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Can nectary structure in Laeliinae promote or constrain nectar secretion?

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Abstract

The orchid subtribe Laeliinae has an assemblage of morphologically diverse taxa. The diversity in floral morphology of its members can be explained in terms of pollination ecology in that this subtribe contains both entomophilous and ornithophilous species. Given the wide range of pollinators, one would expect to find considerable differences in morphology of the floral nectaries. Fully developed nectaries appeared to be entirely non-functional in some taxa. The aim of this work was to compare the micromorphology of the inner nectary spur in selected representatives of Laeliinae in order to ascertain which structural features improve or reduce nectar secretion, and thereby contribute towards the evolutionary success of this subtribe. Here, we investigate the nectary structure of 48 species representing the genera *Prosthechea*, *Encyclia, Epidendrum* and *Dinema*. Of these, the nectary of *Encyclia* was of the narrow-tubular form (cuniculus-type), that of *Prosthechea* and *Dinema* was short and sac-like, whereas both nectary types were present in *Epidendrum*, the former type being the more common. Whereas the nectary of *Dinema* contained nectar, this was either absent or present in nectaries of the other three genera. Statistical analyses of the morphological and micromorphological characters of the nectary revealed that the probability of nectar being present was lower for the long, tubular nectaries (e.g. *Encyclia* and *Epidendrum*), whereas most *Prosthechea* spp. investigated, as well as *Dinema*, possessed sac-like, functional nectaries. Also, all investigated taxa, irrespective of the presence of nectar, shared a thick cuticle and thick epidermal and subepidermal cell walls (in the secretory layer). Analyses also showed that the probability of nectar being present increased with an increase in the thickness of the secretory layer. Furthermore, there was also a greater probability of the epidermal cells lining functional nectaries having a smooth cuticle. The occurrence, or otherwise, of nectar may indicate that the secretory capacity of this group of orchids is plastic, and not limited by structural constraints, thus allowing for the relatively easy turning on and off of the secretory process.

Keywords Orchidaceae, Orchids, Nectaries, Micromorphology

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Introduction

The subtribe Laeliinae Benth. has a Neotropical distribution. Neotropical orchids display great diversity with regard to their habitats, morphology and pollination ecology. Of these orchids, the mega-diverse subtribe Laeliinae, which includes the genera *Epidendrum* L., *Encyclia* Hook., *Prosthechea* Knowles & Westc. and *Dinema* Lindl., shows a wide range of flower morphology and floral rewards that is reflected in the diversity of its pollinators. Although recent molecular investigations provide further data on the phylogeny and floral characteristics of Laeliinae [1–12], the occurrence of rewards and morphological adaptations to pollinators have generally been neglected. There are, however, exceptions to this, such as studies of *Encyclia mapuerae* (Huber) Brade & Pabst [13], *Epidendrum densiflorum* Hook. [14] and *Cattleya cernua* (Lindl.) Van den Berg [5, 15], for which reward production, spur structure and pollination mechanisms are described in detail.

Based on an analysis by [16], the presence of floral nectar in both temperate and tropical orchids can double their reproductive success. Nectar is the most common floral food-reward in Orchidaceae, the rewardless state being regarded as an ancestral character in this enormous family, though seemingly not so in Laeliinae, where the ancestral state, as represented by *Dinema, Encyclia* and *Epidendrum*, is nectariferous [11, 12, 17]. In Orchidaceae, nectar secretion has been repeatedly gained and lost in several lineages [17–22]. For example, independent nectary evolution in *Disa* P.J. Bergius [22] involved both repeated recapitulation of the secretory epidermis, and the acquiring of stomatal nectaries.

In angiosperms, floral nectaries can be associated with any floral structure [23]. In eudicots, CRABS CLAW (CRC), a YABBY-like transcription factor (involved also in gynoecium development), is critical for the initiation and regulation of floral nectary development, but in *Aquilegia* L., the development of nectary spurs was instead found to involve the gene STYLISH [24–26]. Moreover, variation in spur length and shape can be hormonally controlled by mechanisms of cell proliferation and cell expansion, resulting in a range of final spur morphologies [25]. To date, no analogous gene programs for nectary initiation are known for monocots. However, [24] reported that the gene CvSWEET9 is necessary for nectar formation and secretion in the dicot *Cleome violacea* L.

In Orchidaceae, floral nectar may be secreted by cells located on the adaxial surface of the labellum, as in *Maxillaria* Ruiz & Pav. and *Bulbophyllum* Thouars [27, 28] but floral spurs are also common in this family [23, 29]. Labellar spurs may be formed by fusion of the labellum in its basal parts, as in many Orchidiinae [19], or alternatively, it may involve the column-foot and sepals (e.g. *Dendrobium* Sw.). However, in the majority of Laeliinae spp., the floral spur is concealed within the flower (inner spur), and is formed by fusion of the basal part of the labellum, the column and the ovary. This inner floral spur is termed the cuniculus, and occurs as a long and narrow tube that runs alongside the transmitting tract, or frequently, alongside the ovary. In some Laeliinae, however, the inner spur is much shorter and wider, which results in a shallow, sac-like structure. As well as variation in length and shape, spurs may also differ in the structure of their overlying epidermal cells. The epidermis may be glabrous (comprising flattened epidermal cells), papillose or trichomatous [17, 30, 31]. However, the most important difference between spurs, irrespective of their shape, is whether or not nectar is produced. Despite the enormity of subtribe Laeliinae, there is a paucity of data relating to floral food-rewards for most of its taxa, including *Prosthechea*. In most reports concerning Laeliinae, nectar-producing and nectarless species have been distinguished solely by gross macroscopic observation, without detailed inspection of the flower. In *Epidendrum*, which contains about 2000 species, nectar has hitherto been found only in a small number of taxa [17, 20, 32–35]. Cardoso- Gustavson et al. [17] observed that nectar in *Epidendrum* is produced by the smooth epidermal cells lining the spur. Similarly, TEM studies have also demonstrated for a number of species that the epidermal cells lining the cuniculus are secretory and that surface secretion is present irrespective of the shape of these cells or whether the overlying cuticle is ornamented. These species include *E. capricornu* Kraenzl., *E. criniferum* Rchb.f., *E. ciliare* L., *E. pseudepidendrum* Rchb.f. and *E. radicans* Pav. ex Lindl. [30], all of which were previously reported to lack nectar.

Our aim in this study was to investigate and to compare the micromorphology of the inner floral spur of selected species of *Prosthechea*, *Encyclia, Epidendrum* and *Dinema.* These species were selected as models representing both nectariferous and nectarless taxa. We examined this structure at tissue and cellular levels in order to determine structural features that might facilitate or impede nectar secretion. Finally, we subjected the data obtained to statistical analysis, and comparisons were made.

Material and methods

Study design

For this study, we used species of *Prosthechea, Encyclia*, *Epidendrum* and *Dinema* as model taxa for micromorphological and histochemical analyses. The plants used in this study were obtained from the living collections of the Botanic Garden of the University of Warsaw, Poland; the Botanic Garden of Jagiellonian University in Kraków,

Poland; Prague Botanical Garden, Czech Republic; as well as from the collection of Dr Emerson R. Pansarin, housed at the LBMBP Orchid House, FFCLRP-USP, Brazil; from the private collection of Dr Kevin L. Davies at Swansea, UK (prefix KLD); and from Singleton Botanical Gardens, Swansea, UK (prefix S). The list of investigated species with accession numbers is attached as Supplementary material in Table S1. Investigations were undertaken on 19 species of *Prosthechea*; 13 species of *Encyclia*; 15 species of *Epidendrum*; and one species of *Dinema*. We selected species for this research based on our preliminary macroscopic observations.

With the exception of results previously published for *E. mapuerae* [13], all data presented for *Prosthechea* and *Encyclia* are entirely novel. For *Epidendrum,* as well as data previously published [30], new structural data are presented here for *E. centropetalum* Rchb.f., *E.katarunyariku* Hágsater & Wrazidlo and *E. secundum* Jacq. Since the nectary structure of *Prosthechea* has not previously been investigated, we also present data for this genus in diagrammatic form.

The position of the spur and presence of nectar in longitudinally sectioned flowers on the first day of anthesis were determined for each species by means of a Nikon SMZ100 stereomicroscope. For these investigations, five to seven flowers were used. Identification of osmophore tissue in fragrant species was undertaken by means of intra vitam staining of entire flowers with neutral red (NR) according to Vogel [36]. Stained areas were subsequently investigated by means of light microscopy for volatile compounds using NADI reagent [37], as well as NR under UV. Images of the osmophores are provided as Supplementary Fig. S1. The structure of the tissues surrounding the lumen of the spur was subsequently examined using bright-field light microscopy (LM), Nomarski differential interference microscopy (NDIM), fluorescence microscopy (FM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) according to the methods published previously [30, 38]. Detailed micrometry and photomicrography of secretory structures were performed by means of a high-resolution digital camera and NIS software (Nikon). Results in Table S1 represent the means of five replicates.

Statistical analysis

For statistical analyses, we used R software [39]. Using generalised linear model (GLM) with binomial distribution of the response variable, we investigated what effect the following nectary micromorphological characters had on the probability that nectar was present: overall shape (cuniculus vs. sac-like); the type of epidermal cells (flattened vs. papillose/trichomatous); the occurrence of starch (present vs. absent); cuticular ornamentation (present vs. absent); thickness of the outer tangential wall of epidermal cells with cuticle (as outer tangential cell wall); thickness of the tangential walls of subepidermal cells; and finally, the thickness of the secretory layers (epidermis plus subepidermal cells possessing dense cytoplasm and thick cell walls, irrespective of the occurrence of nectar).

Using the same GLM method and a similar set of seven predictors, we assessed the probability of the presence of nectar in these nectaries. Note that instead of simply using the presence of nectar in nectaries as a criterion, we used the presence of the cuniculus-type of nectary in the global GLM. Furthermore, we undertook a more detailed analysis of the individual genera based on the potential of nectar production relative to the thickness of the outer tangential epidermal cell wall; the thickness of cell walls in the subepidermal layer; and the thickness of the secretory layer. Since nectary characters in *Prosthechea* proved to be almost uniform relative to the presence of nectar, this genus could not be included in the analysis, as was also the case for monotypic *Dinema*. Thus, for each of the two genera that permitted us to build a model, namely, *Epidendrum* and *Encyclia*, we constructed a single GLM with binomial distribution of the response variable.

Employing linear regression (LR), and assuming the close-to-normal distribution of response variables, we assessed what effect nectary micromorphological features might have on the thickness of epidermal and subepidermal cell walls, as well as on the thickness of the secretory layer. In considering the thickness of the epidermal cell wall, the global LR comprised four predictors, namely: the presence of nectar (present vs. absent); type of nectary (cuniculus vs. sac); cuticular ornamentation (present vs. absent); and the type of epidermis (flat vs. papillose/trichomatous). With regard to the thickness of subepidermal cell walls, we noted three explanatory variables in the global LR, namely: the presence of nectar; a cuniculus-type nectary; and the thickness of the epidermal cell wall. In accounting for the thickness of the secretory layer, the global LR comprised three predictors: namely, the presence of nectar; a cuniculus-type nectary; and the presence of starch in secretory cells.

In calculating the variance inflation factors (VIF), we checked for collinearity between all the predictors included in each global GLM and LR performed. However, we found no collinear explanatory variables, as indicated by VIF values of less than five for each predictor in each model. Using the *MuMIn::dredge()* function [40], we reduced all global GLMs and LRs containing all assumed predictors to minimize AICc, and we built final models based on all predictors included in a set of models with ΔAICc of less than two. It is worth noting that in evaluating the significance of the results, our priority

was the effect of sample sizes, not *P*-values. We chose this approach because *P*-values can be heavily influenced by sample size (i.e. low *P*-values for small sample sizes, and high *P*-values for large sample sizes), leading to the misinterpretation of ecologically important results by regarding them to be statistically insignificant [41]. Consequently, we visualised the results of the final models using marginal responses, i.e. predicted values assuming effects of all other explanatory variables at a constant (mean) level, using the Tukey posteriori test with studentized adjustment for multiple comparisons and the *emmeans::emmeans()* function [42] for categorical predictors, and *ggeffects::ggpredict()* function [43] for continuous variables.

Results

Micromorphology

In all the investigated species, the cuniculus and sac-like nectaries were located at the base of the column and labellum (Fig. 1A-I). Irrespective of the presence of nectar, the investigated species of *Prosthechea* possessed a sac-like nectary that extended as far as the base of the perianth (Fig. 1A-C), with a single exception, *P. chacoensis* (Rchb.f.) W.E. Higgins. Here, as in *Encyclia,* the nectary ran beneath the perianth segments (Fig. 1D-F). In *Epidendrum*, the narrow cuniculus ran alongside the ovary (Fig. 1G-H), except in the case of the short nectary of *E. centropetalum* (Fig. 1I). In *Prosthechea* and *Encyclia*, multicellular trichomes (Figs. 1A-B and 2A) were present at the base of the labellum close to the entrance to the nectary which, in some instances, was partly occluded by a tuft of hairs (Fig. 1A-B). The labellar trichomes of fragrant species, such as *P. fragrans* (Sw.) W.E. Higgins, *P. aemula* (Lindl.) W.E. Higgins and *P. radiata* (Lindl.) W.E. Higgins, selectively accumulated neutral red, contained lipid droplets that stained with Sudan IV and gave a positive reaction with the NADI test. Such droplets were also present in the cytoplasm of the flattened epidermal cells (Fig.S1. A-F). In nectarless *P. calamaria* (Lindl.) W.E. Higgins, the trichomes and subepidermal parenchyma cells contained numerous starch grains, but lipids were absent from the chloroplasts of the subepidermal parenchyma (Fig. S1. G-H). The epidermal cells lining the sac-like nectary of *Prosthechea* were either uniformly papillose or trichomatous, according to whether they occurred on the upper or lower part of the sac. Close to the nectary entrance, they were papillose and possessed a striate cuticle, whereas at the sac base, they were dorsoventrally flattened with a smooth or weakly ornamented cuticle (Figs. 2B-H, 3A-D). In *Encyclia,* the epidermis was uniformly papillose (Fig. 3E-F), whereas in *Epidendrum*, it was mainly papillose, but occasionally trichomatous (Fig. 2I-J, Supplementary Table 1). In all the investigated species, the epidermal and subepidermal parenchyma cells had thick cellulosic cell walls (Figs. 3A-H; 4A-I; 5A-F), and in particular, thick tangential walls. This secretory layer of thick-walled cells varied considerably in depth, consisting of a single layer of epidermal and subepidermal parenchyma cells, as in *E. alata* (Bateman) Schltr. (Fig. 3E-F), or several layers, as in the remaining investigated species (Figs. 3A-D, G-H; 4A-I, K). Irrespective of the occurrence of nectar, the thick outer tangential cell walls of the epidermis possessed a thick cuticle (Figs. 3A-B; 4A-J; 5A-F). Surface secretion (nectar) was visible in hand-cut sections of living material (Fig. 4D, E, H, K) and also in TEM images of fixed material (Fig. 5D-E). The cuticle was continuous (without ruptures), but irregular and patchy in structure, and contained strongly osmiophilic areas (Fig. 5A-F). The cuticle of nectarless, but strongly fragrant *E. cordigera* (Kunth) Dressler contained small lipid droplets (Fig. 5F) and its plastids contained plastoglobuli (Fig. 5B). The plastids of nectariferous and fragrant *P. baculus* (Rchb.f.) W.E. Higgins and *P. chondylobulbon* (A. Rich & Galeotti) W.E. Higgins (Fig. 5G) also contained numerous plastoglobuli, and very small grains of starch were occasionally visible in the secretory layer, whereas, by contrast, the plastids of *P. aemula* contained numerous starch grains, but plastoglobuli were scarce (Fig. 5H). The characters and measurements for the investigated species are summarized as supplementary material in Table S1.

Data analysis

In considering the shape of the nectary, the final GLM consisted of five predictors (Table 1). In the cuniculus-type nectary, the percentage probability for the presence of a papillose/trichomatous epidermis (Fig. 6A) and ornamented cuticle (Fig. 6 B) was 33% and 15% greater, respectively, in terms of nectar absence (Fig. 6 C).

With regard to the percentage probability of starch in the nectariferous secretory layer, this was 30% lower in the cuniculus-type nectary than in its sac-like counterpart (Fig. 6D), and decreased from 92% for a secretory layer 20 µm thick to 30% for a thicker secretory layer of 110 µm (Fig. 6E).

The final GLM predicting the probability of the presence of nectar in nectaries comprised seven predictors (Table 1). The percentage probability for the presence of nectar was 3% lower in the case of the cuniculus-type nectary as compared to the sac-like nectary (Fig. 7A); 20% lower for the papillose/trichomatous epidermis compared to flattened epidermal cells (Fig. 7B); 18% lower for an ornamented cuticle compared to one lacking ornamentation (Fig. 7C); and 10% greater for the presence of starch in secretory cells as compared to starch-less cells (Fig. 7D). The percentage probability for the presence of

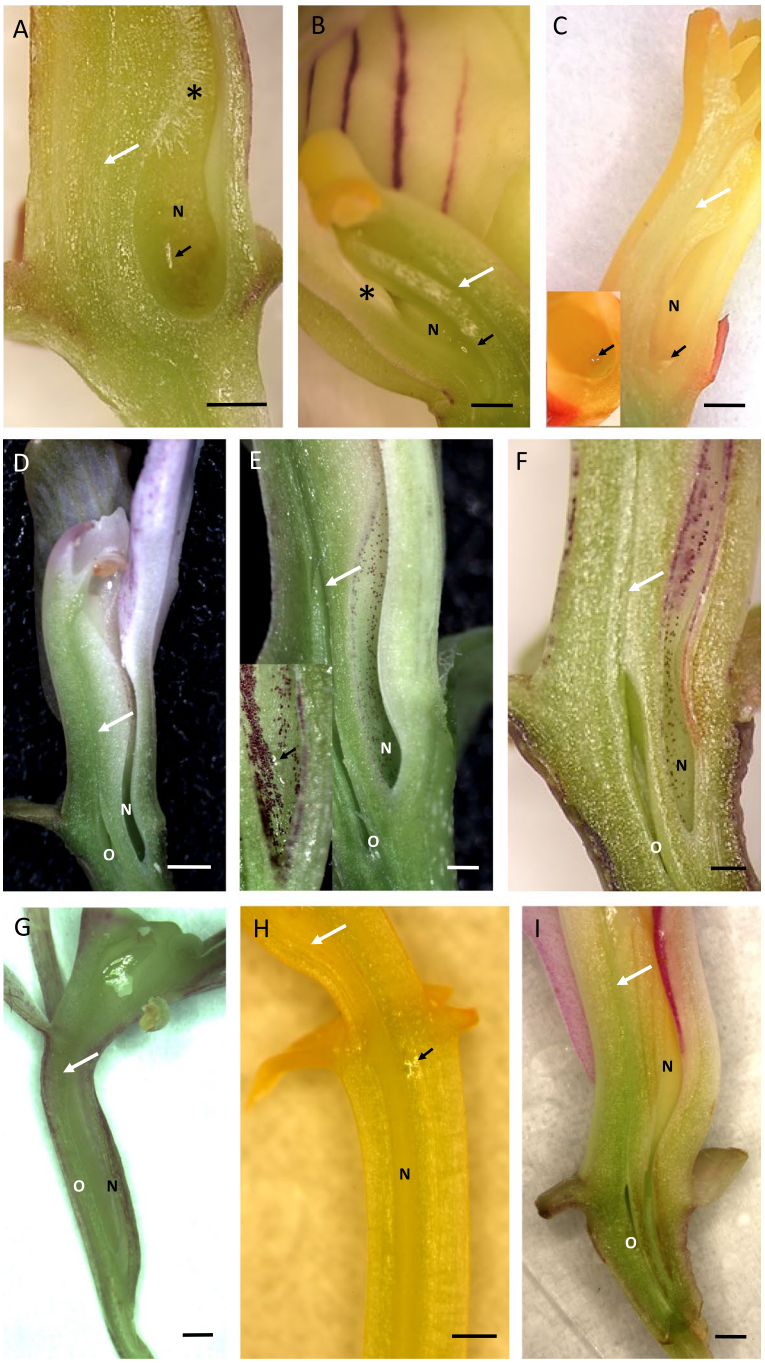

Fig. 1 Half flowers of Laeliinae showing form and position of nectaries. Black arrows indicate droplets of nectar, white arrows indicate stylar canal, N = nectary, O = ovary. A-F Sac-like nectaries; G-I Cuniculus-type nectaries. **A**—*Prosthechea livida*; **B**—*P. radiata*. In **A** and **B**, a tuft of trichomes is indicated by an asterisk; C—*P. vitellina*. Insert shows droplet of nectar at base of nectary (arrow); **D**—*Encyclia santanae*; **E**—*E. argentinensis*. Insert shows droplets of nectar at base of nectary (arrow); **F**—*E. guatemalensis*; **G**—*Epidendrum porpax*; **H**—*E. katarun-yariku*; **I**—*E. centropetalum*. Scale bars: **A**—**C**, **E**, **F** and **I** = 0.5 mm, **D** = 5 mm, **G**, **H** = 1 mm

nectar increased with increasing thickness of the secretory layer from 29% for 20.00 µm to 46% for 110.00 µm (Fig. 7E), as well as with the increasing thickness of the outer tangential epidermal cell wall and cuticle layer from 25% for 2.00 µm to 77% for 13.00 µm (Fig. 7F). Conversely, it diminished slightly with an increase in the

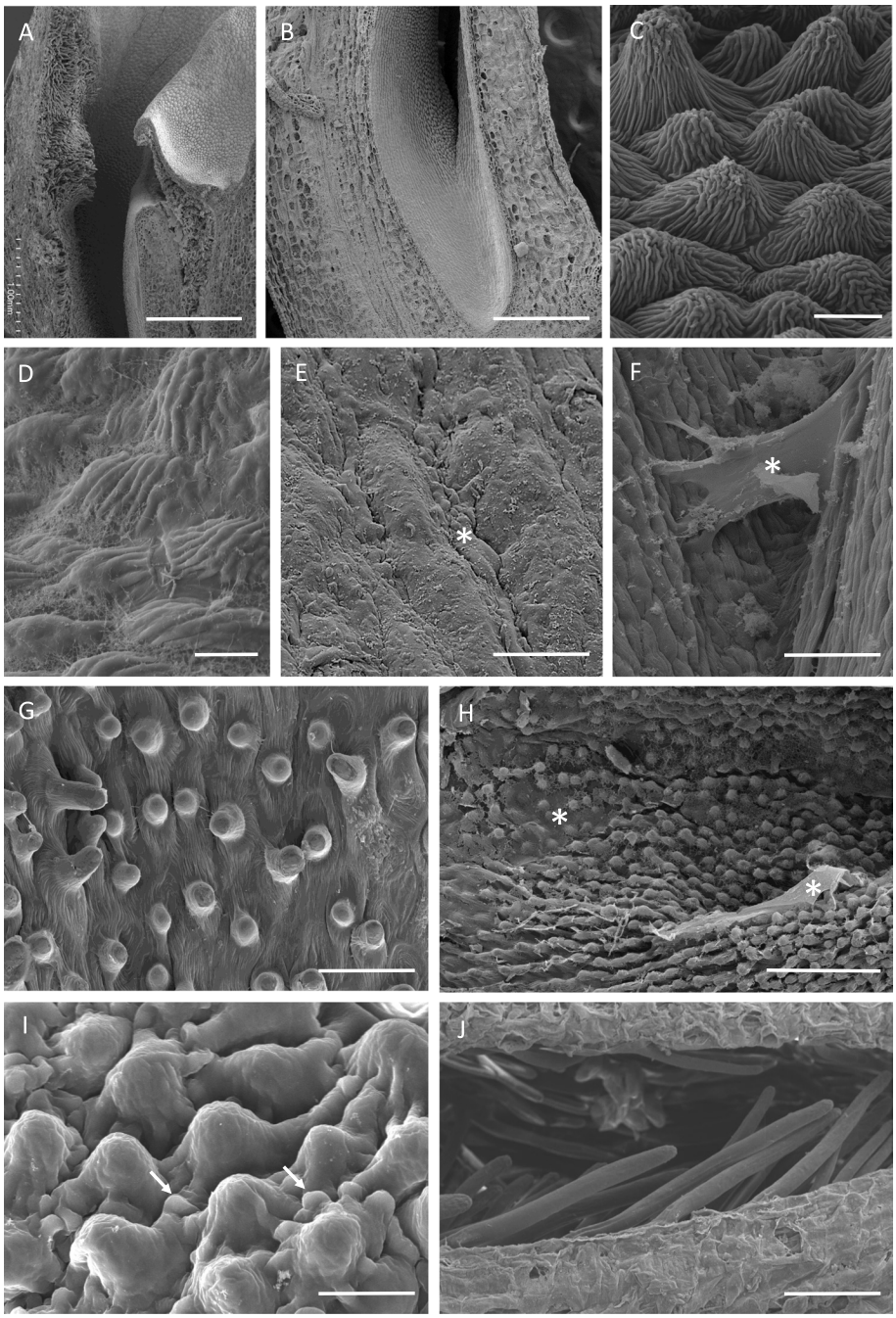

Fig. 2 Detail of sac-like nectaries and cuniculus, showing variously shaped epidermal cells, SEM. **A**—Trichomes at the entrance to the nectary of *Prosthechea radiata*; **B**—Nectary of *P. vitellina*; **C**—**D**. Nectary of *P. cochleata*. **C**- Papillose epidermal cells with striate cuticle at the upper part of the nectary; **D**—Flattened epidermal cells with striate cuticle at the base of the nectary; **E**—Epidermis with secretory residues (asterisk) at the base of the nectary of *P. vitellina*; **F**—Base of nectary of *P. radiata* showing secretion (asterisk); **G**—Nectary trichomes of *P. prismatocarpa*; **H**—Papillose nectary surface of *P. aemula*. Secretion is marked with an asterisk; **I**—Papillose cuniculus surface of *Epidendrum katarun-yariku*. Arrows indicate blistered cuticle; **J**—Trichomes within cuniculus of *E. radicans*. Scale bars: **A** = 1 mm, **B** = 500 µm, **C**, **E**, **I** = 20 µm, **D** = 10 µm, **F** = 30 µm, **G** = 50 µm, **H**, **J** = 100 µm

thickness of the cell walls of the subepidermal parenchyma from 34% for ~1.00 µm to 33% for 12.50 µm (Fig. 7G).

In considering the probability of the presence of nectar in the nectaries of *Epidendrum*, the final GLM comprised three predictors (Table 1). The percentage probability for

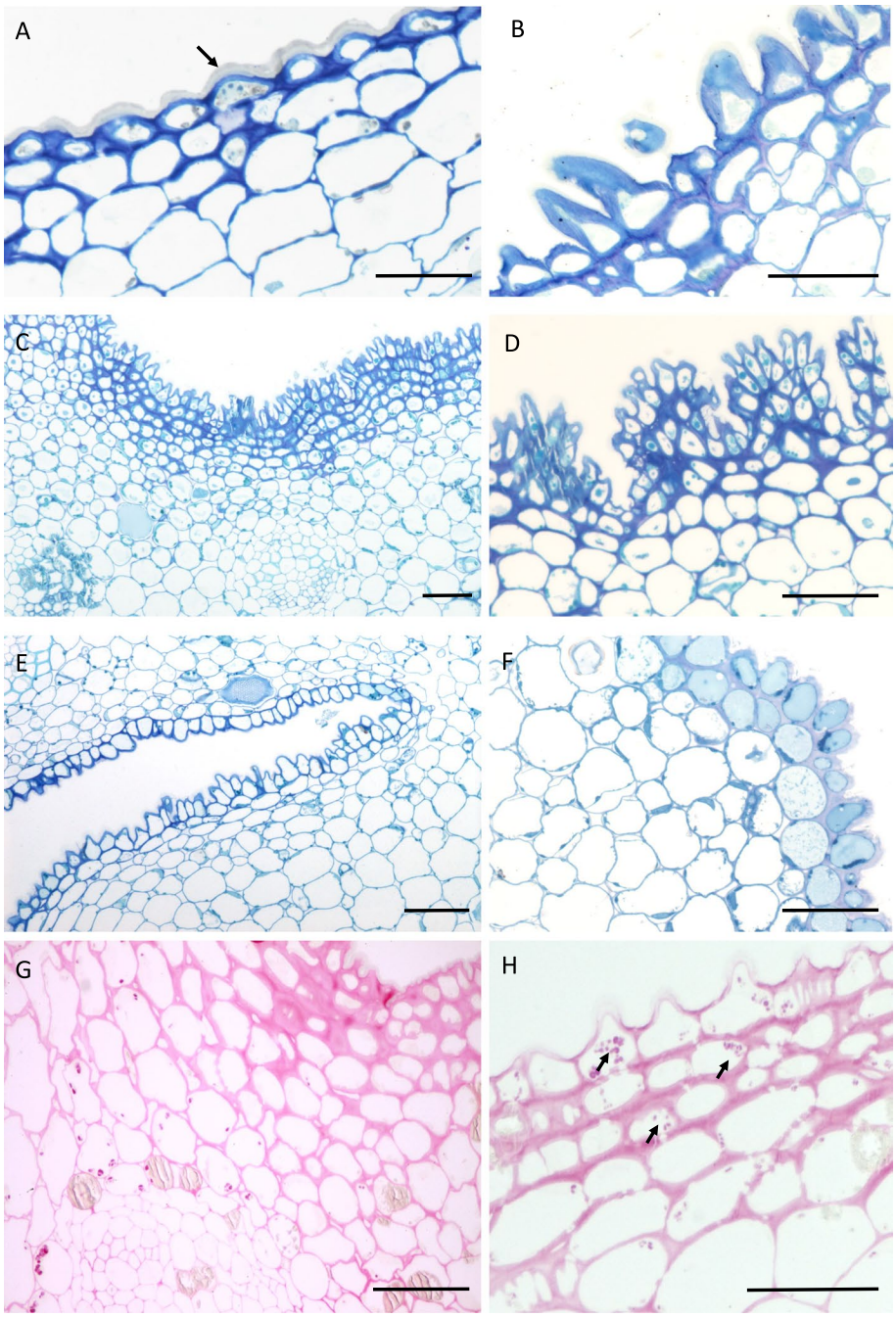

Fig. 3 Anatomy and histochemistry of nectaries, LM. Note the thick cell walls of the secretory layer. **A**—**F** Nectary tissue following staining with TBO and G-H, the PAS reaction. **A**—Flattened epidermal cells of *Prosthechea vitellina*. Thick cuticle indicated by arrow; **B**—Epidermal trichomes of *P. prismatocarpa*; **C**—**D**—Papillose ridges of *P. chondylobulbon*; **E**—**F**—Papillose surface of the nectary of *Encyclia alata*. Note thin walls of cells in subepidermal layer; **G**—Starch grains of *Prosthechea cochleata* concentrated close to a nectary vascular bundle; **H**—Starch grains (arrows) are present in the secretory layer of *P. aemula* (arrows). Note that the subepidermis is collenchymatous. Scale bars: **A**—**H** = 50 µm

the presence of nectar increased with increasing thickness of the secretory layer (from 26% for 22.30 µm to 70% for 65.20 µm; Fig. 7H), and increasing thickness of the outer tangential cell wall of the epidermis and cuticle layer (from 11% for 0.82 µm to 94% for 9.91 µm; Fig. 7I), but diminished with increasing thickness of the cell walls of the subepidermal parenchyma (from 58% for 1.00 µm to 19% for 12.50 µm; Fig. 7J). Our analysis found no relationship between the micromorphological features of the nectary and the presence of nectar in *Encyclia* (Table 1).

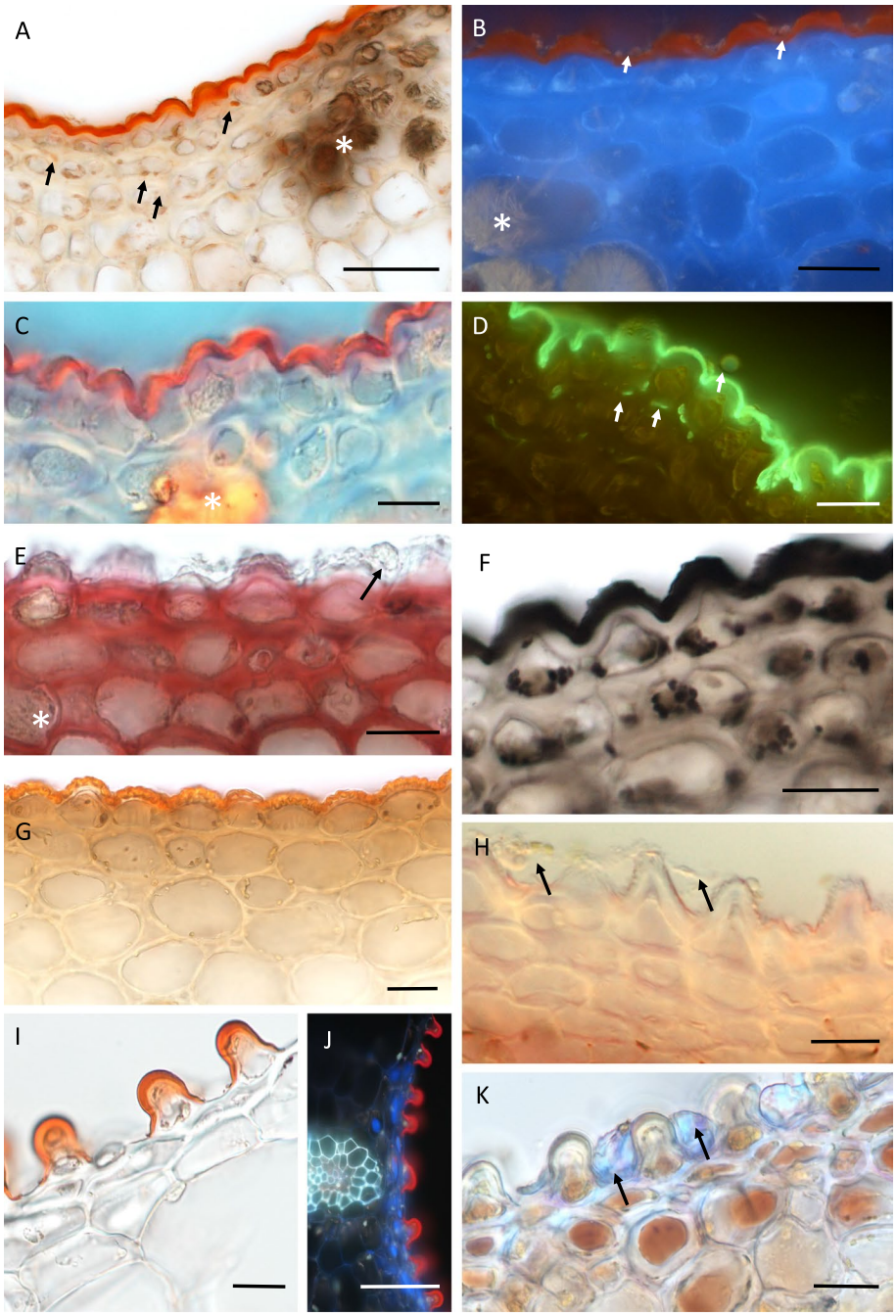

Fig. 4 Epidermal cell walls of nectariferous tissue, LM. **A**—Cuticle of *Prosthechea radiata* stained with Sudan IV, the volatile oils indicated by arrows; **B**—Nectary of *P. vitellina* stained with Sudan IV and observed with UV. Note the thick, red-stained cuticle and residues of surface secretion (arrows); **C**—Nectary of *P. baculus* showing thick cell walls and thick cuticle, NDIM. Asterisks in **A**, **B** and **C** represent fragnettin – the so-called 'flavonoid' crystals typical of the genus *Prosthechea*. **D**—Section through nectary of *P. cochleata* stained with Auramine O. The cuticle appears green and droplets of volatile oils are marked by arrows; **E**—**F** Nectary of *P. vitellina*. **E**—The thick, cellulose—pectin cell walls stain red with ruthenium red. Note the surface secretion (arrow). **F**—Section stained with OsO_4. Note the darkly stained cuticle and plastids; **G**—Cuniculus of *Encyclia cordigera*. The epidermal cells possess a thick, ridged cuticle; **H**—Papillose epidermis of *E. guatemalensis*. Note the surface secretion (arrows) NDIM; **I**—**K** Cuniculus of *Epidendrum katarun-yariku*. **I**—Epidermal papillae with thick cuticle stained with Sudan IV; **J**—Section stained with Sudan IV and observed with UV. The cuticle appears red; **K**—Hand-cut section of unstained, living nectary tissue. Note that the surface secretion has accumulated between the epidermal papillae, NDIM. Scale bars **A**, **J** = 50 µm, **B**—**I**, **K** = 20 µm

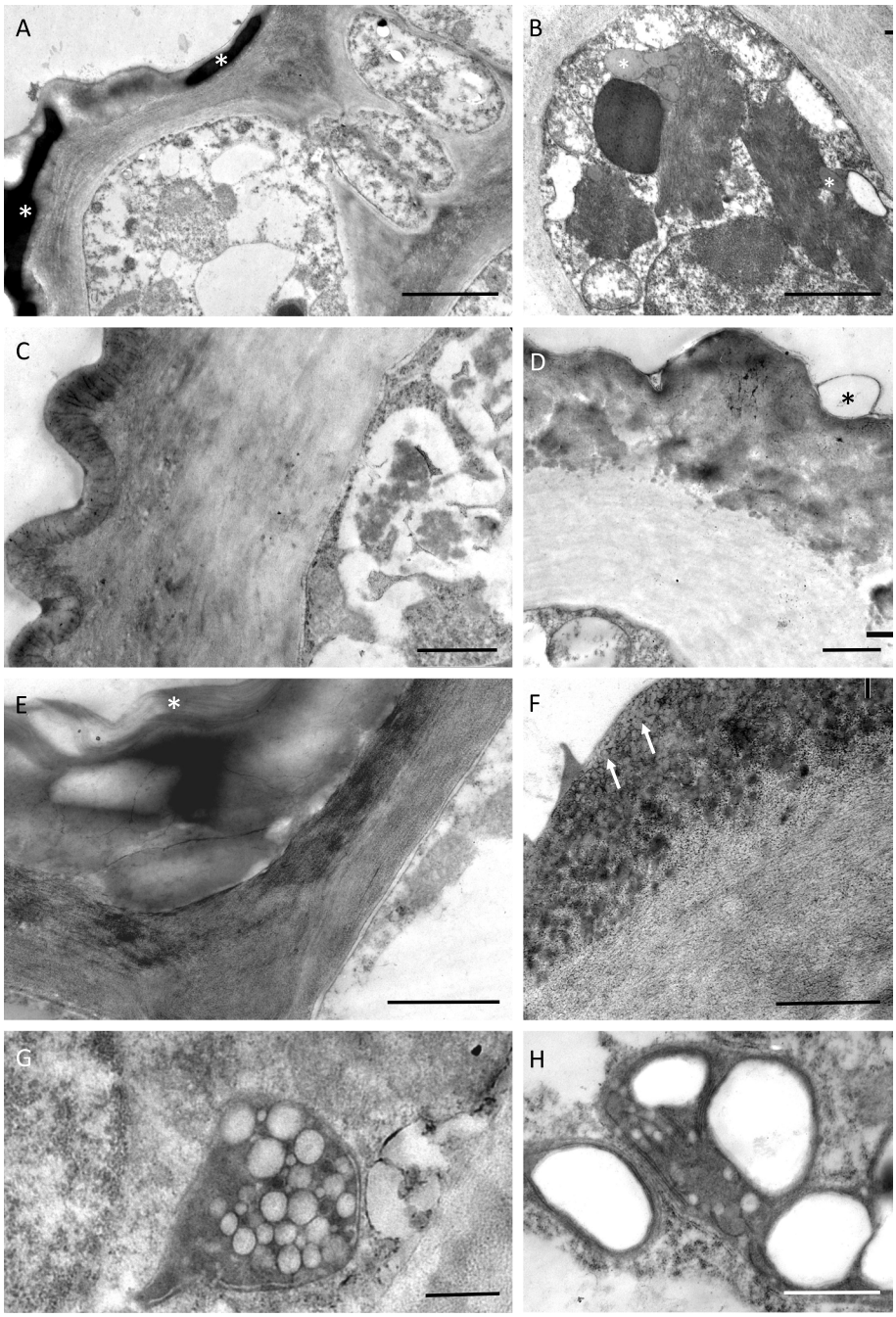

Fig. 5 Ultrastructure of nectary cells in nectariferous and nectarless species of Laeliinae. TEM. **A** – Nectary epidermis of nectariferous *Prosthechea aemula*. Note the dense cytoplasm, thick periclinal cell walls and the osmiophilic regions in the cuticle (asterisks); **B**—Protoplast of nectary cell of nectarless *Encyclia cordigera*. The plastids contain lipid droplets (asterisks), and a large osmiophilic body is present; **C**—Outer tangential wall of nectary epidermal cell of *Prosthechea prismatocarpa*. Numerous micro-channels are visible in the cuticle; **D**—Outer tangential cell wall of nectary epidermal cell of *P. cochleata*. The cuticle has a patchy texture and surface secretion (asterisk) is visible; **E**—A thick, patchy cuticle is present in *P. glumacea*. The surface secretion is marked by an asterisk; **F**—Outer tangential cell wall of nectary of *E. cordigera*. The outermost part of the cuticle contains numerous lipid droplets (arrows); **G**—Lipid droplets in plastid of *P. chondylobulbon*; **H**—Starch in plastid of *P. aemula*. Scale bars **A**—**B** = 2 µm, **C**-**E**, **H** = 1 µm, **F**- **G** = 0.5 µm

Table 1 Final model parameters for testing the effect of nectary micromorphological features on the presence of a cuniculus-type nectary, the presence of nectar (independent of genus—"all data" in Table 1), the presence of nectar at the genus level, and the thickness of tangential cell walls in the epidermis, in the subepidermal layer, and the thickness of the secretory layer

Response variable	Predictor	Estimate	SE	z/t	Pr(>|z/t|)
Cuniculus-like nectary presence	(Intercept)	0.407	1.101	0.370	0.711
	Nectar presence = absent	0.612	0.861	0.711	0.477
	Epidermis type = papillose/trichomatous	1.387	0.829	1.674	0.094
	Cuticle ornamentation = present	1.423	0.843	1.688	0.091
	Starch = present	−1.224	0.837	−1.463	0.143
	Thickness of secretory layer	−0.037	0.021	−1.689	0.091
Nectar presence (all data)	(Intercept)	2.570	1.763	1.458	0.144
	Cuticle ornamentation = present	−2.223	1.322	−1.669	0.095
	Epidermis type: other = (papillose/trichomatous)	−2.416	1.200	−2.013	0.044
	Starch = present	1.320	0.876	1.506	0.132
	Thickness of outer tangential cell wall of epidermis + cuticle	0.222	0.263	0.846	0.397
	Thickness of cell wall in subepidermal layer	−0.005	0.269	−0.020	0.984
	Thickness of secretory layer	0.008	0.025	−0.313	0.754
Nectar presence (genus = *Epidendrum*)	(Intercept)	−3.728	2.693	−1.384	0.166
	Thickness of outer tangential cell wall of epidermis + cuticle	0.539	0.680	0.793	0.428
	Thickness of cell wall in subepidermal layer	−0.153	0.570	−0.269	0.788
	Thickness of secretory layer	0.043	0.051	0.860	0.390
Nectar presence (genus = *Encyclia*)	(Intercept)	−0.154	0.556	−0.277	0.782
Thickness of outer tangential cell wall of epidermis + cuticle	(Intercept)	3.438	0.677	5.077	< 0.001
	Cuticle ornamentation = present	1.754	0.683	2.565	0.013
	Nectary type = sac-like	0.411	0.628	0.655	0.515
	Nectar presence = absent	−0.639	0.656	−0.974	0.334
Thickness of cell walls in subepidermal layer	(Intercept)	0.610	0.383	1.591	0.118
	Thickness of outer tangential cell wall of epidermis + cuticle	0.700	0.075	9.278	< 0.001
Thickness of secretory layer	(Intercept)	44.917	2.962	15.164	< 0.001
	Nectary type = sac-like	7.281	4.641	1.569	0.123

The final LR testing for the effect of micromorphological features of the nectary on the thickness of the outer tangential epidermal cell wall with cuticular layer resulted in three explanatory variables (Table 1). This layer was 1.75 µm thicker when the cuticle was ornamented (compared with a cuticle lacking ornamentation; Fig. 8A); 0.42 µm thinner for cuniculus-like nectaries (compared with sac-like nectaries; Fig. 8B); and 0.64 µm thinner in nectaries lacking nectar (compared with those containing nectar; Fig. 8C). The final LR testing for the effect of nectary micromorphological features both on the thickness of cell walls in the subepidermal layer, and on the thickness of the secretory layer resulted in one predictor each (Table 1; Fig. 8D-E). Whereas the thickness of cell walls of the subepidermal layer increased with increasing thickness of the outer tangential epidermal cell wall and cuticular layer from 2.01 µm to 9.72 µm and 2.00 µm to 13.00 µm, respectively (Fig. 8D), the secretory layer was 7.56 µm thinner for cuniculus-like nectaries, as compared with sac-like nectaries (Fig. 8E).

Discussion

The possession of functional and nectarless nectaries by genera in which both tubular (cuniculus) and sac-like nectaries occur demonstrated that nectar production is not related to the shape of the nectary. However, the percentage probability for the occurrence of nectar was lower for the cuniculus-type nectary typical of *Epidendrum* and *Encyclia* than for the shorter, sac-like nectary, which was more easily accessible to less specialized pollinators. Species possessing long, tubular nectaries (cuniculus) have the potential to evolve a deceptive pollination system and thus reduce the expenditure of material and energy resources required for nectar secretion, as was noted by Silveira et al. [14] for *E. densiflorum* Hook. In this species the fragrance probably mimics that of plants that are a source of alkaloids for

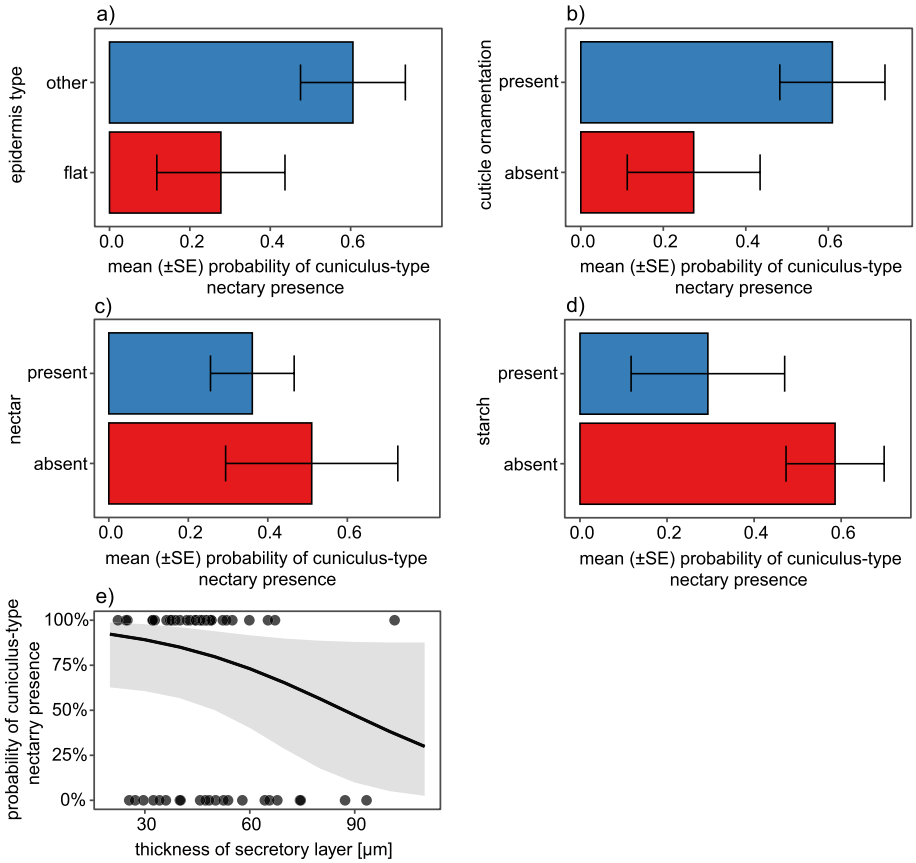

Fig. 6 Visualisation of final GLM predicting the probability of the presence of a cuniculus- type nectary based on: (**a**) epidermis type; (**b**) the presence of cuticular ornamentation; (**c**) the presence of nectar; (**d**) the presence of starch and (**e**) the thickness of the secretory layer. For model parameters see Table 1

lepidopteran pollinators [14]. However, the study demonstrated low reproductive success in this deceptive species. Food deception has also been reported in four species of Brazilian *Cattleya* Lindl. [44]. Conversely, and contrary to our expectations based on the small volume of published literature available on the subject, as well as our preliminary investigations, most *Prosthechea* spp. investigated were nectariferous, except for *P. calamaria* and *P. garciana* (Garay & Dunst.) W.E. Higgins, both of which had sac-like nectaries. It would appear that the presence of nectar is an ancestral state in *Prosthechea* and *Encyclia* since the basal line, as represented by *Dinema polybulbon* Lindl. [12], among others, is nectariferous. In studies by Cardoso-Gustavson et al. [17], the presence of nectar was also indicated as the most common ancestral state for most *Epidendrum* spp. In African *Disa*, with its various strategies of pollination, and contrary to the Laeliinae investigated here, the evolution of nectar production from rewardless ancestors may be due to limited pollinator visits to flowers, resulting in the selection of nectar-producing individuals that invest relatively little resources, but achieve greater reproductive success [22] Similarly, Pansarin et al. [45] have also shown the evolution of a reward-producing clade (i.e. *Cleistes* Rich. ex Lindl.) from rewardless Pogonieae Pfitzer ancestors.

Our investigations indicate that fragrant species, such as *P. fragrans*, *P. radiata* and other members of *Prosthechea* (representatives of the *Anacheilium* Hoffmanns. group), have osmophore tissue that attracts male euglossine bees as pollinators (pers. observation Emerson Pansarin). However, in cases where male euglossine bees are attracted to flowers by their fragrances, they must firstly seek out nectar, as previously demonstrated for *Vanilla* Plum. ex Mill. [(e.g.) 46]. The presence of fragrance-producing labellar tissue has also been reported for some species of *Encyclia* by Lipińska et al. [11], as well as by Del Mazo Cancino and Damon [47], but they did not state whether those species investigated produced nectar.

In our analysis, all the investigated taxa, irrespective of the presence of nectar, shared thick epidermal and subepidermal cell walls (in the secretory layer) and epidermis

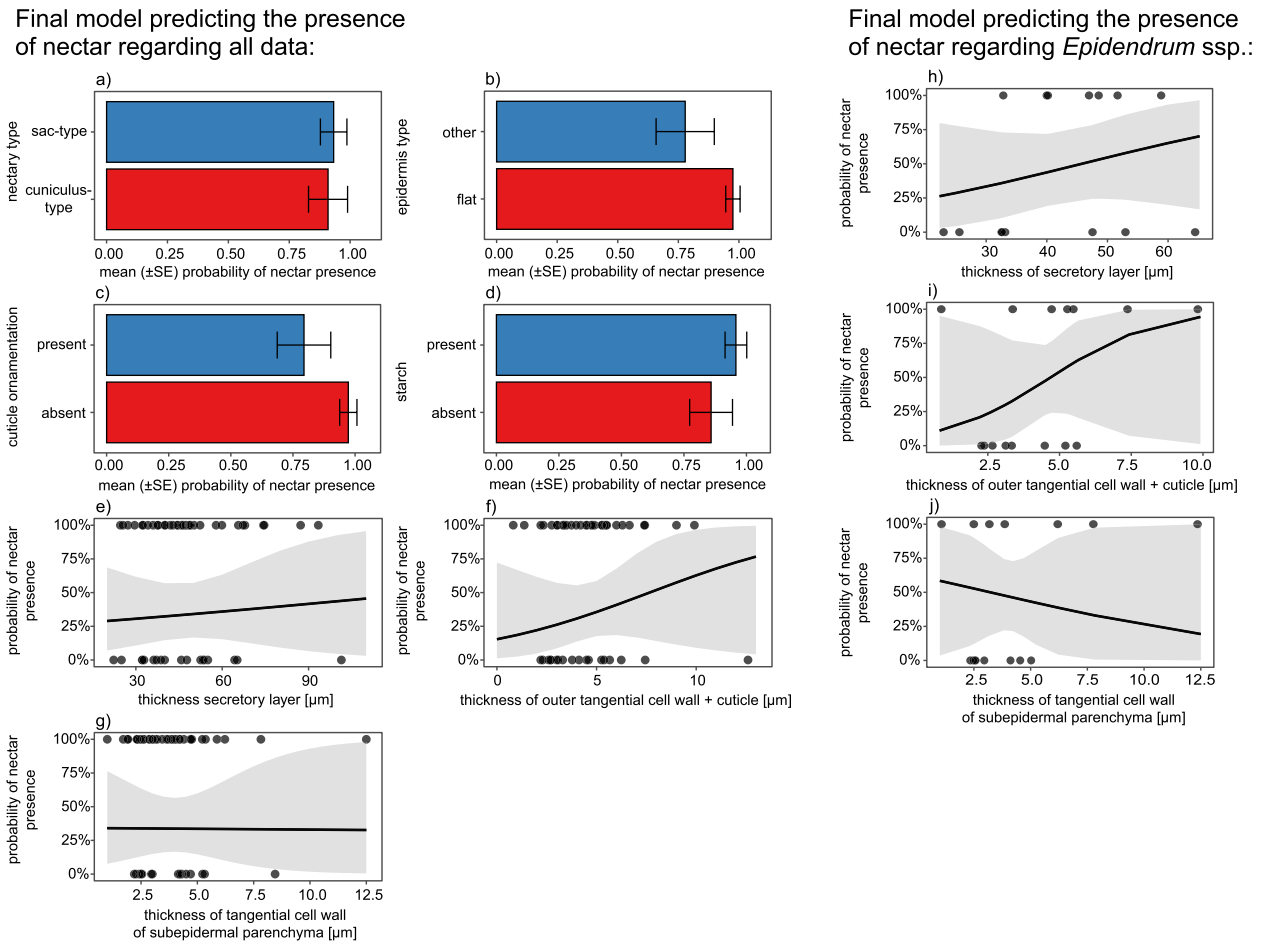

Fig. 7 Visualisation of final model predicting the probability of the presence of nectar in nectaries performed for all data based on: (**a**) nectary type; (**b**) epidermis type; (**c**) the presence of cuticular ornamentation; (**d**) the presence of starch; (**e**) the thickness of the secretory layer; (**f**) the thickness of the outer tangential cell wall with cuticle and (**g**) the thickness of the tangential cell wall of the subepidermal parenchyma, and final model developed for the genus *Epidendrum* based on: (**h**) the thickness of the secretory layer; (**i**) the thickness of the outer tangential cell wall with cuticle, and the thickness of the tangential cell wall of the subepidermal parenchyma (**j**). For model parameters see Table 1

with a thick cuticle. These features are also present in other representatives of Laeliinae, such as nectariferous *Barkeria scandens* (Lex.) Dressler & Halb., *B. whartoniana* (C. Schweinf.) Soto Arenas [48] or *B. melanocaulon* A. Rich & Galeotti and *B. skinneri* (Bateman ex Lindl.) Paxton (Małgorzata Stpiczyńska, unpublished data), as well as in *Brassavola flagellaris* Barb. Rodr. [49], *B. cucullata* (L.) R.Br. and *B. nodosa* (L.) Lindl. (Małgorzata Stpiczyńska, unpublished data). The presence of thick cell walls has also been recorded e.g. for the nectary cells of *Maxillaria coccinea* (Jacq.) L.O. Williams [27] and in the cells of the wide and short nectary spur of *Dendrobium finisterrae* Schltr. [50], where this feature was considered typical of ornithophilous species. However, literature searches indicate that most Laeliinae investigated to date are visited by insects and bird visitors have been reported more rarely [13, 21, 50, 51 and references therein;]. Therefore, a correlation between the presence of thick cellulosic cell walls in the nectary cells and ornithophily seems unlikely in this case. Instead, the thickened walls, as in the case of typical collenchyma, probably function both in the mechanical support of the labellum and that of the nectary lumen, be the nectary tubular or sac-like. Thick-walled cells observed in the nectary spurs may represent a shared structural adaptation.

GLM analysis, however, revealed that the thickness of the outer tangential epidermal cell wall is associated with nectar secretion. Our data are congruent with previous observations that neither thick cell walls nor a thick cuticle limit the transport of nectar to the surface of the nectary, and this has been shown for *Barkeria* Knowles & Westc. [48]. Based on our analysis, the probability of nectar being present increases with increased thickness of the secretory layer. The latter is usually characterized by

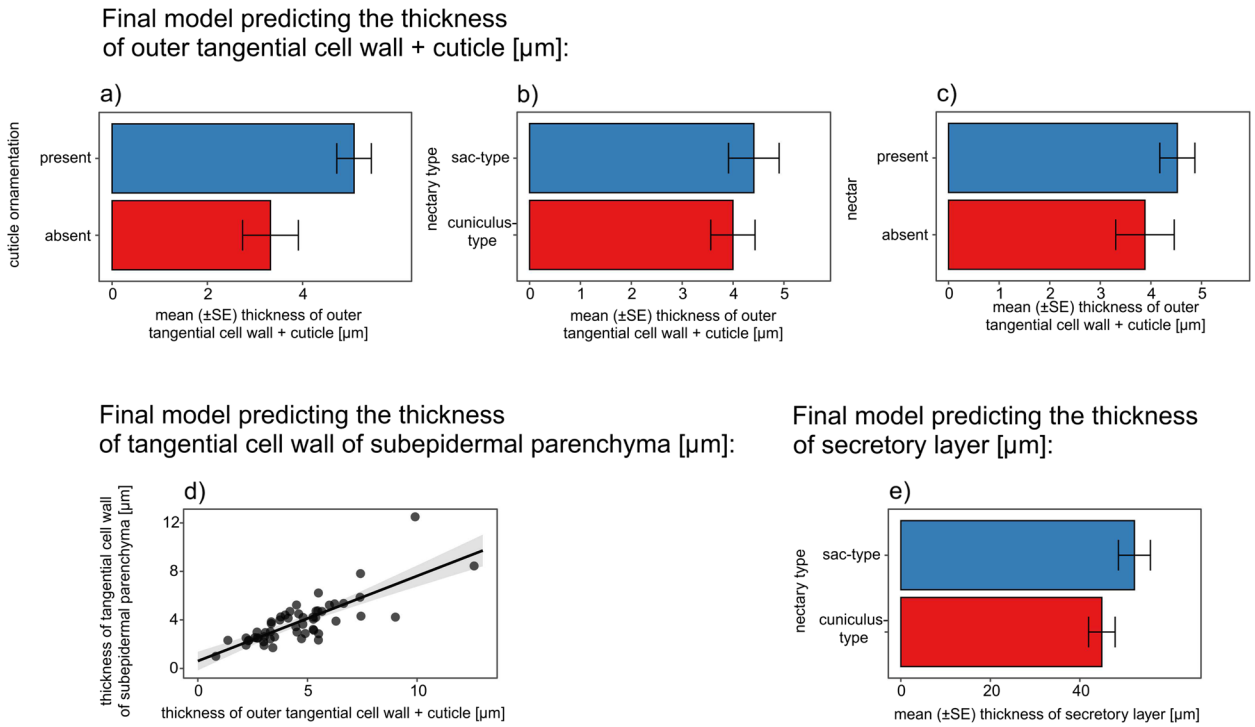

Fig. 8 Visualisation of final LRs testing the effect of the micromorphological features of nectaries on: (**a**-**c**) the thickness of the epidermal layer; (**d**) the thickness of the tangential cell wall of the subepidermal layer, and (**e**) the thickness of the secretory layer. For model parameters see Table 1

thick cell walls, an exception being nectariferous *E. alata*, which has only one layer of secretory parenchyma.

In the investigated taxa, the epidermis of the cuniculus or sac-like nectary was composed of flattened, papillose or trichomatous cells, and no stomata were observed. Lipińska et al. [11] hypothesized that stomata present on the labellum of *Encyclia* might be engaged in the secretion of fragrance or nectar, but our investigations did not support this. In *Prosthechea*, in particular, it was possible to observe a gradation in the type of epidermal cell, with papillose and flattened epidermal cells occurring in the upper and basal regions of the nectary, respectively. These flattened cells were coated with secretion probably produced by cells located deep in the nectary. However, these observations cannot be used to support the claim that nectary cell shape in *Prosthechea* is correlated with secretory capacity, since the former, and their gradation, are likely to be determined by the shape and mechanical properties of the sac-like nectary. Conversely, in *Encyclia* (even though we found no relationship between micromorphological features and the presence of nectar here), and *Epidendrum* (where nectarless species predominate), the cuniculus was lined in most cases with papillose/trichomatous cells. Similar results were obtained by Cardoso-Gustavson [17], who demonstrated that an unornamented epidermis was present in nectariferous species of *Epidendrum*, whereas a trichomatous epidermis was present in food-deceptive species. The presence of papillae or trichomes may also facilitate deceptive pollination strategies by means of tactile stimuli. Conversely, and contrary to our results for Laeliinae, in the model species of European Orchidinae investigated by Bell et al. [19], all of which possessed a floral spur, nectar secretion was negatively correlated with cuticular striations and positively correlated with the presence of papillae. It should be noted that long papillae similar to those described by Bell et al. [19] e.g. in *Platanthera chlorantha* (Custer) Rchb., are referred to as trichomes in the present study (e.g. in *E. radicans*). This distinction is largely based on the length of these structures and whether they are unicellular or multicellular. Indeed, our previous studies [30] have shown that papillae and trichomes may co-exist and intergrade on the same floral structure. Bell et al. [19] propose that large papillae, by their mere presence in nectar-secreting species, may provide tactile cues for pollinators.

Another feature common to all the species investigated here was a smooth or striate, thick cuticle, with the probability of a smooth cuticle being greater in nectariferous species. It is possible that a thick cuticle may limit the evaporation of nectar in dry environments, but not impede the cuticular passage of nectar. In

Barkeria, the cell walls and thick cuticle contain obvious micro-channels that enable the transport of nectar onto the cell surface [48]. It is speculated that in some nectariferous *Encyclia* and *Prosthechea* spp., the tuft of trichomes that partly occludes the entrance to the nectary may additionally function as protection against water loss by evaporation, as in Trichocereeae cacti [52, 53 for general information], which can, in turn, affect the properties of the nectar, as well as protecting the flower against nectar-robbers.

The present study did not find substantial differences in the cellular ultrastructure of nectariferous and nectarless species, just the expected organelle complement typical of secretory cells. Most of the investigated species were also fragrant, and those that did not secrete even minute volumes of nectar, nonetheless produced volatile oils. These oils were visible as droplets on the cuticle of certain species such as nectarless *E. cordigera*. Species with functional nectaries also contained starch more frequently than those of nectarless species, which is not surprising considering that hydrolysis of starch is a source of nectar sugars and metabolic energy [54 and references therein]. This polysaccharide, however, was uncommon in some species such as highly fragrant *P. chondylobulbon*, whose plastids, instead, contained numerous droplets of lipid that probably function as precursors of volatile compounds. Starch is usually present in fragrance-producing species, but it is likely that during anthesis, much of it is metabolized [55, 56]. This could be the reason for the lack of large amounts of starch in the analyzed species. Even so, the possibility that the complete absence or presence of only a minute volume of secreted nectar might be compensated for by the secretion of pollinator-attracting volatiles, cannot be entirely dismissed, especially since such a transition from a nectar-based pollination system to another resource-based strategy, has previously been documented for *Jacaranda oxyphylla* Cham., Bignoniaceae Juss [57].

Our results may indicate that the type of nectary, the occurrence or otherwise of nectar and modifications to the secretory layer, especially to the outer tangential wall of the epidermis, and the presence of cuticular ornamentation, all reflect pollinator selection and the pollination ecology of the individual species. It would thus appear that during the evolution of Laeliinae, the ancestral nectariferous floral nectary diversified and the nectarless condition became more common, as observed for the cuniculus-type of nectary of *Epidendrum* and *Encyclia*. Meanwhile, other representative species remained nectariferous, with a concomitant increase in the thickness of the outer tangential epidermal wall of the nectary, a greater frequency in the occurrence of a smooth cuticle (vs. striate), and an increased thickness to the walls of the secretory layer.

It should be emphasized that regardless of nectary morphology (cuniculus vs. sac-like nectary), no substantial differences were observed between the nectary micromorphology of nectariferous and nectarless species. This may indicate that the capacity for nectar secretion in this group of orchids is plastic, and not limited by structural constraints, thus facilitating the turning on and off of secretion and adapting the nectary to a range of diverse pollination strategies that have contributed to the evolutionary success of this subtribe.

Conclusions

We found that in the investigated Laeliinae, there is a slight relationship between the capacity for nectar secretion and aspects of nectary structure, such as the thickness of the tangential cell walls, the surface of the cuticle, or the thickness of the secretory layer. In Laeliinae, there appears to be an evolutionary trend from nectariferous ancestors towards seemingly nectarless flowers that nonetheless still retain their inner floral spur (cuniculus) and produce meagre volumes of nectar, just sufficient to encourage visits by potential pollinators, and this, in turn, has probably contributed towards the success of this group of orchids. Furthermore, the capacity for nectar secretion in Laeliinae is plastic and appears not to be impeded by structural barriers, thus facilitating an adaptational shift towards a range of diverse pollination strategies, largely due to its effect on pollinator selection. This improves the ability of these plants to occupy diverse ecological niches, contributing, in turn, to the evolutionary success of the subtribe. Finally, the application of nectary studies of this kind to other plant taxa also has the potential to provide valuable insights into their evolution and pollination ecology.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06810-5.

Supplementary Material 1: Fig. S1. Osmophore and trichomes of *Prosthechea*. A - B. Labellum of *P. fragrans*. A – Control; B - Following treatment with NR. Basal trichomes are marked with a star; C - F. *P. radiata*. C - Trichomes at the entrance to the nectary stained with Sudan IV. Lipid droplets (volatiles) are marked by arrows; D - Lipid droplets in cytoplasm of epidermal and subepidermal cells; E - Trichomes of P. radiata treated with NADI reagent. Arrows indicate droplets of volatile compounds. F - Section of labellum stained with NR and observed under UV. Arrows indicate droplets of volatile compounds. G - *P. calamaria*. Starch-laden trichomes and parenchyma at the base of labellum following staining with IKI (iodine/potassium iodide solution); H - Section of labellum, as observed with UV. Note the chloroplasts in parenchyma cells and blue

cuticle overlying the trichomes. Scale bars: A, B = 5 mm, C = 50 µm D =10 µm, E = 20 µm, F, G, H = 100 µm

Supplementary Material 2.

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Authors' contributions
MS: conceptualisation, methodology, lead investigation, data analysis, writing—original draft and final editing of the main manuscript text, preparation of Figs. 1, 2, 3, 4, 5; KLD: investigation, writing—original draft and final editing of the main manuscript text; KS: investigation; BP: investigation; MW: supporting investigation; PCz: statistical data analysis, writing—original draft of the statistical analysis section of the manuscript, preparation of Figs. 6, 7, 8. All the authors reviewed the manuscript.

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Data availability
Data is provided within the manuscript. All additional images and the table supporting the presented results with the accession numbers of cultivated plants are included as supplementary files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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