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Liparis crenulata (Orchidaceae, Liparidinae) – chemical and morphological study of the flower in the context of the pollination process

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Summary: Liparis is a large genus comprising ca 300 species, mainly pantropical in distribution. The lips of *Liparis* flowers, like other members of the subtribe Liparidinae, are downwardly directed and serve as a landing platform for pollinators. The pollination and floral morphology (especially micromorphology) of *Liparis* are still insufficiently investigated. Field observations have revealed so far that the flowers are visited by small flies, midges, fruit flies, other small dipterans, ants, spiders and mites. However, none of these have been recorded to pollinate these flowers effectively.
Preliminary observations revealed the presence of small droplets on the lip surface of *Liparis crenulata*. As further research revealed, this is the first time that nectar secretion has been recorded in this species. Liquid secretion collected from the lip surface and from whole flowers was subjected to sequential organic solvent extraction and gas chromatography-mass spectrometry (GC-MS). To support our observations, we also investigated floral parts and their role in pollination by means of the scanning electron microscope (SEM) and transmission electron microscope (TEM). The presence of viscid droplets on the lip of *Liparis crenulata* flowers as well as the presence of a food reward, are reported for the first time.

Keywords: gas chromatography, morphology, nectar; scanning electron microscopy, transmission electron microscopy

Pollination strategies in Orchidales Raf. are amongst the most advanced in the plant kingdom, especially in the context of adaptation to different forms of pollination by animals (mostly entomophily). These are reflected in the morphology and physiology of the orchid flowers. The lip displays the greatest modification and can be used by visiting insects as a landing platform and / or act as a long- and / or short-distance signal of rewards that are either offered or potentially offered. Also, the reproductive structures in this plant group are highly adapted to pollination by animals. This includes, for example, reduction of the number of stamens and stigmatic surfaces from 3 to 2 or mostly 1, fusion of the pistil with the stamen to form the gynostemium and the clumping of pollen into 2 or more packages (pollinia) equipped with various structures that facilitate their transport as entire units by insects. All these mechanisms prevent the orchid flower from being self-pollinated. Self-pollination is interpreted as an evolutionary disadvantage. However, in some orchids, there are some system modifications that enable autogamy, when suitable pollinators are scarce or absent and/or under adverse growing conditions. As van der Pijl & Dodson (1966) state that self-pollination occurs in approximately 3% of orchid species. Catling (1990) estimates that this can be even as much as 5–20%.

Liparis is a large genus comprising ca 300 species. The genus is mainly pantropical in distribution with some representatives occurring in temperate regions of the northern and southern hemispheres. These orchids are epiphytic, terrestrial or lithophytic, forming colonies of various

sizes. The lips of *Liparis* flowers, like other members of the subtribe Liparidinae, are downwardly directed and serve as a landing platform for pollinators.

The pollination and floral morphology (especially micromorphology) of *Liparis* are still insufficiently investigated. Over nearly the past 30 years, no work has been published describing pollination mechanisms in the representatives of this genus. Field observations have revealed so far that the flowers are visited by small flies, midges (Kaiser 1993; Margońska pers. obs.), fruit flies (Feldmann & Barré 2001; Margońska pers. obs.), other small dipterans, ants, spiders and mites (Margońska pers. obs.). However, none of these arthropods have been recorded to pollinate flowers effectively.

In cultivation, small flies and other small dipterans (such as e.g. Culicidae: *Culex* sp. or Sciaridae: *Bradysia* sp., *Lycoriella* sp., *Sciara* sp.) have been observed on several occasions visiting *Liparis* flowers (H. Petersen, cited by Christensen 1994; Margońska pers. obs.).

The flowers of *Liparis reflexa* Lindl. smell of rotten egg yolk and it is known that they are frequently (and effectively) pollinated by Sarcophagidae (Wallace 1974) or Mycetophilidae flies (Adams & Lawson 1993). Similarly, *Liparis coelogynoides* F. Muell. possesses flowers with a urine-like odour and these are also known to be pollinated by 'small flies' (Bishop 1996).

Kaiser (1993) investigated the unusual floral scent of *L. viridiflora* (*Stichorkis viridiflora* (Bl.) Marg., Szlach. & Kułak) and identified 25 compounds, the most abundant being (E, E)-farnesene (65%, giving sperm-like notes to the odour), as well as (E)-7-methyl-1,6-dioxaspiro-(4,5)-decane (8.1%), benzaldehyde (1.8%), (E,Z)-2,6-nonadienal and benzyl alcohol (both 1.5%), limonene (1.3%), pinene (1.1%), methyl benzoate and (E)-β-farnesene (both 1%). Aldehydes such as (E)-2-nonenal and (E,Z)-2,6-nonadienal are reminiscent of the smell of cucumber and *Viola* leaves. Francke et al. (1977) noted the interesting fact that spiroketals like the two diastereoisomers of chalcogran occur as biologically active compounds in the pheromone of the bark beetle *Pityogenes chalcographus*. Kaiser (1993) emphasized the remarkably large quantities of (E)- and (Z)- isomers of 7-methyl-1,6-dioxaspiro-(4,5)-decane present in the scent of *L. viridiflora*. Furthermore, Francke et al. (1977) recognized this compound to occur in a pentane extract of a pheromone obtained from female workers of *Paravespula vulgaris*. Kaiser (1993) observed also that other *Liparis* species (*Liparis sensu stricto* and especially *sensu Stichorkis*) exuded a range of unusual scents reminiscent of e.g. algae, yeast, crustaceans, etc., which may also attract flies and midges. He recorded that their scent has a similar spectrum to that of *Eria* species. (Margońska et al. 2012/2013).

The fragrances of some *Liparis* flowers are sometimes described as resembling that of cucumber or sweet cucumber. In our opinion, the smell is the result of coumarin compounds present in the tissues of these plants, and this is released after e.g. mechanical damage caused by even the slightest touch or the breaking of inflorescences or flowers.

Liparis crenulata (Bl.) Lindl. (in Gen. Sp. Orchid. Pl. 30. 1830, *Stichorkis crenulata* (Bl.) Marg., Szlach. & Kułak, in Szlach., Marg. & Kułak, Acta Soc. Bot. Poloniae 77(1): 37. 2008) is a wet-mountain epiphyte recorded from Sumatra and Java between altitudes of 800–2200 m. At higher elevations and in colder conditions, it also grows as a terrestrial. This orchid usually forms colonies in shaded or semi-shaded areas of forests. A single, unifoliate pseudobulb produces seasonally a single, suberect to horizontal inflorescence that is often longer than top of the leaf. A spike is the

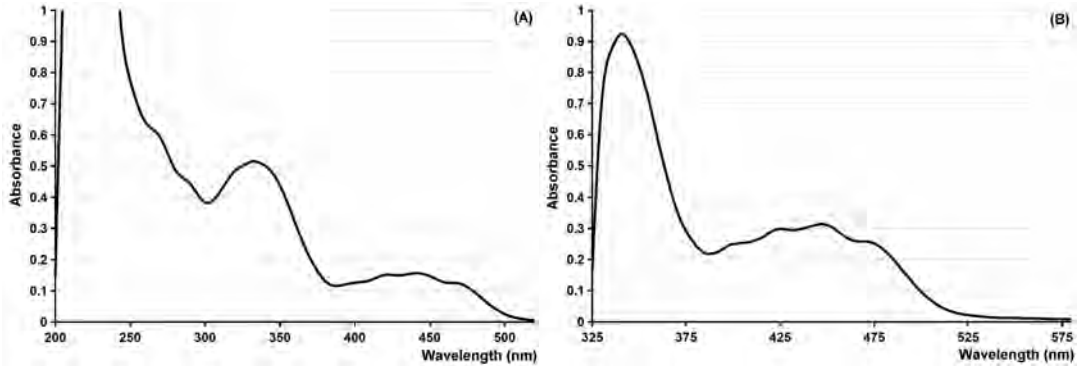

Figure 1. Reconstructed absorption spectra of the *Liparis crenulata* flower extract, recorded in methanol (A) and acetone containing 20% water (B).

typical inflorescence of *Liparis* species and this bears numerous flowers that in *L. crenulata* are relatively large for the genus (1.6–2 cm long). The flowers are shiny, with brick-red to brownish red tepals and an intense red lip (Fig. 8A).

Age-related changes in the floral morphology are typical of representatives of Malaxidinae and Liparidineae. The sepals and petals become folded back, making the lip more prominent.

The lip is divided into two distinct and well-developed parts. These are the hypochile (the proximal part, channeled, narrower than longer and thicker than the epichile) and the epichile (wider than longer, distally almost flabellate, forming a landing platform for insects). The boundary between the hypochile and epichile is arcuate and recurved, becoming more distinct at anthesis (Fig. 8A). Near the base of the hypochile, an anvil-shaped callus is present (Fig. 8B). In many Liparidinae flowers, there is a central thickening running from the lip callus/calli or from the base to nearly the tip of the lip. The central thickening is smooth, shiny, and at least slightly more intensely colored (Fig. 8B). This structure is termed a pseudonectary (sometimes also called a 'nectar mimic'). We confirmed that it fulfills a role in insect attraction and secretion, and possibly functions as an olfactory area (not yet proven in this case). This is the very first part of the flower that is explored by insects just after landing on the lip (the first author's observations, unpublished data). Moreover, it promises more than it actually provides. The epichile in this species is flattened and obcordate in shape. A small appendix is present within the distal cleft. The margin of the epichile is minutely ciliated (Fig. 8C).

Preliminary observations revealed the presence of small droplets on the lip surface of *Liparis crenulata*. As further research revealed, this is the first time for liquid (nectar) secretion to be recorded in this species. Liquid secretion collected from the lip surface and from whole flowers was subjected to sequential organic solvent extraction and gas chromatography-mass spectrometry (GC-MS). To support our observations, we also investigated floral parts and their role in pollination by means of scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Materials and methods

Plants of *Liparis crenulata* (Liparidinae) have been cultivated by Lipińska since 2013 in the glasshouses of Gdańsk University. They flowered regularly every season since then. The flowering

season *in situ* ranges mostly from November (December) to February. Other cultivated plants of this species are usually reported to flower between September and February. Those of the corresponding author flower between November and January. The flowering period usually lasts about 4 weeks. Specimens of the cultivated species were regularly preserved at each stage of anthesis as both pressed and spirit samples in Copenhagen Mixture (collection: UGDA-ML839217, voucher: ML839217) and photographed by Margońska. SEM and TEM analyses were performed using juvenile, but fully developed flowers collected early in the morning.

Identification of the plants was confirmed by the first author according to classical taxonomy methods, and with reference to the original taxonomic material, such as type specimens and protologues (by Margońska).

All observations were carried out on living plants (throughout anthesis) grown in the glasshouses of Gdańsk University. Floral macroscopic structures were investigated using a light stereoscopic microscope.

For chemical analyses, the liquid secretion of ca 20 flowers was carefully collected using several small pads of glass wool and then extracted in 10 ml methanol. Whole flowers were subjected to sequential organic solvent extraction. First, non-polar compounds were isolated in 10 ml dichloromethane for 20 sec., then carbohydrates were extracted by dipping flowers for 30 sec. in 10 ml methanol. Extracts were then stored at 4°C prior to analysis. The dichloromethane extract was concentrated to ca 0.3 ml under a stream of nitrogen. Triplicate samples were then analyzed using gas chromatography-mass spectrometry (GC-MS), which was performed using a Shimadzu QP-2010SE system (Shimadzu, Kyoto, Japan), equipped with a 30 × 0.25 mm i.d., film thickness 0.25 µm, ZB-5ms capillary column (Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The split ratio was 1:10, and the injection volume was 1 µl. The injector and GC-MS interface temperatures were maintained at 310°C. Electron ionization (electron energy 70 eV, ion source temperature 200°C) was used. The column temperature was programmed from 30°C (isothermal for 3 min) to 180°C at 4°C min^{-1}, then from 180°C to 310°C at 8°C min^{-1}, and then maintained at 310°C for 12 min. Methanolic extracts were subjected to the same procedure after trimethylsilylation. An aliquot of each extract was evaporated to dryness under a gentle stream of nitrogen and 0.1 ml BSTFA + TMCS (99:1; Sigma Aldrich) was added. Samples were kept at 60°C for 30 min. Identification of non-polar compounds in dichloromethane extract was based on the retention order on non-polar stationary phase in GC-MS analysis and on mass spectra recorded in both the case of volatiles (Adams 2007) and non-volatile components (Becker et al. 1983; Carlson & Mackley 1985; Ginzel et al. 2006; Howard et al. 2003; Nelson & Leopold 2003; Volova et al. 2003). The relative composition of non-polar compounds was determined based on the peak area of each compound and separately for the volatile and non-volatile fractions of the extract. The sugar fraction (the liquid secreted by the lip) was analyzed using nuclear magnetic resonance spectroscopy (NMR). Methanolic extracts were evaporated to dryness under a stream of nitrogen. Samples were subsequently dissolved in 0.75 ml 99% D_2O (Sigma-Aldrich, USA) and ^1H NMR spectra recorded at 25°C using a Bruker Avance III 500 MHz spectrometer (Bruker BioSpin AG Switzerland). Chemical shifts were referenced to an internal standard of acetone (δ_H 2.225; δ_C 31.45).

About 20 flowers were degreased (30 sec in 20 ml n-hexane) and pigments extracted twice using 20 ml 5% acetic acid in methanol (15 min in the ultrasonic bath). The solvent was then removed under reduced pressure and pigments were re-dissolved in methanol or acetone. Samples

were kept at 4°C until analyzed. UV-VIS spectra were recorded using Beckman DU 650 UV-Vis spectrophotometer (Beckman, Brea, California, USA) in methanol (wavelength range of 200–800 nm) and acetone containing 0%, 20% and 40% water (320–800 nm). The respective pure solvent was used as a blank for each treatment.

Samples for scanning electron microscopy (SEM) were preserved in 2.5% GA and 2.5% PFA in 0.05M cacodylate buffer (pH 7.0). Following dehydration in an ethanol series, they were dried by the critical point method using liquid CO_2 and coated with gold and observed by means of a Philips XL-30 scanning electron 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). Postfixation overnight in 1% OsO_4 in cacodylate buffer. Following 1-hour staining in 1% uranyl acetate in distilled water, the samples were dehydrated by means of a graded acetone series and embedded in Spurr's resin. Ultrathin sections (60 nm) were cut using a Leica UC7 ultramicrotome. Sections were examined by means of an FEI Tecnai Spirit BioTWIN transmission electron microscope at 120 kV.

Margońska was responsible for planning the research, observing living flowers in the field, preparing the morphological description (at different levels, from macro to micro), photographing the living flowers and taking some SEM images, analysis of the results and discussion. Haliński and Czerwicka conducted the chemical analysis and contributed to the discussion. Davies' consultative input contributed significantly to the manuscript. Narajczyk prepared samples, took TEM images and contributed to the preliminary analysis of the results. Łuszczek prepared samples for SEM and undertook photography. Lipińska was responsible for cultivating and observing the plants, preparing the manuscript and contribiuting to the analysis of the final results and discussion and preparing the figures.

Results

In most species of Malaxidinae and Liparidineae, the flower color usually changes with age. However, in the case of *Liparis crenulata*, we did not observe this phenomenon throughout the whole of anthesis. The color of the flowers remained essentially unchanged – the tepals being brick red to brownish red, whereas the lip remained an intense red (Fig. 8A). The central thickening is the only part that is glossier than the rest of the lip surface and is almost of the same intense red color (Fig. 8B). However, the red pigment of the lip dissolved into the preserving liquid (Copenhagen Mixture) and unusually, this process was surprisingly long, lasting for several weeks.

We observed in this species, for the first time, the presence of small, viscid droplets on the lip surface of fresh flowers, and these were located close to and beneath the callus and below its surroundings (Fig. 8B).

Observations of fresh flowers revealed that the anvil-shaped callus and/or adjacent regions beneath it are the source of a liquid secretion. As in many other Liparidinae, the lip has a longitudinal central thickening running from the lip base/basal callus nearly to the tip of the epichile. In this species, the central thickening runs from below the basal callus to the boundary between the hypochile and the epichile and occurs in a distinct depression (Fig. 8B). The liquid secretion appears to flow downwards along the lip surface and gathers in the depression before it flows further down onto the distal part of the lip's central thickening where it eventually evaporates (at

late anthesis). The viscid droplets gently removed from the young lip in the evening were replaced by new ones in the early morning (albeit in smaller volumes). In older flowers, the liquid droplets evaporated and left small residues on the surface of the lip epidermis.

The marginal cilia appear as bright pointed structures. Cilia arranged spheres are present along the crenulate edges of the lip from the boundary between the hypochile and epichile (where they are brighter and longer) to the distal part of the epichile. They are absent at the distal cleft, where the margins are entire. At the boundary between the edges of the ciliate epichile and the distal cleft, irregular structures of intermediate form between crenulate and smooth margins are present. Small clusters of short cilia occur on the apical part of the lip (Fig. 8C).

A small, oblong appendix occurs at the base of the distal cleft. Its adaxial surface is composed of cells containing raphides of calcium oxalate (Fig. 8C).

We did not notice the presence of any scent perceptible to humans in fresh flowers of this species.

Chemical analyses

The results of GC-MS analyses of the non-polar components isolated from *Liparis crenulata* flowers are given in Table 1, together with the main diagnostic ions used for the identification of the compounds. Two fractions were detected: a volatile fraction consisting of six monoterpenes and a non-volatile fraction, composed of saturated and unsaturated straight-chain hydrocarbons containing 21–31 carbon atoms and trace quantities of several steroids. The identification of the main fraction component (*n*-pentadecatriene) was tentative. Its elution was shortly prior to that of monounsaturated and saturated C_{25} compounds during the non-polar stationary phase of GC analysis and the close similarity of its fragmentation pattern during mass spectrography resembled those reported for shorter-chain nonadecatriene (Becker et al. 1983), both of which confirm its identity as *n*-pentadecatriene. However, the lack of a molecular ion signal on the mass spectrogram renders the identification tentative.

The GC-MS analysis of methanolic extracts (the lip liquid secretion) revealed only the presence of sugars. Consequently, these extracts were further analyzed using NMR spectroscopy. The results confirmed the presence of three saccharides: sucrose, glucose and fructose. Their molar ratio was 1:0.5:0.9 in extracts of whole flowers and 1:0.3:0.7 in a solution of the isolated secretion. Consequently, the relative composition of the sugar fraction (% by weight) was: 58% sucrose, 15% glucose and 27% fructose in extracts from whole flowers and 66% sucrose, 10% glucose and 24% fructose in the isolated floral secretion.

Therefore, we report for the first time that flowers of *Liparis crenulata* do not only attract insects by their color and scent, but also offer them relatively small quantities of food reward, nectar.

The extracts contained a water-insoluble yellow-orange pigment. The extraction of red pigment present in plant tissues could not be completed using 5% acetic acid in methanol. Furthermore, during sample storage, sedimentation of small amounts of red pigment of unknown properties and structure was observed. Spectra detected by UV-VIS spectrophotometry, obtained for the samples in methanol and acetone containing 20% water are presented in Fig. 1A and B, respectively. The amount of water present in the acetone did not affect the appearance of the spectra. The presence of bands at wavelengths exceeding 600 nm, such as those observed in the chlorophyll *a* and *b* spectra, was not confirmed.

Table 1. *Liparis crenulata.* The relative composition of the volatile and non-volatile fractions of the dichloromethane extract derived from *Liparis* flowers. The amount is given as a percent of the fraction (mean values ± standard deviation; n = 3). The main diagnostic ions from the GC-MS analysis are given with their relative abundances in parentheses; a molecular ion is given in bold.

Compound	RT (min)	Amount (%)	Main diagnostic ions (m/z)
Volatiles			
Myrcene	14.165	0.90 ± 0.16	93(100), 69(97), 53(39), 91(34), 79(24)
α-Terpinene	15.121	0.65 ± 0.02	93(100), 121(69), 79(53), 77(53), **136(48)**, 91(36)
Limonene	15.625	88.82 ± 0.50	68(100), 67(89), 93(61), 79(48), 53(40), 77 (34), 91(31)
γ-Terpinene	16.876	2.41 ± 0.31	93(100), 91(54), 77(47), 79(37), 51(23), **136(22)**, 121(22)
Terpinen-4-ol	21.558	6.12 ± 0.34	71(100), 93(56), 55(49), 111(34), 67(33), 69(30), 53(27)
α-Terpineol	22.083	1.10 ± 0.17	59(100), 81(53), 93(39), 121(34), 79(33), 77(31), 136(26)
Non-volatiles[a]			
21:0	47.010	0.88 ± 0.14	57(100), 71(58), 85(42), **296(3)**
23:1	49.319	0.46 ± 0.18	55(100), 57(90), 69(81), 83(73), **322(32)**
23:0	49.622	3.75 ± 0.40	57(100), 71(55), 85(36), **324(3)**
25:3[b]	51.432	31.11 ± 2.18	79(100), 67(82), 55(62), 95(41), 108(39)
25:1	51.622	0.59 ± 0.11	55(100), 69(73), 57(58), 83(46), 97(43), **350(5)**
25:1	51.699	1.03 ± 0.11	55(100), 57(98), 69(87), 97(77), 108(62), **350(9)**
25:0	51.885	1.50 ± 0.13	57(100), 71(45), 85(29), **352(1)**
27:1	53.698	1.22 ± 0.02	55(100), 57(72), 69(68), 83(64), 97(61), **378(15)**
27:1	53.768	10.61 ± 0.47	55(100), 57(85), 69(76), 83(65), 97(58), **378(4)**
27:0	53.908	3.56 ± 0.76	57(100), 71(61), 85(43), **380(2)**
28:0	54.857	0.76 ± 0.13	57(100), 71(74), 55(26), 85(26), **394(6)**
29:2	55.501	0.74 ± 0.04	57(100), 55(84), 83(65), 97(64), 69(57), **404(3)**
29:1	55.585	1.25 ± 0.06	57(100), 55(84), 69(68), 83(66), 97(64), **406(14)**
29:1	55.658	18.99 ± 0.21	57(100), 55(92), 69(72), 97(72), 83(69), **406(8)**
29:0	55.771	12.20 ± 1.37	57(100), 71(58), 55(36), 85(36), **408(2)**
31:0	57.544	2.59 ± 0.34	57(100), 71(75), 85(51), 55(37), **436(2)**
Unidentified steroid	58.688	1.88 ± 0.19	55(100), 69(90), 78(55), 105(53). 255(44), 315(16), 398(25)
Ergosta-7,22-dien-3-ol	60.034	3.28 ± 0.09	69(76), 55(74), 79(60), 271(100), 314(29), 383(26), **398(11)**
Ergost-7-en-3-ol	60.118	3.60 ± 0.13	55(100), 79(60), 91(66), 105(58), 213(33), 229(30), 255(71), **400(97)**

[a] Hydrocarbons described in format X:Y. X – total number of carbon atoms, Y – the number of double bonds; the location and configuration of double bonds were not determined.
[b] Tentative identification, see text for details.

Scanning electron microscopy (SEM)

Dorsal sepal. Both abaxial (outer) and adaxial (internal) surfaces of the dorsal sepal are glabrous and composed of regularly arranged, elongated epidermal cells, with a smooth cuticle (Fig. 2A). Both surfaces of the entire sepal are covered with small globules (ca 0.5–1.5 µm in diameter) (Fig. 2B and 2C) and much larger globules (ca 10–20 µm in diameter) of a viscid substance (Fig. 2C and 2D). Similar residues are observed on the remaining tepals, including the lip. The larger globules are more numerous on the distal part of the sepal.

Neither stomata nor epidermal trichomes were observed on the abaxial surface in the case of the dorsal sepal.

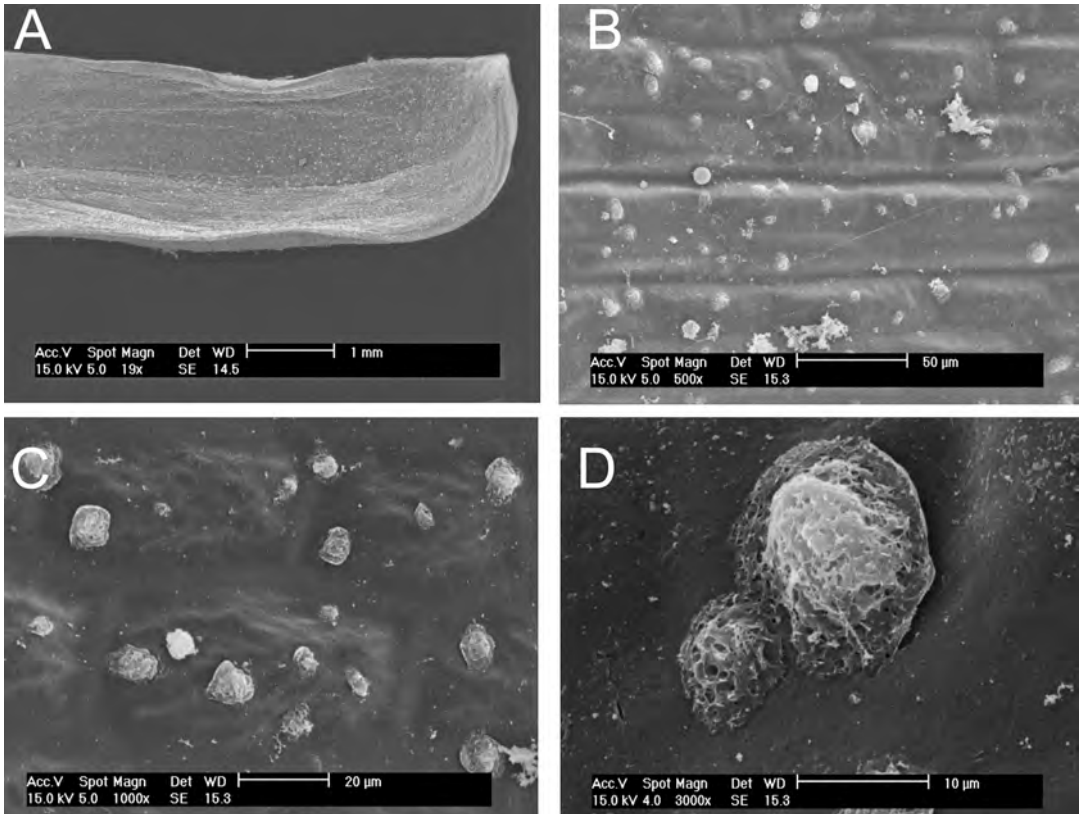

Figure 2. *Liparis crenulata*. Scanning electron microscopy. Dorsal sepal: Abaxial (external) glabrous surface (A) comprising regular, elongated epidermal cells, with smooth cuticle (B). Smooth cuticle coated with small (B and C) and much bigger globules (C and D).

The entire adaxial surface of the dorsal sepal possesses long, few-celled trichomes. Both the smaller and larger globules are irregularly distributed on cells of the trichomes.

Lateral sepals. Both abaxial (outer) (Fig. 3A) and adaxial (inner) (Fig. 3B) surfaces of the lateral sepals are similar to those of the dorsal sepal, with regularly arranged, elongated epidermal cells, with a smooth cuticle, coated with small globules and much larger viscid globules.

Neither stomata nor epidermal trichomes were observed on the abaxial surface of lateral sepals. Small quantities of secretory residues are located on the apical part of lateral sepals (Fig. 3C).

Like the dorsal sepal, the entire adaxial surface of the lateral sepals possesses long, usually 2(or more)-celled trichomes (Fig. 3D). The basal cells of trichomes are at least two-fold narrower than the others, forming a stalk-like structure. Again, the surface of the trichomes is irregularly coated with small and larger viscid globules.

Petals. Both abaxial (outer) (Fig. 4A, black arrow) and adaxial (inner) (Fig. 4A, white arrow) surfaces of the petals resemble those of the sepals, and consist of regular, elongated epidermal cells with a smooth cuticle. Small amounts of secretory residues are located at the apex of the abaxial surface of the petals (Fig. 4B).

The entire adaxial surface is coated with long, usually 2-celled trichomes. The basal cells of the trichomes are elongated and narrower than the upper ones (Fig. 4B, C). Both the cells of

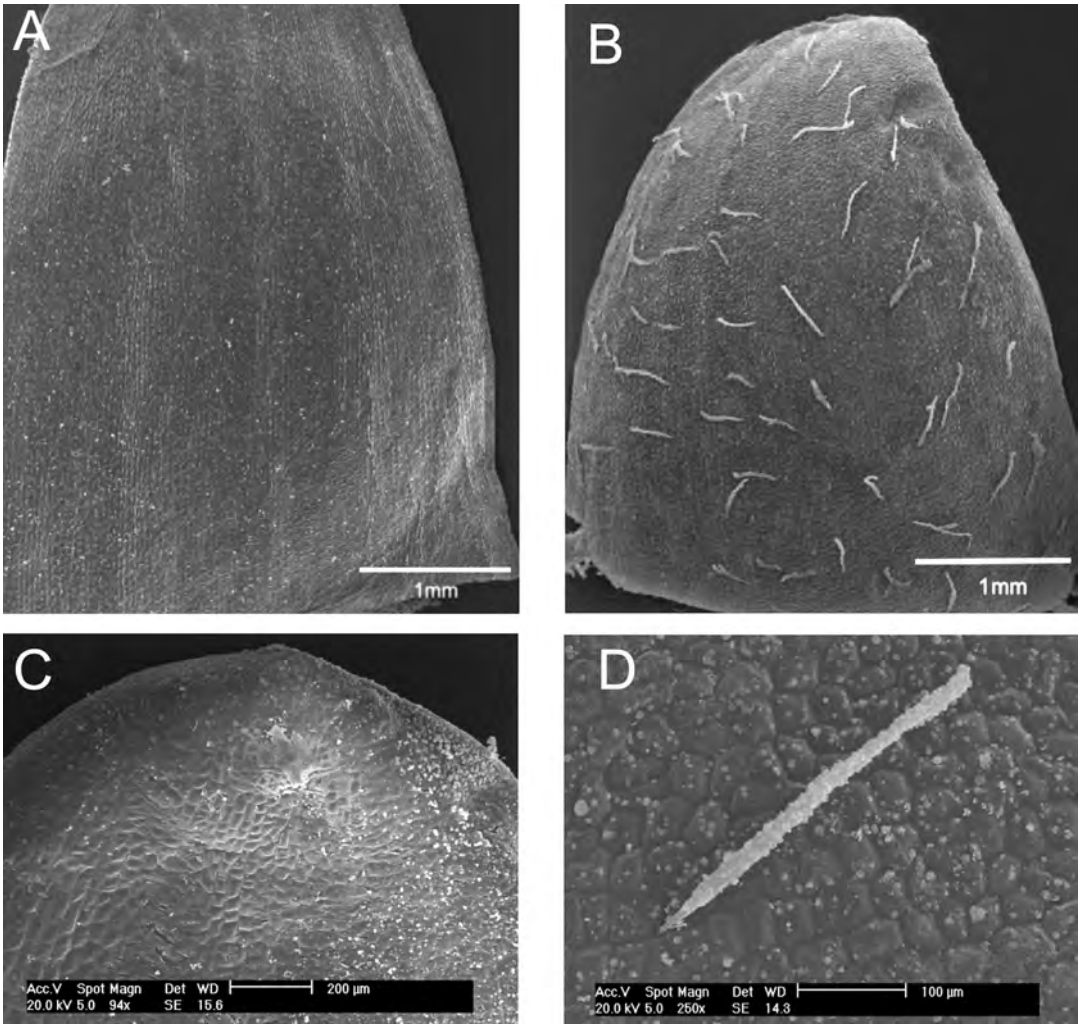

Figure 3. *Liparis crenulata*. Scanning electron microscopy. Lateral sepals: Abaxial (external) glabrous (A) and adaxial (inner) surfaces with trichomes. A small amount of secreted substance occurs on their external apical parts (C). Few-celled trichomes (D).

trichomes and surfaces of petals are covered with both small and viscid larger globules. Stomata are rarely present at the apices of the petals (Fig. 4D).

Lip. Both abaxial (outer) and adaxial (inner) lip surfaces including all their various structures are coated with small globules and larger viscid globules (Fig. 5D).

Hypochile (Fig. 5A). An anvil-shaped callus is present at the base of the hypochile and consists of regularly arranged, more isodiametric cells with a smooth or slightly concave apical surface (Fig. 5B and 5C). A similar form of epidermal cells is also present bordering the lip's central thickening, particularly in its basal half (Fig. 5B). The cuticle and epidermal cells are directed slightly obliquely or nearly transversely to the long axis of the lip. The middle part of the hypochile, just below the base of callus, is concave (Fig. 5B), and contains the basal part of the lip's central thickening. The epidermis of this central thickening is composed of more or less elongated, flat, epidermal cells with a gently undulate, wrinkled cuticle (Fig. 5F). Both epidermal

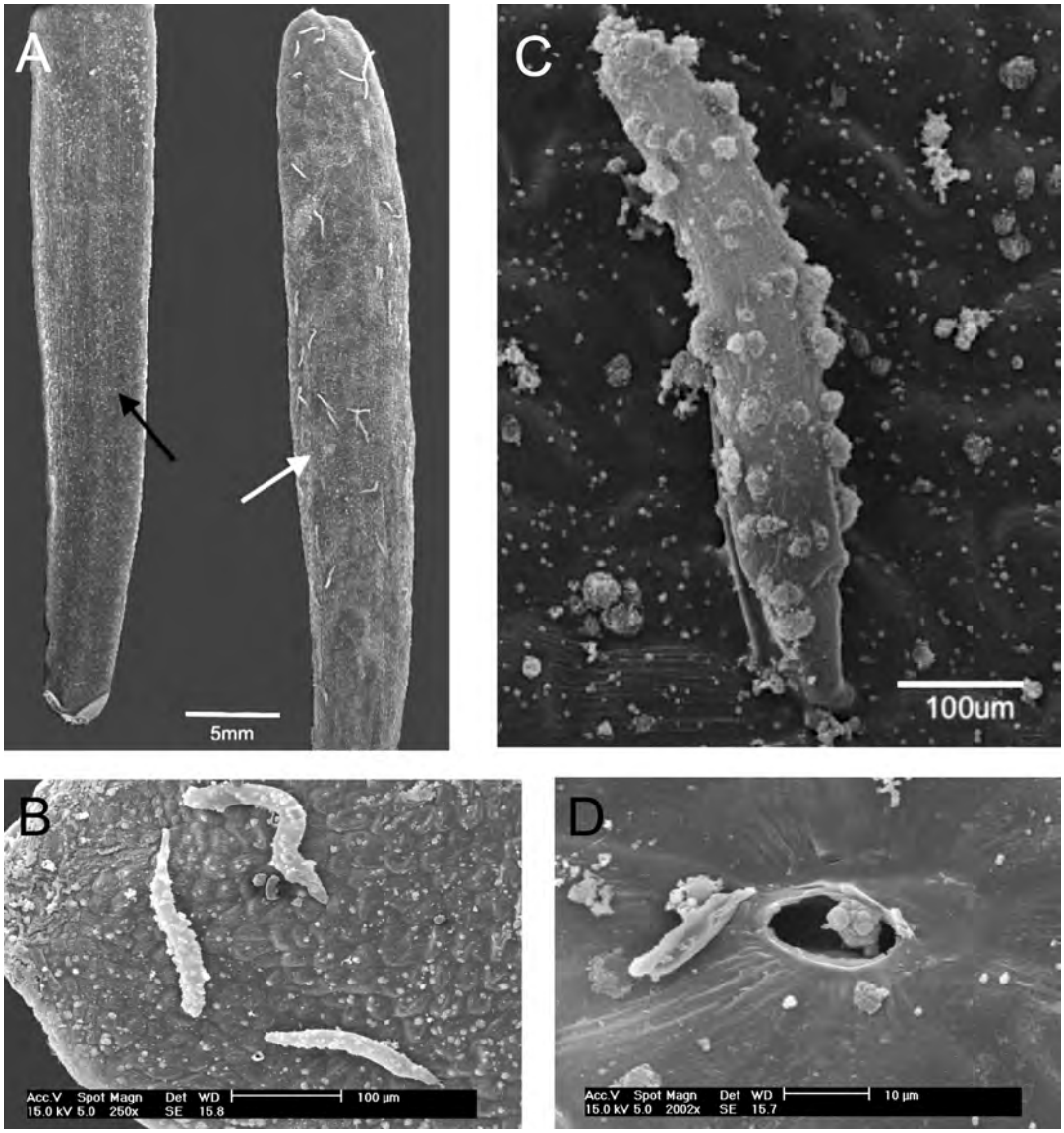

Figure 4. *Liparis crenulata*. Scanning electron microscopy. Petals: Abaxial (external) glabrous (A, black arrow) and adaxial (inner) surfaces with trichomes (A, white arrow). A small amount of secreted substance occurs on their external apical part (B). Few-celled trichome (B and C). Smooth cuticle including trichomes covered with small (B, C and D) and large globules (B, C and D). Secreting stomata at tip of the petals (D).

cells and their cuticulare striae are orientated parallel to the long axis of the lip. A small number of secretory residues are present on the lateral parts of the central thickening of the lip (Fig. 5E).

The basal auricles of the lip and the lateral parts of the chanalled section of the hypochile comprise distinctly elongated, regularly arranged epidermal cells with distinctly longitudinal cuticular striae (Fig. 5F). These cells are usually orientated obliquely to the long axis of the lip.

Epichile (Fig. 6A). The part of the lip is generally obcordate. Distally it has a central deep cleft containing a small appendix (ca 200 µm long). The whole epichile margin, except for the cleft, is crenulate and ruffled (Fig. 6B – lateral margin and Fig. 6C – distal margin). The single-celled

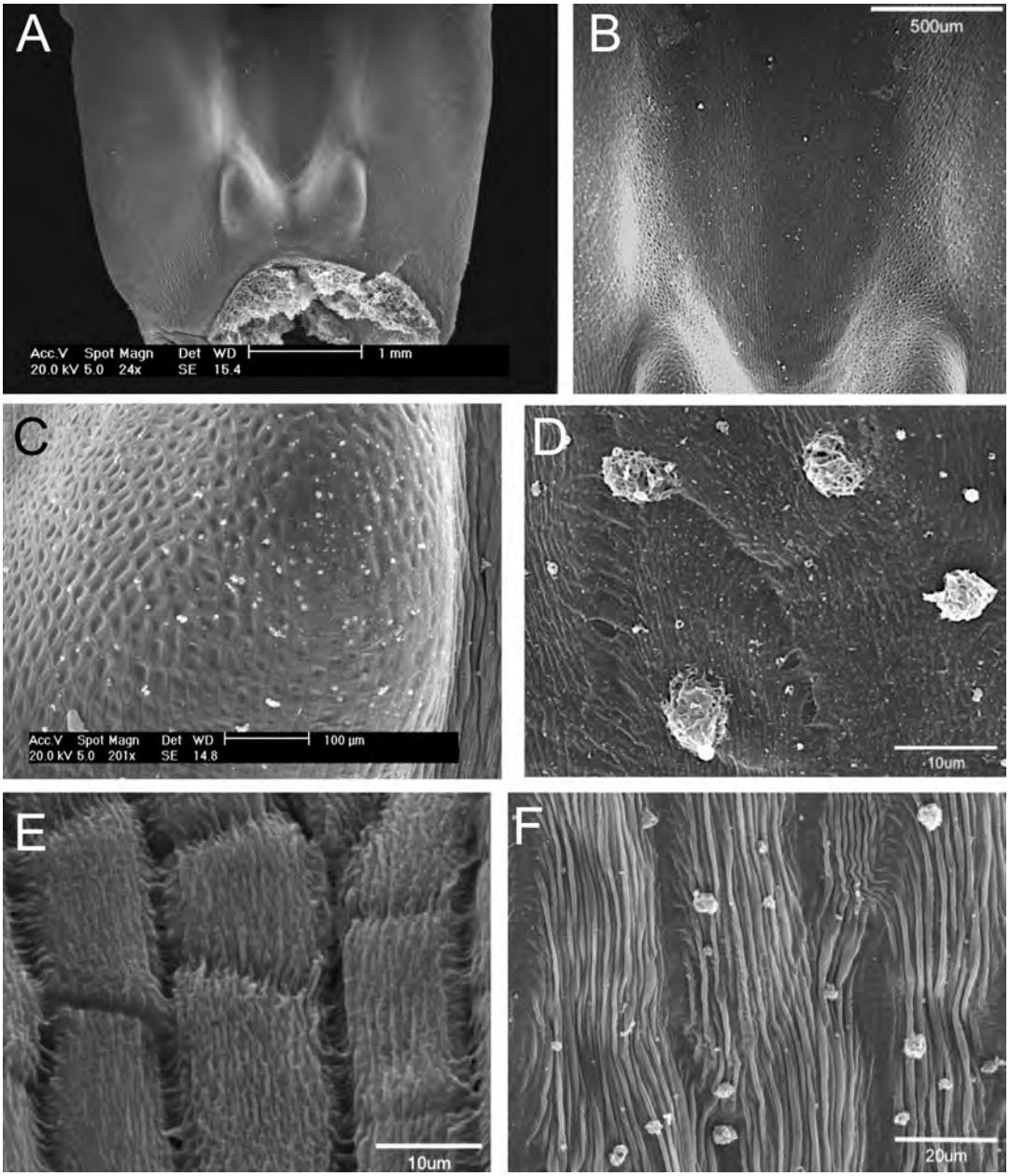

Figure 5. *Liparis crenulata*. Scanning electron microscopy. Lip: Hypochile – basal lip auricles and anvil-shaped callus (A). Lip central thickening concavity with nearly isodiametric, smooth or slightly concave apical surface cells (B and C). Adaxial (inner) surfaces of whole lip coated with globules (D). Epidermal cells of hypochile part of central thickening with gently undulating wrinkled cuticle (E, F).

cilia (up to ca. 100–150 µm long) have a distinctly and densely, longitudinally wrinkled cuticle (Fig. 6D) and are coated with small globules and much larger globules of viscid material. At the boundary between the ciliate margin of the epichile and the smooth margin of the cleft a few irregular tooth-like structures are present (Fig. 6E). Apically they bare clusters of several, smaller cilia (Fig. 6F). The internal margins of the cleft comprise only slightly elongated epidermal cells (Fig. 7A). The cuticle of the epidermal cells near the cleft part of the epichile is strongly sculptured

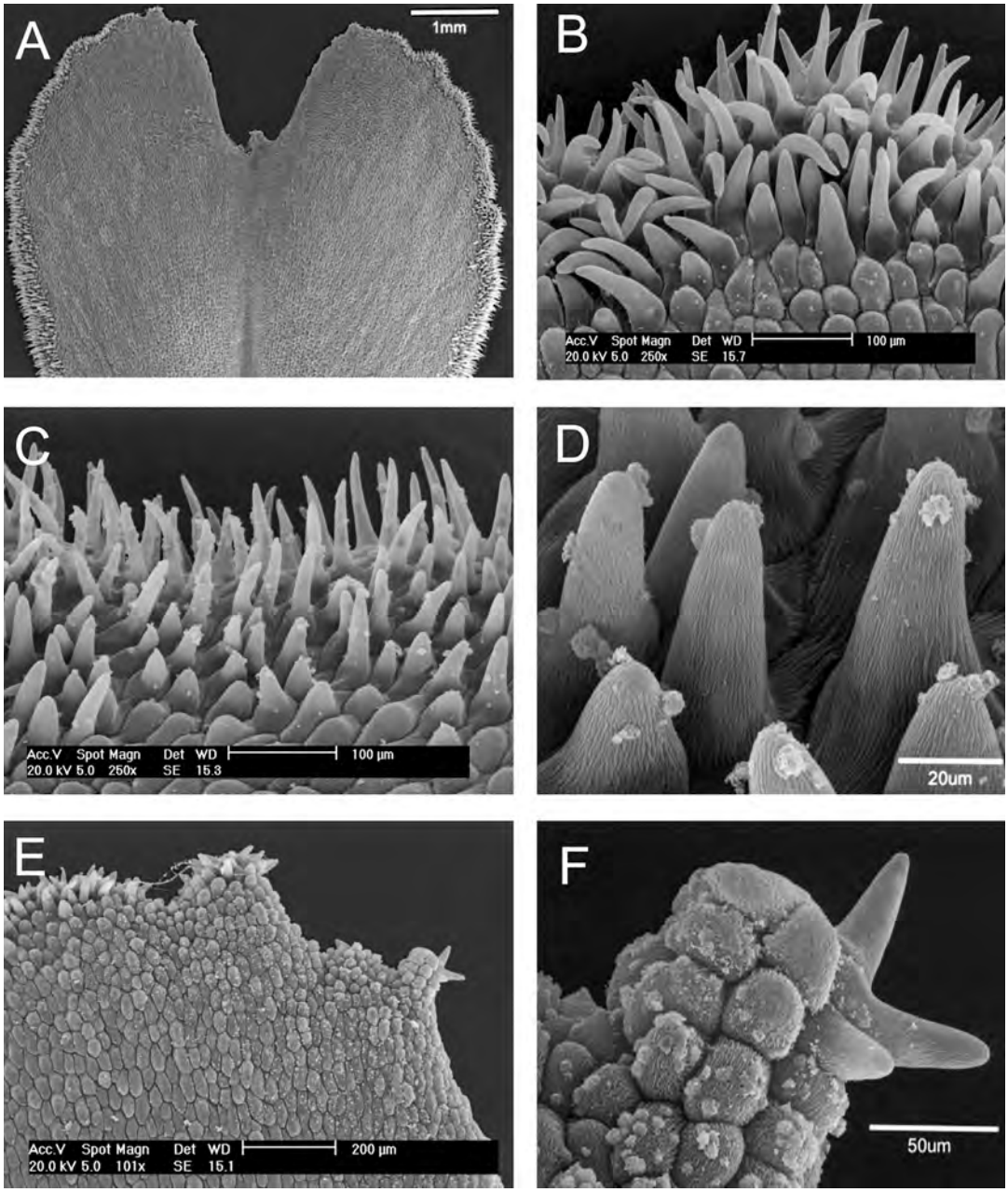

Figure 6. *Liparis crenulata*. Scanning electron microscopy. Lip: Epichile (A). Distinctly both crenulate and spherically ruffled lateral margin of the epichile (B). Slightly crenulate and spherically ruffled distal margin of the epichile (C). The single-celled cilia of the epichile margin with densely, longitudinally wrinkled cuticle and globular secretion residues (D). The boundary between the ciliate edges of the epichile and smooth margins of the distal cleft (E) with ciliate irregular tooth-like structures (F, magnification).

or coated with significant quantities of secretory residues deposited as globules, especially along the cell borders (Fig. 7A). The adaxial surface of the appendix is densely coated with elongated, thin, needle-like raphides (ca 40 µm long) (Fig. 7B), which appear to have been released from idioblasts. Secretory residues of different sizes occur upon crystals. The secretion probably originates from the cuticle of epidermal raphide cells.

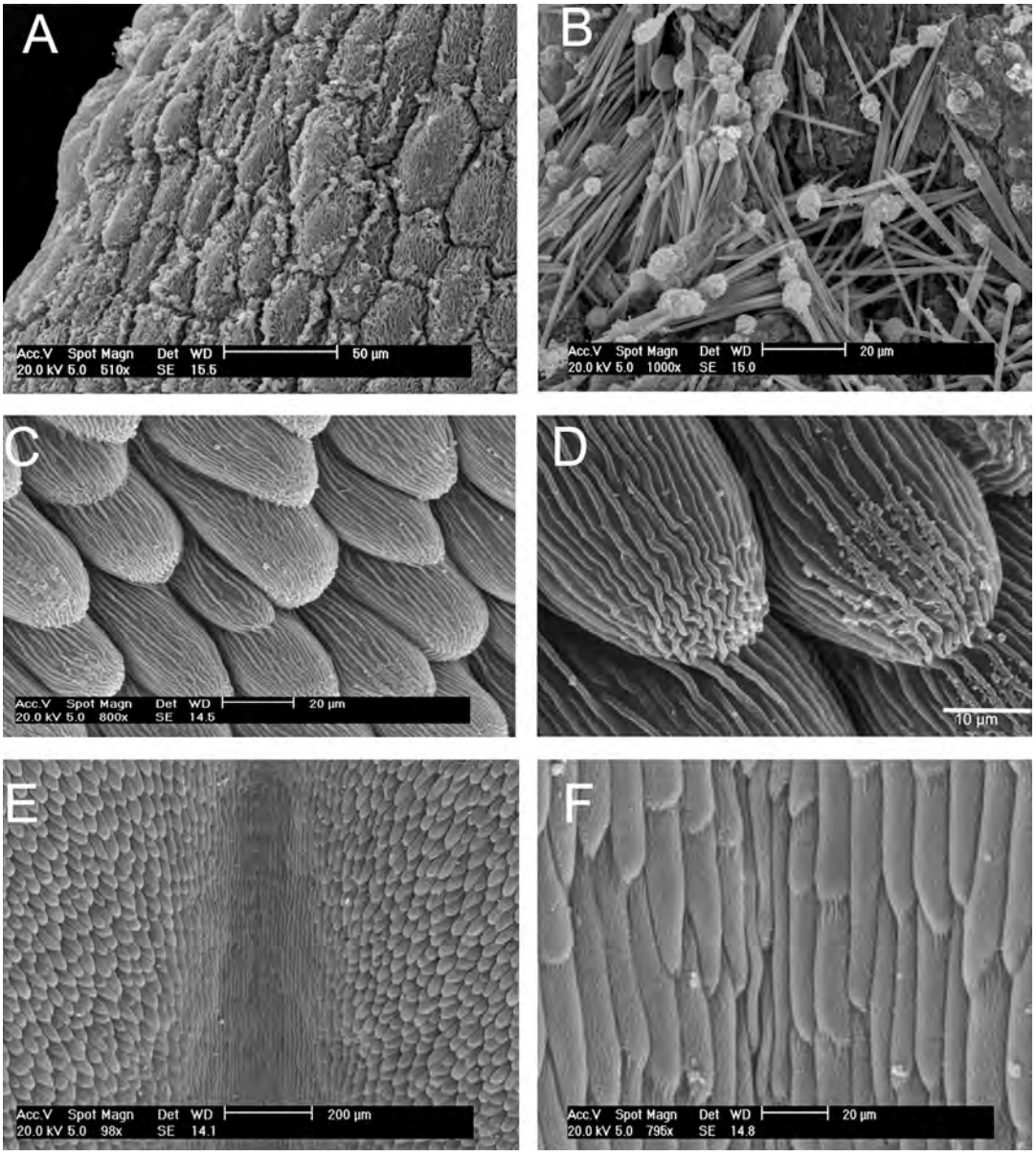

Figure 7. *Liparis crenulata*. Scanning electron microscopy. Marginal cells of cleft part of epichile and their neighboring epidermal cells with richly wrinkled cuticle and a significant amount of the residue along shared cell walls (A). The appendix at the base of the cleft is rich in needle-like raphides, released from the epidermal cells (B). Finger-like, prominent epidermal cells or papillae of the adaxial (inner) surface of epichile with strongly, roused cuticular striae (C) and small amounts of secretion at their apices (D). The central thickening at the epichile part of the lip (E). Epidermal cells of the central thickening overlap each other (F).

Almost the whole surface of the epichile, except for the central thickening, is clothed with finger-like, prominent epidermal cells or papillae (Fig. 7C). These cells have a strongly, longitudinally striate cuticle. Additionally, small amounts of secretory residues are located at the tip of these cells (Fig. 7D). The cells are orientated obliquely relative to the epichile part of the lip's central thickening (Fig. 7E). Epidermal cells of the central thickening are elongated, slightly convex and overlap each other; their cuticle is gently undulated and wrinkled (Fig. 7F). Stronger cuticular

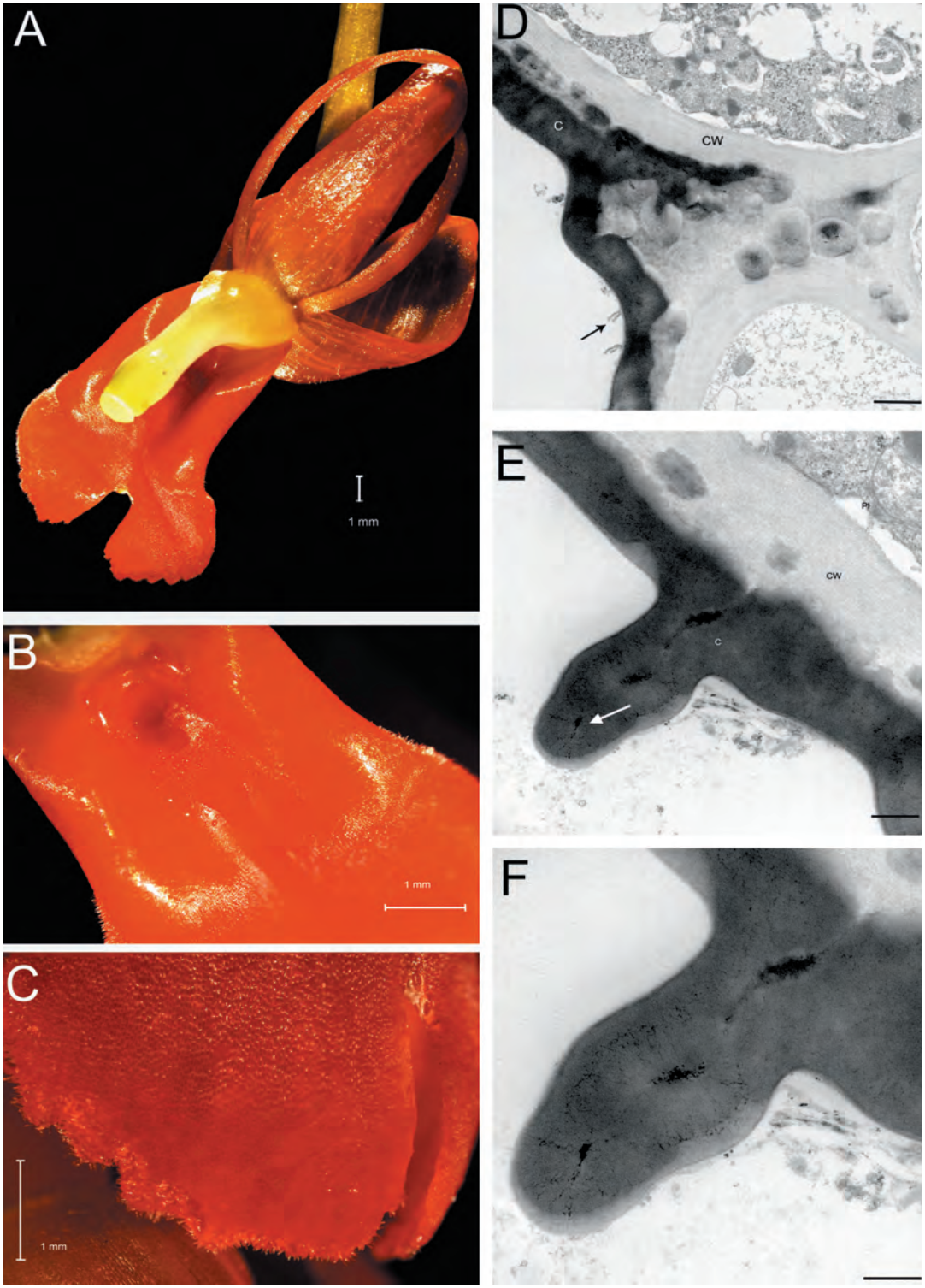

Figure 8. *Liparis crenulata*. Living young flower (A; photo Margońska). Lip of fresh young flower, the central thickening with drops of liquid below the basal callus (B; photo Margońska). Crenulate and papillate margin of distal part of the epichile (C; photo Margońska). Transmission electron microscopy: ultrastructure of cell of hypochile basal callus (D). Heterogeneous cell wall covered with cuticle. Notice the small amount of secreted substance (black arrow; E) and the diffuse network in the cuticle (F). Scale bars: A–C = 1 mm; D–F = 1 µm.

ornamentation was observed only at their narrow boundaries. Small amounts of secretory residues were recorded on the central thickening of the lip apex.

Gynostemium. This is typical of representatives of *Liparis* section *Blepharoglossum* Schltr. However, in samples prepared for SEM analysis, the contents of the interior of the stigma were seen. The base of the stigma is covered with several layers of tissue comprising individual elongated and vertically oriented cells. The tissue appears to originate from the lower part of the stigma concavity but often does not extend to its tip. Its distal/upper margins are eroded and its cells almost free. The cells are coated with secretory residues, probably of stigmatic fluid.

Transmission electron microscopy (TEM)

Lip. Secretory activity was observed over the entire surface of the lip. The cell walls of the lip epidermal cells were covered with an amorphous cuticle.

Small quantities of secretory residues were observed on the basal callus of the hypochile (black arrow, Fig. 8D–F). Moreover, the cuticle was reticulate comprising a diffuse network of electron-dense fibrillar material (white arrow Fig. 8E).

The epidermal cells of the labellum are typical of highly metabolic secretory cells and possess an organelle complement characteristic of tissues involved in lipid biosynthesis. They are nucleate and vacuolated (Figs 8D; 9A) and contain abundant arrays of smooth endoplasmic reticulum (SER) with distended cisternae (Fig. 9A–C). Numerous mitochondria with well-developed cristae (Fig. 9A, C, D) are also present, together with occasional dictyosomes (Fig. 9A). Outgrowths are present on the inner surface of the outer cell wall of the epidermis (Fig. 9A–C), and since the plasmalemma follows the contours of this structure, it too is undulate in section and greatly increases the total area of the secretory surface. Electron-dense, osmiophilic, amorphous, spherical bodies and small secretory vesicles accumulated in the cytoplasm close to the plasmalemma (Fig. 9D) before traversing the cell wall (Fig. 8E) and accumulating between the cell wall and the cuticle (Figs 8D; 9D). The cuticle comprises an outer homogeneous layer and an inner reticulate layer (Fig. 8C–D) and the greatest accumulations of osmiophilic (probably lipid) material coincide with the position of the cuticular ridges (Fig. 9D). The latter possess a central cuticular microchannel into which the secreted material passes (Figs 8E, F; 9E, F) before traversing the outer layer of the cuticle and being deposited on its surface (Figs 8D–F; 9A–F).

Discussion

The color of the flower of *Liparis crenulata* is the main signal for the attraction of pollinators. A change in the color of the flowers also seems to play an important role in signalling the state of flower readiness to receive pollinators. However, any change in color with respect to that part of the light spectrum perceived by humans (ca 390–700 nm) is only slightly visible in the species.

The absorption spectra obtained for the extracts (Fig. 1A, B) are quite similar to those obtained for a number of carotenoids, including β-carotene, with three barely distinct maxima between 400 nm and 500 nm (Lichtenthaler & Buschmann 2001; Zsila et al. 2001). The presence of an additional pronounced peak at 332 nm, observed in spectra recorded in each of the solvents used in the study, was, however, unexpected. Similar spectra were reported for lycopene, when aqueous acetone was used as a solvent (Hempel et al. 2016) and this can be explained by the formation of aggregates in a hydrophilic environment. In the current study, however, this

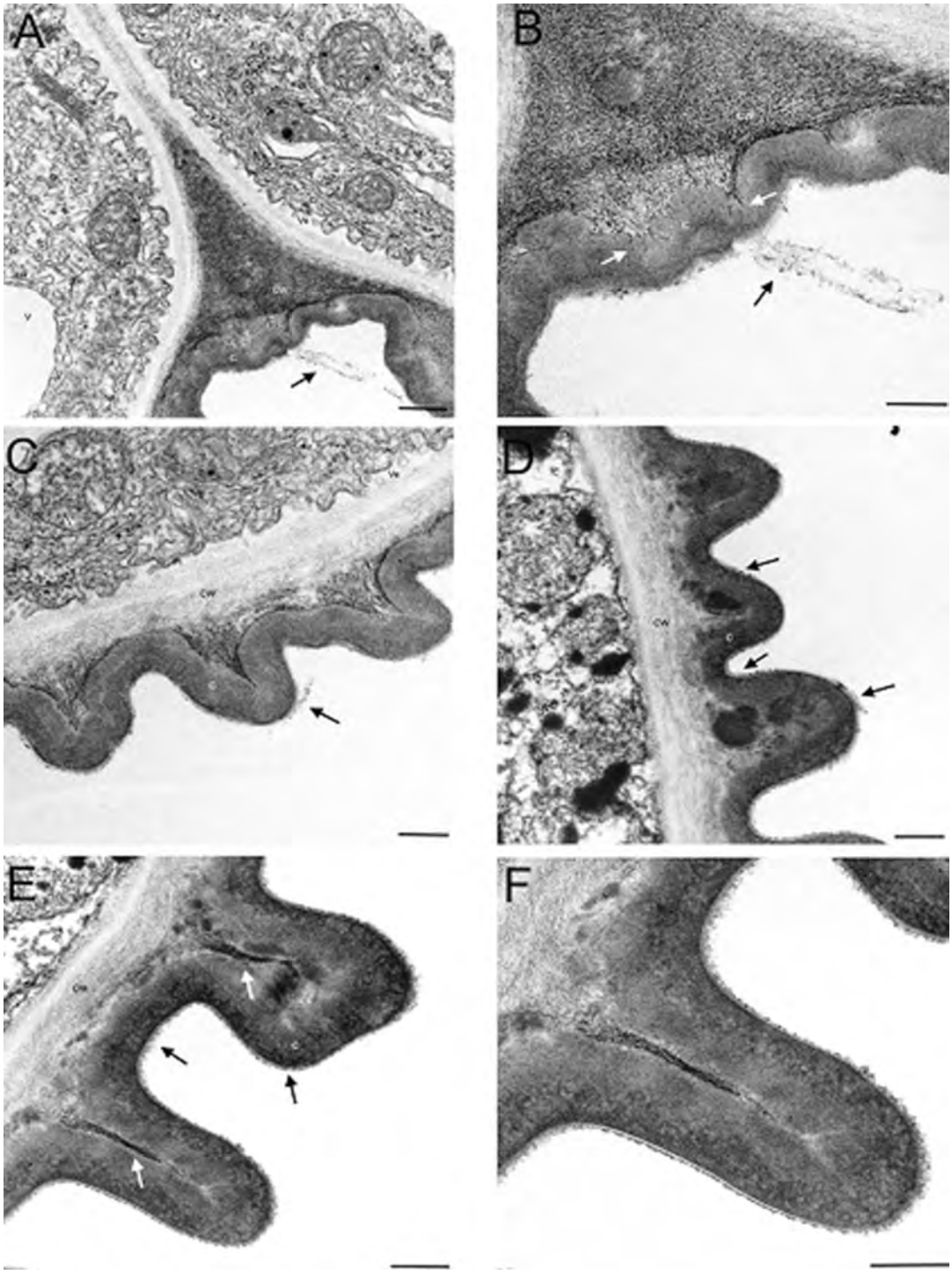

Figure 9. *Liparis crenulata*. Transmission electron microscopy. Ultrastructure of epichile. Cytoplasm with dictyosome, vacuole, mitochondrion and SER membranes (A). Notice vesicles between plasmalemma and cell wall and the diffuse network in the cuticle (white arrows) (B). Cell with mitochondrion smooth endoplasmic reticulum (SER) and vesicles. Black arrow indicates secreted substance (C, C–cuticle, CW–cell wall, M–mitochondrion, D–dictyosome, V–vacuole, Ve–vesicle, ER–endoplasmic reticulum). Ultrastructure of lip marginal cell. Amorphous cuticle with residues of secreted substances (black arrow) (D). Diffuse network in the cuticle (white arrow) with secreted substance (black arrow) in microchannel of cuticle (E and magnification F). Scale bars = 1 µm.

additional peak occurred regardless of how much water was present in the solvent. It is possible that the presence of residual acetic acid, which was not removed from the extracts, could result in such a spectrum. There may have been a shift of maxima in the 400–510 nm range from their original values of 447 nm, 474 nm and 506 nm, respectively recorded for lycopene (Hempel et al. 2016) to 425 nm, 447 nm and 472 nm (see Fig. 1B). Although lycopene is responsible for the red color of ripe tomato fruit, it is also present in other organs of different plant species (Zhu et al. 2010). Moreover, this pigment is insoluble in water, like that isolated from *Liparis crenulata* flowers. The slightly different absorption spectrum obtained in the current study may indicate the presence of other carotenoids of similar structure. Whilst, the red color of *Liparis crenulata* flowers would support this hypothesis, the extracts obtained were actually yellow, indicting the presence of other pigments. Further investigations including the application of chromatographic and more advanced spectroscopic techniques are required in order to determine the actual structure of floral pigments in *Liparis crenulata*.

As previously mentioned, no scent perceptible to humans was detected.

The chemical composition of the volatile fraction of the dichloromethane extract is relatively simple and typical of many higher plants. It is greatly dominated by limonene (almost 90% of the whole fraction), which is usually characteristic of plant species pollinated by bees (Dudareva & Pichersky 2006). It should be noted that the composition of the fraction, containing monoterpenes only, is very different from that reported by Kaiser (1993), which was dominated by sesquiterpenes and several less typical compounds representing different chemical classes. However, much more detailed direct analyses of volatiles, utilizing, for example solid-phase microextraction (SPME), are required to describe fully the floral scent composition of *Liparis crenulata*. The non-volatile fraction of the same extract was dominated by saturated and unsaturated straight-chain hydrocarbons, including compounds whose structures contained two or three double bonds. These are possibly present on the surface of the flower cuticle: whereas saturated compounds with an odd number of C-atoms are amongst the most common components of cuticular waxes (Kunst & Samuels 2003); their unsaturated analogs are much less typical. Interestingly, it has been well established that *Liparis crenulata* is commonly visited by Diptera flies (Margońska, pers. obs.). Several triunsaturated hydrocarbons, including pentacosatriene, which was tentatively identified in the current study, were also found in the cuticular lipids of stable flies *Stomoxys calcitrans* (Carlson & Mackley 1985). Their biological role was, however, not determined. The importance of mono- and diunsaturated hydrocarbons in mate choice has also been described for *Drosophila serrata* (Howard et al. 2003). Mono- and triunsaturated hydrocarbons have also been reported to function as sex pheromones for several other groups of insects (Becker et al. 1983; Ginzel et al. 2006). Therefore, it is possible that the atypical profile for the floral cuticular hydrocarbons of *Liparis* is associated with the pollination mechanism of this species. This hypothesis, however, requires confirmation based on more detailed studies.

The composition of the sugar fraction was similar in both the flower exudate and the whole flower extract, the most abundant sugar being sucrose (58–66% by weight). This suggests the presence of sucrose-dominant nectar, as has been reported, for example, in many orchid species from the temperate forests of South America (Chalcoff et al. 2006).

Consequently, for the first time we have shown that flowers of *Liparis crenulata* attract pollinators not only by means of color and scent, but also by offering them a small amount of food reward, namely nectar.

Small globules and much larger globules of viscid material coating the flower (see scanning electron micrographs) are, in our opinion, artifacts rather than residues of any natural secretions (Figs 2B, C; 5D).

Scanning electron microscope observations revealed that small quantities of secretory residues occurred on apical parts of sepals and petals (Figs 3C; 4B). Also, rare secretory stomata were present on the distal parts of petals (Fig. 4D). Long, few-celled trichomes occurred on the entire adaxial surface of the sepals and petals (Figs 3B, D; 4B, C). Similar secretory residues and trichomes have been reported for the tepal surface of many representatives of Liparidineae and Malaxidineae (Margońska pers. obs.).

The sepals and petals become folded back, making the lip more prominent during anthesis, but this does not significantly increase the attention of visiting insects.

Trichomes were absent from the entire surface of the lip. Scanning and transmission electron microscope results confirmed, however, that secretory activity occurs over the entire surface of the lip, with drops of nectar accumulating at the basal calli and, according to our observations, it therefore seems that secretion by the epidermal cells of the lip occurs exclusively through the cuticle.

The role of the anvil-shaped callus and its adjacent tissues (especially below the callus), as sources of nectar secretion, was confirmed mainly by transmission electron microscopy (Figs 8; 9).

The central part of the hypochile just below the base of the callus is concave (Figs 8A, B; 5B). This, together with the shape of the lip's epidermal cells, their arrangement relative to the long axis of the lip and the structure of their cuticle (Figs 8A–C; 5B, C) suggest that they can cause the nectar droplets to become confluent. The largest droplets gather in the concave depression of the hypochile, and here they pass to the central thickening. Maximum secretion of nectar droplets in fresh flowers occurred during early anthesis. Later, only small traces of nectar were visible along the central thickening.

The lateral and distal edges of the epichile (Figs 6C; 8C) are crenulate and ruffled. The cilia possess a strongly, longitudinally wrinkled cuticle. The epichile surface between the margins and the central thickening is densely clothed with finger-like epidermal cells or papillae which are prominent and obliquely oriented relative to the long axis of the lip. They display a secretory activity visible under the scanning electron microscope. The secretion of a viscid substance by both epidermal structures was confirmed by transmission electron microscope studies.

The role of the marginal cilia and the finger-like epidermal cells or papillae of the epichile is still uncertain. They may be involved in the production of olfactory compounds or provide tactile stimuli (Figs 8; 9). The finger-like epidermal cells may also participate in channeling the flow of nectar along central thickening of the lip.

The layer of tissue present on the concave stigma is visible in SEM images. Similar structures were observed during SEM analysis of other *Liparis* species (*L. pallida*, *L. robusta*, *L. cespitosa*, *L. gibbosa*, etc.). In living flowers, the stigmatic surface is smooth, glossy and coated with stigmatic fluid. In our opinion the structure of the cells of this highly hydrated tissue becomes clear only following dehydration in appropriate solvents which dissolve the overlying stigmatic fluid.

Some species of subtribes Malaxidinae and Liparidinae (Margońska et al. 2012/2013) are known to demonstrate facultative self-pollination, which occurs at late anthesis. In both groups,

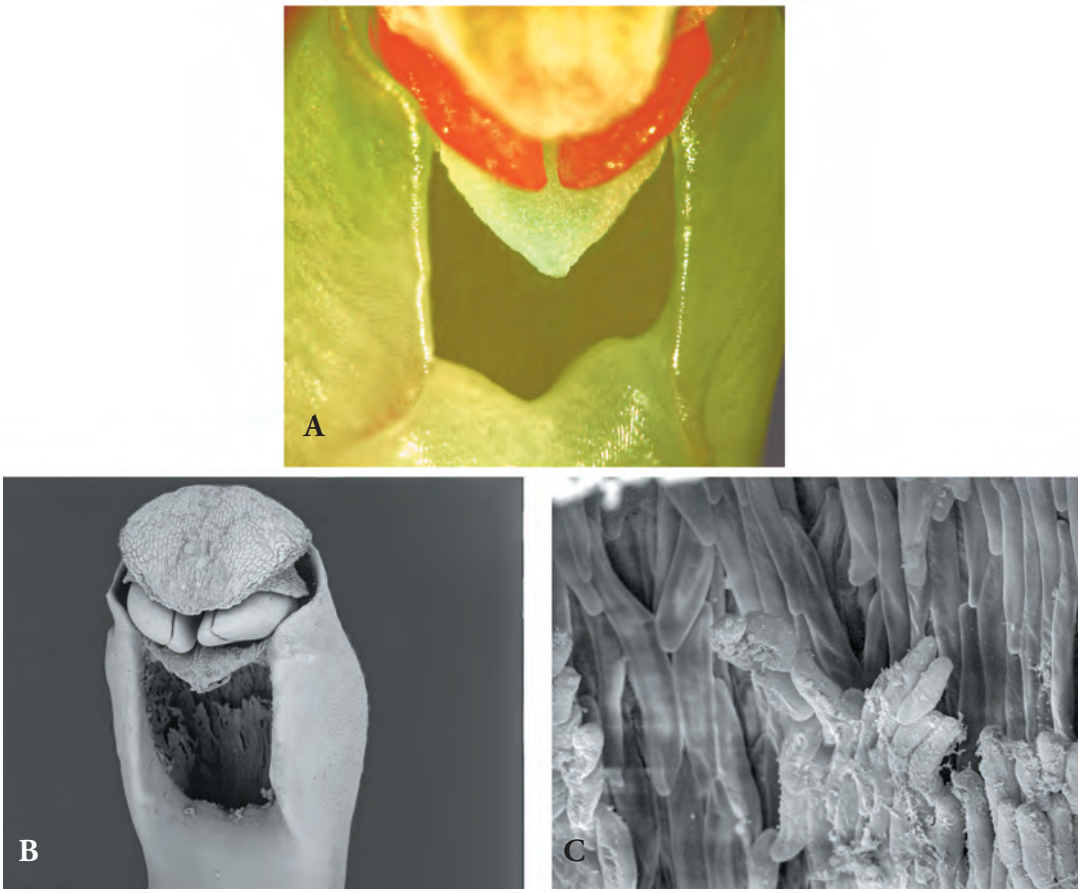

Figure 10. *Liparis crenulata*. Gynostemium. Light microscopy of stigmatic surface(A). Scanning electron microscopy of gynostemium showing stigma, pollinia and anther cap (B). Elongated cells of stigmatic surface (C).

the entire pollinia usually fall or slide down onto the stigma, resulting in self-pollination. This is possible, since the pollinia (owing to the close proximity of anther and stigma) are often still attached by caudicules to the top of the rostellum. This mechanism of self-pollination is also known for several other representatives of Orchidaceae Juss. (e.g. Catling 1990; Liu et al. 2006). However, we did not observe this phenomenon in cultivated *Liparis crenulata*.

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