



STAMINODIAL NECTARY STRUCTURE IN TWO *PULSATILLA* (L.) SPECIES

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In plants belonging to the Ranunculaceae the floral nectaries may differ in origin, location in the flower, shape and structure. In many cases they are defined as modified tepals or modified stamens. The nectary organs in this family are frequently termed "honey leaves," and staminodial origin is attributed to them. Gynopleural and receptacular nectaries are rarely found in Ranunculaceae. To date there are no reports on the structure of the nectary organs in plants of the genus *Pulsatilla*. We used light and scanning electron microscopy to study the location and structure of the nectaries in *Pulsatilla slavica* and *P. vulgaris* flowers. The staminodial nectaries were found to be nectar-secreting organs. The number of stamens per flower (102-398) increases with plant age. The share of staminodes is 12–15%. The staminodes are composed of a filament and a modified head. They are green due to the presence of chloroplasts in the epidermal and parenchymal cells. The parenchymal cells are in a loose arrangement. Stomata (3–20), through which nectar exudation occurred, were found only in the abaxial epidermis of the staminode head. The stomata are evenly distributed and have well-developed outer cuticular ledges. Some of them are immature during nectar secretion, with their pores covered by a layer of cuticle. During the activity of the nectariferous organs in the flowers, primary (on the staminode surface) and secondary nectar (at the base of tepals) are presented. The staminodes of the two *Pulsatilla* species show similar structural features and have similar shares in the androecium.

Key words: Staminodial nectaries, structure, micromorphology, nectar exudation, *Pulsatilla slavica*, *P. vulgaris*.

INTRODUCTION

Species of the genus *Pulsatilla* (Ranunculaceae) are found in natural stands in the Northern Hemisphere. Most are encountered among plants in xerothermic communities. Seven species of this genus grow in the wild in Poland, and have been under strict protection for many years (Szafer et al., 1986; Piękoś-Mirkowa and Mirek, 2003).

Pulsatilla vulgaris Mill. occurs on sunny stony slopes in lowlands and foothills. *Pulsatilla slavica* Reuss. is an endemic plant found in mountains. It grows in Slovakia as well as in Poland in the Tatra and Pieniny Mountains on sunny limestone and dolomite rock. Both species produce violet flowers, and in Poland they flower from April to May (Rutkowski, 1998; Piękoś-Mirkowa and Mirek, 2003).

In terms of pollination ecology, due to their large number of stamens, pasque flowers are treated by many authors as exclusively polleniferous flowers which do not produce nectar, similarly to other Ranunculaceae (Szafer and Wojtusiakowa, 1969; Koltowski, 2006; Lipiński, 2010). However,

some papers report that in pasque flowers the nectar is secreted by staminodial nectaries located at the base of the androecium (Kugler, 1970; Kratochwil, 1988a,b; Barth, 1991).

Pasque flowers are visited by insects seeking pollen and nectar, such as honey bees and bumblebees, and also by butterflies feeding only on nectar (Kugler, 1970). Kratochwil (1988a,b) stated that *P. vulgaris* flowers are pollinated also by *Hymenoptera* of the genera *Andrena* and *Lasioglossum*.

In the literature we found no data on nectary structure in *Pulsatilla* flowers. In this study we located and counted the floral nectaries in two species, *Pulsatilla slavica* and *P. vulgaris*, and compared their structure.

MATERIALS AND METHODS

We examined 10-year-old specimens of *P. slavica* (n=5) and 1-year-old and 4-year-old *P. vulgaris* plants (n=6) obtained from the collection of the

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Botanical Garden of Maria Curie-Skłodowska University in Lublin, Poland. In fresh flowers at the stage of pollen release we counted the stamens and staminodes in 1-year-old (15 flowers) and 4-year-old (11 flowers) *P. vulgaris* plants and in *P. slavica* (23 flowers). We calculated the percentage of nectaries in the androecium, and used stereoscopic microscopy (SM), light microscopy (LM), and scanning electron microscopy (SEM) to reveal the structural details of the perianth and androecium, with special attention to the nectar-secreting structures in both species.

STEREOSCOPIC MICROSCOPY (SM)

We located the nectar-secreting organs, analyzed their morphology, and examined the site of accumulation of attractant secretions, using an SMT 800 microscope for observations and a Nikon Coolpix 4500 digital camera for photographic documentation.

LIGHT MICROSCOPY (LM)

We examined transverse and longitudinal sections of fresh staminodes and stamens. Semi-permanent glycerol-mounted slides were prepared. To detect the presence of starch in the chloroplasts, staminode sections were treated with Lugol's iodine (IKI). We located and counted the stomata on the staminode surface, observed the structure of the epidermis and parenchyma, located the stomata on the stamens, and examined the epidermis of the tepals. Observations and images were made with a Nikon Eclipse 400 microscope.

SCANNING ELECTRON MICROSCOPY (SEM)

SEM was used to observe the surface of the staminodes, stamens, and tepals. Portions of young flowers (with stamens not shedding pollen) and of flowers collected at the end of anthesis (90% of stamens empty of pollen) were sampled. Excised staminodes, stamens and portions of tepals were fixed in 4% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.0) at 4°C for 12 h. Then the material was washed twice with the same buffer for 15 min and dehydrated in an acetone series (30, 50, 70, 90, 100%; 30 min each). After dehydration the plant samples were critical-point dried in liquid CO₂ (Bal-Tec CPD 030 critical point dryer) and coated with gold-palladium (Polaron SC 7640 sputter coater). The fixed plant material was observed and photographed in a Tesla BS-300 SEM equipped with a Tescan attachment for digital processing of microscopic images.

RESULTS

NECTAR SECRETION

As in other Ranunculaceae species, in the flowers of *Pulsatilla* there are numerous stamens and pistils, arranged spirally on the elongated receptacle. In *P. slavica* (Fig. 1a–c) and in *P. vulgaris* the yellow stamens clearly contrast with the violet perianth and gynoecium.

Bees and butterflies collecting nectar were observed in the flowers of *P. vulgaris*, and ants were also noted in *P. slavica* flowers (Fig. 1c). Tiny nectar droplets accumulated on the abaxial surface of staminodes and on the adaxial surface of tepals, opposite the staminodes. Later, merged droplets formed a substantial layer which accumulated in a narrow depression between the base of the lower parts of the androecium and the tepals (Fig. 1g), and nectar was retained by the basal parts of the tepals.

In the protogynous flowers of *Pulsatilla*, nectar is secreted by the staminodes, functioning as nectaries. The staminodes are located at the base of the androecium (Fig. 1d–i). They are much smaller than the stamens (Fig. 1e,f) and of simplified structure (Fig. 1h–n). Glistening droplets of secretion were visible on many staminodes (Fig. 1l,o–s) in younger flowers, at maturity of the gynoecium, and in older flowers at pollen shedding.

NUMBER OF STAMENS AND STAMINODES

The number of stamens in particular flowers of *P. vulgaris* and *P. slavica* varies greatly (Tab. 1). In *P. vulgaris* the number was associated with the age of flower-producing plants: an average 173 stamens per flower in younger plants (1 year old), and 295 in 4-year-old plants. The maximum difference in number of stamens between flowers of older individuals was as much as 255, and 123 in younger ones. For all age groups taken together, in *P. vulgaris* the average number of stamens (234) was as in *P. slavica* (237). The range of variation in number of stamens exceeded 160 in *P. slavica* flowers.

In both *Pulsatilla* species the androecium was composed of stamens of different lengths. The shortest stamens were in the basal part of the androecium, near the staminodes (Fig. 1c–f). The number of staminodes in the flowers of both *Pulsatilla* species accounted for 12–15% of the total number of stamens (Tab. 1). In flowers of younger *P. vulgaris* the average number of staminodes was 31, and 41 in those of older ones. In *P. slavica*, flowers had 34 staminodes on average. There were large differences in number of staminodes per flower between *P. vulgaris* (26–51) and *P. slavica* (16–46).

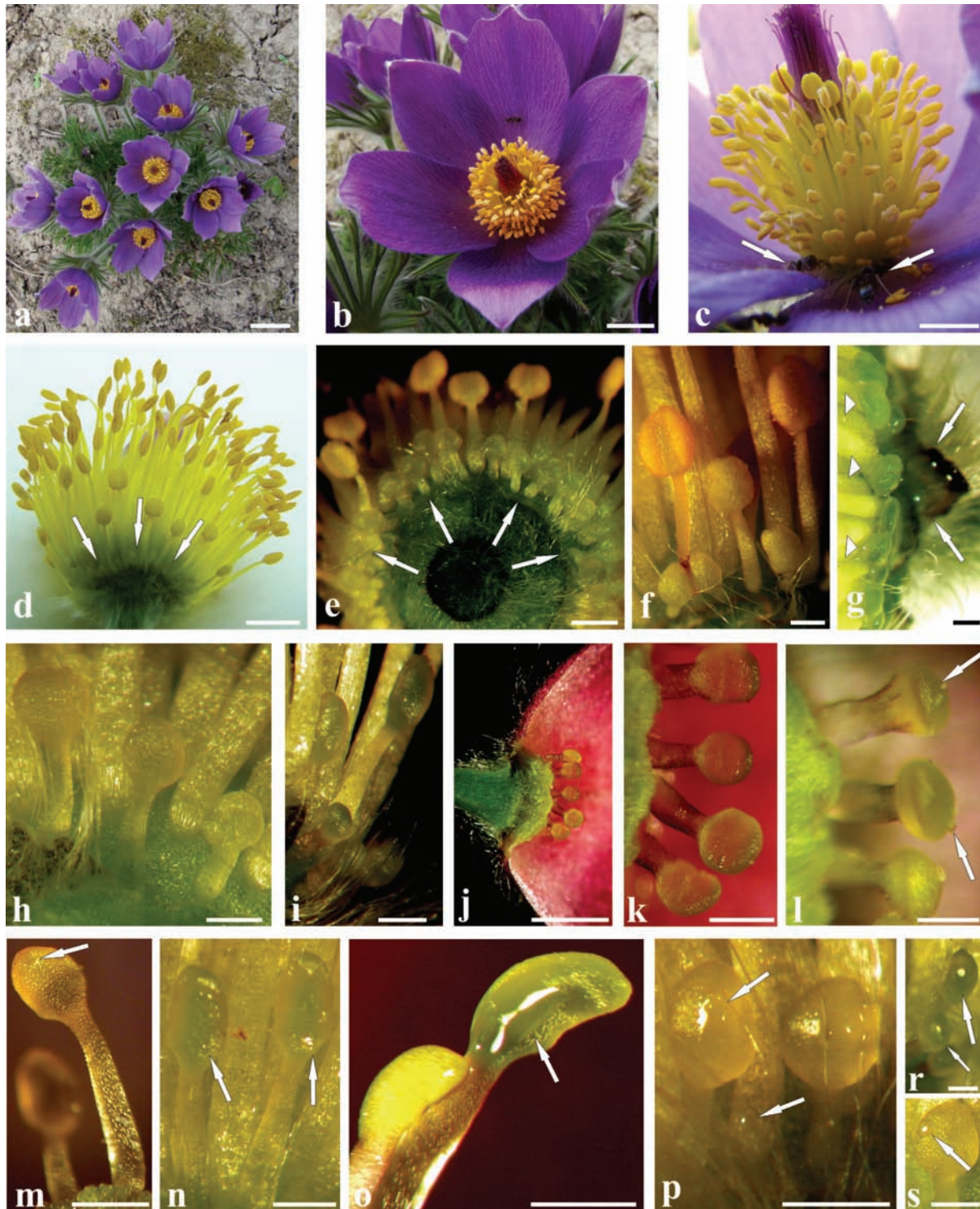


Fig. 1. Location of staminodes in *Pulsatilla slavica* flowers. (a) Plant at full bloom, (b) Androecium with many stamens also presents color attractants, (c) Ants foraging on nectar at base of androecium (arrows), (d) Staminodes are located in lower part of androecium (arrows), (e) Staminodes viewed from the direction of the pedicle (arrows), (f) Stamens and staminodes differ in height, (g) Staminodes viewed from above (arrowheads) and nectar accumulated at base of staminodes (arrows), (h, i) Staminodes of varying size, (j, k) The shortest staminodes seen against a tepal, (l–s) Glistening nectar droplets on surface of staminode heads (arrows). Bars = 1 mm (a, i), 5 mm (b), 2,5 mm (c), 2 mm (d, j), 2 mm (e), 500 μ m (f, g, h, k–s).

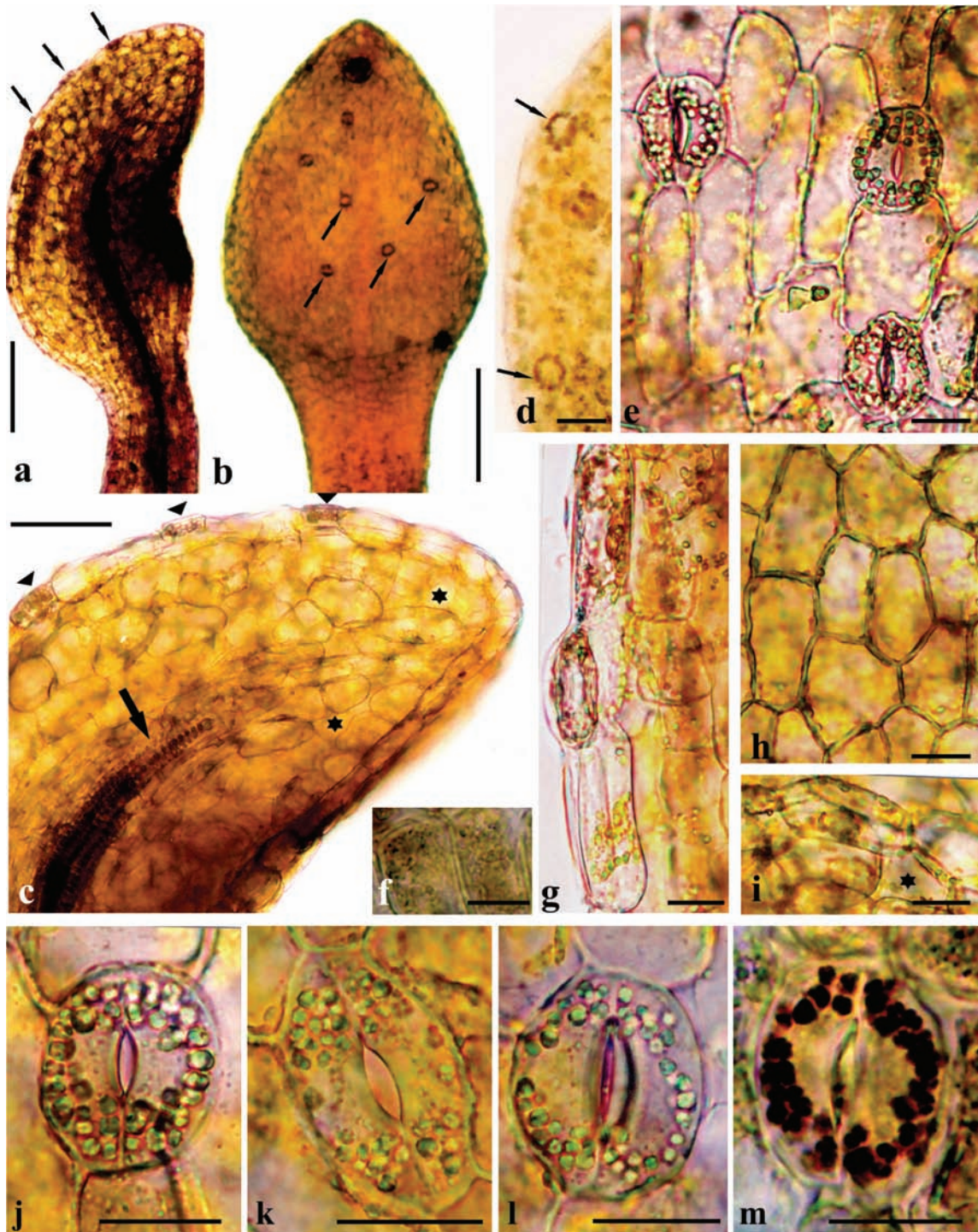


Fig. 2. Fragments of *Pulsatilla slavica* staminodes. (a) Longitudinal sections of staminode with stomata visible on abaxial surface (arrows), (b) Abaxial part of staminode. Stomata (arrows) with stained starch grains (IKI), (c) Fragment of longitudinal section of staminode. Stomata visible on epidermis (arrowheads), large intercellular spaces in parenchyma (asterisks), and vascular bundle containing xylem and phloem elements (arrow), (d, e, g) Fragments of abaxial epidermis in staminodal head with stomata visible; (d) slide IKI-stained, (f) Parenchymal cells of staminode with starch grains visible, IKI-stained, (h) Cells of adaxial epidermis, (i) Parenchyma in staminodal head containing chloroplasts. Large intercellular space (asterisk), (j-m) Stomata located on abaxial epidermis of staminode; (m) slide IKI-stained. Dark starch grains visible in chloroplasts of stomatal and other cells of epidermis. Bar = 200 μ m (a, b), 100 μ m (c), 50 μ m (d), 20 μ m (e-m).

TABLE 1. Number of stamens and staminodes and number of stomata per staminode in the flowers of two *Pulsatilla* species; n – number of flowers

Species	Number of stamens		Number of staminodes		Percentage of staminodes in stamens	Number of stomata per staminode
	min. – max.	mean	min. – max.	mean		
<i>Pulsatilla vulgaris</i>	1-year-old plant (n=15)	102 – 225	173	26 – 36	31	7 – 10
	4-year-old plant (n=11)	143 – 398	295	28 – 51	41	$\bar{x} = 8$
<i>Pulsatilla slavica</i>	10-year-old plant (n=23)	160 – 326	237	16 – 46	34	3 – 20 $\bar{x} = 8$

NECTARY ANATOMY (LM)

In both *Pulsatilla* species the staminodes were composed of a relatively short filament (0.34–2.01 mm) with the upper part resembling a head (0.31–0.70 mm wide) (Fig. 1h,m), usually flattened and taking different shapes (Fig. 4a,f,h). The filaments clearly varied in thickness (Fig. 3k).

In *P. slavica* the staminode heads were generally wider and rounder than in *P. vulgaris*. In *P. vulgaris* some staminodes resembled stamens morphologically. The adaxial surface of the staminode head was slightly concave in both *Pulsatilla* species; the abaxial part was marked by a distinct protrusion (Fig. 2a). The staminodes in *P. vulgaris* flowers sometimes were only a sharply pointed filament.

The release of nectar onto the staminode surface occurred through stomata in the abaxial epidermis on the convex part of the staminode (Fig. 2a–c). The stomata were in the center (Fig. 2b) and on the edge of the surface (Fig. 2d). They were distributed evenly (Figs. 2b,c,e, 5i) and most frequently parallel to the long axis of the staminode. In *P. slavica* there were 3–20 (8 average) pores on a single staminode, and 7–10 (8 average) in *P. vulgaris* (Tab. 1). In both species the stomata were surrounded by 4–7 epidermal cells; that is, they were of the anomocytic type (Figs. 2e, 5j).

The abaxial epidermal cells most often were elongated (Figs. 2e, 5g,j). The adaxial epidermal cells were nearly isodiametric (Fig. 2h). All the epidermal cells contained chloroplasts (Fig. 2e,g,h), and there were numerous chloroplasts in the guard cells (Fig. 2e,j–l). Starch grains were present in the chloroplasts, usually several grains in each, and they stained deep blue with Lugol's iodine (Fig. 2b,f,m). Most often the stomata were slightly open (Figs. 2e,j–m, 6d,e).

The parenchyma cells in the staminode head varied in shape and contained chloroplasts (Fig. 2c,g,i). Large intercellular spaces were observed (Fig. 2c,i). Unlike the greenish staminodes

containing chloroplasts, the yellow-stained stamen heads had chromoplasts in the epidermal cells and in the parenchyma. A single vascular bundle ran through the central part of the staminode, in which both xylem and phloem elements reached the upper part of the organ (Fig. 2c).

MICROMORPHOLOGY OF FLORAL ELEMENTS (SEM)

Tepals

On the adaxial surface of the pasque flower tepals there were depressions corresponding to the arrangement of vascular bundles, branching off in a fan-shaped pattern from the basal part to the upper part of the organ (Fig. 3a). This surface was devoid of hairs. The outer walls of the epidermal cells were slightly convex (Fig. 3b). Long whitish trichomes were on the abaxial surface, growing from massive pillars made up of several epidermal cells (Fig. 3 c,d). These trichomes were unicellular.

Stamens

The stamen in *Pulsatilla* was composed of a long filament and an oval flattened anther with 4 pollen sacs (Fig. 3e,g). Morphological differences between the stamen and staminode are presented in Figure 3e. The epidermal cells of the filament (Fig. 3f) and stamen head (Fig. 3g) were markedly elongated. Stomata occurred only in the connective tissue region on both the abaxial and adaxial surfaces of the anther (Fig. 3h,i).

Staminodes

The staminodes in younger flowers were smaller than in older ones. The upper portion of the staminodes was convex on the abaxial side (Fig. 3j) and concave on the adaxial side (Fig. 3k,l). In many staminodes, visible on the adaxial surface were the expanded central parts formed by the connective tissue and the small edge ones probably

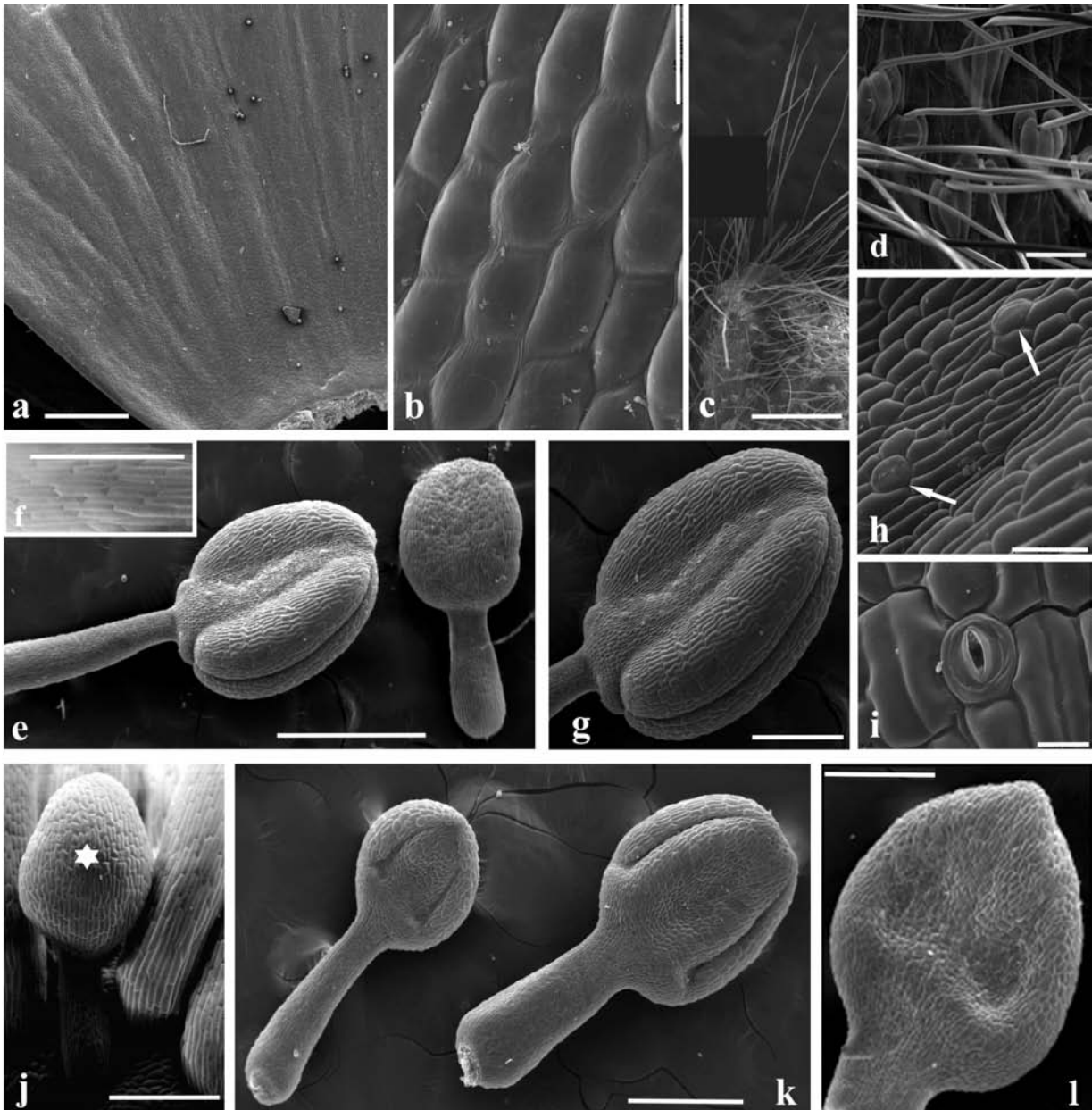


Fig. 3. Fragments of perianth and staminodes of *Pulsatilla slavica* (SEM). (a) Lower part of tepal (adaxial surface), (b) Epidermis from adaxial surface of tepal, (c) Fragments of lower part of tepal (abaxial surface) with long trichomes, (d) Non-glandular trichomes growing from expanded pillar on abaxial surface of tepal, (e) Fragment of stamen and staminode from open flowers (before pollen shedding), (f) Portion of stamen filament, (g) Anther, (h) Fragment of adaxial surface of connective tissue with stomata visible (arrows), (i) Stoma on connective tissue surface, (j) Staminode – convex abaxial part (asterisk), (k–l) Staminodes – concave adaxial part. Bar = 1000 µm (a, c, d), 40 µm (b), 900 µm (e, f), 400 µm (g), 60 µm (h), 20 µm (i), 500 µm (j, l), 300 µm (k).

developed from pollen sacs (Fig. 3 k). In older pollen-shedding flowers the staminodes were of different sizes (Fig. 4a). Stomata occurred in the abaxial epidermis of both small (Fig. 4b,e,f) and larger staminode heads (Fig. 4h). In *P. slavica* there were only 3 stomata (Fig. 4g) on a small staminode (Fig. 4f) and up to 20 on larger ones. The

number of stomata on *P. vulgaris* staminodes ranged from 7 to 10 (Tab. 1). On some staminodes there were sphere-like outgrowths, possibly fat bodies, showing liquid content in material dried for SEM examination (Fig. 4b–e). The epidermal cell surface was covered with smooth cuticle (Fig. 4g,i).

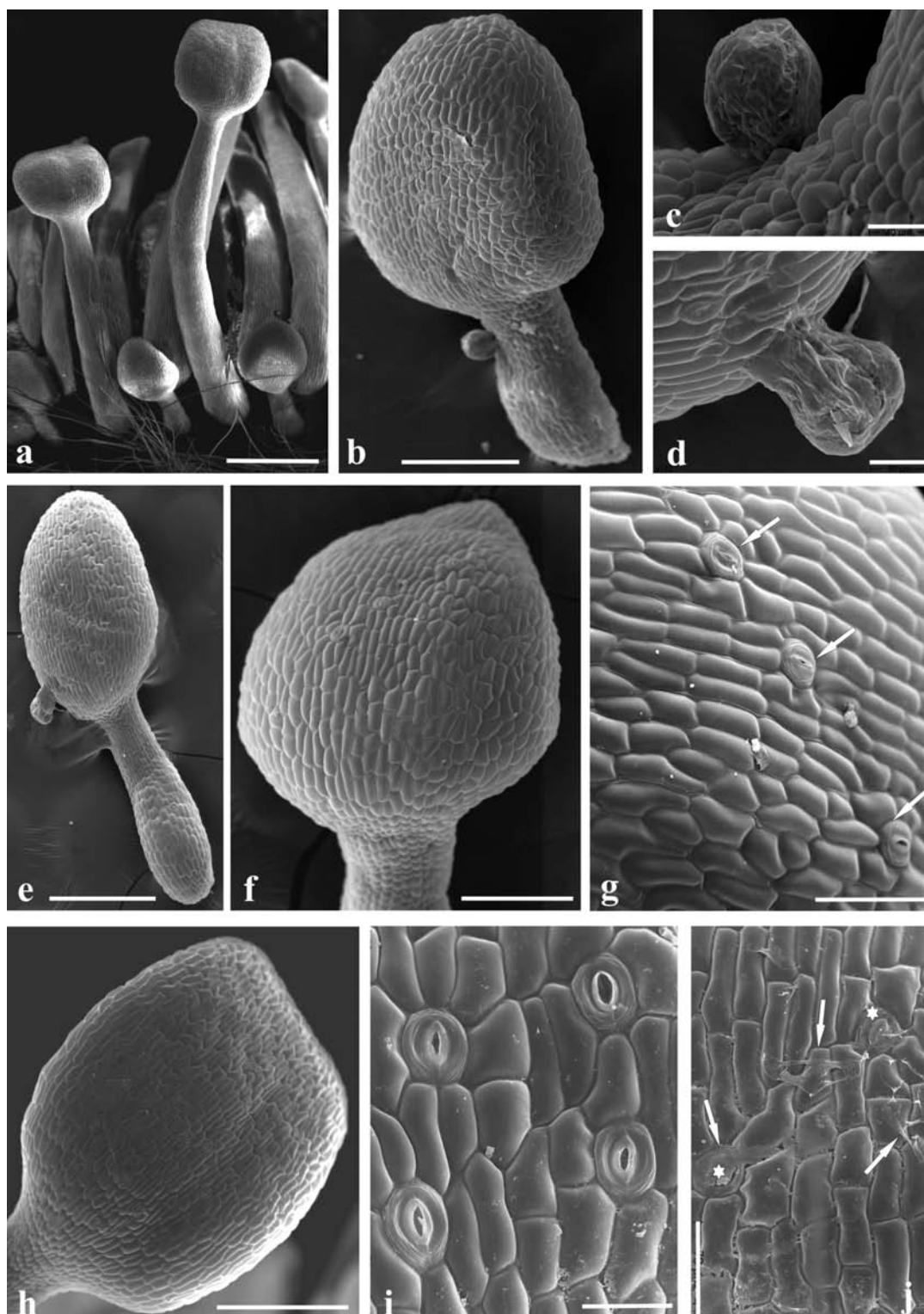


Fig. 4. Staminodes in *P. slavica* flowers. (a) Staminodes of different size in older flowers, (b) Staminode from older flower (stamens almost empty of pollen), (c, d) Fragments of staminodal filament with outgrowth visible, (e, f, h) Staminodes with heads of different shapes, (g) Three stomata on surface of small staminode from Fig. 4f (arrows), (i) Stomata located in abxial epidermis of staminode from Fig. 4h, (j) Fragment of staminodal surface with stomata visible (asterisks) and nectar residues (arrows). Bar = 1000 μ m (a), 300 μ m (b), 60 μ m (c), 40 μ m (d), 500 μ m (e), 200 μ m (f), 9 μ m (g), 400 μ m (h), 50 μ m (i), 70 μ m (j).

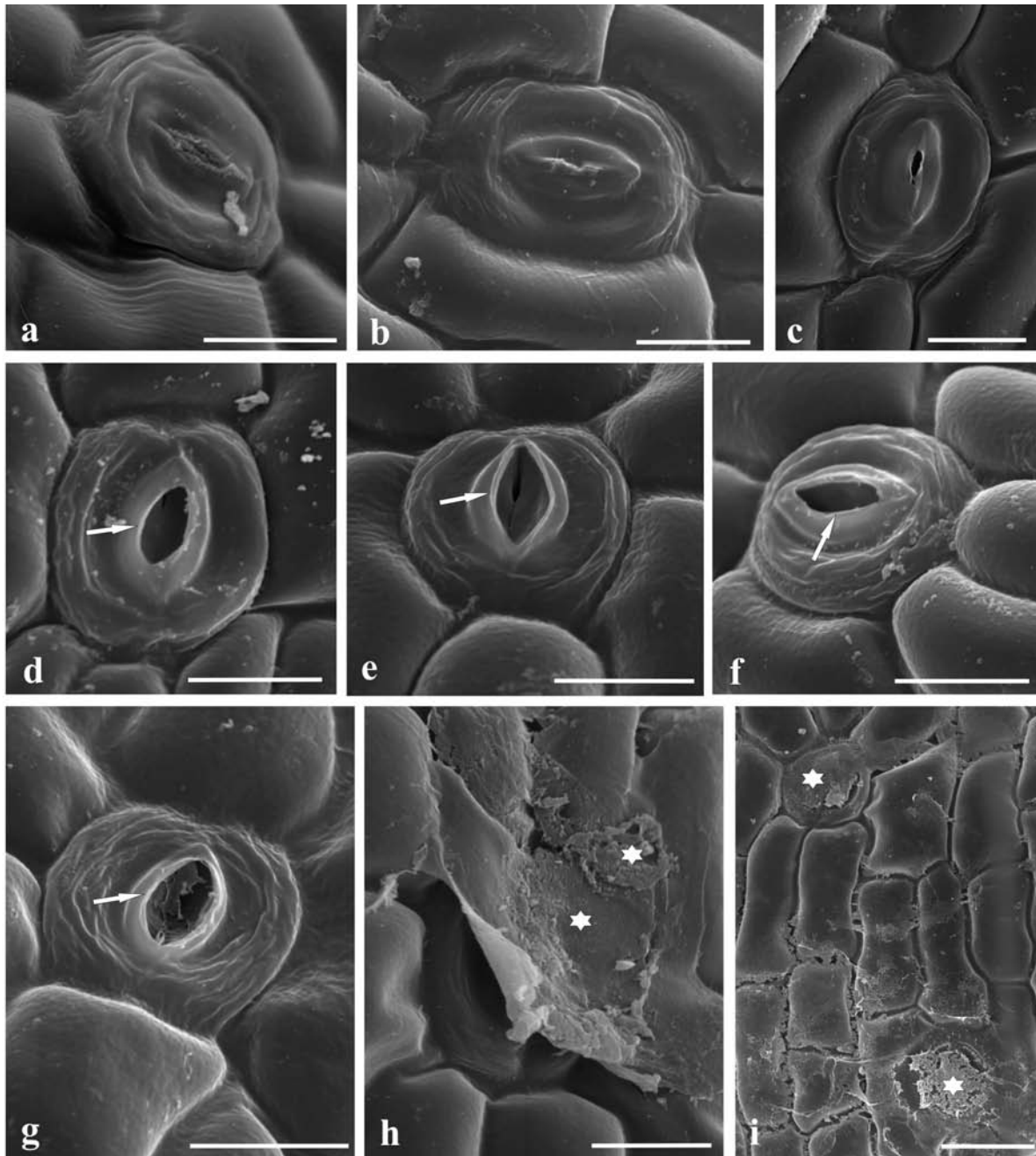


Fig. 5. Stomata on abaxial epidermis of staminode. (**a-g**) Staminodial stomata in different development stages, (**h-i**) Fragments of abaxial epidermis of staminode with nectar residues (asterisks) in vicinity of stomata. Bar = 20 μ m (a-h), 40 μ m (i).

The stomata of the staminodes functioned asynchronously. Closed pores covered by cuticle (Fig. 5a-c) and also partially open pores (Fig. 5d,e) were found on the staminodes in older flowers. Well-developed outer cuticular ledges were visible on most of the stomata. Cuticular striae ran concentrically on the surface of guard cells (Fig. 5a-g). Nectar residues were observed on some parts of the epidermis, mainly next to stomata (Figs. 4j, 6h,i) or within the pore (Fig. 5g).

DISCUSSION

The family Ranunculaceae includes species most frequently pollinated by insects or birds; plants of these species offer them nectar and pollen, or only pollen (Vogel, 1993). *Pulsatilla* produces flowers pollinated primarily by bees (Kugler, 1970), and these flowers are protogynous. Nectar is secreted already at maturity of pistils. Insects seeking nec-

tar must reach the base of the perianth, brushing against the pistil stigmas (Kugler, 1970).

We have observed pasque flowers being visited by butterflies, bumblebees and ants, in addition to bees. In agreement with Kugler (1970), we noted that nectar secretion occurs much earlier than pollen shedding.

Smets (1986) distinguished *nectaria caduca* and *nectaria persistentia* in the family Ranunculaceae. *Nectaria caduca* are associated with floral parts that drop: the petals and androecium. *Nectaria persistentia* are associated with floral parts that do not drop: the sepals, receptacle and gynoecium. He also reported that in most cases in this family the nectar is secreted through the "honey leaves," which are of staminodial origin, that in Ranunculaceae there is nectariferous tissue on the sepals and gynoecium in only a few cases, and that exceptionally some species have staminodial nectaries that develop into receptacular nectaries.

Erbar et al. (1998) and Bernardello (2007) also reported nectaries of different origin, location in the flower, shape, and structure in Ranunculaceae. Nectar is most often produced by the so-called "honey leaves" or "nectary leaves" situated between the perianth and stamens. These nectaries may develop from modified tepals or stamens. Erbar et al. (1998) demonstrated an ontogenetic relationship between the stamens and nectary organs in two genera of this family (*Helleborus*, *Ranunculus*).

Other authors confirm the different origins of the nectaries in Ranunculaceae. Petanidou (2007) reports that in *Ranunculus* the nectaries are located in the lower part of the tepals as "honey pockets." In *Caltha* the nectaries have been placed in the gynopleural type, since the nectariferous trichomes are located on the carpels (Smets and Cresens, 1988). *Aquilegia*, *Delphinium* and *Aconitum* have nectariferous spurs (Erbar et al., 1998). The nectaries in the species of the genus *Helleborus* are modified petals (Vesprini et al., 1999).

Our study shows that nectar production in the flowers of *Pulsatilla* occurs in the staminodes, which, as indicated by their morphology, are modified stamens. The nectar flows out onto the surface of the organ via stomata situated only in the abaxial epidermis of the staminode head. The distribution of stomata in these staminodial nectaries corresponds to the region of connective tissue in stamens, which is significantly expanded in the nectaries. Laterally situated protuberances resembling pollen sacs are noted in many staminodes. The staminode head seems to develop primarily from the expanded connective tissue, with reduction or complete degeneration of the pollen sacs.

Kugler (1970) made a similar suggestion, that in *Pulsatilla* the nectar is secreted by staminodes of

staminal origin, "Honigblätter" in which the connective tissue functions as the nectariferous region, while the anthers are retarded. Esau (1973) stated that most genera of entomophilous plants of the order Ranunculales have nectaries derived from modified stamens or staminodes.

Proctor et al. (1996) claim that in *Pulsatilla* the nectar is secreted at the base of the staminal filaments, but here we have shown that in *Pulsatilla* the nectar is produced in the staminode heads, from whence it flows out onto the epidermis surface via the stomata. According to Dalgren's (1982) classification, the nectaries in *Pulsatilla* should be included among androecial nectaries associated with stamens. Smets (1986) concluded that nectaries associated with stamens or staminodes are the most primitive manifestation of *nectaria caduca*.

Nectar exudation through the stomata of nectaries of different structure has been found in many plants: *Eucalyptus* (Davis, 1997), *Digitalis* (Gaffal et al. 1998), *Ecbalium* (Fahn and Shimony, 2001), *Crataegus* (Weryszko-Chmielewska et al., 2003), *Myosotis* (Weryszko-Chmielewska, 2003), *Rhododendron* (Weryszko-Chmielewska and Chwil, 2007), *Inula* (Sulborska and Weryszko-Chmielewska, 2007) and *Echium* (Chwil and Weryszko-Chmielewska, 2007).

Our observations show that the chloroplast-containing parenchymal cells of the staminodes in *Pulsatilla* flowers produce and secrete nectar. We detected starch grains in the chloroplasts; they probably play a part in nectar production. Nectar exudation onto the epidermis surface occurs through stomata that mature asynchronously. At stamen maturity in the protogynous flowers of *Pulsatilla*, in the nectar-secreting staminodes we found not fully mature stomata with cuticle-covered pores. The nectariferous tissue in the staminode is supplied by a vascular bundle with xylem and phloem elements. Drops on the abaxial surface of the staminode are the primary nectar presentation. Secondary presentation consists of nectar drops or a continuous nectar layer on the tepals or at the base of the tepals and stamens.

Huang et al. (2002) found that the hanging position adopted by *Pulsatilla cernua* flowers during rainfall protects pollen against damage by rain, and the nectar against dilution.

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