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Embryology of *Swertia* (Gentianaceae) relative to taxonomy

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The embryological features of three species of *Swertia* (*s.l.*) – *S. erythrosticta*, *S. franchetiana*, and *S. tetraptera* – were characterized, and the observations were used, together with previously gathered data on other species, to evaluate a recently proposed polyphyly, based on molecular data, of *Swertia s.l.* Comparisons of species within the genus showed that they have diversified embryologically, and there are significant between-species differences. Notable features that vary between species include the number of cell layers that form the anther locule wall, the construction of the wall of the mature anther, tapetum origin, the cell number in mature pollen grains, the structure of the fused margins of the two carpels, the ovule numbers in placental cross-sections, the shape of the mature embryo sac, the degree of ovule curvature, antipodal variation and the presence of a hypostase, and seed appendages. They share characters that are widely distributed in the tribe Gentianeae, such as a dicotyledonous type of anther wall formation, a glandular tapetum with uninucleate cells, simultaneous cytokinesis following the meiosis of the microsporocytes, tetrahedral microspore tetrads, superior, bicarpellary and unilocular ovaries, unitegmic and tenuinucellar ovules, *Polygonum*-type megagametophytes, progamous fertilization, nuclear endosperm, and Solanad-type embryogeny. The presence of variation in embryological characters amongst the species of *Swertia s.l.* strongly supports the view that *Swertia s.l.* is not a monophyletic group. *Frasera* is better separated from *Swertia s.l.* as an independent genus, and is only distantly related to *Swertia s.s.* judging from the numerous differences in embryology. *Swertia tetraptera* is very closely related to *Halenia*, as they show identical embryology.

ADDITIONAL KEYWORDS: development – embryogenesis – megagametogenesis – microgametogenesis – phylogeny – *Swertia erythrosticta* – *Swertia franchetiana* – *Swertia tetraptera* – Swertiinae.

INTRODUCTION

The subtribe Swertiinae of the Gentianaceae–Gentianeae is an almost cosmopolitan group of 14 genera growing mostly in alpine, temperate, or arctic habitats. It is absent only from tropical and subtropical lowlands. *Swertia* L. is the largest genus within the subtribe Swertiinae (Struwe *et al*., 2002), with *c.* 135 species (Chassot, 2000). It is polymorphic and mainly distributed in temperate regions of the Northern Hemisphere.

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As briefly reviewed by Chassot (2000), *Swertia* was first described by Linnaeus in 1753 (Linnaeus, 1753). The circumscription of the genus has often been debated, resulting in disagreements amongst researchers. Part of this debate is a result of the morphological similarities (for example, nectariferous and rotate corolla lobes) amongst the species of *Swertia* and the related genera *Halenia*, *Lomatogonium*, and *Veratrilla*. Despite the similarities with these genera, the definition of *Swertia s.l.* has varied significantly. Many other genera that were once segregated from it, for example *Ophelia*, *Anagallidium*, *Kingdon-Wardia*, and *Frasera* (Walter, 1788; Don, 1836; Marquand, 1929; Grisebach, 1839), are considered synonymous to *Swertia s.l.*, and the circumscription of *Swertia s.l.* has

been defined in the broadest sense, based on the rotate corolla and concave nectariferous lobe (Ho, Liu & Wu, 1988; Shah, 1990, 1992; Ho & Pringle, 1995). A number of local infrageneric classifications have been attempted for *Swertia s.l.* (Nemomissa, 1994), but only three publications offer a treatment of the whole genus (Shah, 1990, 1992; Ho, Xue & Wang, 1994). Shah divided *Swertia s.l.* into two subgenera (*Swertia* and *Ophelia*) and proposed 35 informal groups according to the geographical distribution and morphological similarities. Based on the hypothetical evolutionary polarity of morphological characters, Ho *et al*. (1994) divided *Swertia s.l.* into 11 sections: *Rugosa*, *Swertia*, *Poephila*, *Macranthos*, *Ophelia*, *Spinosisemina*, *Montana*, *Frasera*, *Platynema*, *Kingdon-Wardia*, and *Heteranthos*. However, molecular studies have recently established that *Swertia s.l.* is polyphyletic towards genera of the subtribe Swertiinae, such as *Comastoma*, *Gentianella*, *Gentianopsis*, *Halenia*, *Lomatogonium*, and *Veratrilla* (Yuan & Küpfer, 1995; Chassot *et al*., 2001; Liu, Chen & Lu, 2001; Hagen & Kadereit, 2002; Struwe *et al*., 2002). Some major groups can be recognized in the phylogenetic trees, for example, *Swertia s.s*. *Frasera*, the *Halenia–Swertia tetraptera* complex, and a larger group comprising *Comastoma*, *Gentianella*, *Lomatogonium*, and some *Swertia* species, etc. *Swertia erythrosticta* Maxim. formed part of the *Swertia s.s.* clade. *Swertia tetraptera* Maxim. grouped with *Halenia*, forming a strongly supported monophyletic clade. *Swertia franchetiana* H. Smith was included in a large clade.

Molecular analyses usually agree more with anatomical, embryological, and phytochemical characters, and sometimes even with characters of vegetative morphology (Wagenitz, 1997). Compared with many vegetative and floral characters, embryology shows very little plasticity; variation amongst individual taxa reflects genetic and phylogenetic differences (Tobe, 1989). Significant contributions to the embryology of *Swertia s.l.* include treatments of *S. caroliniensis* (Walt.) Kuntze by McCoy (1949), *S. corymbosa* (Griseb.) C. B. Clarke by Rao (1975), *S. angustifolia* Buch.-Ham. ex D. Don by Maheswari & Lakshminarayana (1977), *S. minor* (Griseb.) Knobl. by Rao & Nagaraj (1982), *S. bifolia* Batal. by Xue (2000), and *S. cincta* Burk. by Xue, Ho & Li (2002). Both *S. angustifolia* and *S. corymbosa* belong to section *Spinosisemina*, whereas *S. bifolia*, *S. caroliniensis*, *S. minor*, and *S. cincta* belong to four different sections: *Rugaso*, *Frasera*, *Ophelia*, and *Platynema*, respectively. With regard to embryology, the literature on *S. erythrosticta*, *S. franchetiana*, and *S. tetraptera* is scanty and fragmental (Liu, Xue & Ho, 1998; Ho, Xue & Liu, 1999; Xue, Ho & Liu, 1999a). The present study provides full descriptions of the embryology of these three species, namely *S. erythrosticta*, *S. franchetiana*, and *S. tetraptera*, which belong to sections *Swertia*, *Ophelia*, and *Heteranthos*, respectively (Table 1).

The data are comparable with those previously presented for *Swertia* species and other genera of Swertiinae, and contribute further towards the resolution of the taxonomic problems of *Swertia s.l.*

MATERIAL AND METHODS

The flowers and floral buds of *S. erythrosticta* (No. XCHY 1995001), *S. franchetiana* (No. XCHY 1995002), and *S. tetraptera* (No. XCHY 1995003), at various developmental stages, were collected in the wild at Xining, Qinghai Province, China, and cultivated at the North-west Plateau Institute of Biology, Chinese Academy of Sciences. Voucher specimens are deposited in the herbarium of the North-west Plateau Institute of Biology (HNWP).

Flowers of all ages from all of the species were fixed in formalin–acetic acid–alcohol (FAA) fixative (five parts formalin to six parts acetic acid and 89 parts 70% alcohol). After staining in Ehrlich's haematoxylin, the materials were embedded in paraffin by the conventional method. Sections (5–10 μm) were cut on a rotary microtome, stained with safranin and fast green, and then observed and photographed under an Olympus BH2 microscope.

RESULTS

Swertia erythrosticta, *S. franchetiana*, and *S. tetraptera* have heterogeneous morphological characters. *Swertia erythrosticta* is a perennial with a solitary stem and few, relatively large pentamerous flowers, bearing a single fimbriate nectary per petal lobe. *Swertia franchetiana* is an annual with a branched stem and many, relatively small pentamerous flowers, bearing two fimbriate nectaries per petal lobe. By comparison, *S. tetraptera* has many apomorphic morphological characters: annual plant with a branched stem and unequal tetramerous flowers, bearing two naked convex nectaries per petal lobe.

Formation of the anther wall

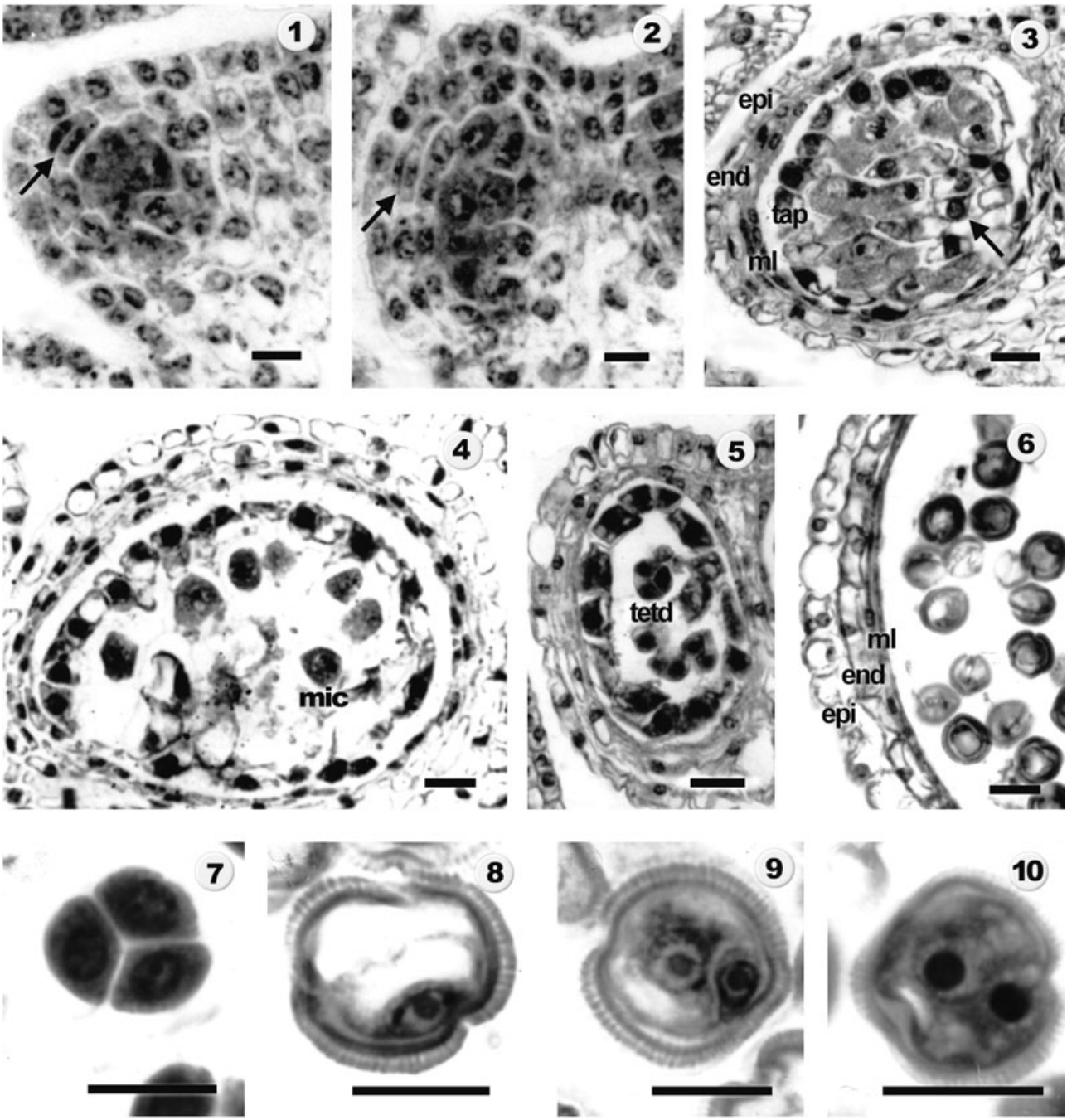
The flowers of *Swertia* are bisexual. The anther has four sporangia, in each of which a row of archesporial cells differentiates just beneath the epidermis. The archesporial cells produce a layer of outer primary parietal cells and a layer of inner sporogenous cells by mitotic division. The cells of the primary parietal layer then divide into two secondary parietal layers (Fig. 1). Cells of the outer layer divide into an endothecium and middle layer (Fig. 2), and cells of the inner layer form the tapetum, with large nuclei,

Table 1. Comparison of embryological characters of *Swertia* L.

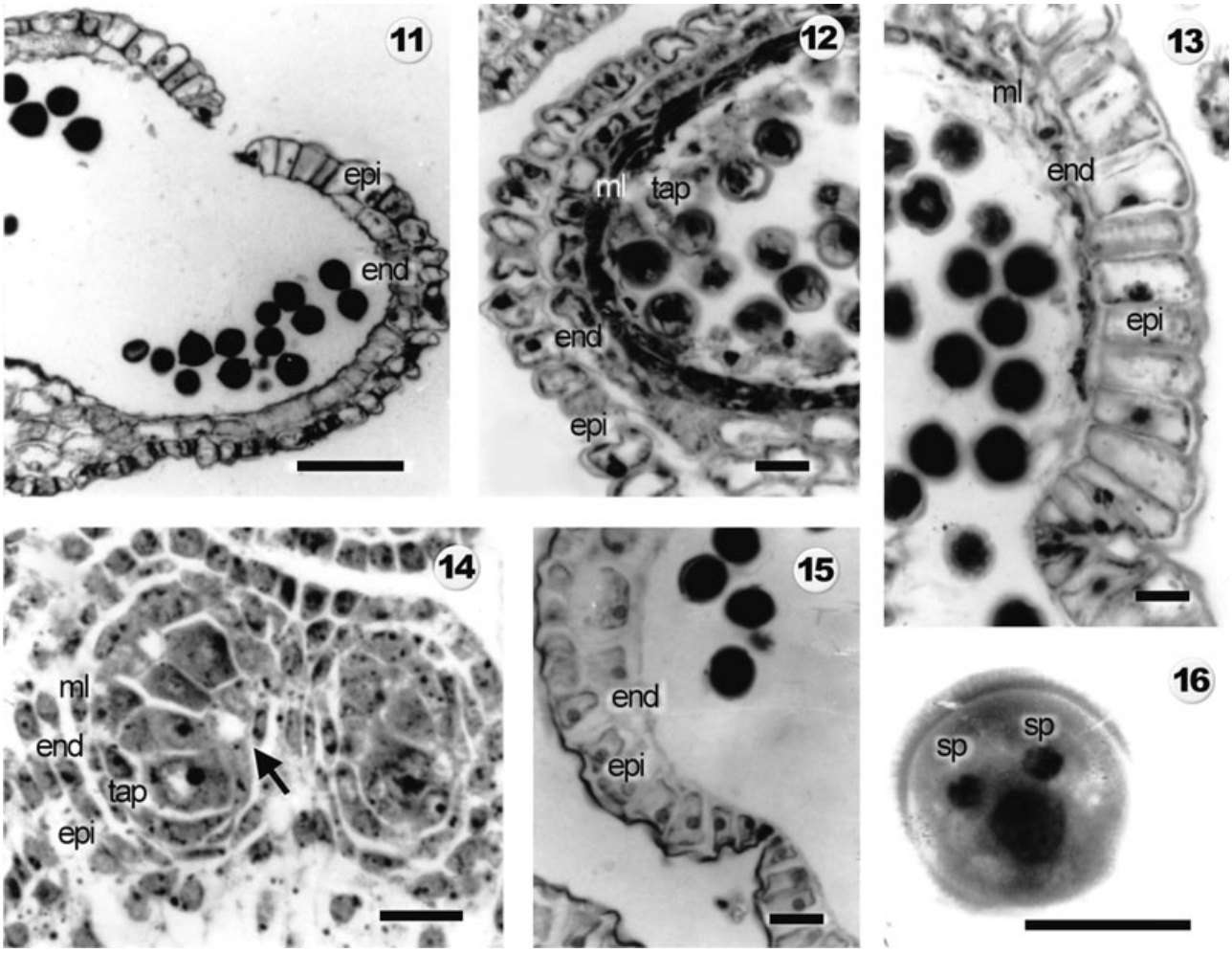
Character	*S. erythrosticta*	*S. franchetiana*	*S. tetraptera*	*S. bifolia*	*S. angustifolia*	*S. caroliniensis*	*S. cincta*	*S. corymbosa*	*S. minor*
Sexuality: bisexual, 1; unisexual, 2	1	1	1	1	1	1	1	1	1
Anthers									
Type of wall development: dicotyledonous, 1; other, 2	1	1	1	1	1	1	1	1	1
Epidermis cell: persistent, 1; fibrous thickening, 2	1	1	1	1	1	1	1	1	1
Endothecium: fibrous thickenings, 1; no fibrous thickenings, 2	2	1	1	1	1	1	1	1	2
Middle layers: number	3	2	1	2–3	1	1–4	2	1	1
ephemeral, 1; persistent and fibrous thickenings, 2	1	1	1	2	2	1	1	1	1
Tapetum: origin: unitary, 1; dual, 2	2	2	2	2	1	1	2	?	2
type: glandular, 1	1	1	1	1	1	1	1	1	1
layer number	2	1	1	2	1	1–4	1	1	1
placentoids: formed, 1; none formed, 2	1	1	1	1	1	1	1	?	1
number of nuclei in cell	1	1	1	1	1	1	1	1	1
Microsporogenesis: cytokinesis: simultaneous, 1; other, 2	1	1	1	1	1	1	1	1	1
Arrangement of microspores: all tetrahedral, 1; nearly always tetrahedral, rarely decussate, 2	1	1	1	1	1	1	1	1	1
Number of cells in a mature pollen grain: three, 1; two, 2	1	2	1	1	1	2	1	2	2
Ovule									
Placenta protrusion: no, 1; yes, 2	1	1	2	1	1	1	1	1	1
Type: anatropous, 1; ana-campylotropous 2; orthotropous, 3	1	1	3	1	1	1	2	1	1

Table 1. *Continued*

Character	*S. erythrosticta*	*S. franchetiana*	*S. tetraptera*	*S. bifolia*	*S. angustifolia*	*S. caroliniensis*	*S. cincta*	*S. corymbosa*	*S. minor*
No. of ovule rows: many rows, 1; 8 rows, 2; 4 rows, 3	1	2	3	1	1	1	1	?	1
Number of integuments: one, 1; two, 2	1	1	1	1	1	1	1	1	1
Nucellus: tenuinucellate, 1; crassinucellate, 2	1	1	1	1	1	1	1	1	1
Shape of megaspore tetrads: linear, 1; other, 2	1	1	1	1	1	1	1	1	1
Type of megagametophyte development: *Polygonum*, 1; other, 2	1	1	1	1	1	1	1	1	1
Hypastase: none formed, 1; formed, 2	1	1	2	1	1	1	1	1	1
No. of antipodal cells: three, 1; many, 2	1	2	1	1	1	1 or 2	2	1	2
Antipodal cells: ephemeral, 1; persistent, 2	1	2	2	1	1	2	2	1	2
Shape of mature embryo sac: ellipsoidal, 1; ovoid, 2	1	1	2	1	1	1	1	1	1
Seed									
Type of endosperm formation: nuclear type, 1	1	1	1	1	1	1	1	1	1
Type of embryogeny: Solanad *Physalis* I, 1; Solanad *Physalis* II, 2	2	2	2	2	2	2	2	2	2
Appendages of seed: winged, 1; wingless, 2	1	2	2	2	2	1	2	2	2
Section	*Swertia*	*Opheila*	*Heteranthos*	*Rugosa*	*Spinosisemina*	*Frasera*	*Platynema*	*Spinosisemina*	*Opheila*
Reference	Ho *et al.* (1999), this study	Liu *et al.* (1998), this study	Xue *et al.* (1999a), this study	Xue (2000)	Maheswari & Lakshminarayana (1977)	McCoy (1949)	Xue *et al.* (2002)	Rao (1975)	Rao & Nagaraj (1982)



Figures 1–10. Anther, microsporogenesis and microgametogenesis. Figs 1, 2. *Swertia erythrosticta*. Fig. 1. Sporogenous cells and primary parietal layer. The arrow shows a cell of the primary parietal layer that has divided into two secondary parietal layers. Scale bar, 20 μm. Fig. 2. The outermost secondary parietal layer, divided into an endothecium and middle layer, showing the dicotyledonous type of wall formation (arrowed). Scale bar, 20 μm. Figs 3–10. *Swertia franchetiana*. Fig. 3. Microspore mother cells and five-cell-layered wall structure: epidermis (epi), endothecium (end), two middle layers (ml), and tapetum (tap). The arrow shows the tapetum of dual origin and the placentoids formed. Scale bar, 20 μm. Fig. 4. Microsporocyte (mic) and anther wall, with initiation of degeneration of tapetal cells. Scale bar, 20 μm. Fig. 5. Tetrahedral tetrad (tetd) and anther wall. Scale bar, 20 μm. Fig. 6. Single-nucleate pollen grains and four-cell-layered wall structure: epidermis (epi), endothecium (end), and two middle layers (ml). Scale bar, 20 μm. Fig. 7. Tetrahedral tetrad. Scale bar, 20 μm. Fig. 8. Pollen grain with a single large vacuole, showing the nucleus pressed to the wall. Scale bar, 20 μm. Fig. 9. Premature binucleate pollen grain. Scale bar, 20 μm. Fig. 10. Mature binucleate pollen grain. Scale bar, 20 μm.



Figures 11–16. Anther and microsporogenesis. Fig. 11. Anther wall before shedding in *Swertia franchetiana*, showing persistent epidermis (epi) and fibrous thickenings of the endothecium (end). Scale bar, 100 μm. Figs 12, 13. *Swertia erythrosticta*. Fig. 12. Anther wall of single-nucleate pollen grain, showing seven-cell-layered wall structure: an epidermis (epi), an endothecium (end), three middle layers (ml), and two disintegrating tapeta (tap). Scale bar, 20 μm. Fig. 13. Anther wall before shedding, showing columnar elongation of the epidermis (epi), reduced endothecium (end), and persistent middle layers (ml). Scale bar, 20 μm. Figs 14–16. *Swertia tetraptera*. Fig. 14. Microspore mother cells and four-cell-layered wall structure: epidermis (epi), endothecium (end), middle layer (ml), and tapetum (tap). The arrow shows the tapetum of dual origin. Scale bar, 20 μm. Fig. 15. Anther wall before shedding, showing persistent epidermis (epi) and fibrous thickenings of the endothecium (end). Scale bar, 20 μm. Fig. 16. Three-celled pollen grains (sp., sperm). Scale bar, 20 μm.

dense cytoplasm, and various shapes (Fig. 3). The middle layers have a common histogenetic origin with the endothecium. Therefore, the formation of the anther wall is of the dicotyledonous type (Davis, 1966). In *S. erythrosticta*, the middle layer ultimately becomes three-layered through periclinal divisions (Fig. 12), whereas, in *S. franchetiana* (Figs 3, 4), it becomes two-layered and, in *S. tetraptera*, it remains single-layered (Fig. 14). Although the cells of the tapetum divide into two layers in *S. erythrosticta,* they remain single-layered in *S. franchetiana* and *S. tetraptera*. The anther wall prior to maturation usually comprises seven cell layers (an epidermis, an endothecium, three middle layers, and two tapetal layers) in *S. erythrosticta* (Fig. 12), whereas, in *S. franchetiana*, it comprises five cell layers (an epidermis, an endothecium, two middle layers, and a tapetum) (Fig. 3) and, in *S. tetraptera*, it comprises four cell layers (an epidermis, an endothecium, a middle layer, and a tapetum) (Fig. 14).

The tapetum is of dual origin: one part towards the protuberant region is contributed by the parietal

layer, and the other (towards the inner half of the loculus) is contributed by connective tissue. Cells of the tapetum on the connective side show radial elongation or periclinal division and intrude into the anther locule to form 'placentoids' (Steffen & Landmann, 1958) (Fig. 3). Tapetal cells are uninucleate throughout their development. At about the time of pollen tetrad formation, the walls of the tapetal cells become indistinct and the tapetal cells degenerate (Figs 4, 5). The tapetal cells degenerate completely by the uninucleate pollen grain stage (Fig. 6). All the tapetal cells degenerate at their original sites, and degenerating tapetum nuclei in the middle of the anther locule originate from the early differentiation protruding of the tapetum (Figs 3, 4); therefore, the tapetum is of, or similar to, the glandular type.

The middle layers are ephemeral and degenerate during the time of meiosis, when the cells become flattened and disintegrate. Only a few flattened remains of the middle layers are present in *S. franchetiana* and *S. tetraptera* at the uninucleate pollen stage (Fig. 6), whereas, in *S. erythrosticta*, the middle layers are more persistent and the remains of the cells are still present at the three-cell pollen stage (Fig. 13).

The epidermal cells are stretched radially, whereas the cells of the endothecium become more or less enlarged. Eventually, the endothecium develops fibrous thickenings in *S. franchetiana* and *S. tetraptera*, but such thickenings are absent in *S. erythrosticta*. The epidermal cells form columns in *S. erythrosticta* (Fig. 13). Thus, the walls of the mature anthers of *S. franchetiana* and *S. tetraptera* consist of the persistent epidermis and fibrous endothecium (Figs 11, 15), whereas those of *S. erythrosticta* consist of the persistent columnar epidermis, with no fibrous endothecium, and remains of the middle layers (Fig. 13).

Microsporogenesis and microgametogenesis

The many-celled sporogenous tissue develops from the archespore, its cells becoming microsporocytes when mitotic divisions end (Fig. 2). They are arranged in three to four layers, are multisided, and have a large nucleus. Meiosis progresses asynchronously in the loculi of one anther and the anthers of one flower. Meiosis in each microsporocyte results in a microspore tetrad (Fig. 5). Cytokinesis is of the simultaneous type, and the resultant tetrads are tetrahedral (Figs 5, 7).

Microspores separate from the tetrad as free, uninucleate microspores, each of which has dense cytoplasm with a prominent and centrally placed nucleus (Fig. 5). As the central vacuole develops, the nucleus takes up a peripheral position (Figs 6, 8). The first division of the microspore nucleus results in the formation of two unequal cells: a large vegetative cell and a smaller generative one. The generative cell is initially lens-shaped and located at the radial wall of the pollen (Fig. 9). The generative cell changes its shape from initially orbicular to elongate as it moves into the lumen of the vegetative cell (Fig. 10). The generative cell gives rise to two sperm by mitosis in both *S. erythrosticta* and *S. tetraptera*. Their pollen grains are thus three-celled at the time of anther dehiscence (Fig. 16). However, in *S. franchetiana*, the generative cell does not undergo mitotic division before anther dehiscence and the pollen grain is two-celled at the time of anther dehiscence.

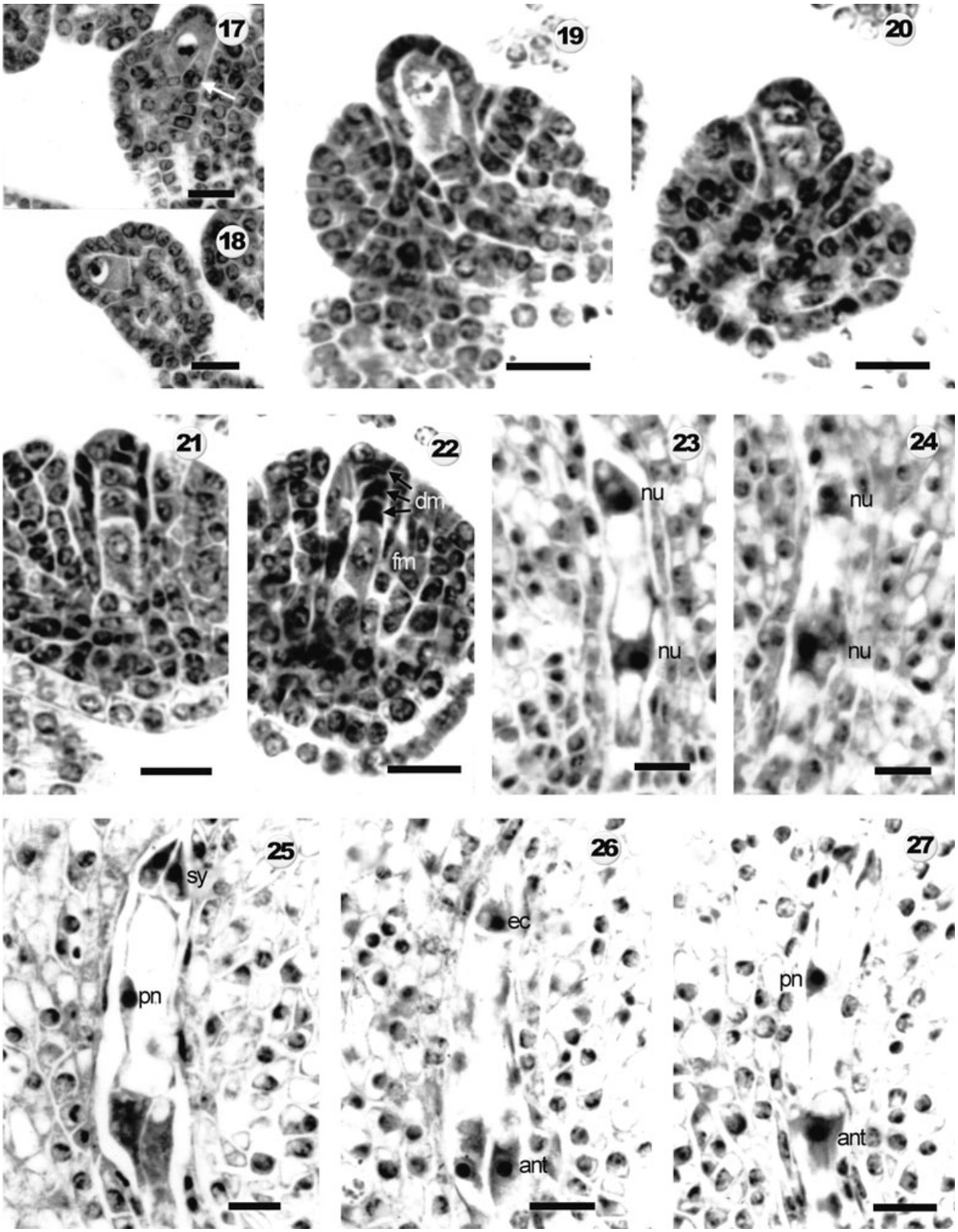
Development of female embryonic structures

The gynoecium is unilocular and composed of two carpels. In *S. tetraptera*, the fused margin of the two carpels enlarges and protrudes extensively into the ovary locule, but is absent in *S. franchetiana* and *S. erythrosticta* (Figs 30, 38, 41). There are many, eight, and four rows of ovules in transections of the ovaries of *S. erythrosticta*, *S. franchetiana*, and *S. tetraptera*, respectively (Figs 30, 38, 41).

In the area in which the ovule arises, the placenta can be recognized as consisting of three zones: epidermal, subepidermal, and central. The epidermal and subepidermal cells mainly divide anticlinally, resulting in two parallel layers. The ovular primordium arises through periclinal divisions of cells in the third to fourth layers of the placenta. One large cell that is differentiated in its subepidermal layer becomes an archesporial cell (Figs 17, 18). The ovular primordium consists of epidermal and subepidermal layers and one axial cell row under the archesporial cell. The differentiation of ovule structures begins with periclinal divisions of the epidermal cells of the ovular primordium. These divisions initiate formation of the integument, which begins to differentiate by periclinal divisions of the epidermal cell layer of the ovule primordium at the archesporial stage (Figs 17, 18). The ovules are unitegmic (Fig. 19). The integument, with between four and six cell layers, forms the micropyle (Fig. 19). During the differentiation of the integument, the ovules begin to bend in *S. erythrosticta* and *S. franchetiana*, completing their curvature when the embryo sac reaches the eight-nucleate stage. However, the ovules in *S. tetraptera* bend very little during development. Therefore, the ovules are anatropous in *S. franchetiana* and *S. erythrosticta*, and orthotropous in *S. tetraptera* (Fig. 39).

Megasporogenesis and megagametogenesis

The archesporium is one-celled and transforms into a megasporocyte without cutting off the parietal cell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Figures 17–27. Megasporogenesis and megagametogenesis of *Swertia franchetiana*. Figs 17, 18. Sporogenous cell and initiation and development of integument (arrowed). Scale bar, 20 μm. Fig. 19. A unitegmic ovule and megasporocyte. Scale bar, 20 μm. Fig. 20. Anaphase II of the first meiosis division in microsporocytes. Scale bar, 20 μm. Fig. 21. Linear megaspore tetrad. Scale bar, 20 μm. Fig. 22. The functional chalazal megaspore (fm), with the other three degenerating megaspores (dm). A single-nucleate embryo sac and the other three degenerating megaspores. Scale bar, 20 μm. Figs 23, 24. Consecutive transections of a four-nucleate embryo sac (nu, nucleus). Scale bar, 20 μm. Figs 25–27. Consecutive transections of an eight-nucleate embryo sac, showing an egg cell (ec), two synergids (sy), two polar nuclei (pn), and three antipodal cells (ant). Scale bar, 20 μm.

(Fig. 19). Thus, the ovule is tenuinucellate (Fig. 19). The megasporocyte undergoes meiosis to produce a linear tetrad of megaspores (Figs 20, 21). Although the three micropylar megaspores of the tetrad eventually degenerate, the chalazal megaspore becomes functional (Fig. 22). The functional megaspore develops successively into a two-, four- (Figs 23, 24) and, finally, eight-nucleate embryo sac (Figs 25–27) by three mitotic divisions. Thus, the mode of embryo sac formation is of the *Polygonum* type. The three micropylar nuclei become the egg and two synergids, collectively comprising the egg apparatus. The two median nuclei become the polar nuclei, whereas the chalazal nuclei become the three antipodals (Figs 25–27). The polar nuclei fuse at the centre, and the resulting secondary nucleus moves close to the egg apparatus (Figs 28, 29).

In the mature eight-nucleate embryo sac, the egg cell can be distinguished by a nucleus at the chalazal end and a large vacuole at the micropylar end. The two synergids can be identified by their nuclei at the micropylar end and a large vacuole at the chalazal end (Fig. 28). The three antipodal cells in *S. franchetiana* divide to form five to eight cells (Figs 28, 29), whereas, in *S. erythrosticta* and *S. tetraptera*, they do not divide (Fig. 39). The antipodal cells in *S. franchetiana* and *S. tetraptera* persist until the stage of a four-celled pro-embryo (Fig. 35), whereas, in *S. erythrosticta*, they degenerate at the two-celled pro-embryo stage (Fig. 37). Mature embryo sacs of *S. franchetiana* and *S. erythrosticta* are ellipsoid (Fig. 28), whereas those of *S. tetraptera* are ovoid (Fig. 39). The hypostase has only been found in *S. tetraptera*. It is differentiated as a dome of cytoplasmic thin-walled cells below the embryo sac during megagametogenesis (Fig. 40).

Fertilization

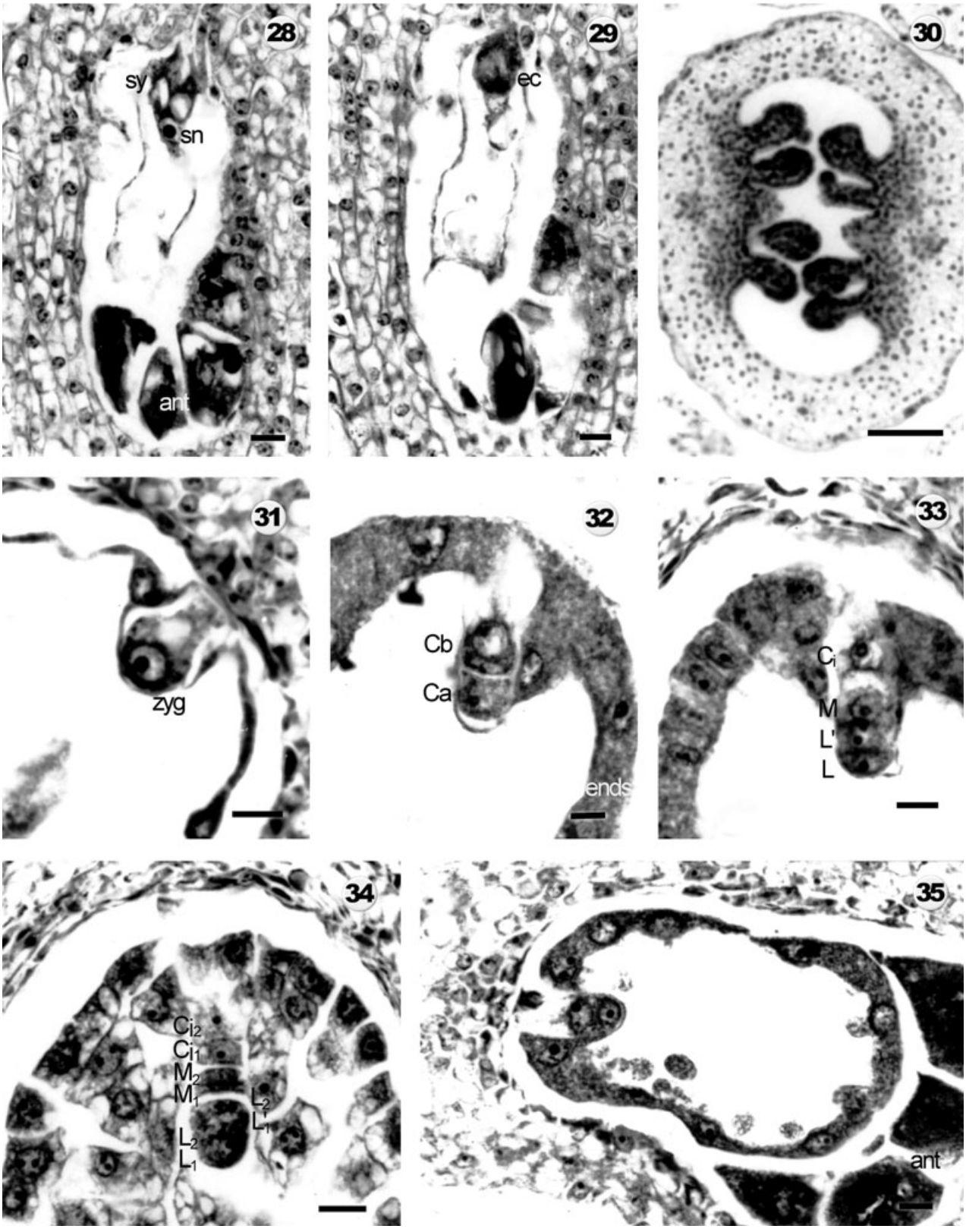
Fertilization is porogamous. The pollen tube enters the megagametophyte via one of the synergids and discharges two sperm. One of the sperm fuses with the egg nucleus, forming the zygote, and the other with the secondary nucleus, forming the primary endosperm nucleus. The primary endosperm nucleus is larger than the zygote nucleus. The first division of the primary endosperm nucleus precedes that of the zygote (Fig. 31).

Development of endosperm

Endosperm formation is of the nuclear type (Figs 31–33). The primary endosperm nucleus gives rise to two free endosperm nuclei. A large number of free nuclei form by a series of divisions of the two free endosperm nuclei (Figs 32, 33). At the multicelled pro-embryo stage, wall formation of endosperm cells initiates from the micropylar end and progresses to the chalazal end; the amount of cellular endosperm increases from the periphery to the centre of the embryo sac and surrounds the pro-embryo (Fig. 34). A few of the endosperm cells are absorbed by the embryo during its development (Fig. 36).

Embryogenesis and seed coat development

The zygote has a large nucleus, conspicuous nucleolus, dense cytoplasm, and large vacuole (Fig. 31). It divides transversely, forming a terminal cell (Ca) and a basal cell (Cb) (Fig. 32). Ca and Cb undergo transverse divisions, forming a linear pro-embryo with four cells, designated L, L′, M, and Ci (Fig. 33). The L, L′, M, and Ci cells divide transversely, forming a linear pro-embryo with eight cells, designated L_1, L_2, L'_1, L'_2, M_1, M_2, Ci_1 and Ci_2 (Fig. 34). The L_1 and L_2 cells divide vertically and transversely, forming primordia of the cotyledons (Pco), stem apex (Pvt), and hypocotyls (Phy). By vertical and transverse divisions, the L'_1 and L'_2 cells give rise to primordia of the central cylinder of the stem (Icc), central cylinder of the root (Iec), and root cap (Co). The M_1, M_2, Ci_1, and Ci_2 cells produce the suspensor (S) by vertical and transverse divisions. Thus, in these three species, the Cb cell of the two-celled pro-embryo does not contribute to the formation of the entire dicotyledonary embryo. The L cell of the four-celled pro-embryo contributes to the development of the cotyledons, stem apex, and hypocotyls. After three cycles of divisions, the pro-embryo is composed of eight cells. The embryogeny corresponds to the *Physalis* II variation of the Solanad type (Johansen, 1950).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31
zyg
32
Cb
Ca
ends
33
Ci
M
L'
L
34
Ci2
Ci1
M2
M1
L2
L1
L2
L1
35
ant

Figures 28–35. Mature embryo sac, development of embryo and endosperm, ovule type, and mature embryo sac shape of *Swertia franchetiana*. Figs 28, 29. Consecutive transections of a mature embryo sac, showing an egg cell (ec), two synergids (sy), secondary nucleus (sn), and antipodal cells (ant); ellipsoid shape of mature embryo sac. Scale bar, 20 μm. Fig. 30. Transverse section showing eight rows of ovules. Scale bar, 100 μm. Fig. 31. Zygote (zyg). Scale bar, 20 μm. Fig. 32. The terminal (Ca) and basal (Cb) cells, showing a two-celled pro-embryo and free endosperm nuclei (ends). Scale bar, 20 μm. Fig. 33. A linear four-celled (L, L′ M, Ci) pro-embryo. Scale bar, 20 μm. Fig. 34. A linear eight-celled (L_1, L_2, L'_1, L'_2, M_1, M_2, Ci_1, Ci_2) pro-embryo. Walls formed in free endosperm nuclei. Scale bar, 20 μm. Fig. 35. The antipodal cells (ant) at the two-celled pro-embryo stage. Scale bar, 20 μm.

In mature seeds, the embryo is spherical (Fig. 36). During the development of the embryo sac, the nucellus is resorbed at an early stage and thus does not contribute to the formation of the seed coat. Only the outer layer of the integument differentiates into an anatomically characteristic structure, whereas the other layers of the integument become compressed and later fully resorbed. In *S. erythrosticta* only, integument cells at both the microphylar and chalazal ends divide, forming an outgrowth, which gives rise to the wing (Fig. 37).

DISCUSSION

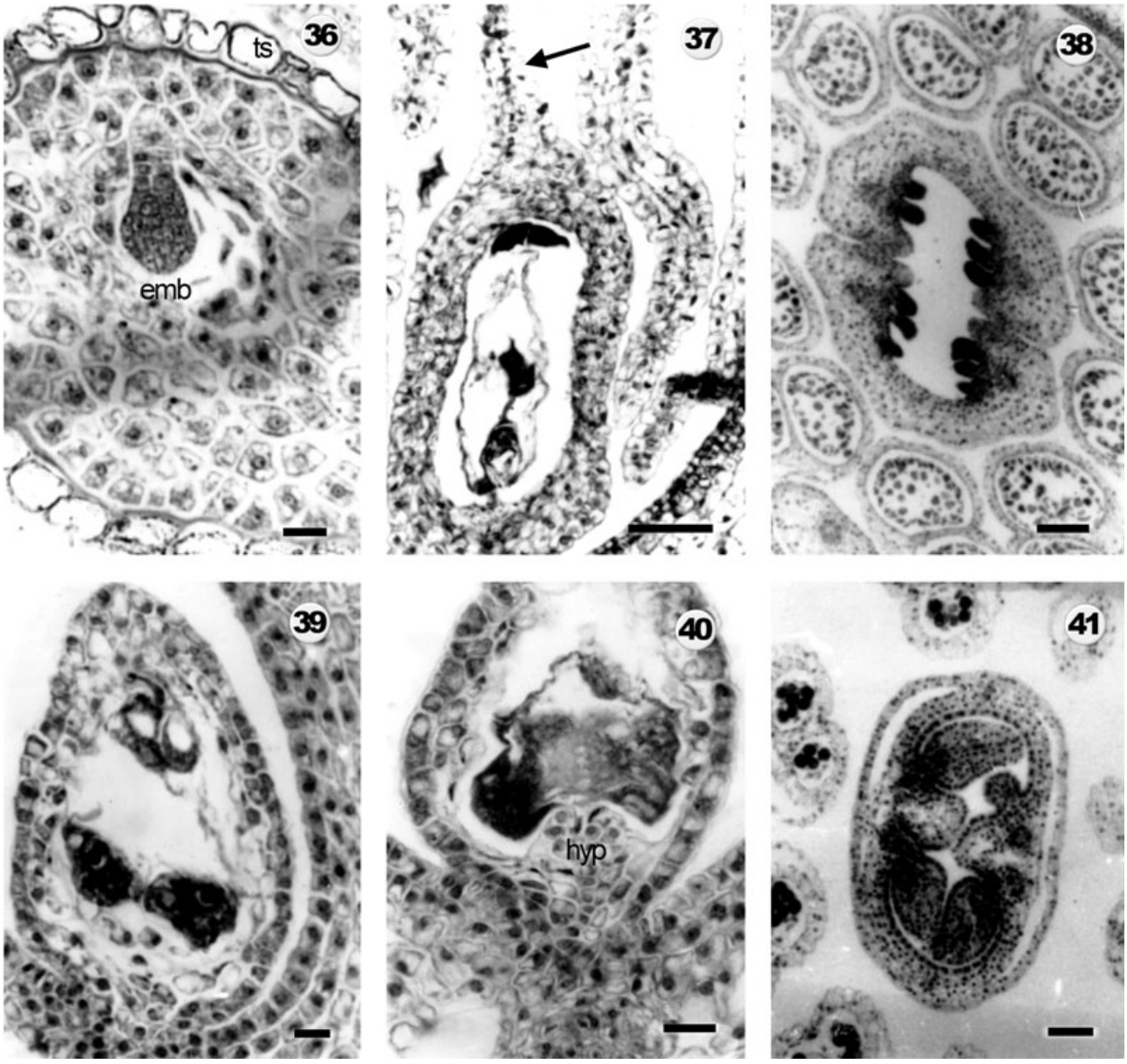
Comparisons of *Swertia s.l.* embryology

The embryological features of species within the genus *Swertia s.l.* can be compared by considering previously reported observations on features of *S. angustifolia*, *S. bifolia*, *S. caroliniensis*, *S. cincta*, *S. corymbosa*, and *S. minor*, and our findings on the embryology of *S. erythrosticta*, *S. franchetiana*, and *S. tetraptera* (Table 1). The features shared by all of the species include: anthers with four sporangia; a dicotyledonous type of anther wall formation; a glandular tapetum with uninucleate cells; simultaneous cytokinesis following meiosis of the microsporocytes; tetrahedral microspore tetrads; superior, bicarpellary and unilocular ovaries; unitegmic and tenuinucellar ovules; *Polygonum*-type megagametophytes; progamous fertilization; nuclear endosperm; and a *Physalis* II variation of Solanad-type embryogeny.

In addition to the shared embryological features, variable features are found in distinct combinations in these taxa (Table 1). Characters varying within the genus include: the number of cell layers that form the anther locule wall; structure of the fibrous thickenings of the endothecium and shape of the epidermal cells of mature anthers; unitary or dual origin of tapetal cells; three- or two-celled pollen grains; protrusion or not of the fused margins of the two carpels; ovule numbers in placental cross-sections; ellipsoid or ovoid shape of the mature embryo sac; degree of ovule curvature; antipodal variation; presence of a hypostase; and seed appendages.

Embryologically, the nine species of *Swertia s.l.* compared are highly diverse, showing species-specific combinations of characters. High degrees of interspecies variation in *Swertia s.l.* may be the result of species radiation or habitat fragmentation (Hagen & Kadereit, 2000; Lienert, Diemer & Schmid, 2002a; Lienert *et al*., 2002b). They all share certain embryological features that are widely distributed in Gentianaceae, such as a dicotyledonous type of anther wall formation, simultaneous cytokinesis, unitegmic and tenuinucellar ovules, *Polygonum*-type megagametophytes, and nuclear endosperm (Johri, Ambegaokar & Srivastava, 1992). By contrast, some major characteristics vary between species, such as the cell number in pollen grains, the degree of ovule curvature, and the shape of the mature embryo sac. In addition, there are other differences in anther development and structure.

The species of *Swertia s.l.* studied here represent seven of the 11 sections into which Ho *et al*. (1994) subdivided the genus (Table 1): sections *Rugosa* (*S. bifolia*), *Swertia* (*S. erythrosticta*), *Frasera* (*S. caroliniensis*), *Opheila* (*S. franchetiana*, *S. minor*), *Spinosisemina* (*S. angustifolia*, *S. corymbosa*), *Heteranthos* (*S. tetraptera*), and *Platynema* (*S. cincta*). However, sections *Poephila*, *Macranthos*, *Montana*, and *Kingdon-Wardia* are not represented. Embryological data are not congruent with the sections of Ho *et al*. (1994): for example, *S. franchetiana* and *S. minor* belong to section *Opheila*, but several embryological differences exist between them (Table 1). Unlike *S. minor*, the former has fibrous thickenings of the endothecium, two middle layers, and eight rows of ovules in placental cross-section. The embryological features are not constant within section *Spinosisemina*: *S. angustifolia* differs from *S. corymbosa* by the possession of persistent fibrous thickenings in the middle layers and three cells in a mature pollen grain. For *S. bifolia* and *S. erythrosticta*, the situation is nearly the opposite. Embryologically, they are similar in all characteristics studied, except for the following (Table 1): the endothecium in *S. bifolia* develops fibrillar thickenings, whereas, in *S. erythrosticta*, it does not, and the middle layer develops fibrillar thickenings and is persistent in *S. bifolia*, whereas it is ephemeral and crushed in the mature anther in *S. erythrosticta*.



Figures 36–41. Mature embryo sac, embryo, and endosperm, ovule type, and mature embryo sac shape. Fig. 36. *Swertia franchetiana*, showing embryo (emb) at the globular-shaped stage and testa when seeds are released from the capsule. Scale bar, 20 μm. Figs 37, 38. *Swertia erythrosticta*. Fig. 37. Antipodal cells and seed wing (arrowed). Scale bar, 100 μm. Fig. 38. Transverse section showing many rows of ovules. Scale bar, 100 μm. Figs 39–41. *Swertia tetraptera*. Fig. 39. Embryo sac after fertilization, showing orthotropous ovule, antipodal cells, and ovoid embryo sac. Scale bar, 20 μm. Fig. 40. Hypostase (hyp). Scale bar, 20 μm. Fig. 41. Transverse section showing four rows of ovules and extensive protrusion of the fused carpels. Scale bar, 100 μm.

EMBRYOLOGICAL COMPARISON OF *SWERTIA S.L.* AND OTHER GENERA

As mentioned above, *Swertia s.l.* has been defined as polyphyletic, on the basis of molecular data, and must be considered in the context of other genera of the Swertiinae. Based on molecular data, the Gentianinae comprises *Crawfurdia*, *Gentiana*, and *Tripterospermum*, and the Swertiinae contains *Bartonia*, *Comastoma*, *Frasera*, *Gentianella*, *Gentianopsis*, *Halenia*, *Jaeschkea*, *Latouchea*, *Lomatogonium*, *Megacodon*, *Obolaria*, *Pterygocalyx*, *Swertia*, and *Veratrilla* (Hagen & Kadereit, 2002; Struwe *et al*., 2002). Substantial efforts have been made to char-

acterize the embryology of the tribe Gentianeae in recent years. As well as the investigations of taxa in *Swertia s.l.*, these efforts have included studies on *Comastoma* by Liu & Ho (1996a), *Crawfurdia* by Chen *et al*. (2000a), *Gentiana* by Ho & Liu (1999) and Ho *et al.* (2000), *Gentianella* by Liu & Ho (1996b), *Gentianopsis* by Liu & Ho (1997), *Halenia* by Xue, Ho & Liu (1999b), *Lomatogonium* by Ho & Liu (2001), *Megacodon* by Xue & Li (2005), *Ptergyocalyx* by Chen, Ho & Hong (1998), *Tripterospermum* by Chen *et al*. (1999, 2000b), and *Veratrilla* by Xue & Li (2005). Most of the embryological features are compared in Table 2. Common features of the *Swertia s.l.* species investigated, which are also shared by other taxa in the tribe Gentianeae, include: anther tetrasporangiate; anther wall formation of dicotyledonous type; glandular tapetum; simultaneous cytokinesis following meiosis of the microsporocytes; tetrahedral microspore tetrads; superior, bicarpellary and unilocular ovaries; unitegmic and tenuinucellar ovules; *Polygonum*-type megagametophytes; progamous fertilization; nuclear endosperm; and Solanad-type embryogeny. As shown in Table 2, the variable features of *Swertia* listed earlier all occur in other genera, for example, the number of cell layers of the anther wall; construction of the wall of the mature anther; cell numbers in mature pollen grains; structure of the fused margins of the two carpels; the number of ovules in placental cross-sections; shape of the mature embryo sac; ovule type; presence of a hypostase; antipodal characteristics; and seed appendages. In addition to these variable characters, there are five features (sexuality, number of nuclei in tapetal cells, arrangement of microspores, functional megaspore, and type of embryogeny) that vary in the tribe Gentianeae.

According to the reviewed embryological data related to the taxa in the tribe Gentianeae, *Halenia* resembles *S. tetraptera* in most anther, ovule, and seed characters. In addition to sharing all the common embryological characters of the tribe Gentianeae, *S. tetraptera* resembles *Halenia* in certain features, including: fibrous thickening of the endothecium; uninucleate tapetal cells with dual origin; three-celled pollen grains; four rows of orthotropous ovules with parietal placentation; enlargement and protrusion of the fused margins of the two carpels; ovoid shape of the mature embryo sac; persistent hypastase; and smooth seeds without wings. Furthermore, the following combination of three embryological features was found only in *S. tetraptera* and *Halenia* of the Swertiinae: enlargement and protrusion of the fused margins of the two carpels in the ovary locule; orthotropous ovules; and the developed hypostase.

Taxonomic conclusions

Embryology is strongly in support of the current view that *Swertia s.l.* should not be considered as a monophyletic group (Yuan & Küpfer, 1995; Chassot *et al*., 2001; Liu *et al*., 2001; Hagen & Kadereit, 2002; Struwe *et al*., 2002). The differences that exist in embryological characters amongst *Swertia s.l.* species, such as the cell number in pollen grains, the degree of ovule curvature, and the shape of the mature embryo sac, are definitely major embryological features, probably ought to attract more attention, and are considered to be constant at the genus or even family level (Palser, 1975; Tobe, 1989; Johri *et al*., 1992). These many and important embryological differences that exist between species of *Swertia s.l.* are absolutely incompatible with the one-genus view. As embryological data do not support *Swertia s.l.*, this implies that it should be separated into different genera. The separation of *Swertia s.l.* into several genera accords with Cave's rule: the embryological characters of the species within a genus are constant (Cave, 1953).

As only nine of the 135 *Swertia s.l.* species have been studied embryologically, any evaluation based on embryology of the phylogenetic trends recognized within *Swertia s.l.* by Chassot *et al*. (2001) and Hagen & Kadereit (2002) would be premature. An analysis including more species of *Swertia s.l.* is necessary to clarify the phylogenetic and taxonomic status of the genus.

Swertia erythrosticta and *S. bifolia* are quite closely related, because of their overall embryological similarity in comparison with the other embryologically known species of *Swertia s.l.* These two species belong to sections *Swertia* and *Rogusa*. According to Hagen & Kadereit (2002), *Swertia s.s.* is a very large group, including much of the sections *Rugosa, Swertia*, and *Montana*, and contains mostly perennials with two fimbriate nectaries per petal lobe, but single or naked nectarines also occur. However, because the embryology of section *Montana* is unknown, generalization about the embryological synapomorphies of *Swertia s.s.* cannot be made. The slight variations described earlier, for example, fibrous thickening of the endothecium or not, and ephemeral middle layers (*S. erythrosticta*) or persistent middle layers with fibrous thickenings (*S. bifolia*), have no pattern and appear as species characters at most.

The genus *Frasera* was established by Walter (1788) with *F. caroliniensis* (= *S. caroliniensis*) as its type. Embryologically, there are some differences between *S. caroliniensis* and *S. bifolia*/*S. erythrosticta*, namely, one to four middle layers and one to four tapetal layers, whose cells are unitary in origin, two-celled pollen grains, and three or many

Table 2. Comparison of embryological characters of *Swertia* with other taxa of the tribe Gentianeae

	Subtribe Swertiinae									Subtribe Gentianinae		
Character	*Megacodon*	*Swertia*	*Halenia*	*Veratrilla*	*Gentianella*	*Comastoma*	*Lomatogonium*	*Gentianopsis*	*Pterygocalyx*	*Gentiana*	*Crawfurdia*	*Tripterospermum*
Sexuality: bisexual, 1; unisexual, 2	1	1	1	2	1	1	1	1	1	1	1	1
Anthers												
Type of wall development: dicotyledonous, 1; other, 2	1	1	1	1	1	1	1	1	1	1	1	1
Epidermis cell: persistent, 1; fibrous thickening, 2	1	1	1	2	2	2	1	2	2	1	2	1
Endothecium: fibrous thickenings, 1; no fibrous thickenings, 2	1	1	1	2	2	2	1	2	1	1	2	1
Middle layers: number	Many	1, 2, 3, or 4	2	2	2	1	1	2	1	2	1	1 or 2
ephemeral, 1; persistent and fibrous thickenings, 2	2	1 or 2	1	1	1	1	1	1	1	1	1	1
Tapetum origin: unitary, 1; dual, 2	2	1 or 2	2	2	1	1	2	2	2	2	1	1
type: glandular, 1	1	1	1	1	1	1	1	1	1	1	1	1
layer number	2	1	1	1	1	1	2	1	1–2	1	1	1
placentoids: formed, 1; not formed, 2	1	1	1	1	2	2	1 (2)	1	1	1	2	1, 2
number of nuclei in cell	1	1	1	1	1 (2)	2 (1)	1 (2)	2 (1)	2 (1)	2	1	1
Microsporogenesis: cytokinesis: simultaneous, 1; other, 2	1	1	1	1	1	1	1	1	1	1	1	1
Arrangement of microspores: all tetrahedral, 1; nearly always tetrahedral, rarely decussate, 2	2	1	1	2	1	1	1	1	1	1	1	1
Number of cells in a mature pollen grain: three, 1; two, 2	1	1 or 2	1	2	1	1	1	1	1	1 or 2	1	1
Ovule												
Placenta protrusion: no, 1; yes, 2	1	1 or 2	2	1	1	1	1	1	1	1	1	1

Type: anatropous, 1; ana-campylotropous 2; orthotropous, 3; hemianatropous, 4; campylotropous, 5; hypertropous, 6	1	1, 2, or 3	3	2	4	1	5	1	1	1	6	1, 6
Functional megaspore: first chalazal, 1; second to fourth chalazal, 2	1, 2	1	1	1	1	1	1	1	1	1, 2	1	1
No. of ovule rows: many rows, 1; 8 rows, 2; 4 rows, 3	1	1, 2 or 3	3	3	3	2	1	1	1	1	1, 2, 3	1, 2, 3
Number of integuments: one, 1; two, 2	1	1	1	1	1	1	1	1	1	1	1	1
Nucellus: tenuinucellate, 1; crassinucellate, 2	1	1	1	1	1	1	1	1	1	1	1	1
Shape of megaspore tetrads: linear, 1; others, 2	1	1	1	1	1	1	1	1	1	1	1	1
Type of megagametophyte development: *Polygonum*, 1; other, 2	1	1	1	1	1	1	1	1	1	1	1	1
Hypastase: none formed, 1; formed, 2	1	1 or 2	2	2	1	1	1	1	1	1	1	1
No. of antipodal cells: three, 1; many, 2	1	1 or 2	1	1	2	2	2	1 (2)	1	1 (2)	1	1
Antipodal cells: ephemeral, 1; persistent, 2	1	1 or 2	2	1	2	2	2	2	2	2	2	2
Shape of mature embryo sac: ellipsoid, 1; ovoid, 2	1	1 or 2	2	1	1	1	1	1	1	1		1
Seed												
Type of endosperm formation: nuclear type, 1	1	1	1	1	1	1	1	1	1	1	1	1
Type of embryogeny: Solanad *Physalis* I, 1; Solanad *Physalis* II, 2	?	2	2	2	2	2	2	2	2	1	1	1
Appendages of seed: winged, 1; wingless, 2	2	1 or 2	2	1	2	2	2	2	2	1 or 2	1	2
References	Xue & Li (2005)	See Table 1	Xue *et al.* (1999b)	Xue & Li (2005)	Liu & Ho (1996b)	Liu & Ho (1996a)	Ho & Liu (2001)	Liu & Ho (1997)	Chen *et al.* (1998)	Ho & Liu (1999)	Chen *et al.* (2000a)	Chen *et al.* (1999); Chen *et al.* (2000b)

persistent antipodal cells. This indicates that *Frasera* should not be considered as a section or group of *Swertia s.l.* (for example, Shah, 1992; Ho *et al*., 1994), but as a genus in its own right, as maintained by, for example, Toyokuni (1965), Threadgill & Baskin (1978), Wood & Weaver (1982), and Hagen & Kadereit (2002), as embryological characters are constant throughout a genus (Cave, 1953). Because of its unique combination of four-merous flowers, mostly one nectary per petal, weakly connate but not decurrent leaf bases, a filiform style, some gross morphological distinctions of pollen characters, and a basic chromosome number of 13 (Hitchcock, 1959; Nilsson, 1967; Wood & Weaver, 1982; Pringle, 1990), *Frasera* must be taxonomically accepted. However, embryology indicates more. As a section under *Swertia s.l*., section *Frasera* was considered to be closely related to section *Swertia* because of the sharing of seed characters (see, for example, Ho *et al*., 1994). The number and importance of embryological differences indicate that *Frasera* belongs in a major part of Swertiinae distant from the major part of *Swertia s.s.* This conclusion agrees fully with the results from molecular studies (Struwe *et al*., 2002).

The recognition of annual species of *Swertia* with often four-merous flowers as section *Ophelia* (Gilg, 1895), or even as a distinct genus (Toyokuni, 1963; Grossheim, 1967), does not appear to be justified, because species of such morphology sampled by authors (*S. angustifolia*, *S. corymbosa*, *S. franchetiana*, and *S. minor*) have strikingly different embryological characters, such as the number of cell layers that form the anther locule wall, construction of the wall of the mature anther, cell number in mature pollen grains, ovule number in placental cross-sections, and antipodal variation. The diverse embryology existing within this group supports the view that it is a loose group, despite similarities in gross morphology (Chassot *et al*., 2001; Hagen & Kadereit, 2002; Struwe *et al*., 2002).

Embryology is strikingly uniform throughout *S. tetraptera* and *Halenia*, by contrast with the large variation in gross morphology. *Halenia* is the only genus in the Gentianaceae to have a spur on each petal lobe, and therefore *Halenia* is quite distinct from *Swertia* in this respect. The very uniform embryology throughout the complex strongly supports the current opinion that the species of this complex constitute a coherent group, despite differences in gross morphology. In addition to having the following unique combination of three embryological features, which are considered to be synapomorphies within Swertiinae – enlargement and protrusion of the fused margins of the two carpels in the ovary locule, orthotropous ovules, and a developed hypostase – *S. tetraptera* is the closest living relative of *Halenia*. This conclusion agrees fully with results from molecular studies (Yuan & Küpfer, 1995; Chassot *et al*., 2001; Liu *et al*., 2001; Hagen & Kadereit, 2002; Struwe *et al*., 2002).

At present, the data on embryology cannot be evaluated taxonomically as only one species of *Comastoma* (25), *Gentianella* (250), *Gentianopsis* (24), *Halenia* (80), and *Lomatogonium* (21) have been studied in this regard, and, in the present study, only nine species of *Swertia s.l.* (of the 135 defined by Chassot, 2000) were sampled. An embryological analysis of most groups of *Swertia* s.*l.* and a re-analysis of morphological characters in the context of the Swertiinae are now underway.

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