



Anther wall formation, sporogenesis and gametogenesis of *Tripterospermum chinense* (Gentianaceae), with emphasis on uniformity of characters in *Tripterospermum* and its systematic values

C. Ao¹

Key words

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Abstract *Tripterospermum sensu* T.N.Ho comprises two sections that are readily discriminated morphologically. In the present paper, embryological characters in anther wall formation, sporogenesis and gametogenesis of *Tripterospermum chinense*, a member of sect. *Platyspermum*, are reported. Comparisons based on available data show that as far as the two recognized sections are concerned, embryological characters are uniform in all aspects, disapproving of the splitting of the genus into two sections. The notable differences between *Tripterospermum* and closely related genera support the phylogenetic conclusion that *Tripterospermum* is monophyletic.

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INTRODUCTION

Tripterospermum was established by Blume (1826) based on a single species, *T. trinerve* Blume from Java. Since then, its taxonomic position has long been in dispute, of which the focus is whether it is a valid genus. Clarke (1875) placed it in *Crawfordia* Wall. as a section, followed by Kuznetsov (1894) and Gilg (1895) who viewed it as a subgenus in *Crawfordia*. Marquand (1931, 1937) transferred it to *Gentiana* (Tourn.) L. as a section, but many subsequent authors have retained it as a separate genus (e.g., Ho 1988, Ho & Liu 1990, Ho & Pringle 1995).

Wu (1984) established a new section, sect. *Platyspermum*, which together with sect. *Tripterospermum* is adopted by Ho (1988) in the Flora of China. Sect. *Platyspermum* C.J.Wu and sect. *Tripterospermum* are readily discriminated morphologically. Generally speaking, sect. *Platyspermum* produces capsules and broadly winged seeds, while sect. *Tripterospermum* has berries and narrowly winged or wingless seeds. However, no other evidence supports the recognition of two sections apart from fruits.

Embryological data may provide more than 50 characters associated with anther, ovule and seed development, and thus contribute to a better understanding of character evolution between and within families (Tobe 1989). Embryological characters were decisive in the classification of flowering plants (e.g., Cronquist 1981, 1988). Up to now, only two species of *Tripterospermum* have been investigated embryologically (Chen et al. 2000a, Huang et al. 2009, 2010), and the embryological information on the genus is still inadequate. Thus, an extensive study involving more species is needed in order to arrive at a comprehensive evaluation of the embryological characters in *Tripterospermum* and a discussion of their systematic value.

Tripterospermum chinense (Migo) H.Sm., a member of sect. *Platyspermum*, is a perennial and twining herb, of which the

inflorescence is generally a corymb of 2–3 aggregated flowers. Its Chinese name ‘Double butterflies’ was derived from two couples of decussate leaves arranged like two flying butterflies when the plants are young. It is traditionally used for the treatment of coughs, hemoptysis and pulmonary disease by local inhabitants.

In his synopsis of *Tripterospermum*, Murata (1989) suggests that *T. chinense* is a berry-carrying species, and includes it in sect. *Tripterospermum* as a synonym of *T. lanceolatum* (Hayata) Hara ex Satake. However, here we follow the Flora of China (Ho 1988, Ho & Pringle 1995) in which *T. chinense* is accepted as a valid capsule-bearing species clearly distinct from *T. lanceolatum*.

In the present paper, anther wall formation, sporogenesis and gametogenesis of *T. chinense* is reported. The aim of the present study is to: 1) describe the embryology of *T. chinense*; 2) check the uniformity of embryological characters in *Tripterospermum* to assess the value of the delimitation of the two sections; and 3) evaluate systematic values of embryological characters in discriminating *Tripterospermum* from closely related genera.

MATERIALS AND METHODS

Floral buds at different developmental stages, together with opened flowers were collected from 18 plants growing spontaneously in Shiyang forest spot, Wencheng, Zhejiang, China (27.453°N, 119.505°E, 810 m asl). The material was fixed and stored in FAA (5 ml formalin; 6 ml acetic acid; 89 ml 50 % ethanol), and embedded in paraffin using conventional methods. Sections with a thickness of 8–11 µm were made, stained with Heidenhain’s haematoxylin combined with safranin, observed and photographed under an Olympus BX-51 microscope. Vouchers (Ao Chengqi 10001) and permanent sections were deposited in the Herbarium of Wenzhou University (WZU).

Pollen fertility was estimated according to the method of Majumdar et al. (2004), with propionocarmine replaced by

¹ School of Life and Environmental Sciences, Wenzhou University, Zhejiang 325035, China; e-mail: aocq@wzu.edu.cn.

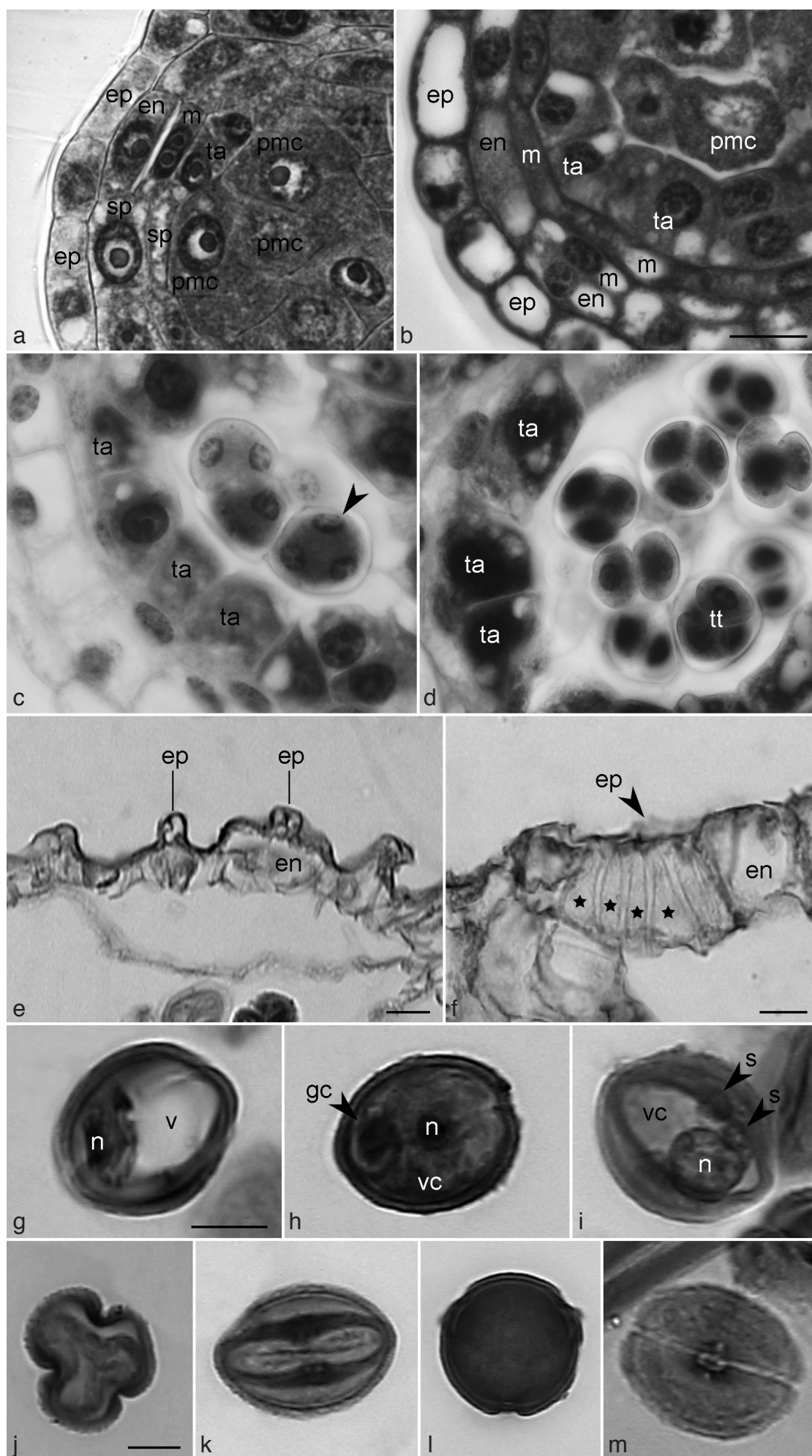


Table 1 Comparison of embryological characters in *Tripterosperrum* and related genera.

| | <i>Gentiana</i> ¹ | <i>Tripterosperrum</i> ² | <i>Crawfordia</i> ³ |
|----------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| Epidermis of anther wall | persistent | degenerated | persistent and fibrously thickening |
| Endothecium of anther wall | persistent and fibrously thickening | persistent and fibrously thickening | degenerated |
| Number of ovule rows | 10–15 | 4 or 8 | 8 |
| Placentae | superficial | parietal | parietal |

¹ Ho & Liu (1999).² The present study.³ Chen et al. (2000b).

acetocarmine. The naturally dehiscent anthers were mounted on slides and at least 200 pollen grains for each anther were examined. The numbers of fully stained pollen grains as well as hyaline grains were counted in the optical field to determine the percentage of fertile grains.

RESULTS

Anther wall formation, microsporogenesis and male gametogenesis

Flowers of *T. chinense* are bisexual and protandrous. Anthers are tetrasporangiate.

At the early stage of development, a row of archesporial cells in each sporangium differentiates under the epidermis. These cells divide periclinally forming outer primary parietal cells and inner primary sporogenous cells. The primary parietal cells divide periclinally and anticlinally forming two layers of the secondary parietal cells (Fig. 1a). The outer secondary parietal cells form a subepidermal endothecium and a middle layer (Fig. 1a). The middle layer has a common origin with the endothecium, so the microsporangial wall formation follows the Dicotyledonous type (Davis 1966). Thus the anther wall is composed of 4–5 layers; from the exterior, the epidermis, endothecium, 1–2 middle layers and tapetum (Fig. 1b). Tapetal cells are uninucleate throughout their development (Fig. 1b, c). At the time of pollen tetrads, the tapetum degenerates *in situ* (Fig. 1d), thus belonging to glandular type. There are one or two middle layers (Fig. 1b) of which cells were often binucleate (Fig. 1a). When the anther wall reaches maturity, the endothecium persists and the cells become pillar and fibrous while the epidermis degenerates (Fig. 1f).

The primary sporogenous cells undergo mitosis, forming secondary sporogenous cells, from which pollen mother cells (PMC) are derived. Meiosis in each PMC leads to a tetrahedral tetrad (Fig. 1d). No cell wall could be detected between the four newly-formed nuclei in telophase of the second division of meiosis in PMCs (Fig. 1c). Clearly the cytokinesis is of the simultaneous type.

Microspores are separated from the tetrad as uninucleate pollen grains. Each microspore has a dense cytoplasm with a prominent and centrally placed nucleus. As the central vacuole develops, the nucleus takes up a peripheral position (Fig. 1g). The first division of the microspore nucleus leads to two unequal

cells, a large vegetative one and a smaller generative one (Fig. 1h). The generative cell gives rise to two sperm cells by mitosis. Pollen grains are 3-celled at time of anther dehiscence (Fig. 1i). Two types of mature pollen grains could be recognized. The first type is deep-tricolporate, with deep lying colpi in polar view (Fig. 1j) and elliptical in equatorial view (Fig. 1k). The second type is shallow-tricolporate, also elliptical in equatorial view (Fig. 1m), but with shallow colpi in polar view (Fig. 1l). The estimation of pollen fertility showed a high percentage of fertile grains (97.5 %), indicating that both deep-tricolporate and shallow-tricolporate pollen grains are fertile.

Megasporogenesis and female gametogenesis

The gynoecium of *T. chinense* is bicarpellary and syncarpous. The ovary is superior and unilocular with two parietal placentae. In cross-section, there are 4 or 8 rows of ovules (Fig. 2a, b). The ovule is unitegmic (Fig. 2c). The integument initiates at the base of nucellus and reaches the top of nucellus to form a microphyle (Fig. 2d). When the hypodermal archesporial cell appears, the whole ovule body begins to reverse (Fig. 2c). With the maturation of embryo sacs, some of the ovules curve further. Hereby two types of ovules viz. anatropous and hypertropous *sensu* Chen et al. (2000a) were detected (Fig. 2a, b).

A single hypodermal archesporial cell differentiates in the young nucellus and functions directly as the megasporocyte (Fig. 2c). Thus the ovule is tenuinucellate. The megasporocyte undergoes meiosis (Fig. 2d), forming a linear tetrad of megaspores. The three micropylar megaspores eventually degenerate, while the chalazal one becomes functional (Fig. 2e). The functional megaspore divides by mitosis, via a binucleate embryo sac (Fig. 2f) and a tetranucleate embryo sac (Fig. 2g, h), to form a mature embryo sac of the *Polygonum* type (Fig. 2i).

DISCUSSION

Systematic implications of embryological characters in Tripterosperrum and related genera

Embryological characters prove to be useful in discriminating *Tripterosperrum* from the closely related genera *Gentiana* and *Crawfordia* (Table 1).

Phylogenetic studies have shown that *Tripterosperrum* is monophyletic (Favre et al. 2010). The notable embryological differences between *Tripterosperrum* and closely related genera

Fig. 1 Anther wall formation, microsporogenesis, microgametogenesis and morphology of pollen grains of *T. chinense*. a. The endothecium and middle layer are derived from the division of outer secondary parietal cells, indicating the development of anther wall follows the Dicotyledonous type; b. the young anther wall composed of epidermis, endothecium, middle layer and tapetum. Note two middle layers and uninucleate cells of tapetum; c. meiosis of pollen mother cells (PMCs). The arrow head points to a PMC at telophase II of meiosis; d. tapetum disintegrates *in situ* (glandular tapetum); e. the epidermis is degenerating and the endothecium has not yet developed fibrous thickenings; f. the epidermis has degenerated and the endothecium has developed fibrous thickenings (*); g. uninucleated microspore with a big vacuole; h. 2-celled pollen grain composed of a vegetative cell and a generative cell; i. 3-celled pollen grain with a vegetative cell and two sperms; j. deep-tricolporate pollen grain in polar view; k. deep-tricolporate pollen grain in equatorial view; l. shallow-tricolporate pollen grain in polar view; m. shallow-tricolporate pollen grain in equatorial view. — Abbreviations used: en = endothecium; ep = epidermis; gc = generative cell; m = middle layer; n = nucleus; pmc = pollen mother cell; s = sperm; sp = secondary parietal cells; ta = tapetum; tt = tetrad; v = vacuole; vc = vegetative cell. — Scale bars: b (also applies to a, c, d) = 25 µm; e = 30 µm; f = 15 µm; g (also applies to h, i), j (also applies to k, l, m) = 10 µm.

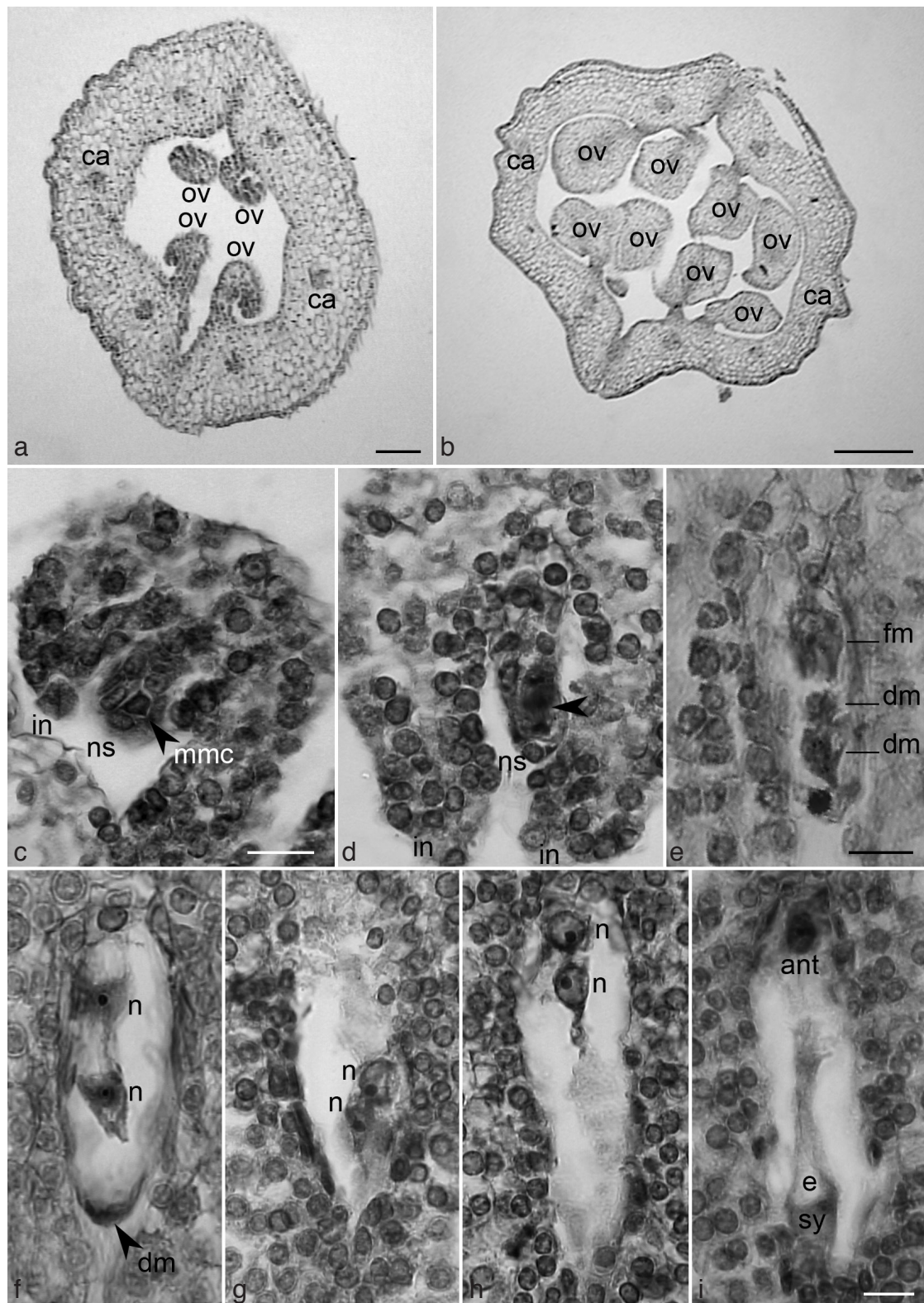


Fig. 2 Transections of young ovaries, megasporogenesis and megagametogenesis. a. Transection of a young ovary shows 4 rows of ovules in the locule; b. transection of another young ovary shows 8 rows of ovules in the locule; c. ovule at the stage of megaspore mother cell (MMC); d. MMC undergoing meiosis (arrow head); e. megaspores at linear tetrad stage; f. binucleate embryo sac and degenerated megaspores at the micropylar end, suggest that the development of the embryo sac follows the *Polygonum* type; g, h. successive sections show a tetranucleate embryo sac; i. mature embryo sac. No polar nucleus is shown in this section. — Abbreviations used: ant = antipodal cells; ca = carpel; dm = degenerated megaspore; e = egg cell; fm = functional megaspore; in = integument; mmc = megaspore mother cell; n = nucleus; ns = nucellus; ov = ovule; sy = synergid. — Scale bars: a = 100 μ m; b = 250 μ m; c (also applies to d) = 30 μ m; e (also applies to f, g, h), i = 15 μ m.

Table 2 Embryological characters in *Tripterospermum* and their consistency at generic level.

| | Sect. <i>Platyspermum</i> | | Sect. <i>Tripterospermum</i> | Character consistency ¹ |
|---|---------------------------------|-----------------------------------|---------------------------------|------------------------------------|
| | <i>T. chinense</i> ² | <i>T. discoideum</i> ³ | <i>T. cordatum</i> ⁴ | |
| Sporangia in each stamen | 4 | 4 | 4 | + |
| Type of anther wall development | dicotyledonous | dicotyledonous | dicotyledonous | + |
| Epidermis | degenerate | degenerate | degenerate | + |
| Endothecium | developing fibrous thickenings | developing fibrous thickenings | developing fibrous thickenings | + |
| Type of tapetum | glandular | glandular | glandular | + |
| Cytokinesis in PMC meiosis | simultaneous | simultaneous | simultaneous | + |
| Predominant shape of microspore tetrads | tetrahedral | tetrahedral | tetrahedral | + |
| Number of cells in a mature pollen | 3 | 2, rarely 3 | 3 | (+) |
| Placentae | parietal | parietal | parietal | + |
| Number of ovule rows | 4 or 8 | 4 | 4 or 8 | (+) |
| Degree of ovule curvature | anatropous, Hypertropous | anatropous | hypertropous | – |
| Number of integuments | 1 | 1 | 1 | + |
| Type of nucellus | tenuinucellate | tenuinucellate | tenuinucellate | + |
| Type of embryo sac | polygonum | polygonum | polygonum | + |

¹ Character consistency: + = always consistent; (+) = basically consistent; – = not consistent.

² The present study.

³ Huang et al. (2009, 2010).

⁴ Chen et al. (2000a).

support the results from phylogenetic and morphological analysis and contribute to the growing evidence that it is a distinct genus.

However, the embryological characters do not support the recognition of the sections in *Tripterospermum*, as they show great consistency over the entire genus (Chen et al. 2000a, Huang et al. 2009, 2010, Table 2): tetrasporangiate anthers, the anther wall development of the Dicotyledonous type, a degenerated epidermis, a fibrous endothecium, the tapetum of the glandular type, the cytokinesis in the microsporocyte meiosis of simultaneous type, tetrads mainly tetrahedral, 3-celled mature pollen grains; a bicarpellary and syncarpous gynoecium, a unilocular ovary with two parietal placentae and 4 rows of unitegmic and tenuinucellate ovules, the embryo sac of Polygonum type.

Although the two sections of *Tripterospermum sensu* T.N.Ho (1988) are readily discriminated morphologically by the presence of capsular or berry-like fruits, it is not known whether these sections are monophyletic. Embryological characters do not support the splitting of the genus into two sections. There is a little discrepancy in the curvature of the ovules, but that is interspecific (*T. discoideum* vs *T. cordatum*) rather than intersectional. The distribution of embryological characters in *Tripterospermum* thus might be taken as evidence that the two sections are unnatural groups, and that the production of berries and the associated changes in dispersal strategy may have appeared several times in the genus. To test this hypothesis, further studies, including more species of both capsular and berried *Tripterospermum* should be undertaken on basis of embryology, gross morphology as well as phylogenetics.

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