 11; Table 4). The measured concentration of the tiglates in the flowers was $\sim$1 µg L^{-1}.

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 behaviours

TABLE 4 *Summary of the coefficients in a mixed model that tests for effects of stimulus compound and concentration on EAG response. The intercept gives the estimate of the coefficient for ethyl tiglate; the other estimates indicate the changes in relation to this reference. A highly significant contribution of stimulus concentration was found. Except for concentration, all 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 that for ethyl tiglate in a concentration-dependent way (see also Fig. 11). The | sign indicates that different intercepts were allowed for each insect. This removes the non-independence of repeated measures taken from the same fly*

Coefficient	Estimate	S.e.	Wald *z*	*P*-value	Significance
Intercept	42·55	3·7627	11·3082	<0·0001	***
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				

*$P \leq 0{\cdot}05$; **$P \leq 0{\cdot}01$; ***$P \leq 0{\cdot}001$.

for several *Drosophila* species (Greenspan and Ferveur, 2000; Villella and Hall, 2008). In *D. 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 or 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 pieces of different fruits. In that study the author found that males of *D. melanogaster* courted females on the feeding site (decaying fruit), whereas 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 specimens caught, including samples of both *D. hydei* and *D. repleta*, belonged to the *D. 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 and O'Grady, 2005). Adults of most species will feed on a range of food sources, but oviposition and larval development are typically more restricted (Carson, 1974). The *repleta* group includes many cosmopolitan species with Neartic and Neotropical distributions, which reportedly use both fruits and cacti as breeding sites (Markow, 1988; Markow and O'Grady, 2005; Markow and O'Grady, 2008; Oliveira *et al.*, 2012). A particular *Drosophila* species may feed and breed exclusively in a resource such as flowers (Brncic, 1983; Markow and O'Grady, 2008). However, with the lack of observed oviposition events, 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 *S. 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 *D. 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 pollinia removal occurs when a fly is pushed against the column once it walks over the lip; while 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 these 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 and Semir, 2001; Albores and 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 species of Diptera to pleurothallid flowers. Blanco and Barboza (2005) supposed that *Lepanthes* spp., 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* spp. to attract pollinators. The use of pheromones, be it sexual or aggregation, might be generalized 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, but evidence for nectar guides is frequently found in more detailed pollination studies in the pleurothallids (Borba and 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 led to an increase in reproductive success. Pollination efficiency was found to be significantly lower in food-deceptive orchids than in rewarding species (Tremblay *et al.*, 2005; Scopece *et al.*, 2010), and

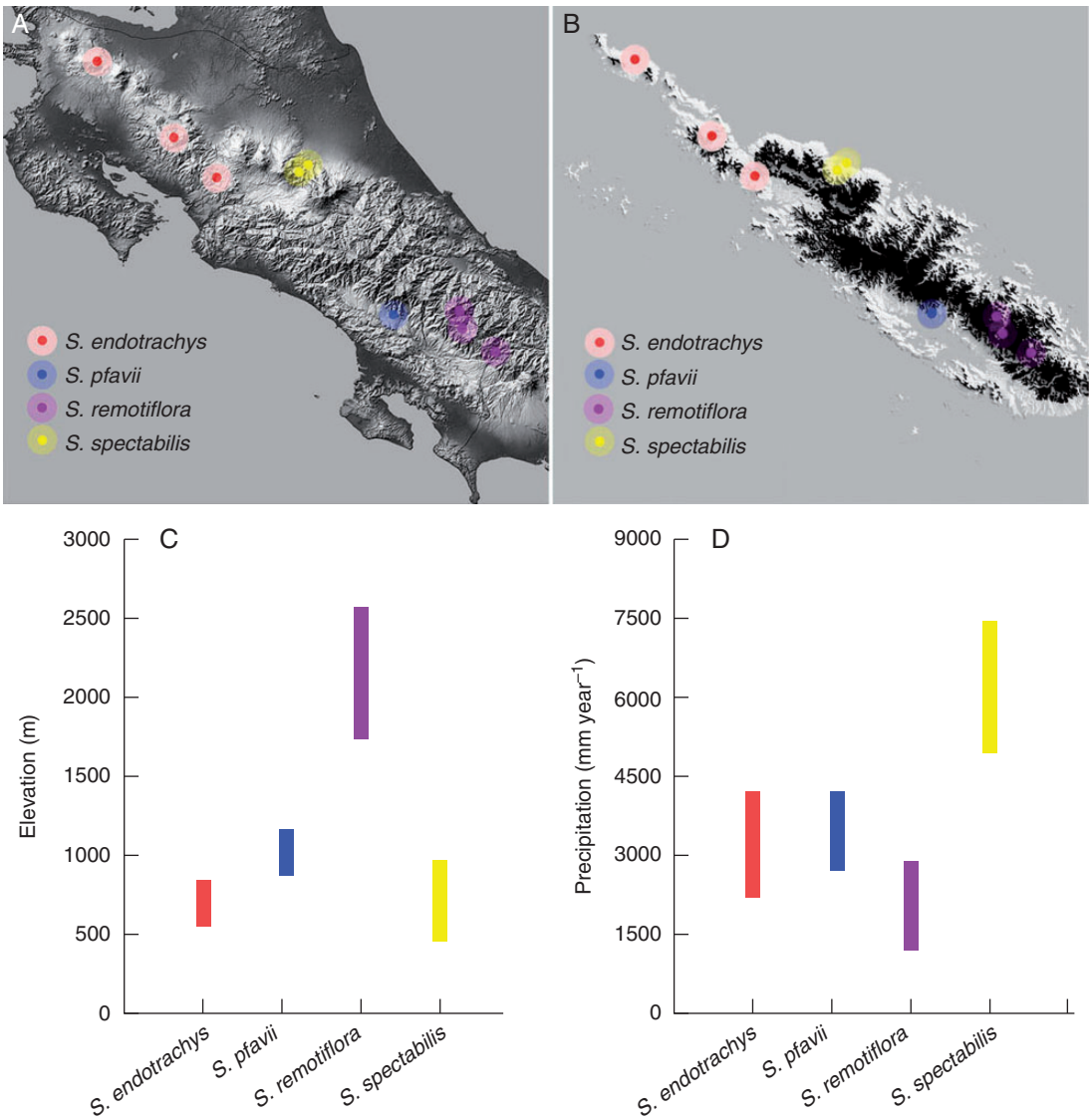

FIG. 12. Distribution and ecological preferences of the *S. endotrachys* group in Costa Rica. (A) Actual known distribution. (B) Distribution with elevations <400 m converted to sea level and elevations >1500 m blackened. (C) Distribution of elevation of found specimens. (D) Distribution of precipitation in the areas where the specimens have been found.

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 whether the number of cases in which orchids are considered non-rewarding is not greatly over-estimated.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Video 1: a fly moving around and sucking on the papilla-rich areas of the adaxial surface of the sepals. Video 2: flies fencing with their fore-legs, flapping their wings and following each other around, and a fly twitching or bending its abdomen. Video 3: flies copulating, an extremely rare event on these orchids. Video 4: a fly liberating itself from the lip/column cavity, removing the anther cap and pollinia in the process.

ACKNOWLEDGEMENTS

We are very grateful to Jorge Warner, director of Lankester Botanical Garden in Costa Rica, and Paul Kessler, director of the Hortus botanicus Leiden, and all their staff for their paramount effort in getting all the collecting and export permits and cultivating the biological material. We wish to thank Marcel Eurlings, Bertie-Joan van Heuven and Rob Langelaan, who contributed greatly in diverse laboratory tests and analyses, Rogier van Vugt and Giovanni Meza, who helped in taking care of the studied plants, Jorge 'Koki' Cambronero, who was

essential in the field, Koos Wubben, who let us use some of his living plants for this study, and André van Roon and Misli Kaya from the University of Applied Sciences Leiden, who helped with the GC–MS analyses. Special thanks to Miriam Contreras, who was of great support and also helped in preparing material for shipment. Young Hae Choi was kind enough to do an analysis of floral compounds for us and go through the manuscript. Many other people, including Diego Bogarín, Maricruz Bonilla, Nicolas Davin, Bobby Driessen, Melania Fernández, Lizbeth Oses, Ton Sijm, Sylvia Strigari and Gerson Villalobos, assisted this project diversely. Two anonymous reviewers made very useful comments on the manuscript and we thank them. We also thank the Costa Rican Ministry of Environment and Energy (MINAE) and its National System of Conservation Areas (SINAC) for issuing the Scientific Passports under which wild species treated in this study were collected. We are in debt to the Vice-Presidency of Research of the University of Costa Rica for providing support through the projects 'Filogenia molecular de las especies de Orchidaceae endémicas de Costa Rica' (814-B1-239) and 'Taxonomía, filogenia molecular, aislamiento reproductivo y diferenciación de nichos de *Specklinia endotrachys*' (814-B3-075). The Alberta Mennega foundation assisted the first author (A.K.) financially in several stages of this research and played a significant role in financing field trips and laboratory work. The Kenniscentrum Plantenstoffen is thanked for their sponsoring of the EAG experiments.

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