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Female gametophyte development in *Sinopodophyllum hexandrum* (Berberidaceae) and its evolutionary significance

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Female gametophyte development of *Sinopodophyllum hexandrum* (Berberidaceae) was studied on materials from different altitudes. The plant displays diversity in the megagametogenesis, and two categories of four female-gametophyte development models are observed, including the monosporous *Polygonum*-type, a new *Sinopodophyllum* type (and its two variants), which is intermediate between the monosporic and bisporic types. The results suggest that the *Polygonum* type is more primitive than the other types, and that the *Sinopodophyllum* type is intermediate between the *Polygonum* type and the *Allium* type. The diversity in the female gametophyte development in *S. hexandrum* reveals megagametogenesis may be influenced by altitude and be of great significance to its adaptability and evolution. In the *Podophyllum* group, *Sinopodophyllum* is more advanced than *Diphylleia* and *Dysosma,* where the female gametophyte development conforms to the *Polygonum* type. In addition, the results of this study directly provide evidence that the disporous *Allium*-type is derived from the monosporous *Polygonum*-type.

Introduction

Embryological information makes great contributions to an understanding of the phylogenetic relationships in plants, and embryological characteristics are at present incorporated in many publications for their value to angiosperm systematics (Cronquist 1981, 1988). However, the systematic value of embryological characteristics has long been persuasively argued (Maheshwari 1950, Palser 1975, Heo & Tobe 1995). Palser (1975) concluded that embryological characteristics are relatively constant at the family level. In families where some embryological variation occurs, genera are constant on the whole.

Megagametophyte development may be of major importance in the determination of relationships within families and genera. Huang and Sheridan (1994, 1996) stated that embryo forms are similar in all systematic groups, and embryogenesis can not provide information of prime importance to the main groups of plant families. However, they considered that at the generic and specific levels, embryogeny is of some systematic value. Few investigations have dealt with variation in embryological characteristics within species. In this study on the female gametophyte development in the Himalayan mayapple *Sinopodophyllum hexandrum* (Berberidaceae) we demonstrate that embryological characteristics are not constant within the species, but vary with different altitudes.

Sinopodophyllum hexandrum is a self-pollinating perennial herb, found at altitudes from 1500 to 4300 m in the eastern Himalayan regions. There are various habitats in this area and the environment is harsh in some regions, but the Himalayan Mayapple shows great tolerance and phenotypic plasticity (Ma & Hu 1996). Traditionally, the Himalayan mayapple and its closely related genera, *Diphylleia*, *Dysosma* and *Podophyllum*, are members of Berberidaceae, but in some classification systems, they are treated in a separate family, Podophyllaceae. There is some dispute about their classification and the relationships among these four genera. Information from an investigation of female gametophyte development may provide some clues toward settling their phylogenic relations.

Sinopodophyllum hexandrum and its relative *Podophyllum peltatum* are the major sources of podophyllotoxin, an effective medicine used to treat genital warts (*condyloma acuminatum*). It is also used as the precursor in the manufacture of the semi-synthetic derivatives etoposide (VP-16), teniposide, and etopophos. Since 1995, CPH 82, a semi-synthetic derivative of podophyllotoxin, is in its third phase of clinical trials for the treatment of rheumatoid arthritis (Lerndal & Svensson 2000). Although many pharmaceutical and biological studies on *S. hexandrum* have been reported, the embryological information is insufficient.

Material and methods

Sinopodophyllum hexandrum is a short-day plant. The flower buds begin to form in the autumn and remain dormant through the winter and early spring. In late spring and early summer, the flower buds resume activity and undergo anthesis. Materials for embryological studies should be collected from the middle of May to the beginning of June. In the present study, the materials were collected in June 2007 and May 2008 at three localities: Bi-ta-hai (alt. 3200 m), Zang-ba (alt. 3750 m) and Tian-e-hu (alt. 4200 m), in the Xiang-ge-li-la county, Yunnan province, PR China. Both entire and partial flower buds and flowers at different development stages were collected and fixed in modified FAA (50% alcohol–glacial acetic acid–formaldehyde = 89:6:5). The fixed materials were stained in Ehrfich's hematoxyfin after being washed with water. The materials were embedded in paraffin after being dehydrated in ten grades of alcohol (mixtures of alcohol and water), cleared with five grades of xylene (mixtures of xylene and alcohol), and serially sectioned at the thickness of 5–8 μm by Microm. The sections were mounted on slides in Neutral balsam, and observed and photographed with the compound microscope Leica DM2000.

Results

Under natural conditions, *S. hexandrum* reproduces mainly through seeds. There is an interval of about five or six years from seed germination to flower development. The plant forms flower buds in autumn when the vegetative growth is complete. At this time, the morphological development of all flower parts is well underway, but the mega- and microsporocytes have not undergone meiosis. In late autumn, the vegetative shoot withers; the flower buds cease development, become dormant, and are enveloped by two succulent bracts. In the following late spring or early summer, when the temperature rises to a threshold, the dormant flower buds rejuvenate again. The Himalayan mayapple grows at ground level. The flowers and leaves of mature plants develop through growth at the base of the plant. When the flower buds just emerge from the ground, the microsporocytes initiate meiosis. There is an interval of about five or six days from the emergence of flowers to the completion of pollination.

The flowering phenology depends on temperature, but not much on precipitation. The earlier the temperature increases, the earlier the flowering occurs. At the altitude of 3200 m, the Himalayan mayapple usually flowers in the middle of May. The higher the altitude, the later the flowering takes place. Generally, when the

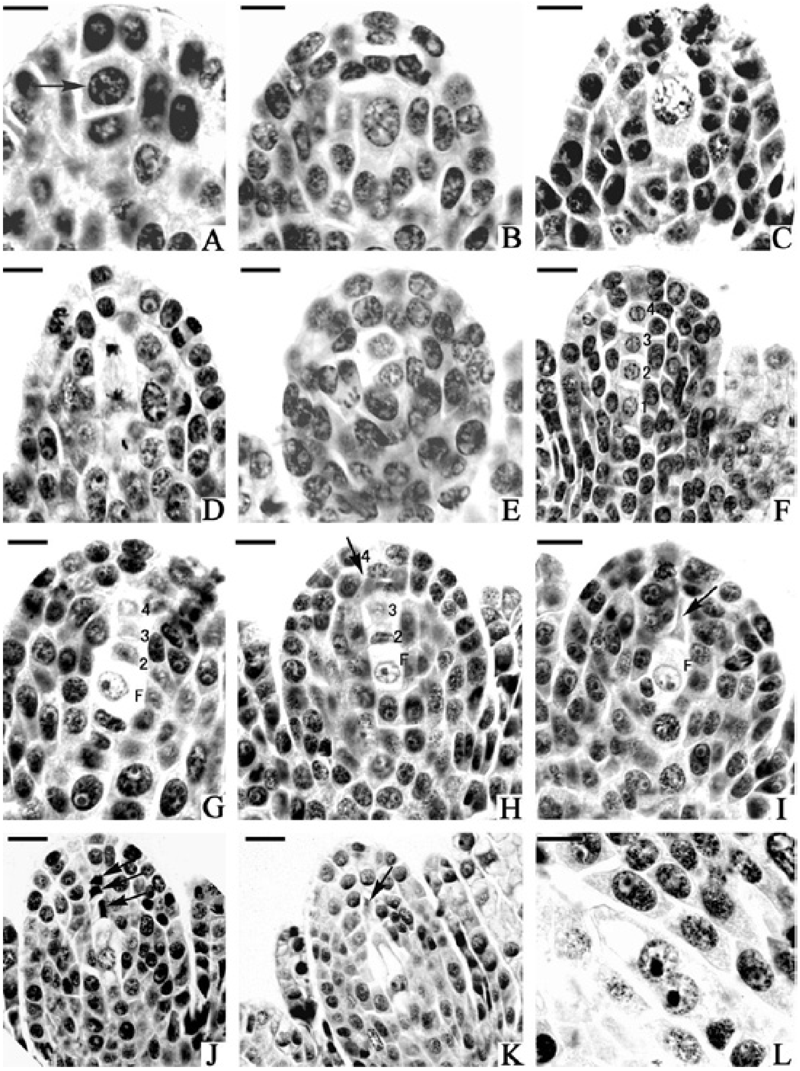

Fig. 1. Female gametophyte development in *Sinopodophyllum hexandrum*: *Polygonum*-type. — **A**: Female archesporium (arrow). — **B**: Megaspore mother cell. — **C**: Megasporocyte at prophase of meiosis. — **D**: Megasporocyte at telophase of meiosis. — **E**: Megaspore dyad, note similar shape and size. — **F**: Linear tetrad of megaspores (1–4). — **G** and **H**: A functional megaspore at chalazal end (F) and three degenerated megaspores at micropylar end (2–4). — **I**: A functional megaspore at chalazal end (F) and traces of three degenerated megaspores at micropylar end (arrow). — **J**: Uninucleate female gametophyte and traces of three degenerated megaspores at micropylar end (arrow). — **K**: Late stage of uninucleate female gametophyte and traces of three degenerated megaspores at micropylar end (arrow). — **L**: Two-nucleate female gametophyte just formed. Scale bars: 10 μm.

altitude increases by 100 m, the blossoming time will delay by three to five days.

The flower is solitary in a terminal position on the aerial stem. It is composed of six sepals in two whorls of three each, six petals in two whorls of three each, six stamens, and a single terminal pistil. The three outer sepals are variable in size and shape. The three inner sepals are elliptic and with a well-developed venation. The six petals are obovate, membranous and have a well developed venation. The carpel has a long style, and it enlarges to assume a sac-like shape. Many ovules grow on the enlarged marginal placenta of the ocular ovary. Ovules in the same locule develop asynchronously. When the carpel margins coalesce, the cytoplast of the cells in the basal part of the ovary thickens and grows rapidly toward the locule to form a conic ovule primordial. Each primordium further develops, and its apex forms the nucellus above the funiculus. Beneath the epidermis at the apex of the nucellus, an archesporial cell differentiates. The cell contains dense cytoplasm and an enlarged nucleus with a large nucleolus (Fig. 1A). The archesporial cell does not divide, but enlarges and directly becomes a megasporocyte. The megasporocyte is much larger than any of the surrounding nucellar cells, and it contains dense

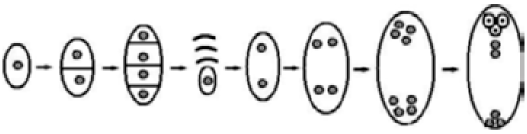

Fig. 2. Schematic representation of the sequence of the *Polygonum*-type female gametophyte development in *Sinopodophyllum hexandrum*.

cytoplasm and a large, distinct nucleus (Fig. 1B). Between the megasporocyte and the nucellar epidermis are one or two nucellar cells derived from the epidermis, so the ovule can be defined as pseudocrassinucellate (Davis 1966).

The megasporocyte first appears in the dormant flower buds and remains unchanged for a long period of time. When the dormancy ends, the nucleus of the vertically elongated megasporocyte, located near the chalazal end of the cell, undergoes meiosis I (Fig. 1C). Female gametophyte development of *S. hexandrum* can be summarized as being of the *Polygonum* type, its new *Sinopodophyllum* type and its two variations, which are defined by the pattern of meiosis I and II.

Polygonum type

The nucleus of the megasporocyte divides by meiosis I (Fig. 1D) followed by cytokinesis to form a dyad with the two cells of equal size (Fig. 1E). Meiosis II in each member of the dyad, followed by cytokinesis, produces a linear tetrad of megaspores initially of equal size (Fig. 1F). The three micropylar megaspores degenerate beginning with the one closest to the nucellar epidermis and proceeding chalazally (Fig. 1G and H). At the same time, the chalazal megaspore enlarges and behaves as functional megaspore (Fig. 1I), which will undergo three successive mitoses to form an eight-nucleate female gametophyte (Fig. 2). Accordingly, the development follows the typical monosporic *Polygonum*-type.

This way of female gametophyte development ensures the existence of tetrad and uninucleate female gametophyte. The functional megaspore enlarges while the vacuoles appear at micropylar and chalazal end, which results in the enlargement of the female gametophyte (Fig. 1J). At this time, the degenerated traces of the three micropylar megaspores are visible. Afterwards, the vacuoles become larger, entering a uninucleate female gametophyte later stage, while the degenerated traces of defunct megaspores can still be seen (Fig. 1K). Then the nucleus of the functional megaspore undergoes mitosis to form two new nuclei, and enters the two-nucleate female gametophyte stage (Fig. 1L).

Sinopodophyllum type

The megaspore mother cell undergoes the first division of meiosis to form a dyad. The dyad members are similar in size and shape. Meiosis II is non-synchronous. The division of the chalazal cell of dyad is earlier than that of the micropylar one (Fig. 3A and B). Cytokinesis follows meiosis, thus to establish a linear tetrad of megaspores, in which the two chalazal cells are larger than the micropylar ones (Fig. 3C). Early in the female gametophyte, the two micropylar megaspores (products of the micropylar dyad member) show signs of degradation (Fig. 3D and E). Afterwards, the two micropylar megaspores degenerate and the two chalazal megaspores (products of the chalazal dyad member) grow (Fig. 3F). Then the wall between the two chalazal megaspores dissolves to form a two-nucleate female gametophyte, while traces of the two micropylar megaspores remain (Fig. 3G and H). The two-nucleate female gametophyte undergoes two successive mitoses to form an eight-nucleate female gametophyte (Fig. 4).

Sinopodophyllum type (1st variation)

In this type, the shape and size of megaspore dyad are similar (Fig. 3I). The chalazal cell continues the second division of meiosis to form two news cells and the micropylar one neither divides nor degenerates, forming the linear triad; two megaspores and a micropylar dyad cell. (Figs. 3J and 5A). Afterwards, the micropylar cell (offspring of the first meiosis) degenerates and the two chalazal cells (offsprings of the second meiosis) enlarge in size (Fig. 5B) while the cell wall between cells dissolves gradually,

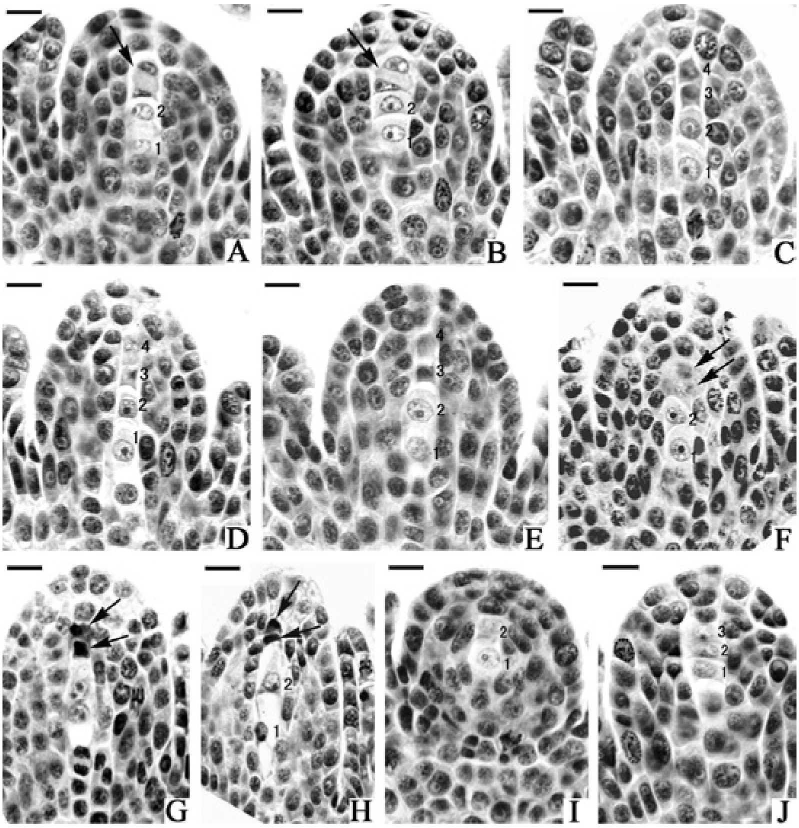

Fig. 3. Female gametophyte development in *Sinopodophyllum hexandrum*: *Sinopodophyllum*-type, 1st variation. — **A** and **B**: Abruption of megaspore dyad. — **C** and **D**: Linear tetrad of megaspores (1–4). — **E**: The two micropylar megaspores degenerate (3 and 4) while the two chalazal megaspores continue to develop (1 and 2). — **F**: Volume of the two chalazal megaspores increases (1 and 2), with traces of the degenerated two micropylar megaspores (arrow). — **G**: Cell wall of the two chalazal megaspores has dissolved, with traces of the two degenerated micropylar megaspores (arrow). — **H**: Two-nucleate female gametophyte, with traces of the two degenerated micropylar megaspores (arrow). — **I**: Megaspore dyad (1 and 2). — **J**: Linear triad of megaspores (1–3). Scale bars: 10 μm.

resulting in a two-nucleate female gametophyte (Fig. 5C) and we can see the trace of the degenerated micropylar cell of the megaspore dyad at this time. The mature female gametophyte develops from this two-nucleate female gametophyte. So it belongs to bisporic type. The two-nucleate female gametophyte undergoes two successive mitoses to form an eight-nucleate female gametophyte (Fig. 6).

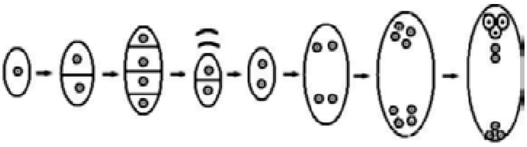

Fig. 4. Schematic representation of the sequence of the *Sinopodophyllum*-type female gametophyte development in *Sinopodophyllum hexandrum*.

Sinopodophyllum type (2nd variation)

The nucleus of the megasporocyte divides by meiosis I followed by cytokinesis to form a dyad with the micropylar member smaller than the chalazal one (Fig. 5D, E and F). The micropylar cell gradually disintegrates (Fig. 5G and H). Then meiosis and the accompanying cytokinesis in the chalazal cell produces two megaspores (Fig. 5I). A disintegration of the wall between the two megaspores gives rise to the bisporic two-nucleate female gametophyte (Fig. 5J). At this time, the degenerated trace of the micropylar cell still exists. In fact, this two-nucleate female gametophyte is equal to two functional megaspore nuclei, which undergo two successive mitoses to form an eight-nucleate female gametophyte (Fig. 7). Therefore, this female gametophyte is very similar to the *Allium* type.

The bisporic type has a marked character that there is no tetrad of megaspores or a uninucleate female gametophyte. The two functional nuclei develop directly into a two-nucleate female gametophyte. At the same time, vacuoles appear

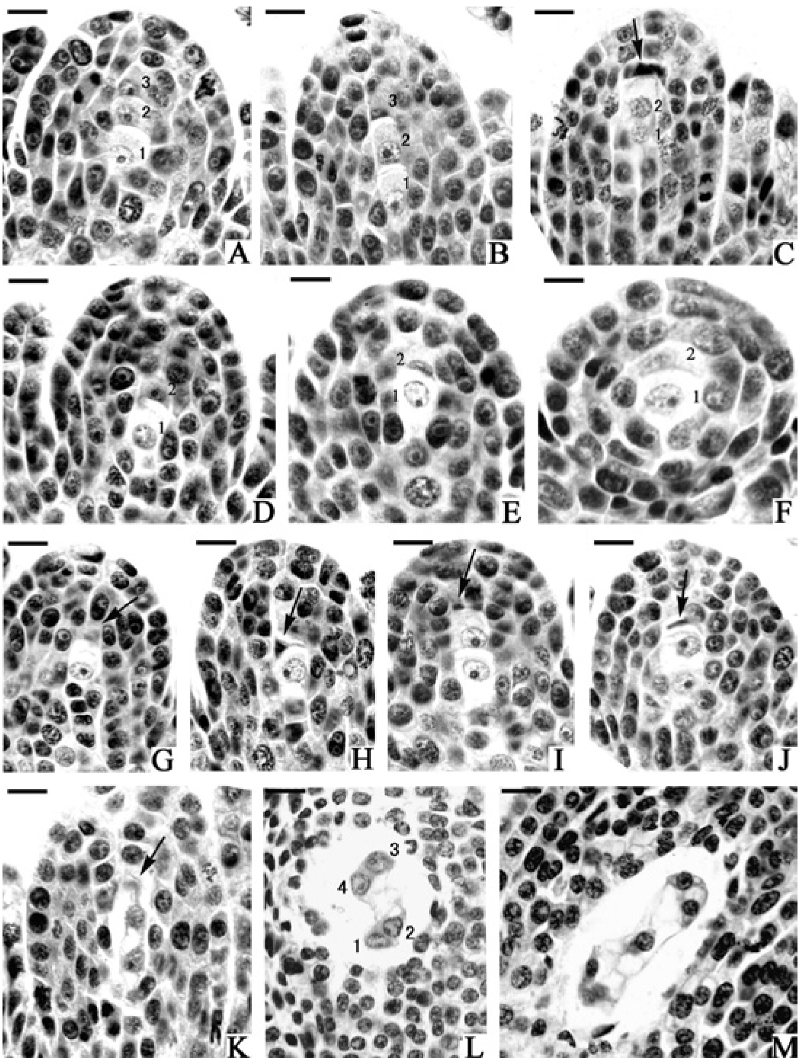

Fig. 5. Female gametophyte development in *Sinopodophyllum hexandrum*: *Sinopodophyllum*-type 1st variation and *Sinopodophyllum*-type 2nd variation. — **A**: Linear triad of megaspores (1–3). — **B**: Triad of megaspores, where the two chalazal two continue to develop (1 and 2) and the micropylar megaspore degenerates (3). — **C**: Cell wall of the two chalazal cells has dissolved, with traces of the degenerated micropylar cell of megaspore dyad (arrow). — **D** – **F**: Megaspore dyad, note different shape and size (1 and 2). — **G**: Micropylar cell of megaspore dyad degenerating (arrow). — **H**: Volume of the chalazal cell of megaspore dyad is aggrandizing, with traces of the degenerated micropylar cell of megaspore dyad (arrow). — **I**: The chalazal cell of megaspore dyad divides into two cells, with traces of the degenerated micropylar cell of megaspore dyad (arrow). — **J**: Cell wall of the two chalazal cells has dissolved, with traces of the degenerated micropylar cell of megaspore dyad (arrow). — **K**: Two-nucleate female gametophyte, with traces of the degenerated micropylar cell of megaspore dyad (arrow). — **L**: Four-nucleate female gametophyte. — **M**: Eight-nucleate female gametophyte. Scale bars: 10 μm.

at both micropylar and chalazal ends, which signals the beginning of the two-nucleate female gametophyte. At this phase the degenerated trace of the micropylar cell can still be observed (Fig. 5K).

The above-mentioned two categories of four female gametophyte development types undergo the four-nucleate (Fig. 5L) and eight-nucleate female gametophyte phases (Fig. 5M) to become mature.

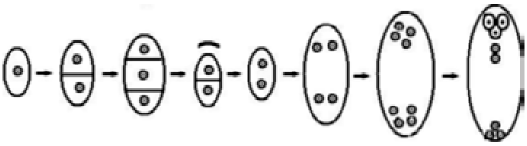

Fig. 6. Schematic representation of the sequence of the *Sinopodophyllum*-type 1st variation in *Sinopodophyllum hexandrum*.

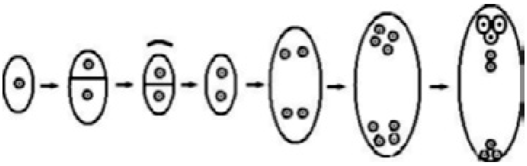

Fig. 7. Schematic representation of the sequence of the *Sinopodophyllum*-type 2nd variation in *Sinopodophyllum hexandrum*.

Discussion

Usually, the megasporegenesis and the formation of the female gametophyte follow a certain pattern in angiosperms. More than 15 different patterns of female gametophyte development have been described; the different patterns arise mainly from variations in cytokinesis during meiosis, number and pattern of mitotic divisions, and pattern of cellularization (Yadegari & Drews 2004). Angiosperms exhibit three main patterns of megasporogenesis, referred to as (1) monosporic, in which the *Polygonum* type is of frequent occurrence, the *Oenothera* type being of rare occurrence, (2) bisporic, in which the *Allium* type is common and the *Endymion* type is uncommon, and (3) tetrasporic, which includes seven forms. The three types differ mainly in whether cell-plate formation occurs after division, thus determining the number of meiotic products that contribute to the formation of the mature female gametophyte. In the monosporic pattern, both meiotic divisions are accompanied by cell plate formation, resulting in four one-nucleate megaspores. Subsequently, three megaspores, generally the micropylar-most megaspores, undergo cell death. In the bisporic pattern, cell plates form after meiosis I but not after meiosis II. The result is two two-nucleate megaspores, one of which degenerates. In the tetrasporic pattern, cell plates fail to form after either meiotic division, resulting in one four-nucleate female gametophyte. Thus, these three patterns give rise to a single functional megaspore that contains one (monosporic), two (bisporic), or four (tetrasporic) meiotic nuclei (Maheshwari 1950, Johri 1992).

In *Sinopodophyllum hexandrum*, two categories of four female-gametophyte development patterns were observed, including the *Polygonum* type and a new *Sinopodophyllum* type and its two variants.

The megaspore mother cell in the *Sinopodophyllum* type normally undergoes meiosis to form a tetrad, and the four megaspores have cell walls and show a typical megaspore tetrad structure. Later on, the two megaspores close to the micropylar end degenerate gradually, and the other two ones close to the chalazal end develop continually into two functional megaspore nuclei with the disappearance of the cell wall. The two-megaspore nuclei migrate to the two poles and undergo two successive mitoses to form an eight-nucleate female gametophyte, and the final female gametophyte structure is similar to that of the *Polygonum* type. This kind of female gametophyte development pattern has never previously been reported for any taxon, and we thus call it the *Sinopodophyllum*-type. It seems that it is a variant of the monosporic type, because the obvious difference between monosporic and the bisporic type is whether there exists a tetrad stage. All monosporic female gametophytes have this stage but the bisporic ones do not. However, from the number of the functional megaspore nuclei, the *Sinopodophyllum* type should belong to the bisporic type.

The development process in the two *Sinopodophyllum* variants is very similar to that in the classical *Allium*-type, the difference lying in megaspore dyad. In the *Sinopodophyllum*-type 1st variation, the micropylar cell neither degenerates nor undergoes the second meiosis and degenerates in the subsequent development process. The two chalazal cells (products of the chalazal dyad member) fuse into a two-nucleated cell and develop into a functional megaspore. In the *Sinopodophyllum*-type 2nd variation, the micropylar cell gradually disintegrates. Then meiosis and the accompanying cytokinesis in the chalazal cell produces two megaspores. A disintegration of the wall between the two megaspores gives rise to the bisporic two-nucleate female gametophyte. Because the mature female gametophyte comes from this two-nucleate functional megaspore, the development accords with the bisporic type. Functional megaspore nuclei undergo two successive mitoses and the final structure of the female gametophyte is similar to that of the *Polygonum* type.

Overall, we consider that the *Sinopodophyllum* type should be the intermediate type between the monosporic and the bisporic types. The existence of the *Sinopodophyllum* type clarifies the evolution of the classical bisporic *Allium*-type development from the monosporic *Polygonum*-type without gaps, the sequence being: *Polygonum*-type → *Sinopodophyllum*-type → *Sinopodophyllum*-type 1st variation → *Sinopodophyllum*-type 2nd variation → *Allium*-type.

In the *Podophyllum* group, there is evidence that the genus *Sinopodophyllum* is the most advanced. Our embryological evidence is in accordance with this notion. In the female gametophyte development, both *Diphylleia* and *Dysosma* belong to the *Polygonum* type (Huang & Ma 2004, Huang *et al.* 2010), and *Sinopodophyllum* belongs to a mixed type, its female gametophyte development contains four development patterns that may result in higher fitness, which we think is closely related with its habitats. In this group, species of *Sinopodophyllum* are the only ones that do not thrive in wildwoods; instead, they are adapted to dry environments and the frigid envirionments at high altitutes. We observed that the proportion of female gametophyte development differs according to altitude. The specimens from lower altitude (3200 m) display four patterns and the proportion of each pattern is just equal, but at higher altitudes the proportion of the *Polygonum* type is reduced, and at the highest altitude (4200 m), the *Sinopodophyllum* type and its two variations are much more frequent than the other types. From that result we regard that the evolution of the *Sinopodophyllum* female gametophyte development has proceeded from the *Polygonum* type to the *Sinopodophyllum* type and then to the *Sinopodophyllum*-type 2nd variation (very similar to the *Allium* type). Individuals with the *Sinopodophyllum*-type 2nd variation appear to thrive in harsher environments. Embryologically the *Sinopodophyllum* type is an intermediate type and *S. hexandrum* with multiple female gametophyte development patterns may have a larger potential evolutionary ability.

Acknowledgements

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