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Liparis stricklandiana (Orchidaceae, Liparidinae) – a morphological study of flower structures in the context of pollination processes

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Summary: The knowledge about pollination and morphology, especially micromorphology, of the flowers of *Liparis* species is still sparse and random. The observations of the living plants and their flowers were carried out during the whole period of anthesis. The morphology of floral elements and their potential pollination functions were analyzed. Observations on flower macrostructures were carried out using a light stereoscopic microscope. Additional analyses by scanning electron microscopy (SEM) as well as transmission electron microscopy (TEM) were performed. The results of the TEM study revealed secretory activity at dorsal and lateral sepals. Secretory activity has been observed on the whole surface of the lip, thus the function of central lip thickening as a floral attractant and secretion area has been proven. During our study, we had recorded the presence of small droplets on the lip surface of *Liparis stricklandiana*, what turned out to be the first observation of such type in this species. It seems that the secretion of the lip epidermis occurs in two ways: through the cuticle and through small cracks of the cuticle. Observations made upon the living flowers let confirm the facultative process of self-pollination in *Liparis stricklandiana*, which occurs at the end of anthesis.

Keywords: micromorphology, electron microscopy, orchids; pollination, secretion

Representatives of Orchidales are well known as one of the most advanced plant groups in the context of adaptation to different forms of zoogamy (mostly entomogamy). The adaptations can be observed in the morphology and physiology of the orchid flowers. The most typical modification concerns one of the tepals of the external whorl of the flower, the so-called labellum. It can be modified, among others, as a landing-platform for visiting insects or as long and short distance signalization of offered or potentially offered floral attractants. The generative structures of orchids are also highly adapted to zoogamy by, for instance, reduction of stamens and stigmatic surfaces to 3, reduction of those still sexually active (3–2 and mostly 1), stamen(s) fusion with pistil into gynostemium or pollen conglomerated into 2 or several packages (pollinia) equipped with various structures, which allow their transport entirely by insects. All these modifications aim to prevent the orchid flower from self-pollination. However, in some orchid groups, system modifications exist which enable autogamy, when suitable pollinators are absent and/or growing conditions are stressful. Self-pollination is estimated to occur in approximately 3% of orchid species (van der Pijl & Dodson 1966) or even 5–20% (Catling 1990).

Materials and methods

The object of the studies was *Liparis stricklandiana* (Liparidinae). The plants have been cultivated by the first author since 2013 in glasshouses of the Gdańsk University and have been blooming since than regularly every year. Flowering season *in situ* ranges between August and October or October and January (except for Hong Kong: January and March). In the living collection of the first author, flowers have been occurring between October and January. Specimens of the cultivated species were regularly preserved (on each stage of anthesis), dried and fixed in

Copenhagen mixture (collection: UGDA-HBM, voucher: HBM 0620013 *ex* KG2010-2347) and photorecorded. Scanning (SEM) and transmission electron microscopy (TEM) analysis were performed using the young, but fully developed flowers collected before early morning water sprinkling.

Initial samples were made available by The Royal Botanic Gardens, Kew (K 2010-2347, non-commercial material supply agreement for live material dispatch no. 6509) in 2013. Determination of the plants was confirmed by the first author based on standard methods of classical taxonomy, with required references to the original taxonomic material such as type-specimens and protologues.

Observations of the living plants and their flowers during whole anthesis period were carried out in the greenhouses of Gdańsk University. Observations of the flowers macrostructures were carried using light stereoscopic microscope by the first author.

For scanning electron microscopy (SEM), flowers were preserved in 2.5% GA and 2.5% PFA in 0.05M cacodylate buffer (pH 7.0), dehydrated in an ethanol series, then dried by critical point method using liquid CO_2, coated with gold and observed in Philips XL-30 microscope.

For transmission electron microscopy (TEM), the lip was fixed overnight in 2.5% GA and 2.5% PFA in 0.05M cacodylate buffer (pH 7.0), post-fixed overnight in 1% OsO_4 in cacodylate buffer. After 1 hour in 1% uranyl acetate in sterile water, the lip was dehydrated with increasing concentrations of acetone and embedded in Spurr's resin. Ultrathin sections (60 nm) were cut on Leica UC7 ultramicrotome. Sections were examined on FEI Tecnai Spirit BioTWIN transmission electron microscope at 120kV.

Theory

The facultative process of self-pollination, occurring at the late anthesis, were confirmed for some species of subtribes Malaxidinae and Liparidinae (Margońska et al. 2012/2013). Owing to the close proximity of anther and stigma, entire pollinia were observed to fall (often still attached by caudicles to the top of rostellum) or slide down onto the stigma, thus resulting in self-pollination in most cases within both groups. This kind of self-pollination mechanism is also known for other representatives of Orchidaceae Juss. (e.g. Catling 1990; Liu et al. 2006).

Liparis is a large, mainly pantropical genus of ca. 300 species with some representatives in temperate regions of the northern and southern hemispheres. Plants are usually autotrophic (except for e.g. *Liparis aphylla* Romero & Garay), terrestrial, lithophytic or epiphytic, forming colonies of various sizes. The lips of representatives of the subtribe Liparidinae are directed downward and serve as a landing place for pollinators.

In the natural environment, small flies, midges (Kaiser 1993; Margońska's own observations), fruit flies (Feldmann & Barré 2001; Margońska's own observations), other small diptera, ants, spiders and bugs (Margońska's own observations) have been observed so far on the *Liparis* flowers. The record of a visit of one of the animals mentioned above, however, is no evidence for an effective pollination.

The knowledge about pollination and morphology (especially micromorphology) of *Liparis* flowers is still rather sparse and random. For example, it is known that *Liparis reflexa* Lindl.

with flowers emitting a scent of stale egg yolk are frequently and effectively pollinated by flies of Sarcophagidae (Wallace 1974) or Mycetophilidae (Adams & Lawson 1993), while flowers of *Liparis coelogynoides* F. Muell. with a urine-like odour are pollinated by 'small flies' (Bishop 1996).

The fragrance of *Liparis* flowers is sometimes recorded as 'cucumber' or 'sweet cucumber' smell. However, it is difficult to determine, whether it was an actual emission of fragrance or rather the odour of the coumarin compounds present in tissues of these plants and released because of e.g. mechanical damages by the slightest touch or by cutting off inflorescences or flowers.

In cultivation, small flies and other small diptera (e.g. Culicidae: *Culex* sp. or Sciaridae: *Bradysia* sp., *Lycoriella* sp., *Sciara* sp.) have been repeatedly observed showing an interest in the flowers of *Liparis* (H. Petersen, cited by Christensen 1994; Margońska's own observations). Nevertheless, because of the incompatibility of pollinator and flower, the visits are not only ineffective (no pollination), but also can even result in fatal consequences for the insect (Fig. 1a; Margońska's own observations; Margońska et al. 2012/2013).

An interesting and rarely confirmed phenomenon is hydrogamy in the form of rain-assisted self-pollination. So far, it has been observed only in some *Liparis* species (Liparidinae**)** from moss-covered mountain cloud forests or peat bogs (e.g. *Liparis hawaiensis* H. Mann, Margońska's own observations, or *Liparis loeselii* (L.) L.C. Rich., see Catling 1990). In this mechanism, rain or condensed water drops fall on the anther, deflect it and move the pollinia on to the stigma. (Margońska et al. 2012/2013).

Liparis stricklandiana Rchb. f. is a lithophytic or epiphytic orchid found in South-East Asia, India (East Himalaya: Sikkim, Assam, Meghalaya), Tibet, Bhutan, China (Guangdong, Guangxi, Guizhou, Yunnan, Hainan, Xizang, Kwangtung, Hong Kong) and in Vietnam (northern part). The plants usually form large mats in shade or semi-shade areas, which are usually wet places, frequently along water courses or cliffs along valleys. Sometimes they are also recorded from open sites.

During our study, we have observed the presence of small droplets at the lip surface of *Liparis stricklandiana*. As further research revealed, this has been the first time of recording secretion in this species. To support our observations, we also analyzed the morphology of flower elements and pollination function.

Results

How complex pollination strategy may be (e.g. pseudocopulation or pseudoantagonism), insects are initially attracted by means of visual and olfactory stimuli (Proctor et al. 1996). Thus, colour effects (human visible light 390–700 nm and long-wave ultraviolet light detectable by insects 315–400 nm) are highly important elements of the orchids' nectar guides, especially as middle distant signals.

In most species of Malaxidinae and Liparidinae, the colour of flowers usually changes with age. In the case of *Liparis stricklandiana*, flowers are freshly and intensely green and get a golden tint (or even nearly ochre colour) at the end of anthesis.

Changes in morphology can also be observed with the age of flowers. Sepals and petals become folded back, making the lip more prominent. The lip distinctly divides into well-developed

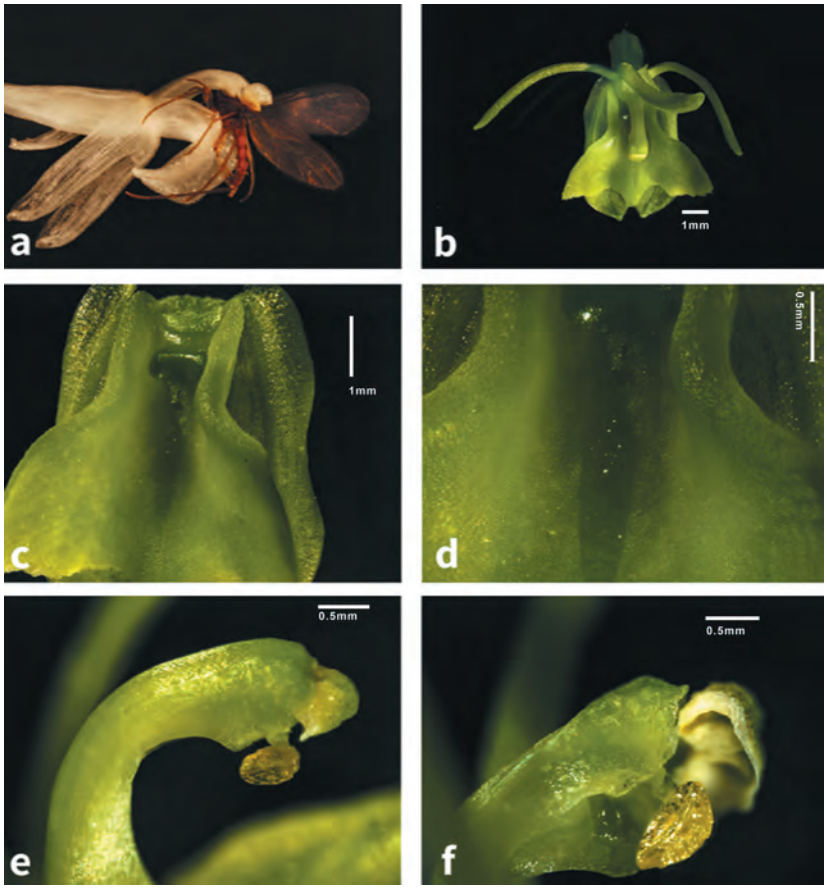

Figure 1. *Liparis viridiflora*. a – flower with trapped insect (Sciaridae), preserved in Copenhagen mixture (Margońska et al. 2012/2013); b – *Liparis stricklandiana*. Living young flower; c – living young lip above both lateral sepals, basal callus and the central thickening with liquid drops; d – living young lip, the central thickening with liquid drops below the basal callus; e – living old gynostemium, arcuating at anthesis and releasing the pollinium; f – living old gynostemium with pollinium near stigma area. Photographs by H. Margońska.

hypochile (the basal part, similar length and width in natural position, wider than long when spread) and epichile (wider than long, nearly flabellate, forming a landing place for insects). The boundary between the hypochile and epichile are recurved, becoming more distinct at anthesis (Fig. 1b).

Distal edge of the lip epichile usually irregularly gains shreds with age (visible on cell's level). Near the lip hypochile base, an anvil-shaped callus is present (Fig. 1c). According to the observation of the callus or / and its surroundings in fresh flowers, it can be a source of little liquid secretions (Fig. 1d). A central thickening runns from the lip callus / calli or the lip base to nearly the top of lip epichile in many Liparidinae. It is smooth, lustrous, at least slightly more intensely coloured (Fig. 1c–d). This structure is called sometimes a 'nectar mimic' or 'pseudonectary'. We confirm its functions as a floral attractant, secretion and maybe olfactory area in this specific species. It is the first part of the flower explored by insects just after landing at the lip (the first author's observations). What is more, it can simulate more secretion, than it actually does in reality.

The liquid secretion in the flowers of *Liparis stricklandiana* seems to flow down along the central thickening before drying, what has been proven (our research by means of SEM and TEM).

When the flowers become more yellowish, drops of liquid are usually dried out and only small traces remain on the surface of epidermis.

Scanning electron microscopy (SEM)

Dorsal and lateral sepal

The adaxial surface of the dorsal and lateral sepals (dorsal sepal, Fig. 2a) is glabrous, built by regular, elongated epidermal cells, with a cuticle slightly curved (dorsal sepal, Fig. 2b). Numerous stomata are present in the whole area, especially along the mid-vein and on the top of the sepal (dorsal sepal, Fig. 2c). Each stoma is surrounded by four isodiametric cells of epidermis (dorsal sepal, Fig. 2d).

Similarily, the abaxial side is built by regular, elongated epidermal cells (dorsal sepal, Fig. 2e). On the whole surface, small bicellular trichomes can be observed. The basal cells of these trichomes are thinner than oblate apices (dorsal sepal, Fig. 2f). At the apical part of the sepals, numerous simple stomata devoid of isodiametric cells of epidermis are observed (dorsal sepal, Fig. 2g–h).

Presence of secretion on the surface of the sepals could not be noticed with SEM.

Lip

The lip consists of two different parts, the hypochile (basal) and the epichile (distal) (Fig. 3a). The callus, placed at the base of the hypochile (Fig. 3b), is covered by elongated epidermal cells with a wrinkled cuticle (Fig. 3c).

The middle part of the lip is formed in the narrow central thickening, contracting gradually to the top (Fig. 3a, e; right side structure). The central thickening is composed of elongated epidermal cells, with a regular longitudinal border of the cuticle. The cells and their cuticle border are situated parallel to the long axis of the lip. Small cracks of cuticle, similar to stomata, can be observed between the neighboring cells. A greater amount of these cracks can be noticed in the area of the basal part of the basal callus (Fig. 3d) than in distal part of the central thickening.

The lateral parts of the lip are covered by regular, isodiametric cells, with a strong undulate cuticle (Fig. 3f). The cuticle borders are directed to the narrow part of the lip (Fig. 3e). The margins are slightly rolling, built by regular cells, which are the same as on the remaining part of the lip (Fig. 3g) and the triangular, pointed top of the lip (Fig. 3h).

Transmission electron microscopy (TEM)

Dorsal and lateral sepals

The results of the TEM study has revealed secretory activity at the dorsal and lateral sepals. The cell walls are covered with an amorphous cuticle (Fig. 4a–b). The secretory substance can be observed upon the cuticle (Fig. 4a–b). Dense cytoplasm contains a large vacuole with different inclusions, amyloplasts and small mitochondria (Fig. 4a). Invaginated plasmalemma includes an electron-dense substance, which is also observed between plasmalemma and cell wall (Fig. 4b, black arrow).

Lip

Secretory activity has been observed on the whole surface of the lip.

The cell walls of the hypochile are covered with an amorphous cuticle with a large amount of residues of secreted substances (Fig. 5a–c). The cytoplasm included amyloplasts, mitochondria

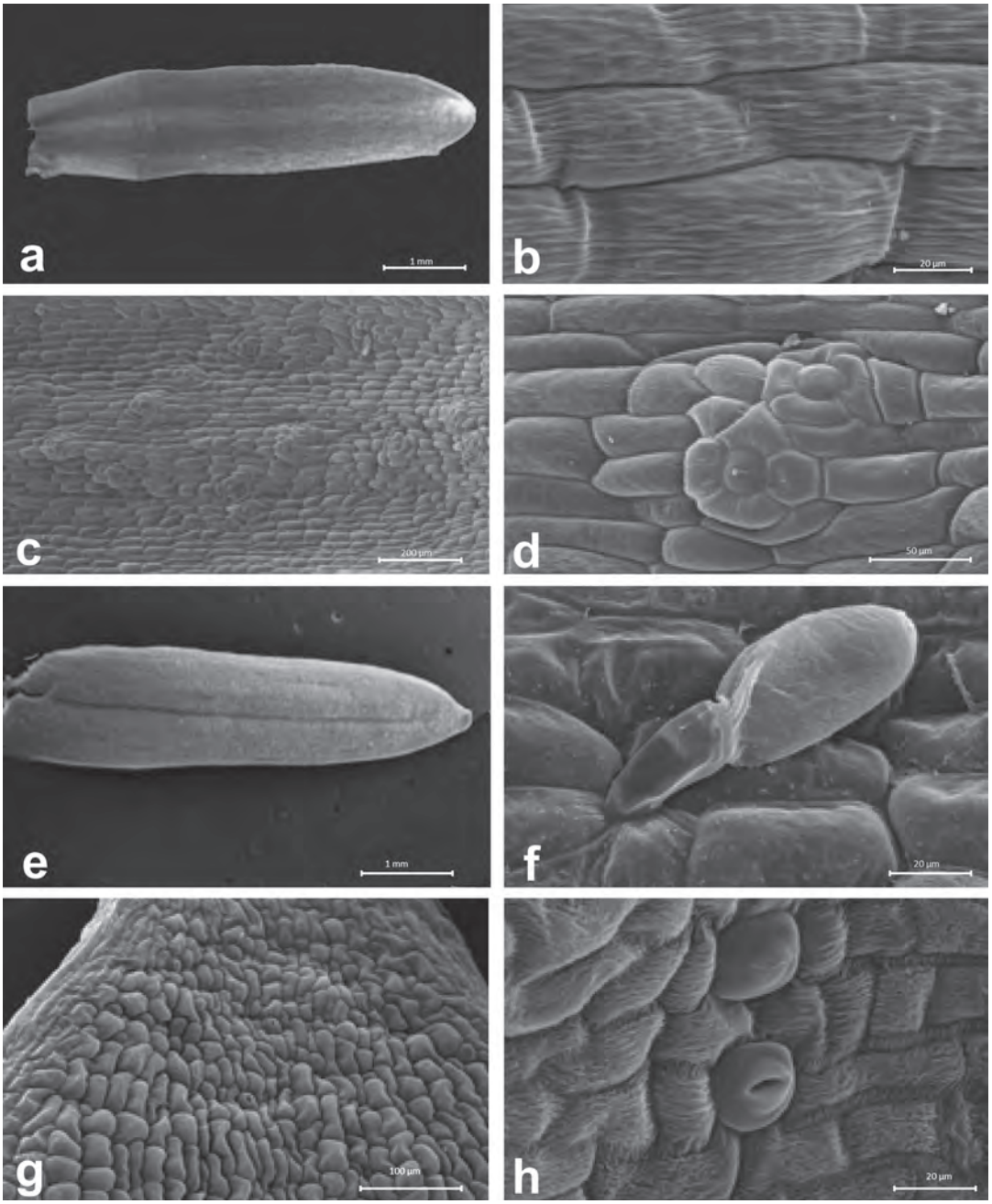

Figure 2. *Liparis stricklandiana* – SEM. Dorsal sepal. a – glabrous inner side of dorsal sepal; b – covered by regular epidermal cells with cuticular border; c – stomata at the top of sepal; d – bounded by isodiametric cells; e – outer side of sepal; f – outer side of sepal with two-celled trichomes; g – numerous stomata located at the apical part of sepal; h – magnification of g. Photographs by D. Łuszczek.

(Fig. 5a–d), small vesicles (Fig. 5b–d, white arrows) and vacuoles with inclusions (Fig. 5a, c). Small, electron-dense vesicles are visible between plasmalemma and cell wall and in the invaginated plasmalemma (Fig. 5a–d, black arrows). The epidermal cells of the epichile are covered by a heterogeneous cuticle (Fig. 6a–c) with small amount of secreted substances (Fig. 6a). Diffuse channels (Fig. 6b, white arrow) and separation of cuticle layers (Fig. 6c, white arrow) are observed.

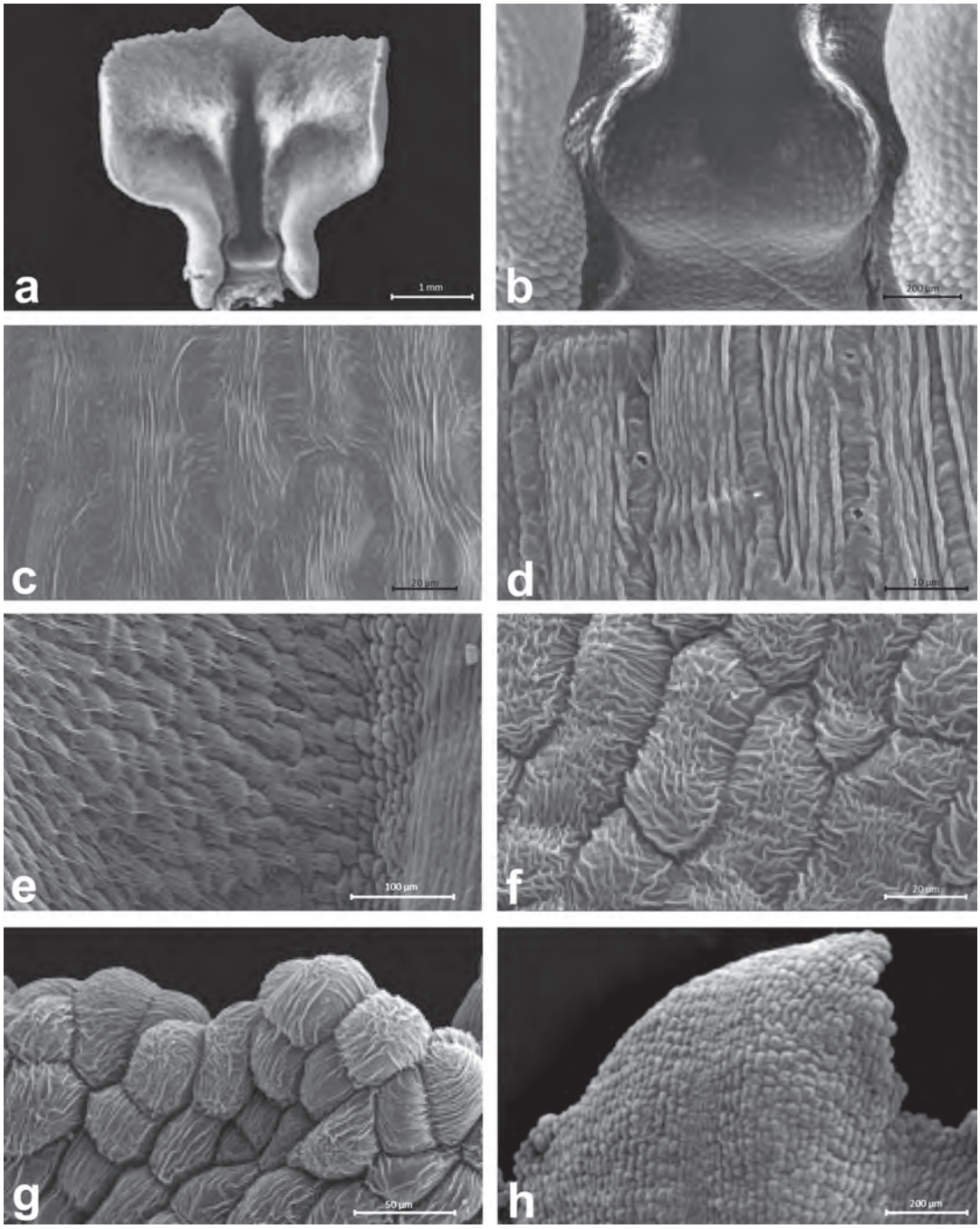

Figure 3. *Liparis stricklandiana* – SEM. Lip. a – blade-like lip with the central thickening well visible (as a narrow path) in the middle; b – two bubbles of callus at the hypochile; c – elongated epidermal cells with cuticular border on the surface of the lip basal callus and d – area at its base, with small cuticular cracks, alike stomata; e – isometric epidermal cell on the side part of lip with f – strong undulate cuticle; g – undulate surface of cells on the edges; h – distal end of epichile. Photos by D. Łuszczek.

Dense cytoplasm contains nucleus, plastid, dictyosomes and mitochondria (Fig. 6a, c–d). Vacuoles with electron-dense material are noticed (Fig. 6a, c). Vesicles are occurring between plasmalemma and cell wall (Fig. 6a, c, black arrows; Fig. 6d) mostly with electron-dense material (black arrows).

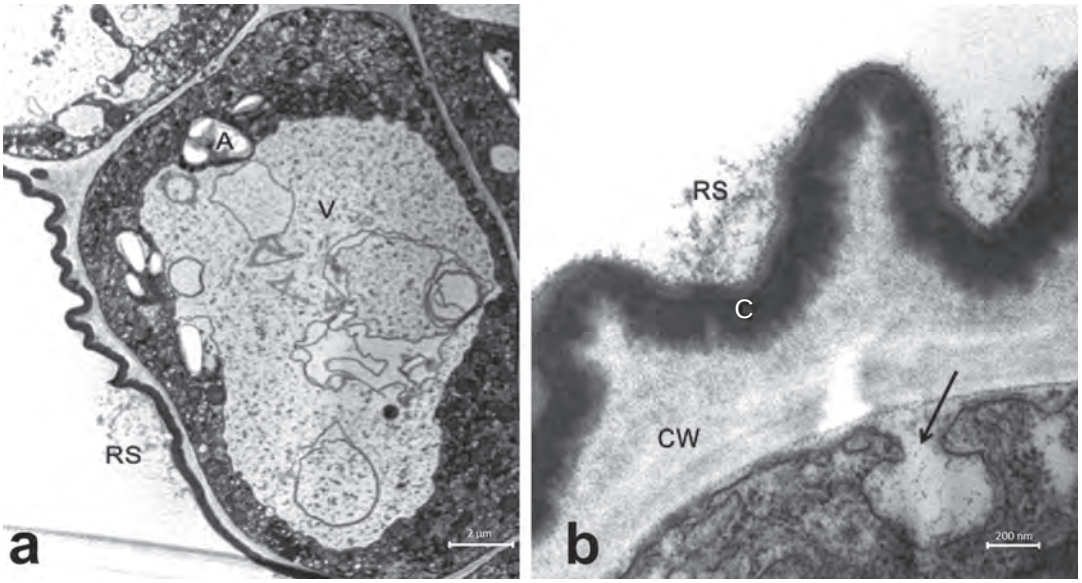

Figure 4. *Liparis stricklandiana* – TEM. Ultrastructure of dorsal sepal. a – cytoplasm containing a large vacuole with inclusions, amyloplasts. Notice secreted substances above cuticle; b – magnification of a, black arrow shows invaginated plasmalemma with electron-dense material. C = cuticle, CW = cell wall, V = vacuole, A = amyloplast, RS = secreted substance. Photos by M. Narajczyk.

Epidermal cells of the lip's margins are covered with a large amount of residues of secreted substances, located above the amorphous cuticle (Fig. 7a, c, d). Dense cytoplasm includes dictyosomes (Fig. 7c–d), amyloplasts (Fig. 7c–d), mitochondria (Fig. 7a–d) and a rough endoplasmic reticulum (Fig. 7b). The endoplasmic reticulum membranes are lying close to mitochondria and plasmalemma (Fig. 7b, white arrow). Invaginated plasmalemma contains electron-dense material and small vesicles (Fig. 7a–b, black arrow).

Discussion

The colour of *Liparis stricklandiana* flowers is the major signal for pollinator's attention. Changing the colour of flowers seems to be also an important element in signaling the state of readiness to effective pollination.

Although observations made by means of scanning electron microscopy did not reveal the residues of secretion (Fig. 2), the transmission electron microscopy results confirmed the presence of secretion activity on the flower surface. The secretory substance was observed upon the amorphous cuticle (Fig. 4a–b) as well as between plasmalemma and cell wall (Fig. 4b, black arrow). Sepals and petals, which are becoming folded back and thus making the lip more prominent during anthesis, do not attract significantly higher attention of the visiting insects. Therefore, it seems possible that the observed secretion of sepals and petals concerns rather olfactoric than food emissions. However, we have not noticed yet any scent in the flowers of examined *Liparis*.

The role of the bicellular trichomes, which have been observed especially at abaxial surface of the sepals, is still unknown (Fig. 2f). They may play a certain role in olfactory processes. Similar structures have been frequently observed on the surface of the tepals in many representatives of Liparidinae and also Malaxidinae (Margońska's own observation).

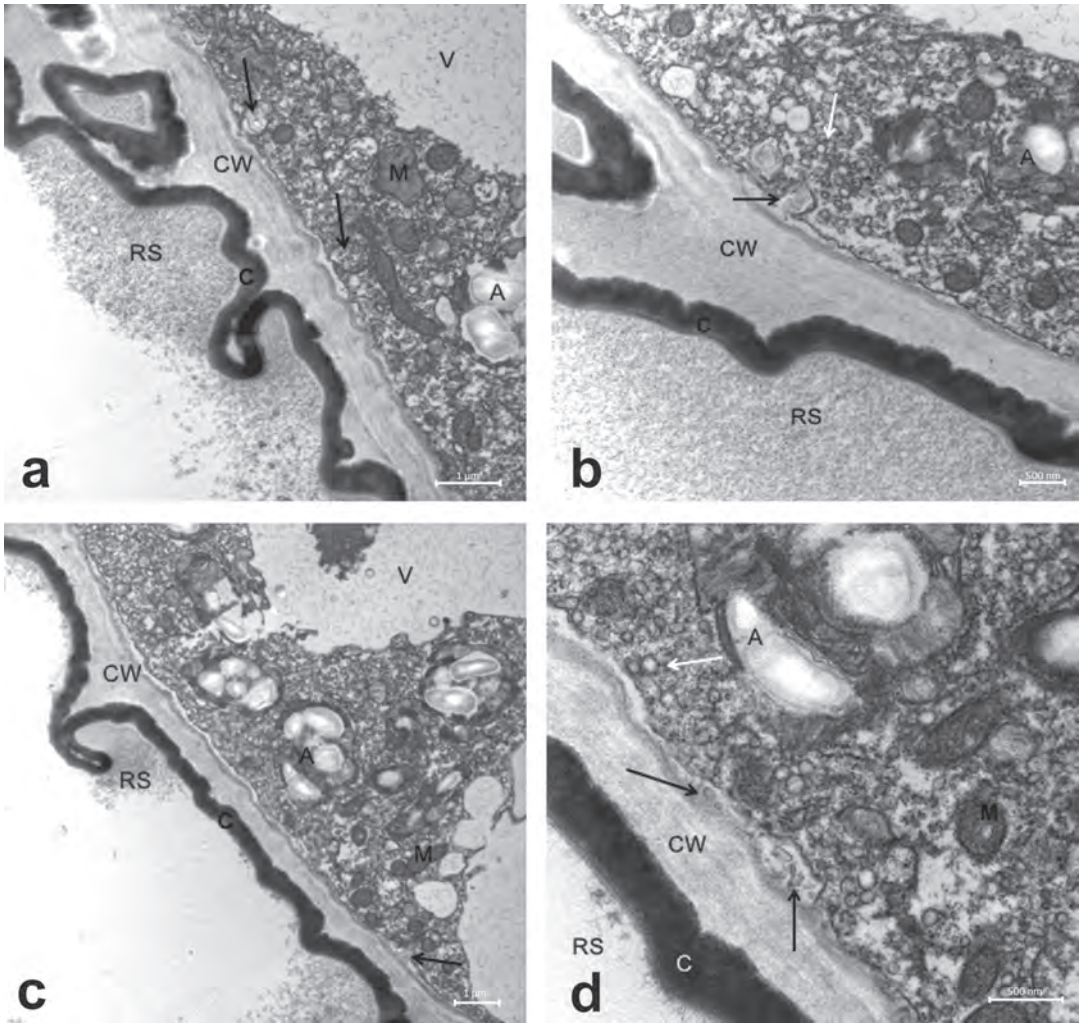

Figure 5. *Liparis stricklandiana* – TEM. Ultrastructure of hypochile. a – secreted substance located above amorphous cuticle. The dense cytoplasm with amyloplasts, mitochondria, large vacuole. Black arrows indicate invaginated plasmalemma, also vesicles between plasmalemma and cell wall. Notice the large amount of small vesicles close to plasmalemma (white arrows) in a–c; d – magnification of c. C = cuticle, CW = cell wall, M = mitochondria, V = vacuole, A = amyloplast, RS = secreted substance. Photos by M. Narajczyk.

The diversity of stomata on the surface of sepals also seems to be interesting. The stomata of the adaxial surface of sepals are surrounded by four isodiametric cells of epidermis (Fig. 2b), whereas those on the abaxial surface are not (Fig. 2h).

Secretory activity has been confirmed on the whole surface of the lip. It seems that the secretion of the lip epidermis occurs in two ways, just through the cuticle or through the small cracks of the cuticle. The surface of the amorphous cuticle of the hypochile is rich in large quantity of residues of secreted substances (Fig. 5a–c). In contrast, the heterogeneous cuticle of the epichile epidermis contains a smaller amount of the secretion, as revealed in SEM analysis (Fig. 6a).

Epidermal cells of the lip's margins are also covered with large amount of the residues of secreted substances, located above the amorphous cuticle. Observations made by transmission electron microscopy confirm the high secretion activity of these epidermal cells, too (Fig. 7a–c).

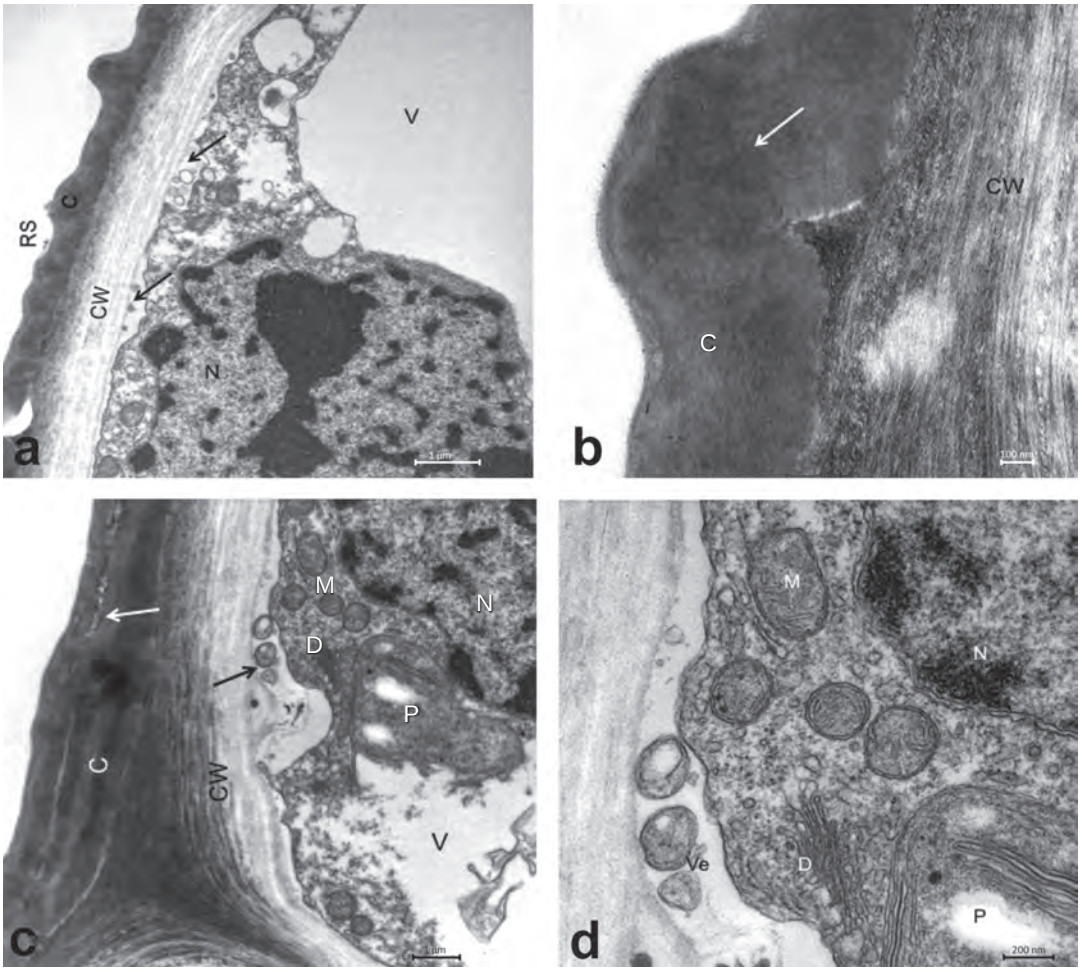

Figure 6. *Liparis stricklandiana*. – TEM. Ultrastructure of epichile. a – cytoplasm with large nucleus, black arrows indicate vesicles between plasmalemma and cell wall; b – diffuse network in cuticle; c – cell with large nucleus, dictyosome, vacuole, plastid and small mitochondria. Black arrow shows electron-dense vesicles between plasmalemma and cell wall. Notice separation of cuticle layers (white arrow); d – magnification of c. C = cuticle, CW = cell wall, M = mitochondria, D = dictyosome, V = vacuole, Ve = vesicle, P = plastid, N = nucleus, RS = secreted substance. Photos by M. Narajczyk.

The structure of the epidermis cells of the lip's marginal parts suggests a possible dripping/exudation of the secretions towards the central part of the lip, then in the direction of the central thickening and finally down the lip (Fig. 3e). Similarly, the lip central thickening, both cells and their cuticle border, situated parallelly to the long axis of the lip, seem to facilitate the leaking down of the secreted liquid (Fig. 3d–e).

The small cracks of cuticle at the central thickening, which are similar to stomata (Fig. 3d), are possible sources of the liquid secretion observed as a little drops on the fresh flowers (Fig. 1d). For the first time, this liquid secretion is confirmed in the flowers of this *Liparis* species. Proportionally, the largest drops are located below the lip's basal callus, where the accumulation of the small cracks is the biggest. Maximum droplet secretions are noticed in the fresh flowers at the early stage of anthesis. Later, when the flowers become already yellowish or golden to ochre, only small traces of the drops remain on the surface of the epidermis of the central thickening. Within a

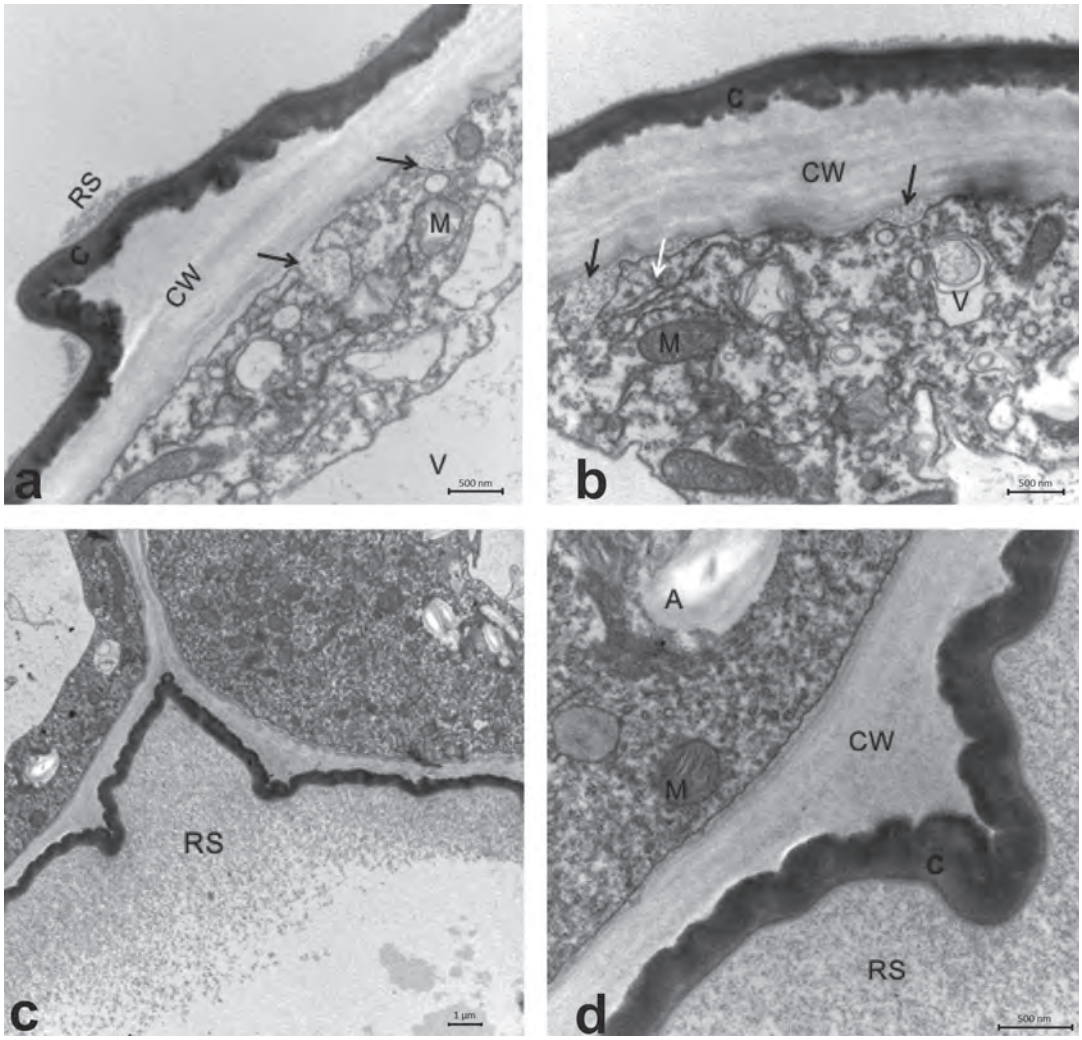

Figure 7. *Liparis stricklandiana*. – TEM. a, b – ultrastructure of lip's margins. Black arrows indicate invaginated plasmalemma with electron-dense material; b – secreted substance located above cuticle; white arrow shows ER membranes located close to plasmalemma; c – notice large amount of secreted substance; d – magnification of c. C = cuticle, CW = cell wall, M = mitochondria, V = vacuole, A = amyloplast, RS = secreted substance. Photos by M. Narajczyk.

single inflorescence, maximum activity of secretion usually concerns a limited number of flowers (only 1–3). Taking also into account the small size of the droplets, it was impossible to collect a sufficient amount of the liquid samples which would have enabled us conducting chemical qualitative analysis. On the basis of the yet unpublished results, obtained during analogous research conducted by us in other species of the genus *Liparis*, it seems to be highly probable that the liquid secretion consists also of polysaccharides. The small cracks of the cuticle may also play a role in the olfactory processes, although this has not been confirmed yet.

The cells of the epichile's distal edges form an irregular dentation, which becomes stronger during anthesis. Cells at the edges (Fig. 3g) and also on the conical end of the epichile (Fig. 3h) have a well visible undulate surface. Our observation made by transmission electron microscopy confirmed the secretion activity of these epidermal cells (Fig. 7a, c, d).

For the first time, observations on the living flowers let us confirm the facultative process of self-pollination in *Liparis stricklandiana*, which occurs at the end of anthesis. The arcuate bend of the column of gynostemium increases and brings the anther closer to the lip during anthesis (Fig. 1e). In this position, the movable anther releases the pollinia by any induction (e.g. air breeze, flower knocking). The entire pollinium, still attached by caudicles to the top of the rostellum, turns down to the stigma cavity (Fig. 1f). Finally the pollinia fall onto the stigma.

Conclusion

Considering that our knowledge about pollination pathways in *Liparis*, as well as in orchids in general, is still insufficient, the presented study represents an important step in better understanding the biology and ecology of this group. Each contribution of such type may support the *in situ* conservation of those highly vulnerable and endangered plants in future.

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