



# COMPARATIVE ANATOMY OF THE LIP SPUR AND ADDITIONAL LATERAL SEPAL SPURS IN A THREE-SPURRED FORM (*F. FUMEAUXIANA*) OF *ANACAMPTIS PYRAMIDALIS*

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*Anacamptis pyramidalis* shows great phenotypic variability. Additional lateral sepal spurs were observed in *f. fumeauxiana*. We used light and scanning electron microscopy to examine the anatomy of the lip spur and additional lateral sepal spur(s). The spurs have the same anatomical structure. We describe the features of the inner and external epidermis, report the presence of an appendix at the base of the additional spur, and discuss the evolutionary context.

**Key words:** *Anacamptis pyramidalis* (L.) L.C. Rich. *f. fumeauxiana* Marg. & A. Kow., anatomy, histochemistry, light microscopy, scanning electron microscopy, lip spur, lateral sepal spur.

## INTRODUCTION

Populations of European-Mediterranean *Anacamptis pyramidalis* show great phenotypic variability of habit, inflorescence shape, flower lip shape, lip spur length and flower color (Bateman and Rudall, 2006b; Margońska and Kowalkowska, 2008). The taxon displays three levels of ploidy: diploids ( $2n = 36$ ), triploids ( $2n = 54$ ) and tetraploids ( $2n = 72$ ) (Bianco et al., 1991; del Prete et al., 1991; Tichy and del Prete, 2001).

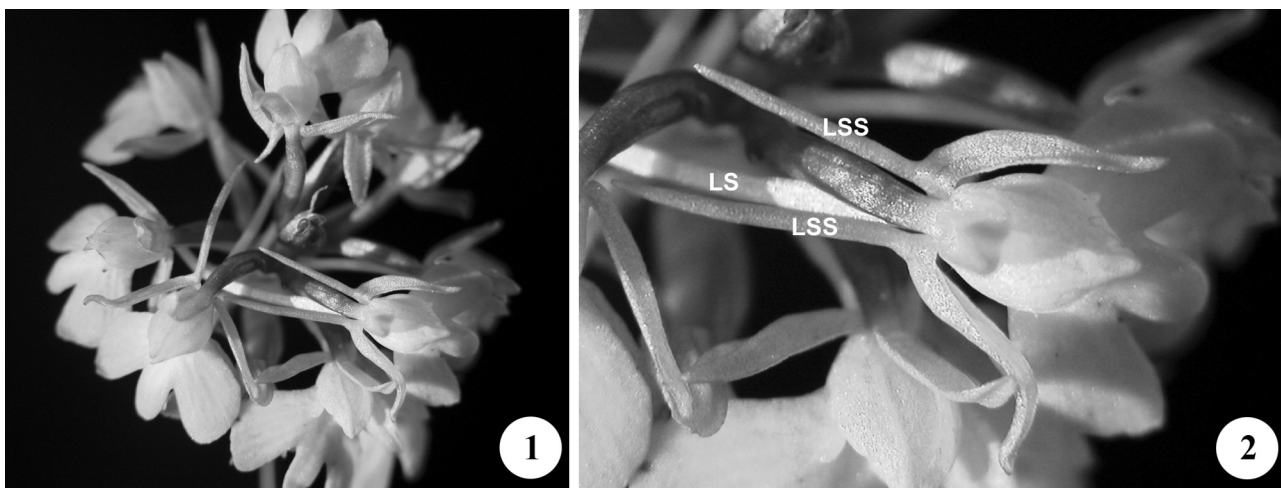
In the Botanical Garden in Geneva, flowers of a few plants of *Anacamptis pyramidalis* were found with a normally developed spur from the lip base and an additional spur developed on one or both lateral sepals (Figs. 1-3). These plants were described as a form – *Anacamptis pyramidalis* (L.) L.C. Rich. *f. fumeauxiana* Marg. & A. Kow. (Margońska and Kowalkowska, 2008, HOLO-, ISO-UGDA-HBM). The spurs are set just from the sepal base and directed back. The shape of the spurs is typical for the species (slender, slightly downcurved tube, formed from the sepal base), but the length can vary, ranging from a small diverticulum up to 10–12 mm long. The remaining parts of the flower are formed normally.

How should we treat the phenomenon of development of additional spurs on lateral sepals? As a key innovation that "opens up new possibilities for the exploitation of new ecological niches and triggers the adaptive radiation to the new pollinators" (Mondragón-Palomino and Theißen, 2008)? In this paper we examine the anatomy of the additional lateral sepal spur, paying special attention to features and substances found in both spurs (lip and lateral sepal), and we compare the features revealed in LM and SEM studies. We also discuss this innovation in an evolutionary context.

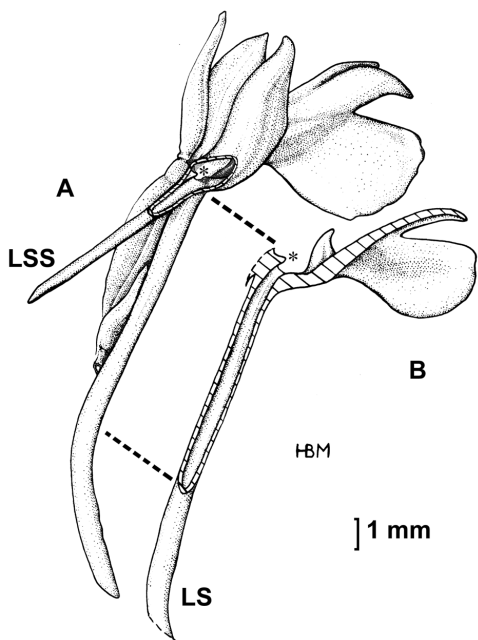
## MATERIALS AND METHODS

Samples were collected in June 2007 from plants cultivated in the Botanical Garden living collection. The plants had been transplanted in April and May from the Soral area (village SE of Geneva, Switzerland) to the Botanical Garden in Geneva. The *Anacamptis* population in the region, although slightly dispersed between agricultural areas, is in good condition. No special morphological aberrations were observed at the stands (in 2007, by Cyrille Latour; in 2008, by

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**Fig. 1.** Inflorescence of *Anacamptis pyramidalis* (L.) L.C. Rich. f. *fumeauxiana* Marg. & A. Kow. **Fig. 2.** Single flower of *Anacamptis pyramidalis* (L.) L.C. Rich. f. *fumeauxiana* Marg. & A. Kow., with lip spur (LS) and two additional lateral sepal spurs (LSS).



**Fig. 3.** Flower in longitudinal cross section at base of lateral sepal spur (a), Longitudinal cross section of lip and lip spur at base (b). Appendixes in both spurs marked with asterisks.

Margońska, Fumeaux and Chautems). In 2007, plants not yet flowering were gathered from several points of a planned bike lane along an existing country road. After construction is completed the plants will be returned to their natural sites.

Fresh flowers at anthesis were observed with a Nikon SMZ1500 stereomicroscope. The plant material was fixed in 2.5% glutaraldehyde (GA) and 2.5% paraformaldehyde (PFA) in 0.05 M cacodylate buffer

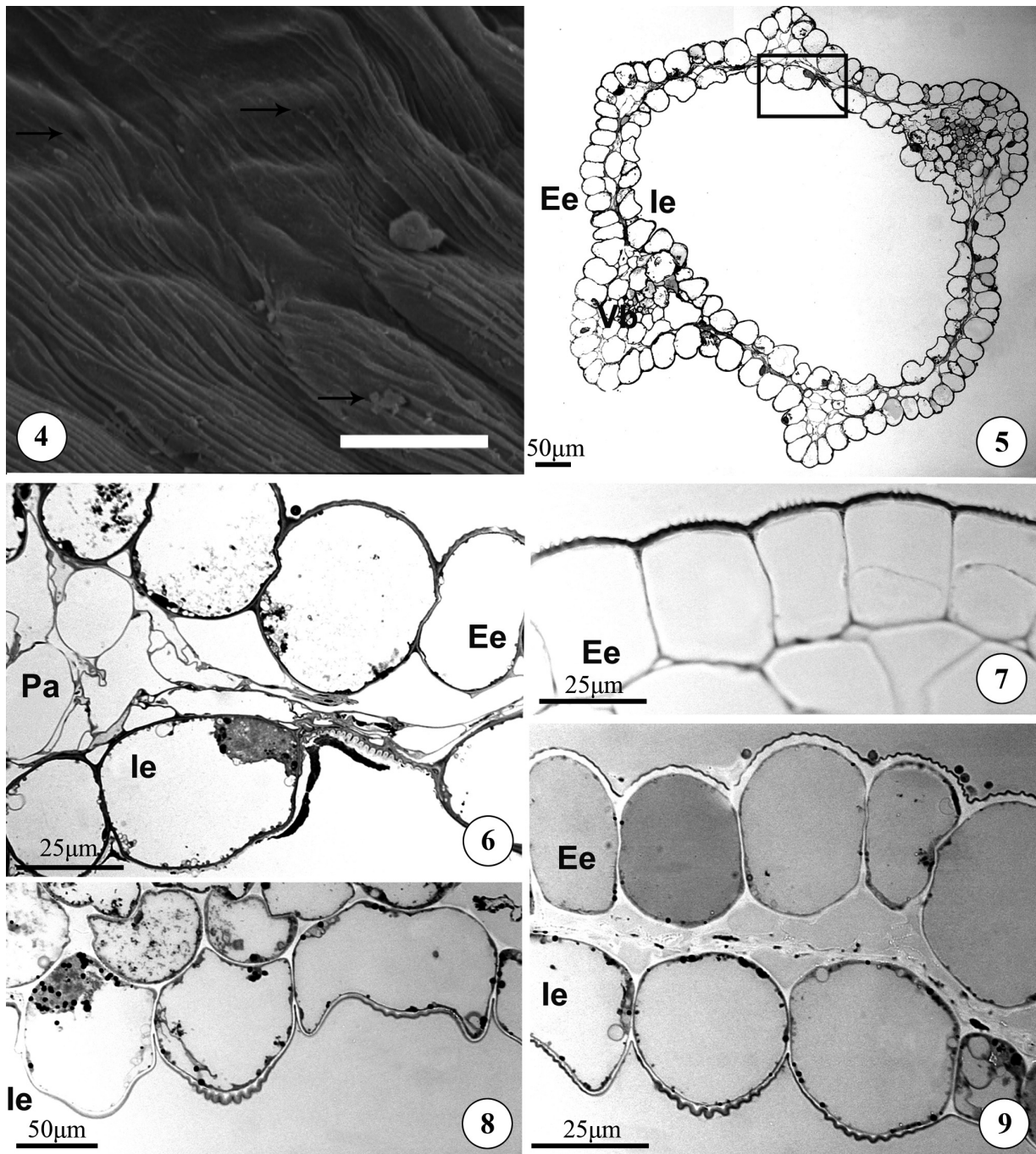
(pH = 7.0) for 4 h at room temperature. The material for LM was rinsed with cacodylate buffer, then dehydrated in acetone. Whole dehydrated material was embedded in epoxy resin (Spurr, 1969). Sections 1  $\mu\text{m}$  thick were cut with glass knives and mounted on glass slides. For LM the material was stained with 0.05% Toluidine Blue O (TBO) for 1 min at 60°C on a hot plate (Feder and O'Brien, 1968; Ruzin, 1999). Aniline Blue Black (ABB, C.I. 20470) was used for detection of water-insoluble proteins (Jensen, 1962). The PAS reaction was used to identify the water-insoluble polysaccharides (Jensen, 1962) and Sudan Black B for lipid localization (Bronner, 1975). The preparations were examined and photographed with a Nikon Eclipse E 800 light microscope and a Nikon DS-5Mc camera using the Lucia Image program.

For scanning electron microscopy, after dehydration in an ethanol series the samples were dried by the critical point method using liquid  $\text{CO}_2$ , coated with gold and observed in a Philips XL-30 SEM at 15 kV accelerating voltage.

## RESULTS

### FLORAL MORPHOLOGY

The lip spur of *Anacamptis pyramidalis* f. *fumeauxiana* is 10–15 mm long and 0.5 mm in diameter. Flowers within the same inflorescence differ in the length of the sepal spur, but their shape and diameter is similar to that of the lip spur (Figs. 1–3). An appendix is present at the lip spur base (Fig. 3, asterisk on LS) and there is an additional lateral sepal spur (Fig. 3, asterisk on LSS). LM and SEM (Fig. 4) revealed small cuticular striations inside the spur epidermis (Figs. 14–15) and some lipid substances on the surface (Fig. 13).



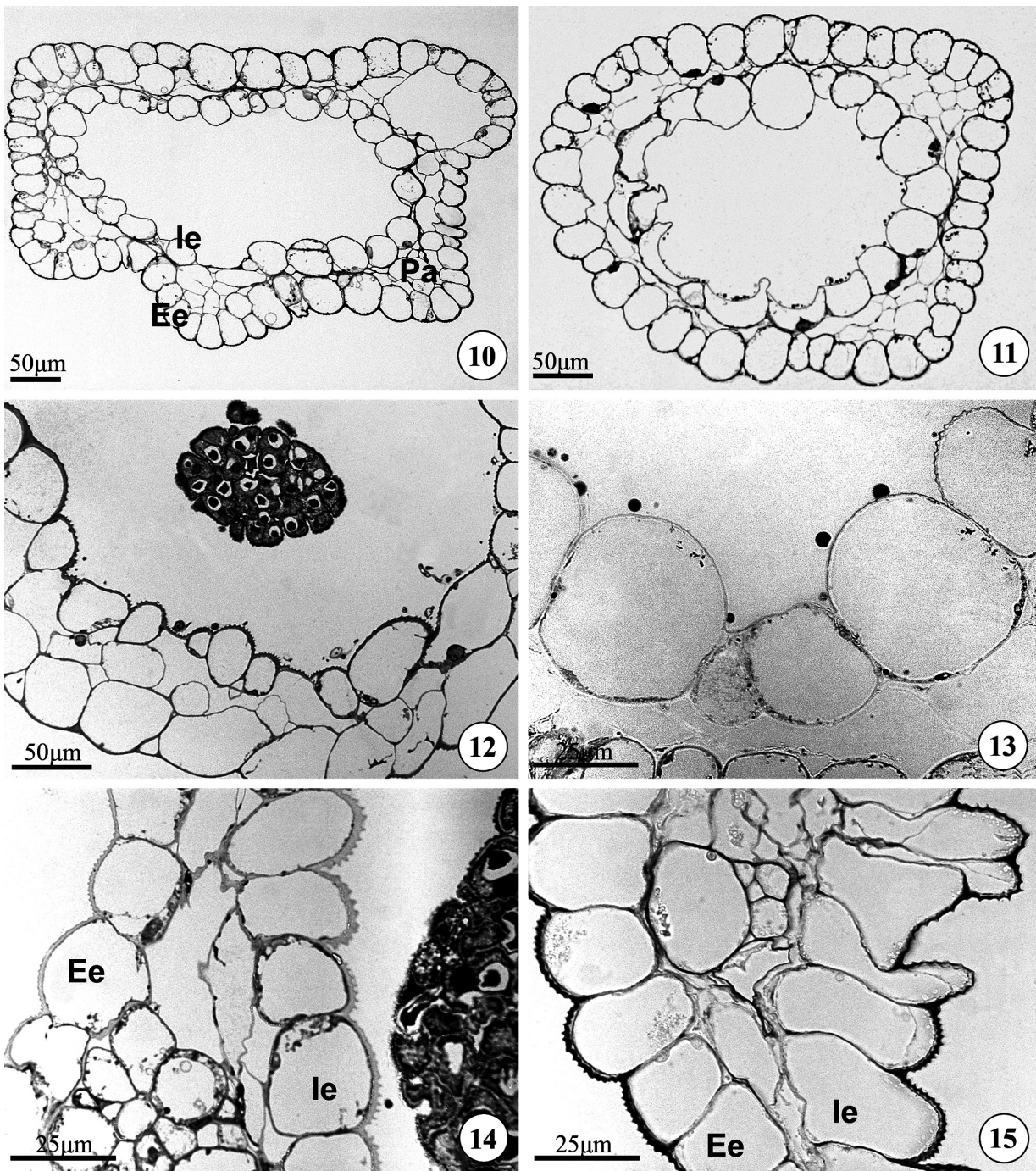
**Figs. 4–9.** Lip spur. **Fig. 4.** Cuticular striations on cells inside lip spur, indicated by arrows (same structure occurs in lateral sepal spur), SEM. Bar = 20 µm. **Fig. 5.** Cross section of spur close to lip base (TBO). **Fig. 6.** Part from Fig. 5 (TBO). **Fig. 7.** Surface of external epidermis intensely staining for polysaccharides (PAS). **Fig. 8.** Undulating cuticle on inner epidermis, lipid droplets around nucleus and near plasmalemma (SBB). **Fig. 9.** Undulating cuticle on inner and external epidermis (SBB). Ie – inner epidermis; Ee – external epidermis; Pa – parenchyma; Vb – vascular bundle.

#### LIP SPUR

LM of the lip spur showed the presence of a single-layered epidermis inside and outside the spur (Fig. 5). Vascular bundles were observed in the parenchyma

close to the lip base: two large bundles and some smaller ones. In open flowers the parenchyma cells were well visible around the vascular bundles. In other parts there were only remains of parenchyma





**Figs. 10–15.** Observations of lateral sepal spur. **Fig. 10.** Cross section of lateral sepal spur at base, behind appendix (TBO). **Fig. 11.** Cross section of lateral sepal spur in middle part (TBO). **Fig. 12.** Cross section of lateral sepal spur and small appendix at base (TBO). **Fig. 13.** Cells of inner epidermis with lipid droplets – part from Fig. 11 (SBB). **Fig. 14.** Undulating cuticle on inner epidermis close to small appendix at base (ABB). **Fig. 15.** Undulating cuticle on both epidermises, intensely staining for polysaccharides (PAS).

cells: crushed cell walls (Fig. 6). The cells of the external epidermis were rounded along the whole length of the spur (Figs. 5–7, 9). A thick irregular undulating cuticle was visible on the external epi-

dermal cells, which stained positively for lipids, proteins and polysaccharides (Figs. 7, 9). The inner epidermis was formed by rounded cells and some flattened cells (Figs. 5, 6, 8, 9). At the spur base

(Figs. 5, 6) collapsed epidermis cells were visible; perhaps the substances on their surfaces are remnants of protoplasts. A few lipid droplets were detected around the nucleus and under the plasmalemma (Figs. 8, 9). Proteins stained similarly on both surfaces of the lip spur.

#### LATERAL SEPAL SPUR

LM of the lateral sepal spurs revealed the same features as examined in the lip spur. The external epidermis was built of a single layer of rounded cells, with a thick undulating cuticle, positively staining for lipids, proteins and polysaccharides (Figs. 10–12, 14–15). In the parenchyma, some single cells around the vascular bundles and some crushed cell walls were noted. In cross section the inner epidermis of the spur behind the appendix consisted of rounded cells (Fig. 10). In the middle part of the spur, more cells were flattened and had numerous lipid droplets on the surface (Fig. 13).

At the base of the additional lateral sepal spur, a small cellular appendix was developed, already known from its presence in the lip spur (Thomé, 1885) (Fig. 12, 14). The appendix was spherical in cross section. The cells stained strongly for proteins and contained dense cytoplasm. A thick layer of cuticle was seen on the surface of the appendix cells (Fig. 14). In cross section, at the site of the appendix (in both spurs) the cells of both epidermises of the lateral sepal spur stained strongly, especially for polysaccharides (Fig. 15).

#### DISCUSSION

*Anacamptis pyramidalis* is an evolutionarily plastic species (del Prete et al., 1991), with variable spur length and diameter. The novelty seen in f. *fumeauxiana* is that the additional spurs developed on the lateral sepals (!), not on petals (lip and petals form inner whorl, sepals form outer whorl). This kind of mutation in the genus *Anacamptis* was not previously known. In other orchid species such as *Plectophora*, additional spurs have been observed on the two lateral petals (Mondragón-Palomino and Theißen, 1991; Rudall and Bateman, 2004), but those were quite different observations. Cozzolino and Widmer (2005) suggested that speciation involves pollinator shifts, which are responsible for changes in floral morphology, color and odor, and in this case the speciation may be based on floral mutations. A feature created in a new location on the organism (as in this form of *Anacamptis*) is called neoheterotopy (Rudall and Bateman, 2004; Bateman and Rudall, 2006a). We suggest that the development of a new additional lateral sepal spur can be explained by a mutation of *TFC* genes

(personal communication, Mondragón-Palomino). Radical changes in flowers may attract different pollinators and cause rapid sympatric speciation (Rudall and Bateman, 2002). Differences in the length, shape orientation and coloration of spurs are commonly associated with differences in pollinators, and they affect reproductive isolation (Box et al., 2008). The question is, what kind of new pollinator in the Alpine region may be attracted by two or three floral spurs?

The entrance to the lip spur is directed by two symmetrical longitudinal calli. Do they not stop or limit access for pollinators? We suspect that the pollinators might only get access to the lateral sepal spurs by accident, because the additional sepal spurs are located beside the lip and are partly covered by lip calli. Bateman and DiMichele (2002) suggested that a single mutation in a developmental gene might have triggered the origin of the '*Nigritella*' floral phenotype from within the *Gymnadenia* clade (Box et al., 2008). Molecular research on the *Anacamptis* form showed that three base pairs differ from the normal type (research by Dr. Piotr Tukałło, unpublished data). Further molecular research is required. We observed that the additional spurs were differentiated in two inflorescences. Rudall and Bateman (2003) consider variability of a feature in a single inflorescence to be an unstable mutation. Do we have a new prospectus or rather a neoheterotopic plant able to generate prospectus (Rudall and Bateman, 2003; Bateman and Rudall, 2006a; Theißen, 2006)? We hope that our present investigations will stimulate more genetic research on *TCF* genes in *Anacamptis*. Biological diversity will be better understood as the mechanisms that bring about key innovations such as the appearance of new feature are explained; this is evolutionary developmental biology ('evo-devo') (after Mondragón-Palomino and Theißen, 2008).

The lip spur and additional spurs on the lateral sepals revealed the same anatomical features. The inner and external epidermises were built of a single layer of cells. The cells of the external epidermis were rounded and covered by a thick undulating cuticle staining positively for lipids, polysaccharides and proteins. We suspect that these delicate elongated spurs may be covered by wax when not desiccated. An undulating cuticle, possibly wax, was also found on some cells of the inner epidermis.

*Anacamptis pyramidalis* is regarded as a food-deceptive orchid (Proctor et al., 1996; Fritz and Nilsson, 1996; Neiland and Wilcock, 1994; 1998; Cozzolino and Widmer, 2005). In our field observations, ants (*Formica* spp.) were visiting the flowers of *Anacamptis* in large numbers; this was not noted previously. Further field observations are needed to discover whether they are effective pollinators for this species. Also, small lipid droplets were detected in the epidermal cells of both spurs. A small cellular



appendix of an unknown role was also noted on both spurs. An appendix in the lip spur was drawn by Thomé (1885). In our studies, an appendix was also observed in the additional lateral sepal spurs. It is not known whether similar appendices occur in orchid species. Ultrastructural study of this form and comparison with the normal type should also help explain the role of spurs and especially of the unusual appendix at the base.

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