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Sporogenesis and gametogenesis in *Sladenia* and their systematic implication

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Sporogenesis and gametogenesis in the monotypic genus *Sladenia* is reported for the first time. The anther wall is of the monocotyledonous type of formation and consists of four-layers, namely a glandular tapetum, one middle layer, an endothecium, and an epidermis. Fibrous thickenings are developed in the epidermis at or near the stage of anther maturity rather than in the endothecium. Successive cytokinesis in microsporogenesis results in tetrahedral or rarely isobilateral tetrads of microspores. Pollen grains are two-celled when shed. The ovule is bitegmic, tenuinucellate, and anatropous. The nucellus degenerates during meiosis of the megaspore mother cell. The micropyle is formed by only the inner integument. Development of the female gametophyte conforms to the Adoxa type. *Sladenia* has been treated either as a member of Actinidiaceae, Dilleniaceae or Theaceae *s.l.*, or as belonging to an independent family, Sladeniaceae. Recent molecular studies have suggested that it belongs to Ternstroemiaceae within the Ericales *s.l*. Several noteworthy embryological characters are distinct from those of the other putatively related families and support the recognition of a separate family, Sladeniaceae. Additional embryological evidence confirms this conclusion. © 2003 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2003, **143**, 305–314.

ADDITIONAL KEYWORDS: Actinidiaceae – anther wall development – Dilleniaceae – embryology – Ericales *s.l.* – ovule development – Ternstroemiaceae – Theaceae *s.s*.

INTRODUCTION

The monotypic genus *Sladenia*, comprising only one species *S. celastrifolia*, is distributed in south-western China, northern Burma, and Thailand. It is an evergreen tree with serrate leaves and axillary dichasial cyme. The bisexual flower has five sepals and five petals. Ten to 13 stamens in one whorl have dilated filaments and sagittate and basifixed anthers with poricidal dehiscence. The superior ovary has an axial placenta with two ovules per locus. The dry fruit has ribs and persistent sepals.

Kurz (1873) originally placed *Sladenia* in Theaceae *s.l*. For over 100 years, its systematic position has been highly controversial, being moved into Dilleniaceae (Gilg, 1893) and later into Actinidiaceae as a subfamily, Sladenioideae (Gilg & Werdermann, 1925; Hutchinson, 1959). Airy Shaw (1965) considered that *Sladenia* differs from Actinidiaceae in morphology and raised it to the family rank, Sladeniaceae. Takhtajan (1987) originally preferred this treatment. Most taxonomists, however, still regarded it as a unique genus (Kobuski, 1951; Melchior, 1964; Dahlgren, 1975, 1980; Cronquist, 1981, 1988; Brummitt, 1992; Takhtajan, 1997) or a subfamily (Thorne, 2000) in Theaceae *s.l.*

These different opinions about familial affinity result from *Sladenia*'s unusual combination of morphological features (Keng, 1962), wood anatomy (Deng & Bass, 1990, 1991), pollen morphology (Wei *et al*., 1999), and cytology (Li, 2001; Li, Liang, & Peng, 2003). This collection of characters differs from that of the families in which it has been tentatively placed. For example, the dichasial cyme, the reduced number of stamens, the dilated filaments, the forked anther, the ribbed dry fruit, the usual lack of the foliar sclereids, the high degree of vessel grouping, the opposite to alternate intertracheary pits, the chromosome number ($x = 12$, $2n = 48$), and karyotype are peculiar in the other related families.

Molecular data support treating two main subfamilies of Theaceae *s.l.* as two separate families, namely Theaceae *s.s.* and Ternstroemiaceae (APG, 1998;

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Prince, 2000; Savolainen *et al*., 2000; Soltis *et al*., 2000; Prince & Parks, 2001). Molecular studies on *Sladenia* have been limited. APG (1998) placed it under 'families of uncertain position', instead of resolving its relationship. More recent studies (Savolainen *et al*., 2000; Anderberg, Rydin & Källersjö, 2002) suggest *Sladenia* be put into Ternstroemiaceae within Ericales *s.l.*, but this needs further confirmation.

This study aims to investigate sporogenesis and gametogenesis of *Sladenia* and to compare them with those of the supposedly related taxa to elucidate its systematic position.

MATERIAL AND METHODS

Floral buds and flowers of *Sladenia celastrifolia* at different developmental stages were collected every 2 weeks from 1998 to 2000. The material was collected from Jingdong and Yimen counties, Yunnan Province, China. The voucher specimens for *Sladenia celastrifolia*, H. Peng 2644 and Li Lu. 1999-5-16 are deposited in Kunming Institute of Botany Herbarium.

Floral material was fixed in FAA (four parts formalin, six parts glacial acetic acid, 90 parts 50% ethanol), dehydrated through an alcohol series, and embedded in paraffin. Sections were prepared at a thickness of 5–8 μm and stained with Heidenhain's iron-alum haematoxylin and Orange G. The anther for a smear preparation was fixed in FAA and transferred to 70% ethanol. The anther smear was stained with acetocarmine in order to clarify successive meiosis. The samples were observed and photographed with an LM Olympus BX51 microscope.

RESULTS

Anther wall development

In the young anther, the outer secondary parietal layer develops directly into an endothecium, whereas the inner produces one middle layer and a tapetum (Fig. 1). The anther wall is composed of four cell layers, including an epidermis, an endothecium, one middle layer, and a tapetum (Fig. 2). Therefore, anther wall formation conforms to the monocotyledonous type (Davis, 1966). As the microspore mother cells begin to undergo meiotic division, the epidermal cells enlarge and the middle layer cells become compressed. The tapetal cells are uninucleate and stay intact, being of the glandular type. At the stage of microspore tetrads, some epidermal cells begin to develop fibrous thickenings on the walls, and the tapetal cells begin to degenerate (Fig. 3). The middle layer almost completely disappears at the microspore stage (Fig. 4). With the further development of microspores, the tapetum gradually collapses. As a result, the mature anther wall is composed of an epidermis and an endothecium at the stage of two-celled pollen grains (Fig. 5). At or near the mature anther stage, further epidermal cells acquire fibrous thickenings on the walls, and endothecial cells begin to degenerate (Fig. 6).

Microsporogenesis and male gametogenesis

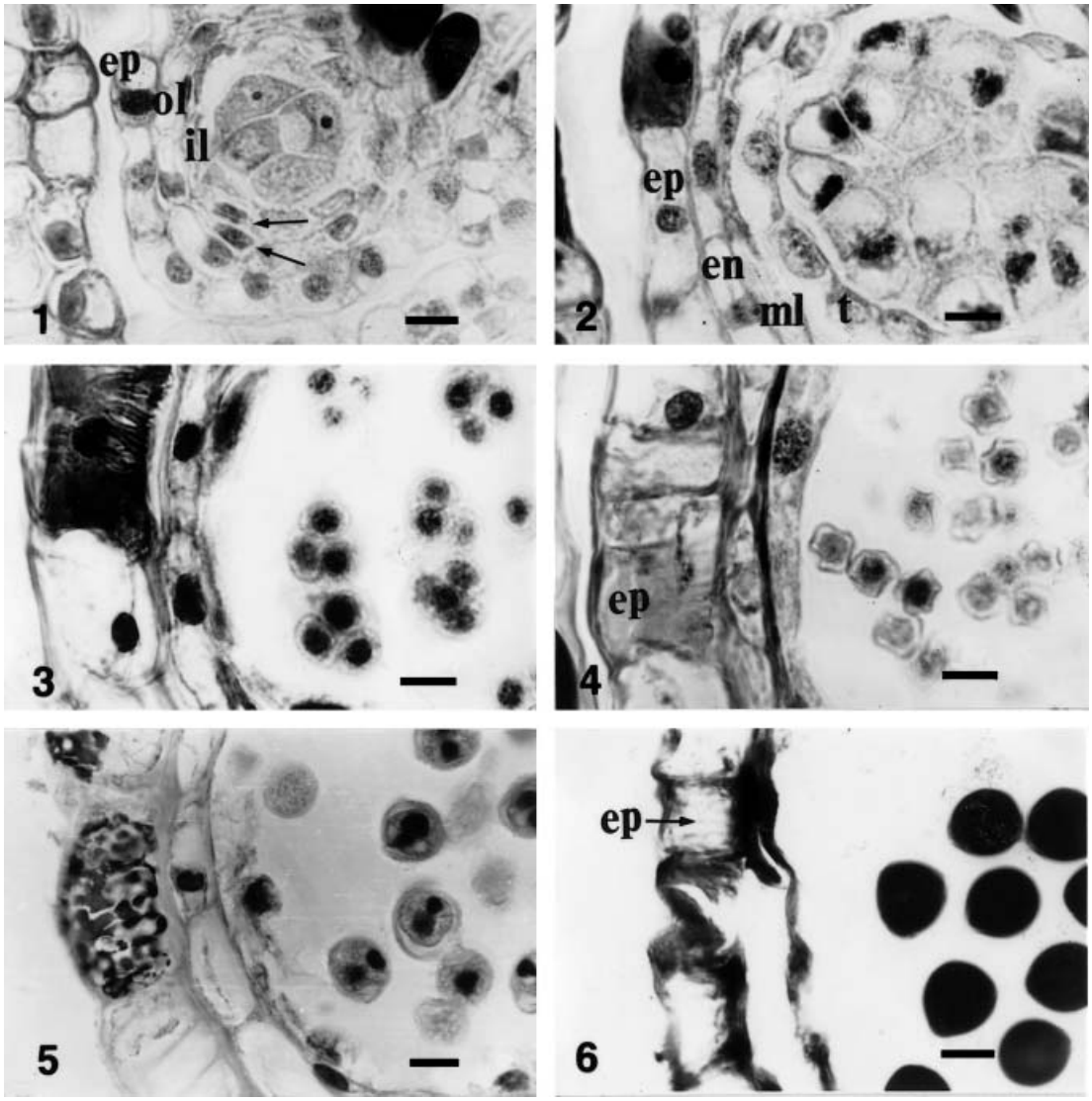
At the early stage, the microspore mother cells are arranged compactly (Fig. 7). Shortly after their maximal enlargement, they undergo meiosis accompanied by successive cytokinesis. After the first meiotic division of the microspore mother cell, a cell wall develops and a dyad is formed (Figs 8–10). The second meiotic division gives rise to a tetrahedral or isobilateral tetrad (Figs 3, 11). Subsequently, uninucleate microspores separate from the tetrads (Figs 4, 12). Mitosis in the microspore gives rise to a large vegetative and a small generative cell (Figs 5, 13). When shed, the pollen grains are two-celled and tricolpate (Fig. 14).

Ovule development

The periclinal division of some cells under the placental epidermis leads to the formation of ovule primordia. The inner integument is initiated from dermal cells at the base of the ovule primordia earlier than the outer one. The outer integument always grows more slowly than the inner. Both integuments have at first two layers of cells, and later become 3–4 layered. The inner integument soon encloses the nucellus and forms the micropyle (Fig. 15). As a hypodermal archesporial cell enlarges directly as a megaspore mother cell, the ovule becomes fully anatropous (Figs 15, 16). Therefore, the ovule is anatropous, bitegmic, and tenuinucellate with a micropyle formed by the inner integument.

Megasporogenesis and female gametogenesis

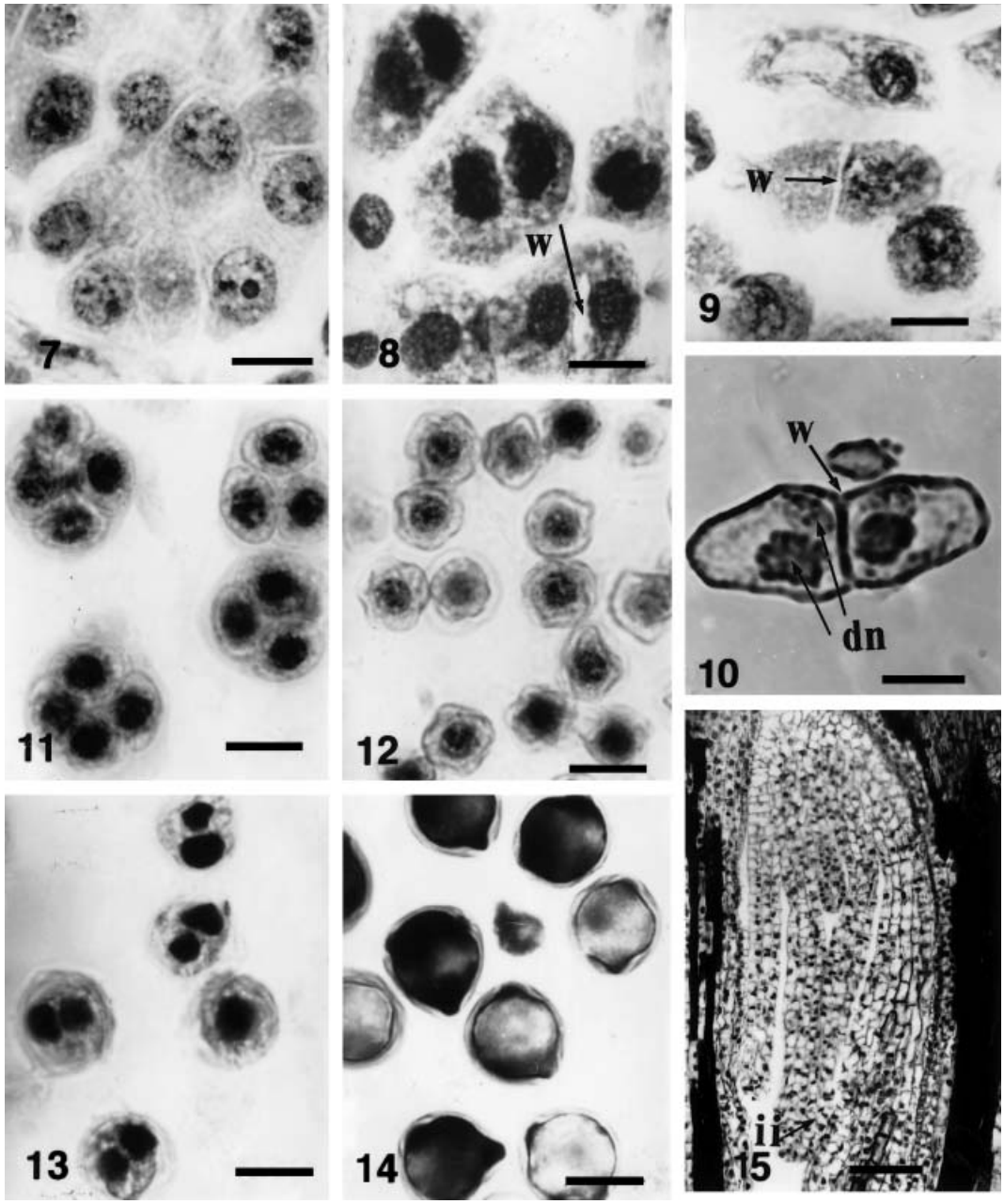
In the tenuinucellate ovule, an archesporial cell functions directly as a megaspore mother cell (Fig. 16). Cytokinesis does not follow the meiotic division in the megaspore mother cell such that there is no dyad or tetrad. The first division produces two megaspore nuclei (Fig. 17), which, respectively, move to each pole (Fig. 18) and undergo the second meiotic division, giving rise to a four-nucleate female gametophyte (Figs 19, 20), in a 2 + 2 arrangement (Fig. 21). An additional division of these nuclei results in an eight-nucleate megagametophyte (Figs 22–24). One nucleus from each pole moves to the centre of the embryo sac to function as the polar nuclei. The three nuclei at the micropylar end differentiate as the egg apparatus, whereas the three nuclei at the chalazal end become



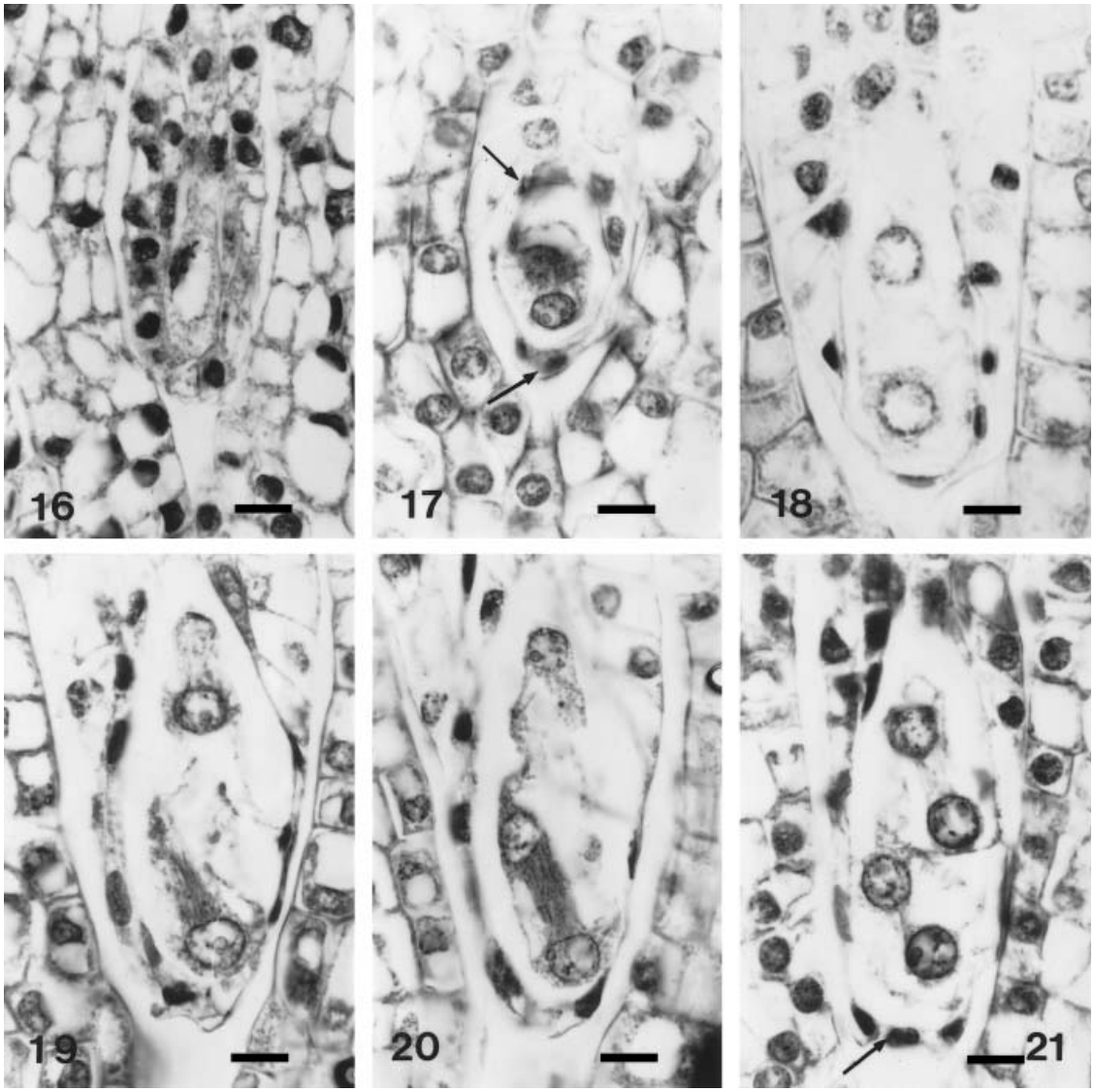
Figures 1–6. Development of anther wall in *Sladenia celastrifolia*. Scale bars = 10 μm. Fig. 1. Three-layered anther wall at pollen mother cell stage, including one epidermal layer, an outer secondary parietal layer, and an inner secondary parietal layer. Note: one inner layer cell has just divided into two daughter cells (arrows), indicating the middle layer and the tapetum from the inner secondary parietal layer cells. Fig. 2. Four-layered anther wall at the stage of meiosis of microsporocytes, showing one epidermal layer, one of endothecium, one middle layer, and a tapetum. Fig. 3. Anther wall at the stage of microspore tetrads, showing enlarged epidermis with some fibrous thickening. Fig. 4. Anther wall at the early stage of microspores, showing absorbed middle layer. Fig. 5. Anther wall at the stage of two-celled pollen with degenerating glandular tapetum. Fig. 6. Anther wall at the stage of mature pollen showing degenerating endothelium and fibrous thickened epidermis. Abbreviations: en, endothelium; ep, epidermis; il, inner secondary parietal layer; ml, middle layer; ol, outer secondary parietal layer; t, tapetum.

the antipodal cells (Figs 25–27). Careful studies involving over 1000 sections of 300 embryo sacs collected during three continuous years show no degenerated megaspores. That is, in *Sladenia*, all four megaspore nuclei produced by the meiotic divisions of a megasporocyte undergo just one more division to form an eight-nucleate female gametophyte and lack any degenerated megaspores. The type of megagametophyte development conforms to the tetrasporic Adoxa type (Maheshwari, 1950; Hu, 1982; Johri, Ambegaokar & Srivastava, 1992).

With the development of the megagametophyte, the nucellar epidermis degenerates gradually as the cell remnants darken. Some cells in the calyx, epidermis of anther wall, anther connective, epidermis of ovary, and style are heavily tanniniferous. None of the fol-



Figures 7–15. Development of male gametophyte in *Sladenia celastrifolia*. All except Fig. 10 represent transverse sections of anthers. Fig. 7. Microspore mother cells. Scale bar = 10 μm. Figs 8–10. Meiosis in the microsporocyte is accompanied by successive cytokinesis. Figs 8, 9. Meiosis I is followed by wall formation. Scale bar = 10 μm. Fig. 10. Smear of fresh anther, showing a dyad with a wall (arrow) and two daughter nuclei (arrows) produced by meiosis II. Scale bar = 40 μm. Fig. 11. Tetrahedral microspores, largely of the tetrahedral type but with some isobilateral types. Scale bar = 10 μm. Fig. 12. Recently dispersed microspores. Scale bar = 10 μm. Fig. 13. Two-celled pollen grains. Scale bar = 10 μm. Fig. 14. Mature tricolporate pollen grains. Scale bar = 10 μm. Fig. 15. Longitudinal section of mature ovule. Anatropous, bitegumental ovule with micropyle formed by inner integument. Scale bar = 70 μm. Abbreviations: dn, daughter nuclei; w, wall; ii, inner integument.

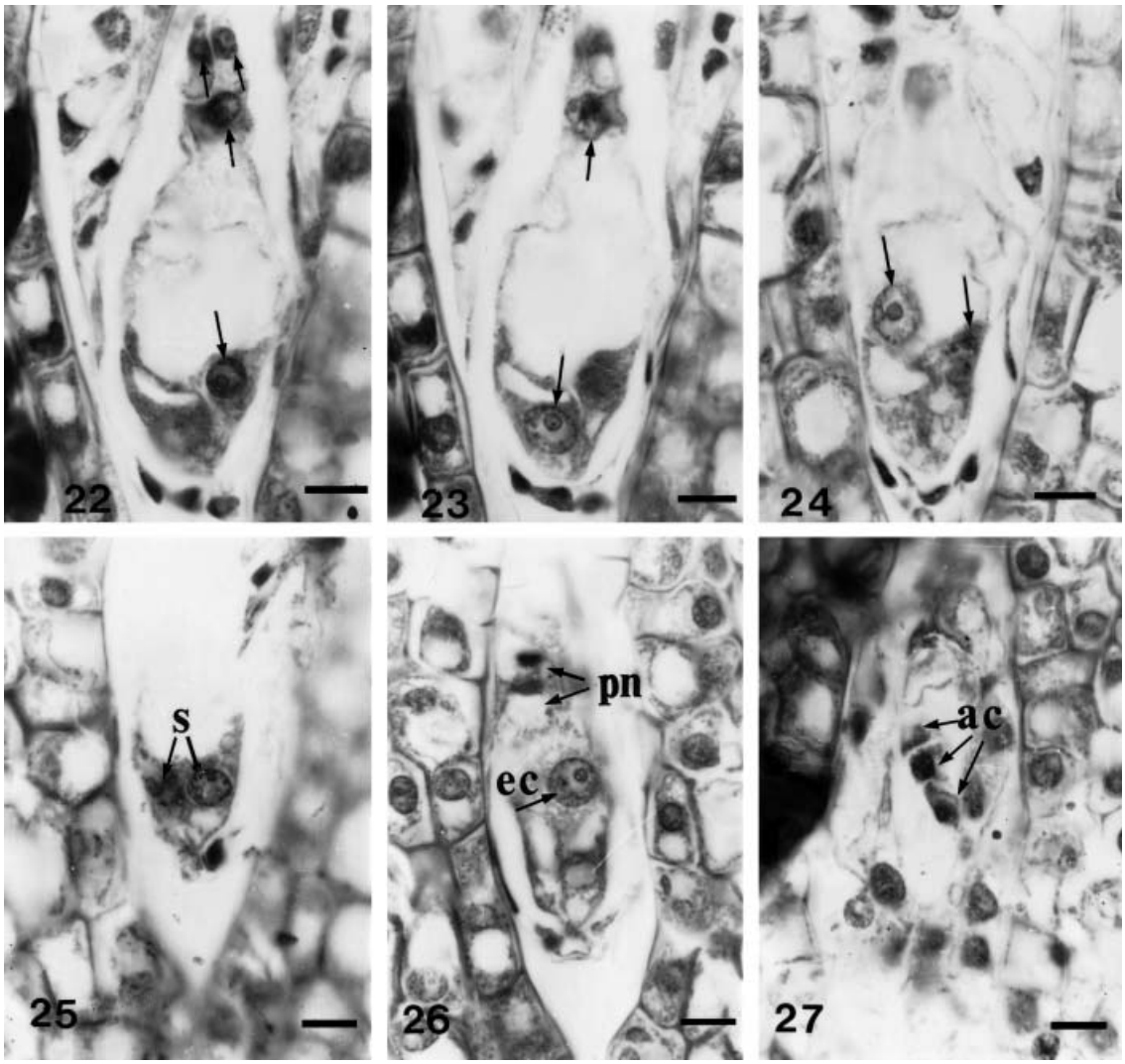


Figures 16–21. Longitudinal sections of ovules. Adoxa type of megagametophyte development. The micropyle is toward the base. Scale bars = 10 μm. Fig. 16. The megaspore mother cell as the functional megasporocyte in the tenuinucellate ovule. Fig. 17. Two-nucleate embryo sac, showing the nucellus starting to degenerate (arrows). Fig. 18. Two nuclei moving to each pole while the embryo sac is extending. Figs 19, 20. Two nuclei at each pole are dividing. Fig. 21. Four-nucleate embryo sac with 2 + 2 arrangement and the remnants of nucellus cells (arrow).

lowing structures has been observed in the ovules: crystals, hypostase, endothelium tapetum, epistase, nucellar beak, nucellar cap, or obturator. Owing to the lack of further development of the megagametophyte in *Sladenia*, the development of endosperm and embryo could not be investigated.

DISCUSSION

Comparative embryology has been an important factor in revealing taxonomic relationships of taxa at higher levels, especially of highly isolated taxa (Palser, 1975; Herr, 1984; Tobe, 1989; Kapil & Bhatnagar, 1991; Johri *et al*., 1992). Comparative data are summarized in Table 1 based on literature for Actinidiaceae (Vijayaraghavan, 1965; An, Cai & Wang, 1983; Zhang, 1987; Johri *et al*., 1992), Dilleniaceae (Rao, 1955, 1957, 1961; Swamy & Periasamy, 1955; Sastri, 1958; Johri *et al*., 1992), Theaceae (Kapil & Sethi, 1963; Cao, 1965; Mathew, 1978; Liu & Zhang, 1983; Li & Cao, 1986; Johri *et al*., 1992; Yang & Ming, 1995; Tsou, 1997), Ternstroemiaceae (Tsou, 1995), and the present study.



Figures 22–27. Longitudinal sections of ovules. Adoxa type of megagametophyte. The micropyle is toward the base. Scale bars = 10 μm. Figs 22–24. Eight-nucleate stage with four nuclei lying at each pole and the remains of nucellus cells. Fig. 22. Three nuclei at the chalaza and one nucleus at the micropyle (arrows). Fig. 23. One nucleus at the chalaza and one at the micropyle (arrows). Fig. 24. The two nuclei at the micropyle (arrows). Figs 25–27. Mature embryo sac with an egg apparatus, two polar nuclei, and the three antipodal cells. Fig. 25. Two synergids at the micropyle. Fig. 26. One egg cell at the micropyle and the two polar nuclei in the middle of the embryo sac. Fig. 27. Three antipodal cells at the chalaza. Abbreviations: ac, antipodal cells, ec, egg cell; pn, polar nuclei; s, synergids.

The comparative study shows several significant embryological features of *Sladenia*, which are markedly different from those of Actinidiaceae, Dilleniaceae, Theaceae *s.s.*, and Ternstroemiacaeae. First, there are four autapomorphies in *Sladenia*: monocotyledonous type of anther wall formation, fibrous thickenings in the epidermis of anthers, successive cytokinesis during microsporocyte meiosis, and the Adoxa type of megagametophyte development. In addition, there are other embryological traits different from those of the four other related families, respectively. These will be discussed in detail below.

Monocotyledonous type of anther wall development

Anther wall development of *Sladenia* is quite different from the four related families, Actinidiaceae, Dilleniaceae, Ternstroemiacaeae, and Theaceae *s.s.* In *Sladenia*, there is only one middle layer in the anther wall, and it is derived from the inner secondary parietal layer. The anther wall formation in *Sladenia* then conforms to the monocotyledonous type. By contrast, in the four related families, there are 2–4 middle layers in the anther walls, and they originate

Table 1. Comparative embryology of *Sladenia* and the four other families

	Sladenia	Actinidiaceae	Dilleniaceae	Ternstroemiaceae	Theaceae
Anther wall formation	Monocotyledonous	Basic	Basic	Basic, rarely dicotyledonous	Basic
Fibrous thickenings in:	Epidermis	Endothecium	Endothecium, rarely epidermis	Endothecium	Endothecium
Middle layer	1 layer	2 layers	2, or 3 layers	1, or 2 layers	2, 3, or 4 layers
Cytokinesis in meiosis	Successive	Simultaneous	Simultaneous	Simultaneous	Simultaneous
Type of anther dehiscence	Poricidal	Longitudinal split and poricidal	Longitudinal split and poricidal	Longitudinal split	Longitudinal split
Pseudopollen	Absent	Absent	Absent	Absent	Present
Ovule	Anatropous	Anatropous	Anatropous to campylotropous	Amphitropous	Anatropous
Nature of nucellus	Tenuinucellate	Tenuinucellate	Crassinucellate	Tenuinucellate	Tenuinucellate
Integument	Bitegmic	Unitegmic	Bitegmic	Bitegmic	Bitegmic
Micropyle formed by:	Inner integument	Unitegument	Both integuments	Inner integument	Inner integument
Hypostase	Absent	Present	Absent	Absent	Present
Type of embryo sac	Adoxa	Polygonum	Polygonum	Polygonum	Polygonum or rarely Allium
Endothelium	Absent	Present	Absent	Present	Present

from both inner and outer secondary parietal layers. This kind of anther wall formation is attributed as the basic type. Although a single middle layer also occurs in *Eurya* of Ternstroemiaceae, it originates from the outer secondary parietal layer (Tsou, 1995). This kind of anther wall development is termed the dicotyledonous type.

Epidermal fibrous thickenings

Secondary thickenings in the anther wall show a discrepancy between *Sladenia* and the four related families. First, in *Sladenia* the fibrous thickenings develop in the epidermal cells, whereas they are absent in the four related families with the exception of *Wormia burbigei* in Dilleniaceae (Rao, 1961). It is highly unusual among angiosperms that fibrous thickenings occur only in the epidermis of the anther wall rather than in the endothecium. A similar feature occurs in some Zingiberaceae, but here the fibrous thickenings also occur in the endothecium and the outer middle layer (Johri *et al*., 1992). Secondly, *Sladenia* has porate anther dehiscence without endothecial thickenings, whereas Theaceae, most Ternstroemiaceae, most Dilleniaceae, and *Actinidia* have longitudinal anther dehiscence with endothecial thickenings. Some Ternstroemiaceae (*Eurya* and some *Cleyra*) and some Dilleniaceae (*Dillenia indica*) are devoid of fibrous thickenings (Rao, 1961; Tsou, 1995).

Successive cytokinesis of microsporocyte meiosis

A difference in meiotic cytokinesis of microspore mother cells is apparent between *Sladenia* and the four related families. In *Sladenia*, meiosis of the microspore mother cell is accompanied by successive cytokinesis. A cell wall is laid down immediately after the first meiotic division and another in each of the two daughter cells after the second meiotic division. By contrast, the four related families are characterized by simultaneous cytokinesis such that no wall is laid down after the first division and the mother cell becomes separated instantaneously into four parts following both the meiotic divisions.

Adoxa type of female gametogenesis

Megagametogenesis of *Sladenia* is quite different from that of the four related families. Female gametophyte development in *Sladenia* is the tetrasporic Adoxa type, whereas in members of the four families it is the monosporic Polygonum type. An exception is the bisporic Allium type in *Camellia* of Theaceae *s.s*. Although these three types finally produce an eight-nucleate embryo sac having a normal egg apparatus, three antipodal cells, and two polar nuclei, they go through different cellular divisions. In the four related families with the Polygonum type, the megasporocyte produces a megaspore tetrad through meiotic division and cytokinesis. The nucleus of the chalazal

megaspore undergoes three consecutive divisions with a resulting eight-nucleate embryo sac. The other three megaspores degenerate. In *Camellia* of the Allium type, the megasporocyte divides to form two dyad cells, of which the upper soon degenerates whereas the lower divides to form two, four and then eight nuclei giving rise to an embryo sac with the usual organization. By contrast, in *Sladenia* of the Adoxa type, a megaspore mother cell produces four megaspore nuclei without cytokinesis after the meiotic divisions. All four megaspore nuclei undergo additional division resulting in an eight-nucleate female gametophyte with neither cytokinesis nor degenerated megaspores. The Adoxa type of female gametophyte is very rare among angiosperms, having been reported with certainty in four genera only (*Adoxa*, *Sambucus*, *Erythronium*, and *Tulipa*) (Maheshwari, 1950; Haig, 1990). The Polygonum type of female gametophyte is the most common in angiosperms and is considered as a primitive embryological character, whereas the Allium type and the Adoxa type are considered derived. The Allium type is more common than the Adoxa type (Palser, 1975).

These embryological features of *Sladenia*, almost absent in the four related families, can be considered as autapomorphies of the genus and as such strongly support segregating it into its own family, Sladeniaceae.

Additional evidence for independence of Sladeniaceae

Besides the features mentioned above, some other embryological characters can be distinguished from those of the four related families, thus providing further evidence for the independence of Sladeniaceae.

Sladenia differs from Actinidiaceae in having bitegmic rather than unitegemic ovules, absence of crystals, hypostase and endothelium tapetum. Van Tieghem (1899) moved *Actinidia* out of Dilleniaceae as a separate family only because of its single integument that is unlike the double integuments in Dilleniaceae (Vijayaraghavan, 1965). Such differences further suggest that *Sladenia* should not be assigned to Actinidiaceae.

Sladenia was once placed in Dilleniaceae (Gilg, 1893), although later authors have not preferred this assignment. There are obviously differences in embryology between them. Dilleniaceae have parietal placentation, crassinucellate campylo-amphitropous ovule, and zigzag micropyles formed by both integuments. By contrast, *Sladenia* possesses an axial placenta, tenuinucellate anatropous ovule, and micropyle formed by inner integument only. These differences argue against including *Sladenia* in Dilleniaceae.

Sladenia and Theaceae *s.s.* are clearly distinguished by embryological characters. The latter is characterized by pseudopollen and stomata in anther connectives, integumentary tapetum, and hypostase. These features are absent in *Sladenia*. Pseudopollen originates from the anther connective cells. It is an autapomorphy of Theaceae *s.s.* (Camellioideae) and has thus far been determined only for this family (Tsou, 1997). Thus, it is highly unlikely that *Sladenia* is allied to the Theaceae *s.s*.

Ternstroemiaceae (Ternstroemioideae) is characterized by a large number of crystals in anther connectives and the anther epidermis, a campylotropous ovule, and antipodal haustorium (Tsou, 1995). These features are absent in *Sladenia*. Molecular study indicates that *Sladenia* is a sister group to Ternstroemiaceae, but it gives low support for the treatment of *Sladenia* as a member of this family (Savolainen *et al*., 2000; Anderberg *et al*., 2002). Indeed, both the autapomorphies and the discrepancy between *Sladenia* and Ternstroemiaceae do not favour this suggestion.

The embryological features reported here suggest that *Sladenia* is markedly distinguished from the presumed closely related families. On the basis of its unique collection of embryological, morphological, and anatomical traits, *Sladenia* should be properly retained in its own family, Sladeniaceae. A cladistic study based on morphological and molecular data will provide further clues to its phylogeny.

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