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Evolution of pollination systems involving edible trichomes in orchids

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Abstract. Most flowering plants need biotic vectors for pollen transfer. Consequently, the emergence and maintenance of floral attributes are driven by selection generated by pollinators. As a result of such selective pressures, new strategies related to pollinator attraction and rewards can arise. In addition to nectar and pollen, pollinators exploit floral perfumes, wax, resins, edible oils and edible trichomes. Edible trichomes have been recorded in several plant families, but most frequently in orchids. However, these food hairs have not been recorded previously among members of the Neotropical Catasetinae. Using studies of flower morphology and anatomy, analyses of rewards, and observation of pollinators and pollination mechanism, this study aims to ascertain the pollination biology of *Cyanaeorchis*, an unusual genus currently recognized within Catasetinae, for which pollinators and rewards are unknown. We also investigate the evolution of floral rewards among the Catasetinae, and the evolution of edible trichomes in orchids more generally. *Cyanaeorchis arundinae* produces food hairs as a reward and is pollinated by bees that collect this food material. No other Catasetinae offer edible trichomes as a reward to pollinators. *Grobya* produces edible oil as a resource, while *Galeandra* is pollinated by nectar deception. The clade containing the genera *Dressleria*, *Mormodes*, *Cycnoches*, *Clowesia* and *Catasetum* offers volatile compounds ('perfumes') as a non-nutritive reward to male euglossine bees. Our data indicate that perfume rewards originated only once in the Catasetinae. Our analyses also suggest that edible trichomes evolved independently five times in Orchidaceae.

Keywords: Edible trichomes; Catasetinae; *Cyanaeorchis*; Epidendroideae; evolution; floral rewards; flower resource.

Introduction

Most flowering plants require biotic vectors for pollen transfer. As a consequence, the emergence and maintenance of floral attributes are determined through selection generated by pollinators. Due to variation in behaviour and life history strategies, different pollinators may impart divergent selection pressures on flowers. As a consequence, flowers have evolved a wide range of traits, such as the production of fragrances (floral 'perfumes'), peculiar flower morphologies and a variety of colour patterns (Sakai 2002). In addition to the flower attributes related to attraction and the correct placement of pollen on the body of the pollinators, plants have evolved a range of floral rewards that are collected by a variety of animals, mainly bees. Hymenopterans are

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the most important pollinator group, of which more than 20 000 species are dependent on rewards produced by flowers (e.g. Danforth *et al.* 2013).

Among orchids, besides nectar and pollen (pollen not forming pollinia; Pansarin and Amaral 2008), bees exploit floral fragrances (species pollinated by male euglossine bees), waxes, edible oils, and edible trichomes (e.g. van der Pijl and Dodson 1966; van der Cingel 2001). Labellar edible trichomes have been recorded in several unrelated epidendroid genera, such as *Eria* (Beck 1914; Davies and Turner 2004*a*), *Maxillaria* (e.g. Davies *et al.* 2000, 2003), *Dendrobium* (Davies and Turner 2004*b*) and *Polystachya* (e.g. Davies *et al.* 2002; Pansarin and Amaral 2006), suggesting that floral reward evolved independently several times in the evolution of the subfamily Epidendroideae. Sometimes the edible trichomes are referred to as pseudopollen, since they consist of a whitish or yellowish farinaceous material that resembles pollen in appearance (e.g. Davies *et al.* 2002; Davies and Turner 2004*b*; Pansarin and Amaral 2006). In *Polystachya*, the pseudopollen is derived from the fragmentation of moniliform and multicellular trichomes into individual cells (Pansarin and Amaral 2006). Food hairs are distinguishable from pseudopollen, since the former remain intact (van der Pijl and Dodson 1966). In fact, edible trichomes are very diverse in structure, appearance, shape and content. Such trichomes usually contain protein bodies (e.g. *Maxillaria* and *Polystachya*), oil droplets (*Maxillaria*) or starch grains (*Dendrobium*; Davies and Turner 2004*b* for a review), and are collected by bees as a nutritive reward. Nevertheless, studies on the pollination biology of orchid species offering edible trichomes as a reward are scarce. So far, conclusive data are available for species of *Maxillaria* (Davies and Turner 2004*b*; Davies *et al.* 2013 for a review on this subject), and three species of *Polystachya*: *P. flavescens* (Goss 1977), and *P. estrellensis* and *P. concreta* (Pansarin and Amaral 2006). Although pseudopollen has been associated with pollination by deception (e.g. Davies *et al.* 2013), at least in Brazilian *Polystachya* a strategy of deception is not involved, since the collected material is nutritive to bees (Pansarin and Amaral 2006).

Although, among orchids, the offering of edible trichomes has been recorded in Polystachyinae, Maxillariinae, Eriinae and Dendrobiinae. Among the eight genera currently recognized within Catasetinae, five (*Catasetum*, *Clowesia*, *Cycnoches*, *Dressleria* and *Mormodes*) are widely known to offer floral odours as rewards; these are gathered exclusively by male euglossine bees (e.g. van der Pijl and Dodson 1966). *Grobya* produces edible oil as a resource, being pollinated by oil-gathering bees (Mickeliunas *et al.* 2006; Pansarin *et al.* 2009). *Galeandra* is pollinated by nectar-seeking bees, although no nectar is produced in the spurs, suggesting that nectar deception is involved (Whitten *et al.* 2014; E. R. Pansarin, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - FFCLRP/USP, Ribeirão Preto, SP, Brasil, pers. obs.). *Cyanaeorchis*, a genus occurring in Brazilian grasslands, is currently considered to belong to the Catasetinae as a sister to *Grobya* (Batista *et al.* 2014; Whitten *et al.* 2014). It is assumed to be pollinated by *Xylocopa* based on its resemblance to the flowers and habit of members of *Eulophia* (Batista *et al.* 2014). *Eulophia* produce nectar as a resource and are pollinated by large nectar-seeking bees (Jürgens *et al.* 2009). However, nothing is known about the pollination biology of *Cyanaeorchis* (Whitten *et al.* 2014).

Using studies of flower morphology, anatomy, histochemistry, analysis of flower rewards, and observations of pollinators and pollination mechanism, this study aims to ascertain the pollination biology of *Cyanaeorchis*, for which the pollinators and floral rewards are unknown. On the basis of the previous assumption that *Xylocopa* acts as a pollinator of *Cyanaeorchis* (Batista *et al.* 2014), our main hypothesis is this species is pollinated by large nectar-seeking bees. Furthermore, this study aims to test the position of *Cyanaeorchis* within Orchidaceae and Catasetinae, using different data sources. We also investigate the evolution of floral rewards among the Catasetinae, and the evolution of the production of edible trichomes in orchids.

Methods

Taxon sampling and study areas for studies on pollination biology of *C. arundinae*

We studied the reproductive biology of *Cyanaeorchis arundinae* in two populations occurring in wet grasslands (vereda vegetation) near the municipality of Uberlândia, in the state of Minas Gerais, southeastern Brazil. One population is located 20 km S from Uberlândia, at the Clube de Caça e Pesca Itororó de Uberlândia (18°60′S; 48°18′W), while the other one occurs 15 km SSW from Uberlândia along the edge of the road to Campo Florido (19°03′S; 48°21′W). The climate in the region is characterized by its high seasonality with a wet season from October to March and a dry season from April to September. The mean annual precipitation is 1482 mm, while the mean temperature is 22.8 °C (Cardoso *et al.* 2009).

Floral features of *C. arundinae*

The duration of flowers of *C. arundinae* was recorded in October 2013 (two plants; five inflorescences; 10 flowers). The morphological features of fresh flowers of the

species studied (10 plants; 10 inflorescences; 10 flowers) were recorded using a binocular stereomicroscope. Measurements were made directly on the floral structures using a Vernier calliper. The morphological study considered the format and size of floral structures, such as sepals, petals, labellum, column, anther and pollinarium, taking possible intra-population variations into account (Faegri and van der Pijl 1979). The production of floral fragrance was verified daily by directly smelling the flowers from blooming to withering.

In order to detect the effect of ultraviolet light (UV) on flowers, 10 fresh flowers (10 plants; 10 inflorescences) were exposed to a 6-watt Longwave UV-A light (365 nm) and observed in a UV light chamber Quimis Q315CE.

Fresh flowers were immersed in 0.1 % (w/v) aqueous neutral red for 20 min in order to localize possible secretory tissues (Dafni 1992). Once stained, they were rinsed in tap water and examined. To characterize the anatomy of the secretory structures, flowers were fixed in buffered neutral formalin (BNF) for 48 h (Lillie 1965), left in the fixative under low vacuum and stored in 70 % ethanol. Floral pieces such as petals, sepals, labellum and column were dehydrated through an ethanol series and embedded in glycol methacrylate. Transverse sections were obtained with a rotary microtome (9–12 μm) and stained with toluidine blue (Sakai 1973).

In order to identify the main classes of chemical compounds present in the flowers, histochemical procedures with labella of fresh flowers using Sudan III plus Sudan Black B, and Lugol's solution were performed to detect total lipids (Pearse 1985) and starch grains (Johansen 1940), respectively. Additionally, transverse sections of the labellum (fresh flowers cut by hand) were stained with Xylidine Ponceau to detect total proteins (O'Brien and McCully 1981), and with Fehling's reagent to identify reducing sugars (Purvis *et al.* 1964). For the histochemical tests appropriate controls were conducted (Pansarin *et al.* 2009). The images of the anatomic structures and the histochemical tests were captured with a Leica DM500 optical microscope equipped with a Leica ICC50 camera connected to a PC running IM50 image analysis software. A specimen collected in the study area was vouchered (Brazil, Minas Gerais, Uberlândia, 05.X.2012, *E.R. Pansarin & A.A. Maciel 1512*) and included at the LBMBP Orchidarium, Biology Department, FFCLRP-USP, University of São Paulo.

Floral visitors and pollination process of *C. arundinae*

Data on pollinators, pollination mechanism and total frequency of pollinators on flowers of *C. arundinae* were recorded during the 2012 and 2013 flowering seasons. Floral visitors were captured for later identification. During the 2012 flowering period, the observations were carried out from 27 September to 1 October and from 25 to 29 October, totalling 75 h. In the 2013 flowering season, the observations were carried out from 23 to 27 September, totalling 72 h. Daily observation in both flowering seasons (2012 and 2013) took place from 0800 to 1400 h. Floral visitors were photographed using a Nikon D-SLR D800 camera and a Micro Nikkor 105mm f2.8 lens.

Insects were collected, identified and deposited in the 'Pollinator Collection' of the LBMBP laboratory of the Department of Biology, FFCLRP-USP, University of São Paulo, Brazil.

Taxon sampling for phylogenetic analysis

Material from *Cyanaeorchis* and members of the tribe Catasetinae were analysed and referred to as the ingroup. They cover all genera currently included within Catasetinae (Batista *et al.* 2014). Species of Apostasioideae, Cypripedioideae, Epidendoideae and Orchidoideae were defined as outgroups. A list of ingroup and outgroup species, voucher and GenBank accession numbers is given in **Supporting Information [Table S1]**.

DNA extraction, amplification and sequencing

Total DNA was extracted from fresh tissues according to a modified CTAB method. Amplifications were carried out using 50 μL PCR reaction volumes. Betaine 5 M was added to the PCR reaction to relax the DNA strands. Primers of 18S (Bult *et al.* 1992), 26S (Kuzoff *et al.* 1998), ITS1, 5.8S and ITS2 (Sun *et al.* 1994), *matK-trnK* (Johnson and Soltis 1995) and *ycf1* (Neubig *et al.* 2009) were used for amplification and sequencing. The 5.8S gene was amplified and sequenced using ITS primers covering the regions ITS1, 5.8S and ITS2 (Sun *et al.* 1994). *Taq* DNA polymerase was added to the PCR reaction mix at 80 °C after a period of 10 min denaturation at 99 °C in the thermocycler. Thirty-five cycles were run according to the following programme: denaturation, 1 min, 94 °C; annealing, 45 s, 64 °C (5.8S and ITS), 48 °C (18S), 58 °C (26S), 51 °C (*matK-trnK*) and 68 °C (*ycf1*); extension, 1 min, 72 °C; final extension, 5 min, 72 °C. Amplified PCR products were purified using GFX PCR columns (GE Health Care). Sequencing reactions were prepared using Big Dye 3.1 (ABI), purified PCR products and the same primers mentioned above. Samples were dehydrated and resuspended with loading dye. Sequences were obtained using an Applied Biosystems automated sequencer model 3100. For sequence editing and assembly of complementary and overlapping sequences, the Sequence Navigator and Autoassembler (Applied Biosystems) software were used. DNA sequences were aligned using the software BioEdit version 5.0.9.

Phylogenetic analyses

Maximum parsimony (MP) analyses were run with software PAUP* version 4.0b5 (Swofford 2001). We performed a combined analysis with 5.8S + 18S + 26S in order to confirm the position of *Cyanaeorchis* within Orchidaceae and to study the evolution of food hairs in orchids. The regions ITS + *matK* + *ycf1* were combined in order to study the phylogenetic relationships within Catasetinae. For the Orchidaceae matrices, the heuristic searches were conducted with 102 taxa for 5.8S, 107 for 18S, 97 for 26S and 87 for the matrix combining the 5.8S + 18S + 26S (nrDNA) regions. In addition, for the Catasetinae matrices, heuristic searches were conducted with 98 taxa for ITS, 24 for *matK-trnK*, 38 for *ycf1* and 106 for the matrix combining the ITS + *matK* + *ycf1* regions. The search strategy for individual and combined data used 1000 replicates of random taxon entry additions, option MULTREES and the tree bisection-reconnection (TBR) swapping algorithm, holding 10 trees per replicate and saving all shortest trees. Support for clades was assessed using 1000 bootstrap replicates (Felsenstein 1985).

Bayesian inference (BI) was analysed with MrBayes version 3.1 (Ronquist and Huelsenbeck 2003), while maximum likelihood (ML) analysis of concatenated loci was run with MEGA7 (Kumar *et al.* 2016). For both BI and ML, a combined data matrix of 5.8S, 18S and 26S (Orchidaceae) containing 87 taxa (2721 characters) was analysed. In addition, a combined matrix of ITS, *matK-trnK* and *ycf1* (for Catasetinae) containing 106 taxa (3509 characters) was analysed. Maximum likelihood was based on the Kimura 2-parameter model (Kimura 1980). Initial trees for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. For BI analysis, the combined matrices were partitioned into three categories (5.8S, 18S and 26S) plus (ITS, *matK-trnK* and *ycf1*), while the optimal model of sequence evolution for each partition was selected using jModelTest (Posada 2008), and under Bayesian information criterion (BIC). The software selected the evolution models SYM+G, SYM+I+G, HKY+G, K80+G for 5.8S, SYM+I+G for 18S, GTR+I+G for both ITS and 26S partitions, and GTR+G, GTR+I for both *matK-trnK* and *ycf1* regions. Four Markov chains were run simultaneously for 3 million generations, with parameters sampled every 100 generations. The consensus tree was calculated after removal of the first 3000 trees, which were considered as 'burn-in'. Posterior probability (PP) values above 0.5 were calculated and mapped onto branches of the consensus tree.

Data on distribution of reward production and pollinators were manually mapped onto a molecular phylogeny in order to establish a hypothesis of parsimonious evolution of pollination in Catasetinae. Data on the production of edible trichomes were also mapped onto phylogenies in order to establish a hypothesis of evolution of this floral reward within Orchidaceae. Data on pollinators and floral rewards in other Catasetinae, such as the production of edible trichomes in Orchidaceae, were obtained from the literature.

Results

Flower features

The flowering period of *C. arundinae* occurs from September to October. The fruits are dehiscent in January. The anthesis is diurnal and the flowers open in succession. In the population studied, each unpollinated flower lasts ca. 4–5 days. The species is exclusively terrestrial. *Cyanaeorchis arundinae* possesses an apical inflorescence, which is an erect raceme with up to five resupinate flowers.

The flowers of *C. arundinae* are predominantly creamy in colour (Fig. 1A). The tests with UV light (365 nm) revealed that the central crest of the labellum absorbs UV light, whereas the apex of the petals reflects UV light (Fig. 1B). Sepals 21–26 × 5.5–13 mm, elliptic, free and creamy. Petals 12–18.5 × 5.5–8 mm, elliptic to lanceolate, free and creamy. Labellum 3-lobed, 12–15 × 10–11 mm, creamy, articulate with the column base. Lateral lobes pronounced, falcate, vinous, involving the column longitudinally. Apical lobe round to ovate, with undulating yellow margins, incurved to reflexed. Apical lobe of the labellum with yellow projections (Fig. 1A and B). Central portion of the labellum with two rectangular protuberances just below the anther and stigma (Fig. 1A). Basal portion of the labellum covered with finger-like trichomes (Fig. 1C–E). Column ca. 10 mm in length, straight, incurved and vinous. Stigmatic cavity ca. 1.8 × 2.2 mm, transversally ovate. Anther ca. 2 mm in length, ovate and vinous. Pollinarium ca. 2 mm in length, with two ovoid and yellow pollinia (ca. 1 mm), and a circular, whitish viscidium.

Flowers of *C. arundinae* produce a sweet fragrance. Fragrance is produced by small papillae on the outer surface of the labellum (Fig. 1D–F). Tests with neutral red were positive, confirming these papillae are labellar osmophores. The tests with neutral red were also positive on trichomes located on the base of the labellum (Fig. 1C). These trichomes are clavate, unicellular and uninucleate (Fig. 2A and B). These clavate trichomes are also rich in starch grains, as confirmed using the Lugol test (Fig. 2B). The tests with Sudan III and Sudan Black

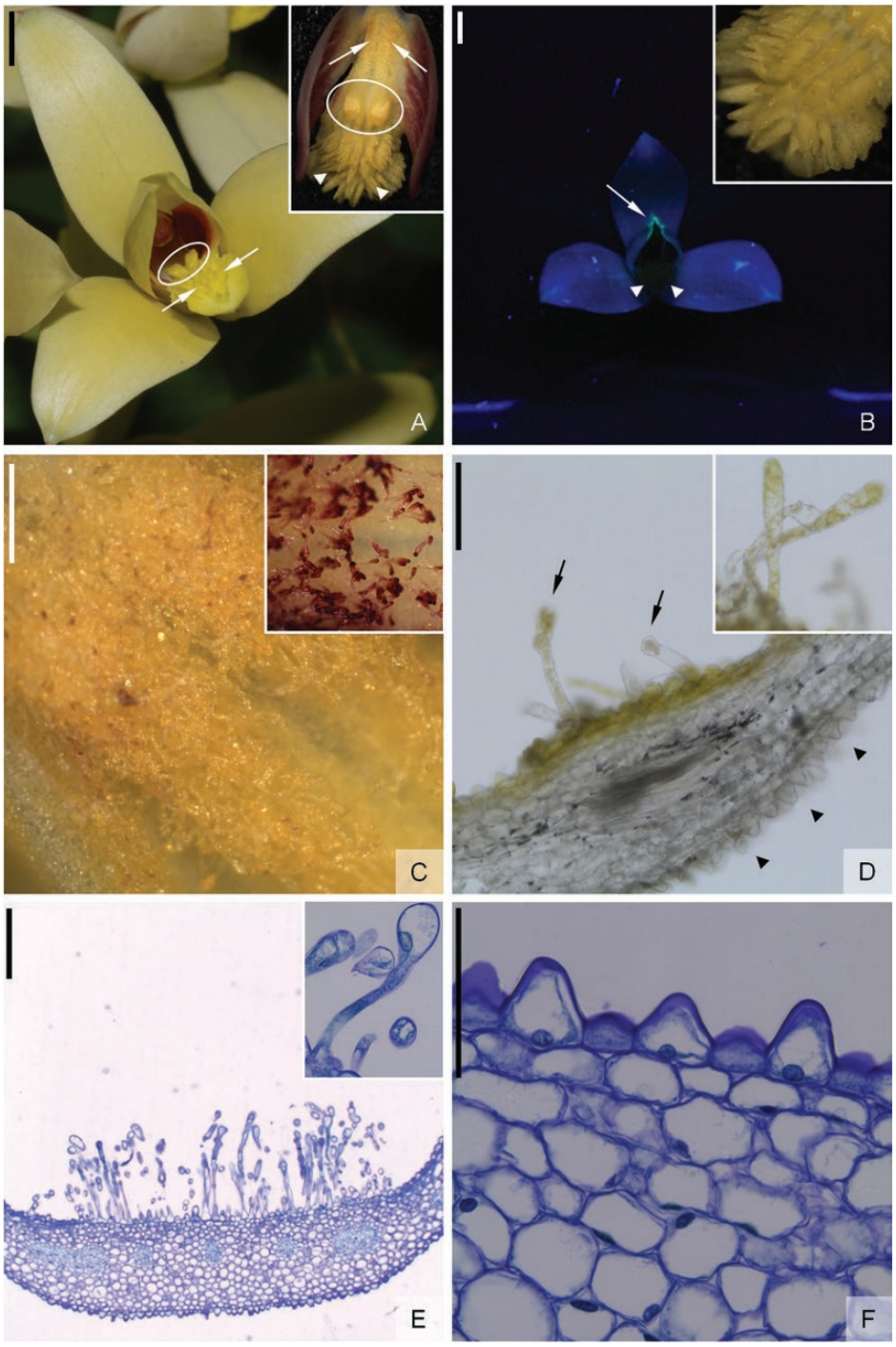

Figure 1. *Cyanaeorchis arundinae*. (A) Flower in front view showing the apex of the labellum with the yellow projections (arrows) and the two trapezoidal protuberances located just below the anther and stigmatic surface (white circle). The detail shows a detached labellum with the yellow projections of the mid-lobe (arrowheads), the two trapezoidal protuberances (white circle) and the clavate trichomes of the lip base (arrows). (B) A flower under UV (325 nm) light. Note the central portion of the labellum absorbs UV light (arrowheads), whereas UV reflection occurs in the apical portion of both petals (arrow). The detail shows the labellar projections without UV light. (C) Detail of the base of the labellum showing the food hairs. The detail shows the clavate trichomes stained with neutral red. (D) Transverse section of the base of the labellum (no coloration). Note the edible trichomes on the adaxial surface (black arrows), and the osmophores on the adaxial surface (arrowheads). (E) Transverse section of the base of the labellum stained with toluidine blue. Note the edible trichomes on the inner surface. The detail shows an isolated unicelullar trichome. (F) Transverse section of the base of the labellum stained with toluidine blue. Note the papillae on the outer surface. Scale bars: A–B = 1 cm; C = 0.5 mm; D = 100 μm; E = 200 μm; F = 50 μm.

Table 1. Bees recorded as floral visitors, pollinators (*), their sex and visitation number on flowers of *Cyanaeorchis arundinae* in the municipality of Uberlândia, southeastern Brazil.

Species	Visits	Pollinator
Apinae, Exomalopsini		
Exomalopsis (*Exomalopsis*) *fulvofasciata* Smith, 1879 ♀	3	*
Apinae, Tetrapediini		
Tetrapedia sp. 1 ♀	1	-
Tetrapedia sp. 2 ♀	1	-
Apinae, Xylocopini		
Ceratina (*Crewella*) sp. ♀	13	*
Halictinae, Augochlorinae		
Augochloropsis sp. 1 ♀	4	-
Augochloropsis sp. 1 ♂	2	-
Augochloropsis sp. 2 ♀	5	*
Halictinae, Halictini		
Pseudagapostemon (*Neagapostemon*) *cyanomelas* Moure, 1958 ♀	8	-
Megachilinae, Anthidiini		
Anthodioctes sp. ♀	5	*
Megachilinae, Megachilini		
Megachile sp. ♀	8	*
Apinae, Meliponini		
Trigona spinipes (Fabricius, 1793) ♀	6	-

B detected the occurrence of oil bodies inside the cells (Fig. 2A), while experiments using Xylidine Ponceau were negative, suggesting the absence of proteins in the labellar trichomes from plants of the studied populations.

Pollinators and pollination mechanisms

In both areas in which our investigation took place, females of several species of bees pollinated *C. arundinae* (Table 1). Visits to flowers were recorded from 0850 to 1335 h. The bees visited from one to three flowers per inflorescence and each visit lasted from 1 to 25 s. During the observations (2012 and 2013 flowering periods), 59 visits were recorded and 11 pollinaria were removed. Visits started with the bee landing on the labellum and forcing their entry headfirst into the straight (up to 4 mm) space formed by the labellum and column. The bees collected edible trichomes on the base of the labellum with their forelegs and afterwards leaved the flowers. Edible trichomes were stored on the hind legs of the bee. Pollinarium deposition occurred when the bee passed the apical portion of the anther. The pollinarium was deposited on the scutum of the bee (Fig. 2c and D). The pollinarium was removed with the anther cap, which fell down after a few seconds (Fig. 2C and D). Pollination occurred when a bee carrying a pollinarium entered headfirst into the floral tube and the pollinium contacted the stigma. The function of the central callus located below the anther and stigma is to lift the bee body and to hinge the anther with the dorsal portion of the bee thorax. Since the flowers of *C. arundinae* closed after 1400 h, nocturnal observations were not performed. A list of floral visitors and pollinators is given in Table 1.

Maximum parsimony tree statistics of isolated and combined regions

Data on tree statistics for individual and combined data sets from MP analyses, including scores of consistency (CI) and retention indices (RI), tree lengths, total numbers of characters in data matrices, phylogenetically informative characters, numbers of variable characters and most parsimonious trees are listed in **Supporting Information [see Table S2]**.

MP analyses of the 5.8S region yielded 81 phylogenetically informative characters, while the 18S and 26S regions yielded 254 and 336 phylogenetically informative characters, respectively. In the MP analyses for Catasetinae, the ITS region yielded 279 phylogenetically informative characters, while the *matK-trnK* and *ycf1* regions yielded 126 and 128 phylogenetically informative characters, respectively. The number of most parsimonious trees was 499 for 5.8S, 1161 for 18S and 2207 for 26S **[see Supporting Information—Table S2]**. In the MP analyses specific for Catasetinae, the number of most parsimonious trees was 829 for ITS, 10 for *matK-trnK* and 48 for *ycf1* region **[see Supporting Information—Table S2]**. Phylogenetic analysis combining sequences from the 5.8S + 18S + 26S (nrDNA) regions resulted in 2984 most parsimonious trees, with 593 informative characters, while the analysis combining sequences of ITS + *matK* + *ycf1* regions resulted in 2414 most parsimonious trees, with 581 informative characters **[see Supporting Information—Table S2]**.

Analyses of isolated and combined data matrices of 5.8S, 18S and 26S for Orchidaceae

In the MP analyses obtained from isolated 5.8S and 18S analyses, Apostasioideae emerged as sister to the Cypripedioideae. In all isolated and combined (MP, BI and ML) analyses, Cypripedioideae appeared as sister to the

Figure 2. (A) Transverse section of the base of the labellum stained with Lugol. Note the starch grains stained dark inside the edible trichomes. The detail shows the starch grains inside the food hairs. (B) Transverse section of the base of the labellum stained with Sudan IV. Note the oil droplets inside the clavate trichomes (arrows). (C) A *Exomalopsis fulvofasciata* leaving a flower with a pollinarium attached to the scutum. Note the pollinarium is removed with the anther cap. (D) A halictid bee abandoning a flower with a pollinarium attached to the scutum (white arrow). The detail shows the Halictidae with a pollinarium (black arrow). Scale bars: A = 50 μm; B = 100 μm; D–E = 1 cm.

Vanilloideae, while the vanilloid clade emerged as sister to the Orchidoideae. Orchidoideae was nested as sister to the large Epidendroideae with strong support (BS 100, PP 1). The MP analyses obtained from isolated 5.8S and 18 yielded a poorly resolved strict consensus tree, with large polytomies embracing the Epidendroideae clades. Conversely, the MP strict consensus tree based on the sequencing of the 26S region, and the analyses (MP, BI and ML) combining the three nuclear regions was almost completely resolved. The topology of the trees based on these analyses was similar (Fig. 3). Differences were found in the topology and the resolution of the larger clades, such as Maxillariinae (Fig. 3). Catasetinae emerged as a monophyletic group with strong support (BS 88) in the isolated 26S analysis, and in all analyses based on the combination of the three (5.8S, 18S and 26S) nrDNA regions, with a BS 81 and PP 99 support in the MP and BI analyses, respectively. Within the Catasetinae, the clade *Cyanaeorchis*/*Grobya* (BS 97, PP 0.99) emerged as sister to the remaining subtribe (Fig. 3). In the MP analysis, the clades *Catasetum*/*Galeandra* (BS 74), *Clowesia* and *Dressleria*/*Mormodes*/*Cycnoches* formed a trichotomy (BS 88). In the BI analysis, the clades within Catasetinae were highly congruent with the MP analysis, but with *Clowesia* emerging as sister to the clade *Catasetum*/*Galeandra* (PP 88) with low PP support (63). The clade *Catasetum*/*Galeandra* emerged as sister to the clade including *Dressleria*/*Mormodes*/*Cycnoches*, whereas *Dressleria* emerged as sister to the clade *Mormodes*/*Cycnoches* (PP 1) with strong support (PP 1). The ML analysis was strongly congruent with the BI analysis (Fig. 3).

According to these results, food hairs have evolved independently at least five times during the evolution of the orchid family (Fig. 3), since this flower reward has previously been recorded in members of the subtribes Dendrobiinae, Eriinae, Maxillariinae and Polystachyinae and, is recorded here for *C. arundinae*, in Catasetinae (Fig. 3).

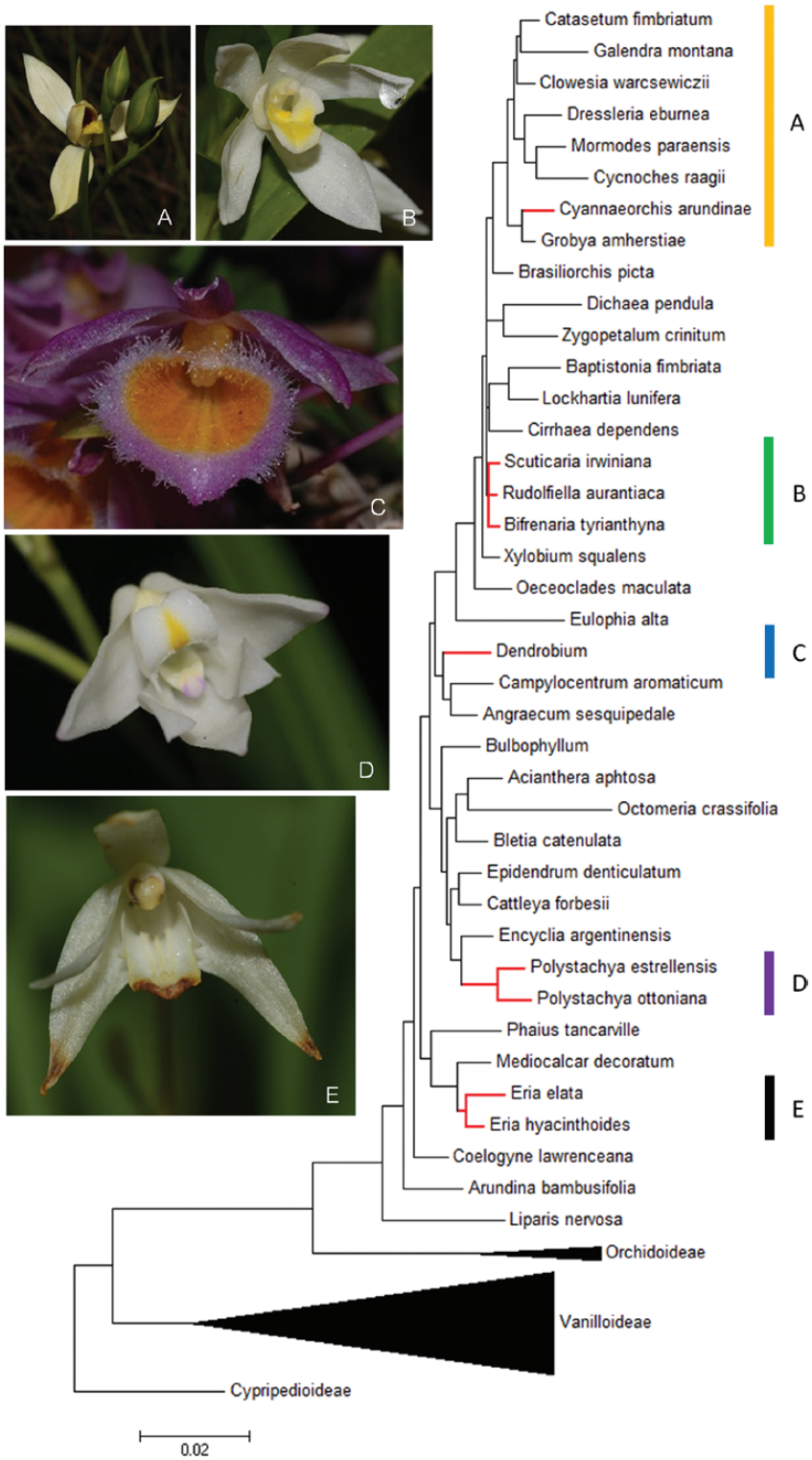

Figure 3. Maximum likelihood analysis of Epidendroideae (Orchidaceae) and outgroups (Cypripedioideae, Vanilloideae and Orchidoideae) based on the combination of the regions 5.8S, 18S and 26S (nrDNA), and Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Vertical coloured bars refer to the clades (not necessarily the taxa) that include species offering food hairs as a resource. Orange = Catasetinae; green = Maxillariinae; blue = Dendrobiinae; purple = Polystachyinae; black = Eriinae. A = *Cyanaeorchis arundinae*; B = *Maxillaria camaridii*; C = *Dendrobium loddigesii*; D = *Polystachya ottoniana*; E = *Eria elata.*

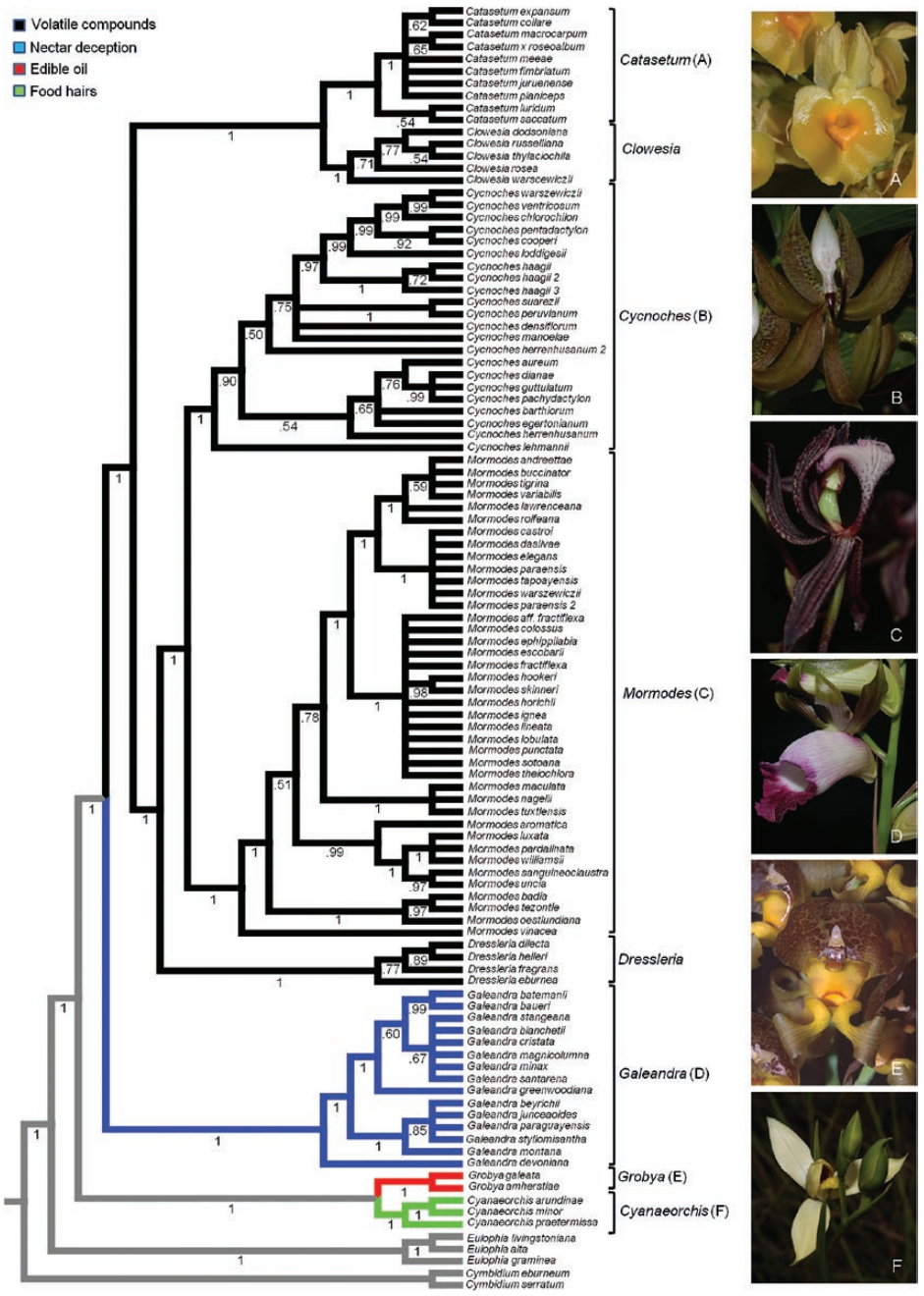

Figure 4. Bayesian inference analysis based on combined ITS (nrDNA), *matK-trnK* and *ycf1* (cpDNA) regions of Catasetinae (Orchidaceae). Posterior probabilities > 0.5 (BI) are given below branches. (A) *Catasetum expansum*; (B) *Cycnoches cooperii*; (C) *Mormodes vinacea*; (D) *Galeandra montana*; (E) *Grobya amherstieae*; (F) *Cyanaeorchis arundinae*. Vertical bars refer to genera within Catasetinae. Gray lines refer to outgroups. Coloured lines = diversity of floral rewards among genera of Catasetinae.

Analyses of isolated and combined data matrices of ITS, *matK-trnK* and *ycf1* for Catasetinae

All analyses obtained from isolated and combined data matrices were strongly congruent in the analyses involving Catasetinae. As in the 5.8S, 18S and 26S analyses, the clade *Cyanaeorchis*/*Grobya* emerged as sister to the remaining Catasetinae with strong support (BS 100, PP 1) in the data matrices based on the combination of the three regions: ITS, *matK-trnK* and *ycf1* (Fig. 4). In all isolated and combined (MP, BI and ML) analyses, *Cyanaeorchis*, which includes species that offer food hairs as a resource, emerged as sister to the oil-offering *Grobya* (Fig. 4). The clade *Cyanaeorchis*/*Grobya* was strongly supported as sister to *Galeandra* (BS 100, PP 1), a genus whose species are pollinated through nectar deception (Fig. 4). The food deceptive *Galeandra* emerged as sister

to a large and well-supported clade (BS 100, PP 1) that produces volatile compounds as a reward and is pollinated exclusively by male euglossine bees (Fig. 4). Within this euglossinophilous clade, *Catasetum*/*Clowesia* (BS 52, PP 1) emerged as sister to *Dressleria* (Fig. 4). *Dressleria* was nested as sister to the clade *Mormodes*/*Cycnoches* (BS 97, PP 1) with strong support (BS 97, PP 1).

Discussion

The flowering period of *C. arundinae*, which occurs from September to October, overlaps with those of other orchids occurring sympatrically in the study areas, such as *Cyrtopodium hatschbachii*, whose flowers offer no reward to pollinators, being pollinated by food deceit (A. A. Maciel, Universidade Federal de Uberlândia, Uberlândia, MG, Brasil, pers. obs.). In contrast, *C. arundinae* produces food hairs as a reward. The production of edible trichomes as a reward has been recorded in *Maxillaria*, as well as *Polystachya*, *Eria* and *Dendrodium* (see Davies *et al.* 2013). Based on its resemblance to flowers of *Eulophia*, *Cyanaeorchis* has been placed among the Eulophiinae (Dressler 1993). However, more recent studies have included this South American genus among the Catasetinae (Batista *et al.* 2014; Whitten *et al.* 2014). The majority of the genera in Catasetinae produce floral fragrances as rewards (e.g. van der Pijl and Dodson 1966; Williams and Dodson 1972; Nunes *et al.* 2017), although edible oils as rewards have been recorded in *Grobya* (Pansarin *et al.* 2009), and nectar deception occurs in *Galeandra* (Whitten *et al.* 2014). The genera *Catasetum*, *Clowesia*, *Cycnoches*, *Dressleria* and *Mormodes* offer floral odours as a resource; these are produced by labellar osmophores (van der Pijl and Dodson 1966; Franken *et al.* 2016). The anatomy of the secretory structures of Catasetinae is poorly known. However, detailed studies of *Cychnoches* and *Catasetum* have revealed that the secretory tissue is epidermal, with several layers of subepidermal parenchyma (Franken *et al.* 2016). In *Grobya*, the edible oil is secreted by elaiophores located on the apex of the labellum and at the column base (Mickeliunas *et al.* 2006). The secretory tissue of the labellar elaiophore is composed of both palisade epidermal cells and elongated unicellular trichomes (Pansarin *et al.* 2009). In addition, the flowers of *Grobya amherstiae* release a honey-like scent produced by epidermal cells distributed along the abaxial surface of the labellum (Pansarin *et al.* 2009). Thus, to the best of our knowledge, this is the first report of edible trichomes in Catasetinae.

The food hairs of *Cyanaeorchis* are rich in starch grains and lipoidal droplets. The occurrence of starch grains has also been recorded in abundance in the labellar trichomes (i.e. pseudopollen) of *Dendrobium unicum* (Davies and Turner 2004*b*), while this carbohydrate is also present in the edible trichomes of some species of *Maxillaria* (see Davies and Turner 2004b). Although our tests with Xylidine Ponceau on the food hairs of *C. arundinae* were negative, indicating the absence of proteins in the specimens from the study site, the pseudopollen of many species of *Polystachya* and *Maxillaria* contain protein bodies. The occurrence of lipid droplets, as recorded here in the edible trichomes of *C. arundinae*, has been more rarely recorded as a component of edible trichomes, but these lipoidal substances have been observed in *Maxillaria* (Davies *et al.* 2000).

As recorded for other genera of Catasetinae (e.g. van der Pijl and Dodson 1966; Williams 1982; Williams and Whitten 1983; Nunes *et al.* 2017), *Cyanaeorchis* is pollinated by bees. Among the bees, undoubtedly male euglossines are the most important pollinators. In fact, the association between male euglossine bees and members of the Catasetinae (i.e. *Catasetum*, *Clowesia*, *Cycnoches*, *Dressleria* and *Mormodes*) has frequently been reported (e.g. Williams 1982; Williams and Whitten 1983; Nunes *et al.* 2017). Genera within Catasetinae (except *Galeandra*, *Grobya* and *Cyanaorchis*), plus Stanhopeinae and some species of Lycastinae, Maxillariinae, Oncidiinae (Ornithocephalinae included) and Zygopetalinae, are pollinated by male euglossine bees that are attracted by volatile compounds produced by the flowers. Besides Orchidaceae, male euglossine bees are known to collect fragrances from several plant families, such as Annonaceae, Araceae, Euphorbiaceae, Gesneriaceae and Solanaceae (e.g. Williams 1982; Teichert *et al.* 2009). The floral odours are composed mainly of terpenes, esters and aromatic compounds, totalling more than 60 known compounds (e.g. Williams and Dodson 1972). In *Grobya*, the lipoidal substances are collected by Tapinotaspidini bees, namely *Paratetrapedia* (Mickeliunas *et al.* 2006) and *Trigonopedia* (E. R. Pansarin, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - FFCLRP/USP, Ribeirão Preto, SP, Brasil, pers. obs.); these bees collect edible oils produced by elaiophores at the apex of the labellum and the base of the column. In *Galeandra*, the pollinators are large social and solitary bees, which search for nectar on flowers (e.g. *G. beyrichii*, *G. minax*, *G. montana*; E. R. Pansarin, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - FFCLRP/USP, Ribeirão Preto, SP, Brasil, pers. obs.). However, the spur is dry and the species are pollinated through food deception (e.g. Whitten *et al.* 2014; E. R. Pansarin, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - FFCLRP/USP, Ribeirão Preto, SP, Brasil, pers. obs.).

Flowers of *C. arundinae* produce edible trichomes as a reward; these are collected by native solitary and social

bees. The presence of oil droplets and starch grains in the food hairs confirms that this material is nutritive to bees, similarly to the pseudopollen of Brazilian species of *Polystachya* (Pansarin and Amaral 2006). Based on their superficial resemblance to pollen grains, these edible trichomes have been considered a form of pollination by food deception (see Davies and Turner 2004*a*, *b*; Davies *et al.* 2013). However, although we do not know the intention of the bees, since this parameter was not tested in this work, bees probably collect pseudopollen because this is a nutritive material and not because it looks like pollen grains. In fact, as presented here for *C. arundinae*, the food hairs are nutritive and actively collected by bees, but do not resemble pollen grains.

Our results suggest that edible trichomes have evolved independently at least five times in Orchidaceae, as this flower reward has been recorded in members of the Eriinae, Dendrobiinae, Maxillariinae and Polytachyinae and, as recorded here, in Catasetinae (i.e. in *Cyanaeorchis*). Edible trichomes have also been reported in other unrelated plant families, such as Calycanthaceae, Nymphaeaceae and Pandanaceae (Faegri and van der Pijl 1979; Cox 1982; Thien *et al.* 2009), suggesting parallel evolution of this flower resource during the evolution of flowering plants. However, to date, no studies have been conducted on this subject.

Our results also reveal that nutritive materials, such as edible oils and food hairs, are found in the clade *Grobya*/*Cyanaeorchis*. The remaining Catasetinae do not offer food material as a reward to pollinators, since members of the clade containing the species of *Galeandra* are pollinated by nectar deception (e.g. Whitten *et al.* 2014; E. R. Pansarin, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - FFCLRP/USP, Ribeirão Preto, SP, Brasil, pers. obs.), and members of the clade containing the genera *Dressleria*, *Mormodes*, *Cycnoches*, *Clowesia* and *Catasetum* produce perfumes as a reward, being pollinated exclusively by male euglossine bees (e.g. van der Pijl and Dodson 1966; Williams 1982; Williams and Whitten 1983; Nunes *et al.* 2017). Thus, according to our data (Fig. 4), the offering of perfumes as a resource evolved only once, in the euglossinophilous clade.

Among orchids, it has been widely suggested that deceptive mechanisms evolved from reward-based pollination systems (Dafni 1984; Ackerman 1986; Nilsson 1992). This assertion has been corroborated in some isolated cases (e.g. Ackerman 1986; Johnson and Nilsson 1999; Johnson 2000), but has rarely been based on a phylogeny (Cozzolino *et al.* 2001; Pansarin *et al.* 2012). Dressler (1981) stated that shifts from rewarding to deceptive pollination systems may have occurred many times during the evolution of orchids. Based on the reconstruction of a phylogeny, Johnson *et al.* (1998) recorded three evolutionary transitions between food-producing and deceptive flowers in *Disa*. However, analyses of pollination strategies mapped onto phylogenetic trees for Orchidinae have not supported this hypothesis, suggesting instead that food deception is the ancestral and predominant pollination strategy in this tribe, and that reward-based pollination evolved independently more than once from food deception (Cozzolino *et al.* 2001). Within the vanilloid tribe Pogonieae (Orchidaceae), reward production has also been tested on the basis of a phylogenetic hypothesis (Pansarin *et al.* 2012). According to this study, ancestors of Pogonieae have given rise to two lineages, one of them spreading into tropical America, eventually originating the extant Neotropical *Cleistes*, with nectariferous flowers, and another one predominantly North American-Asiatic, with pollination based on food deception (Pansarin *et al.* 2012).

Some authors argue that the production of a resource by the flower can be energetically expensive for plants and that in species pollinated through deception, the energy used for reward production could be allocated more usefully for other functions increasing fitness (e.g. Ackerman 1986). Apparently, for this reason, food deceptive lineages have arisen frequently from reward-producing ancestors (e.g. Pansarin *et al.* 2012). The main problem with this hypothesis is that in many orchids, fitness is pollination limited, rather than resource limited (Calvo 1993). Furthermore, although few studies have been performed on this subject (see Pyke 1991), the rate of resource production (i.e. nectar) tends to be lower in short-lived than in long-lived flowers (Johnson and Nilsson 1999; Pansarin *et al.* 2012). Conversely, when compared with mechanisms involving the production of flower resources, deception may reduce the frequency of visits and as a consequence, reproductive success (Dafni 1984; Ackerman 1986; Johnson and Nilsson 1999; Johnson 2000).

Conclusions

Our data show that *Cyanaeorchis* is unique among the Catasetinae in producing food hairs as a reward and is pollinated by bees. *Grobya* produces edible oil as a resource, while the remaining genera within the Catasetinae clade do not offer food material as a reward to pollinators. *Galeandra* is pollinated by nectar deception, and the members of the clade containing the genera *Dressleria*, *Mormodes*, *Cycnoches*, *Clowesia* and *Catasetum* offer perfumes as a non-nutritive reward to male euglossine bees. Our data indicate that perfume rewards evolved only once in the Catasetinae. Our analyses also suggest that edible trichomes evolved independently five times across the orchid family.

Supporting Information

The following additional information is available in the online version of this article—
Table S1. Species of Catasetinae (Orchidaceae) and outgroups included in the molecular studies, vouchers, data collected and GenBank accession numbers.
Table S2. Summary of results of maximum parsimony analyses of Catasetinae (Orchidaceae, Epidendroideae).

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Contributions by the Authors

E.R.P. and A.A.M. developed the idea of the study, performed the experiments and wrote the manuscript; A.A.M. participated in describing the results. All authors read and approved the final version of the manuscript.

Conflicts of Interest

None declared.

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