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A study of microsporogenesis and male gametogenesis in *Psammosilene tunicoides* (Caryophyllaceae)

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Microsporogenesis and male gametogenesis in *Psammosilene tunicoides* (Caryophyllaceae) were studied and described for the first time. This species possesses essential embryological features of Caryophyllaceae, such as tetrasporangiate anthers, fibrous endothecium, glandular tapetum, two tapetal cells, simultaneous cytokinesis of pollen mother cells, and three-celled pollen grains. A majority of the microspores were arranged in tetrahedral tetrads, but rhomboidal tetrads were observed in one population. Notable differences in the tapetum degeneration were observed in different populations. Early or late degeneration may both lead to male sterility and subsequently to reproductive failure. The tapetum was dimorphic in two populations. We also included data concerning the breeding system of the species and discussed its implications for conservation purposes.

Key words: Caryophyllaceae, male gametogenesis, microsporogenesis, population, *Psammosilene tunicoides*, tapetum

Introduction

Psammosilene tunicoides is distributed in relatively narrow ranges of Yunnan, Guizhou, Sichuan, and Xizang, mainly along the Jinsha River and the Yarlung Zangbo River. It grows on sandy or calcareous soil in pine forests, or in rock crevices on mountain slopes, at altitudes between 1500 m and 3800 m. One of its main distribution areas is from middle to northwestern Yunnan (Lu *et al*. 2001). This species is a well-known medicinal herb in southwestern China and has been widely used for more than five hundred years. The main compounds extracted from *P. tunicoides* are saponins and cyclic peptides (Ding *et al*. 2000, Zhong *et al*. 2003).

Research on the morphology and chromosome numbers have supported the position of *Psammosilene* as a monotypic genus in the Caryophyllaceae (Wu & Wu 1945, Pan *et al*. 2004). Such monotypic taxa deserve special attention from the conservational point of view (*see* Rana & Ranade 2009). A long-term uncontrolled usage has caused a reduction in *P. tunicoides* geographic range and population size, and now the species is in a great risk of extinction. It was listed in *China Plant Red Data Book* as a rare and endangered species (Fu 1992).

From the perspective of conservation, it is very important to investigate the reproductive process of *P. tunicoides* because one of the major causes for species' rarity might be the reduction of population size resulting from reproductive failure. Male reproductive processes are necessary for generating seeds that will produce the next plant generation (e.g. Robert 1993). Therefore, the processes play a prominent role in contributing to population maintenance and regeneration of endangered species.

Embryological studies are often useful not only in shedding light on the events of sexual reproduction but also in solving taxonomic and phylogenetic problems (Davis 1966, Tobe 1989, Johri *et al*. 1992). Although the embryology in Caryophyllaceae has received considerable attention (Cook 1909, Davis 1966, Pal & Murthy 1974, Dang & Chinnappa 2007), little anatomical work on the embryology, microsporogenesis and male gametogenesis of *P. tunicoides* has been carried out. Also observations on the breeding system, especially stamens and how they release pollen grains may clarify the male reproductive processes.

Embryological studies were often carried out at or above the generic level and rarely at the population level (Dang & Chinnappa 2007). Populations of *P. tunicoides* in Yunnan grow in a variety of habitats and the phenotypic plasticity may be environmentally induced (Dai *et al*. 2007; Y. Qu unpubl. data), which renders it necessary to compare the embryological and breeding features in populations from different habitats.

In the present study, we focus on the male reproductive process, mainly on microsporogenesis and male gametogenesis, along with some observations of the breeding system. We address the following questions, for systematic purposes (here mainly involving the population study): (1) Are there any striking features in microsporogenesis and male gametogenesis in *P. tunicoides* as compared with those in other Caryophyllaceae species? (2) How do the populations differ in the male reproductive processes and their breeding system? (3) Are there any obstacles during *P. tunicoides* male reproductive processes to incur the species' endangerment? (4) Since it is a self-compatible species, are there different levels of autogamy among populations and how would it affect the populations' breeding success? We expect that the answers to the above questions would assist the understanding of why these populations differ and what impact the differences may have on the species' survival. The study would therefore provide basic information for investigating the reproductive pattern and developing a plan to maintain or enhance the populations of the species. The embryological evidence at population level might be of value for weighing and choosing high-yield germ plasm resources for the planting of good agricultural practice (GAP) of medicinal plants.

Material and methods

Flowers of *P. tunicoides* are hermaphroditic and self-compatible. The inflorescences are dichasia. Typical inflorescences have a primary flower, a secondary flower on either side of the central axis, and a tertiary flower on the side of the lateral axis. *Psammosilene tunicoides* has regular, radially symmetrical flowers. Mature flowers have five free, clawed petals forming a functional tube enclosed by the tubular calyx. The claw has a small fringe at the top. Five stamens are attached on the corolla throat. Ovary is two-celled with two stigmata. Four to seven petals and stamens were occasionally seen in some individuals. Each capsule generally yielded one seed.

From 2003 to 2005, we collected individuals representing six natural populations of *P. tunicoides* from the northwestern, middle and eastern Yunnan (Fig. 1) by random sampling. These individuals were subsequently cultivated in the greenhouse of the Inmol Laboratory of Biotechnology, Baihanchang village, Xiaoshao town, Guandu district, Kunming, Yunnan (altitude 2100 m a.s.l.) for the embryological study. Vouchers were deposited at the Institute of Herb Biotic Resources of Yunnan University (YH). Collectors chose different types of habitats including a grassy slope, calcareous rocky crevices, and a red soil eroded place. The studied populations ranged from 24°N to 28°N and 99°E to 104°E. The localities, sample sizes of the populations, their altitudes, vouchers, and habitats are given in Table 1.

In order to describe the anther development, at least three reproductive structure samples at each stage (from flower buds to fertilized flowers) were collected randomly from 20 plants of each population, before and during the reproductive seasons of 2004 and 2005. Approximately 400 randomly chosen samples at different developmental stages per population were processed. Flowers of all ages were fixed in FAA (5 ml formalin: 6 ml acetic acid: 89 ml 70% (v/v) ethanol), and further dehydrated in a graded ethanol series. Infiltration and embedding in paraffin was done using a conventional method (Yeung & Law 1987, Yang *et al*. 2007). Serial 8 μm sections were cut using a Teichert Autocut microtome, and the sections were stained with Safranin and counterstained with fast green (Berlyn & Miksche 1976), and finally mounted permanently on slides with a cover slip. The stained sections were examined and photographs were obtained using a microscope (BXS, Olympus, Japan).

As to the breeding system, the observations mainly included flower morphology, especially the stamen development and the intimate stigma/anthers contact, which were often associated with self-compatibility. Bagging experiment was used to test the ratio of autogamy.

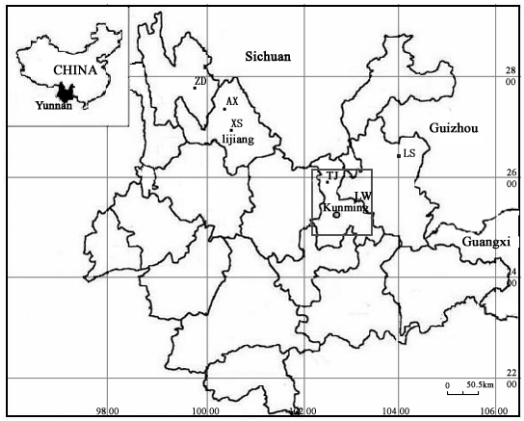

Fig. 1. Distribution of the six sampled natural populations of *Psammosilene tunicoides* in the present study. The locations of the six populations are shown on the map of Yunnan (codes correspond to populations in Table 1).

Results

Formation of anther wall

In early March, the anthers were tetrasporangiate. At an early stage of development, below the epidermis of anthers, archesporial cells differentiated, which were recognizable by their large volume, radial elongation, dense cytoplasm and conspicuous nuclei (Fig. 2a–c). These cells divided into outer primary parietal cells and inner primary sporogenous cells. The primary parietal cells then divided periclinally and anticlinally to form two secondary parietal layers. The outer secondary parietal cells divided into a subepidermal endothecium and middle layers (Fig. 2d). The inner secondary parietal appeared to undergo further divisions to form middle layers and a tapetum. Because the middle layers had a common histogenetic origin with both the

Table 1. Locality, habitat and voucher specimens of the six sampled populations of *Psammosilene tunicoides*.

Population code	Sample size[a]	Locality in Yunnan	Altitude (m)	Habitat	Voucher
AX	20	Axi, Lijiang	1900	calcareous, rocky, and grassy slope	Yu H. (0401) (YH)
TJ	20	Xishan, Kunming	1980	calcareous red-soiled, and eroded place	Yu H. (0402) (YH)
XS	20	Xiangshan, Lijiang	2500	calcareous, rocky and grassy slope	Yu H. (0403) (YH)
LS	20	Luoshui, Xuanwei	1850	calcareous, red-soiled, and eroded place	Yu H. (0404) (YH)
LW	20	Laowushan, Songming	2180	calcareous, rocky and grassy slope	Yu H. (0406) (YH)
ZD	20	Xiaozhongdian, Shangri-la	3320	forest margin on grassy slope	Yu H. (0408) (YH)

[a] numbers of individuals collected and examined for each population.

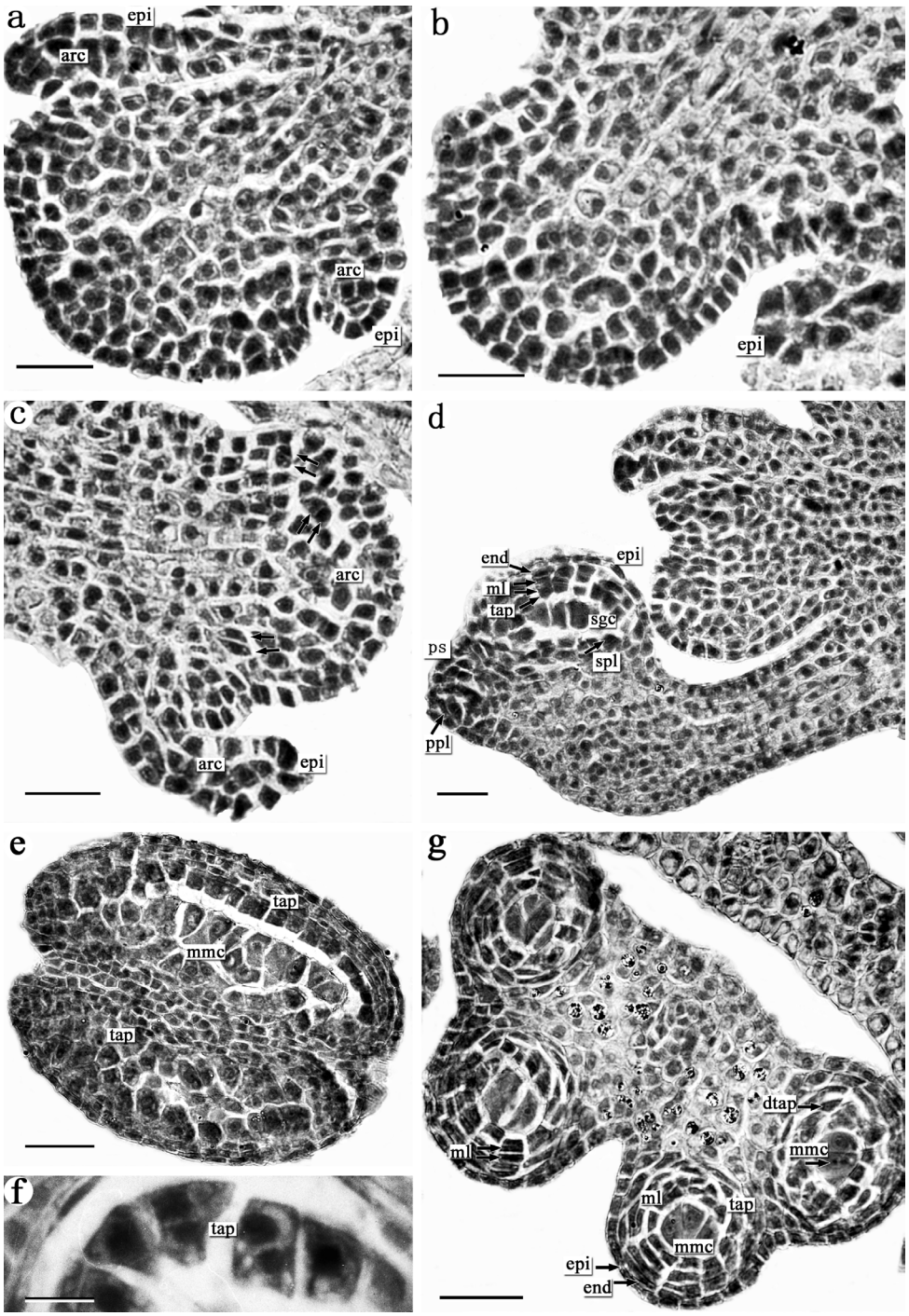

Fig. 2. Anther and microsporogenesis in *Psammosilene tunicoides*. — **a**–**c**: Archesporial cells (arc) and protoderm (epi). — **d**: Pollen sac (ps), sporogenous cells (sgc) and primary parietal layer (ppl). Outer secondary parietal layer (spl) is divided into endothecium (end) and middle layer (ml) (arrows). — **e**–**g**: Sporogenous cell (sgc) and all layers beneath the epidermis. — **e**: Microspore mother cell (mmc) in prophase. — **f**: The tapetal cells (tap) are initially uninucleate, and then binucleate. — **g**: Telophase of meiosis of microspore mother cells. An anther wall composed of epidermis, an endothecium (end), two middle layers (ml) and one or two layers of tapetal cells (initiation of degeneration (dtap) (arrows). Scale bars: 10 μm in **a**–**d** and **g**; 40 μm in **e** and **f**.

endothecium and the tapetum, the microsporangial wall formation conformed to the basic type (David 1966). From the cross section, we saw that the anther wall was comprised of five layers from outer to inner: an epidermis, an endothecium, two middle layers, and a layer of tapetum. Meanwhile, in the loculi the sporogenous cells formed and some of them entered the stage of microspore mother cell (Fig. 2e–g).

From the stage of one-nucleate free microspores to the formation of mature pollen grains, the tapetum degenerated. The thin-walled epidermal cells were longitudinally elongated, forming vesicles later (Fig. 3e and f). The walls of endothecium became thickened after microspore formation (Fig. 3d–f) and when pollen matured, they showed helicoidal thickenings, except in the region of dehiscence (Fig. 4w, arrow). The middle layers were compressed as the anther developed (Fig. 3a–d). However, some cells of the middle layers adjacent to the endothecium swelled and entered the locule, then made contact with the grains during pre-anthesis (Fig. 3e). The rich starch in cells of middle layers was digested and gradually it disappeared in the process, providing nutrition for the pollen development. At late microgametogenesis, tapetum cells and middle layers could not be observed in mature anthers. The anther wall was only composed of vesicle epidermis, and a thickened endothecium (Fig. 4w).

The region of the connective presented idioblasts containing raphides, at the beginning of anther wall development (Fig. 2d). They became richer in the process of the forming of microspore tetrads, and then were found in the vesicle epidermis and they dyed red during pollen maturity (Fig. 4w).

Tapetum characteristics

In sections from all populations, the tapetum of *P. tunicoides* was partly originated from the ground tissue near the connective tissue. Thus the tapetum was of dual origin. The tapetum cells formed by secondary parietal cells surrounding the sporogenous tissue were mononucleated at first, and later became binucleated along the division of the microspores, each having a rounded or ellipsoidal nucleus with dense cytoplasm throughout the development process (Fig. 2e and f). They were often smaller in volume and regular-rectangular, arranging in order around the sporangiole (Figs. 2e and 4a). The tapetum cells formed by connective tissue were larger, nearly rounded, with multiple layers, piling up in the corner of anther locule. They appeared to elongate radially and intrude into the anther locule to form 'placentoids' (Steffen & Landmann 1958) (Fig. 4c and d). This kind of tapetum degenerated later than thate formed by the inner secondary parietal cells, remaining untill the stage of binucleated pollen grains (Fig. 5c and 6f).

The tapetum type varied among populations. At tetrad stage, we found that the tapetal cells degenerated at their original sites in ZD, AX, LS and XS populations. Neither cell fusion nor cell invasion into the loculus took place (Fig. 3a–e). Therefore, the tapetum belonged to typical glandular type as named by Bhojwani and Bhatnagar (1979). In TJ and LW populations, the tapetum with indistinct cell wall and some fluidity migrated to the loculus involving the microspores and surrounding the tetrads (Fig. 5e and f). At vacuolated stages, we also observed tapetal cells between microspores. The tapetum appeared heteromorphic, but there was a distinction between the heteromorphic tapetum as compared to what we observed. The tapetal cells had invaded into loculi before they degenerated because the protoplast remained at the surface of tapetal cells during their invasion into the loculus, indicating that the wall did not break and the tapetum cells went into the loculi due to early mitosis. Besides, the tapetal cells degenerated and were absorbed completely at the stage of mature pollen grains. Thus, the tapetum was heteromorphic and glandular, or dimorphic (Eames 1961, Ho *et al*. 2000).

Interestingly, the initial and end stage of the tapetum degeneration also varied among populations. In LS, ZD and AX populations, the internally tangent wall of tapetum was thickened at the beginning of meiosis of a microspore mother cell. At the stage of metaphase I, the tapetum became bigger and reached its highest development. The cell possessed large volume, dense cytoplasm, less vacuoles, and a big nucleus, an

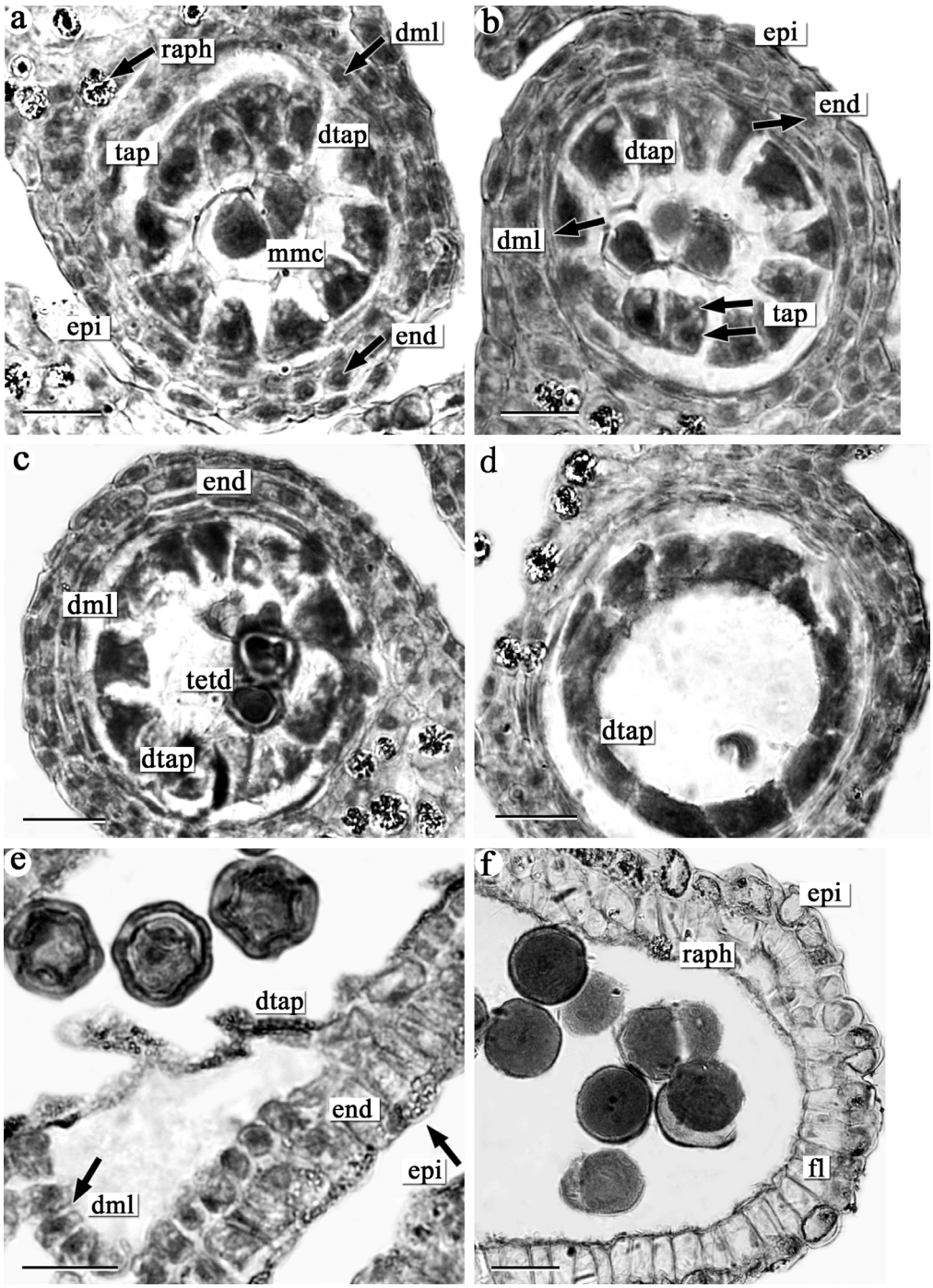

Fig. 3. Anther wall and microsporogenesis in *Psammosilene tunicoides*, transverse sections. — **a**: Microsporocytes enclosed in a callose layer and tapetum cells during division. Raphides (raph) appeared and increased in the connective. — **b**: Microspores during pre-meiotic division. Note: The tapetum cells have large vacuoles and initiation of degeneration in middle layer (arrows) is visible. — **c** and **d**: Microspores arranged in a tetrahedral manner at tetrad stage. Degenerated tapetum and degenerated middle layer. — **e**: Anther wall with two-nucleate pollen grains. Vesicular epidermis, fibrous endothecium and adjacent swelled median layers. — **f**: Anther wall before releasing pollen, fibrous thickened endothecium and persistent vesicular epidermis. Scale bars: 20 μm in **a**–**d**; 30 μm in **e** and **f**.

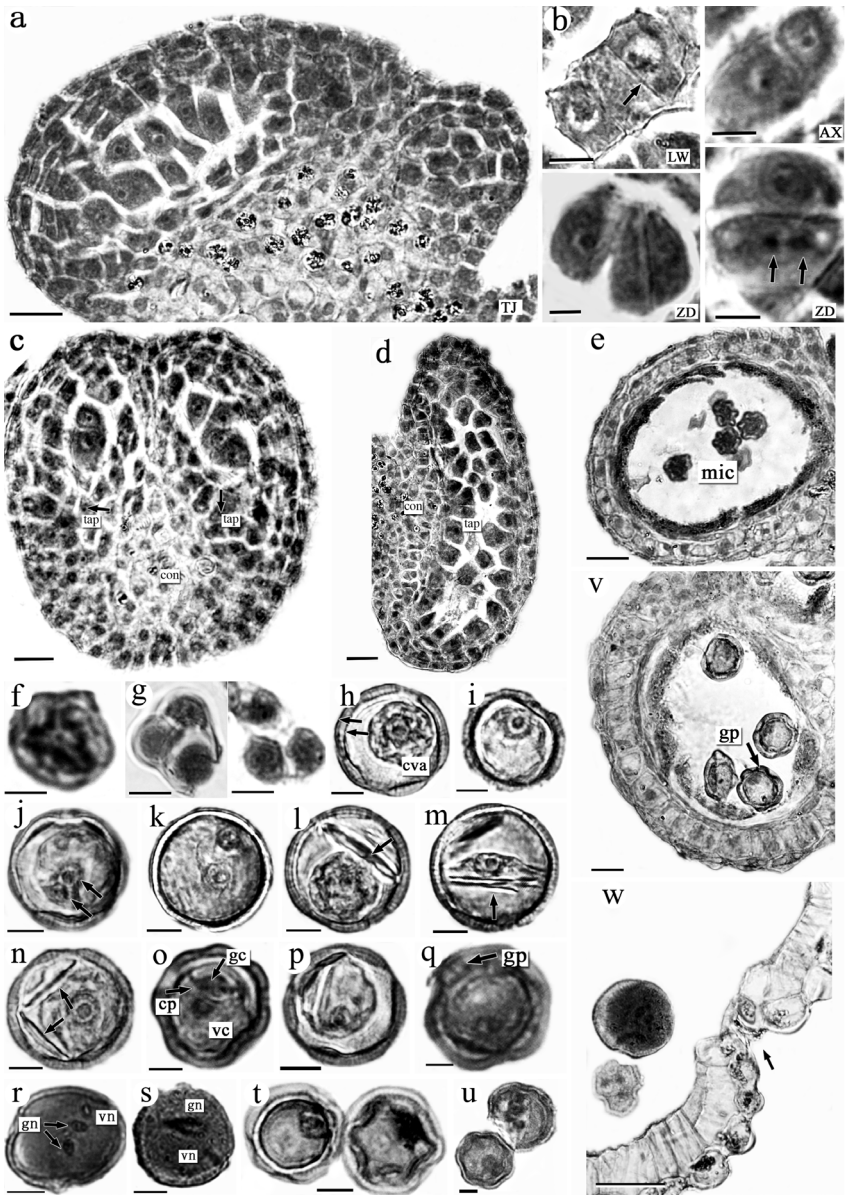

Fig. 4. Microsporogenesis in *Psammosilene tunicoides*, transverse sections. — **a**: Microspore mother cell (mmc) in prophase in population TJ. Unsynchronized development among different sacs and among different microspores in the same sac. — **b**: Microspore mother cells during prophase in populations AX , ZD, and LW. — **c** and **d**: Dual origin tapetum. Tapetum is partly originated from the ground tissue near the connective tissue (arrows). — **e**: Stage of one-nucleate free microspores (mic). — **f**: Telophase I of meiosis of microspore mother cells in anaphase. — **g**: Telophase II tetrahedral tetrad (tetd) and tetrads separated from each other. — **h** and **i**: Anther wall of one-nucleate free microspores with a big central vacuole (cva). — **j** and **k**: Formation of two-nucleate pollen grains. — **l**–**n**: Formation of cell plates (cp) (arrows). — **o**–**q**: Development of generative cell and vegetative cell. — **o**: An equal division of the generative cell producing two sperm cells. Cell plates (cp), clear aperture (gp), generative cell(gc), and vegetative cell (vc) (arrows). — **r** and **s**: Three-nucleate pollen grains with generative nucleus (gn) and vegetative nucleus (vn) (arrows). — **t** and **u**: Un-synchronized phenomena between microspores in the same sac. — **v**: Anther wall of free one-nucleate microspores, showing vesicle epidermis, annular and helical thickening of endothecium, crushed middle layers, degenerated tapetum, and clear aperture (gp) (arrow). — **w**: Part of anther wall near the region of dehiscence (arrow), vesicular epidermal cells, annular and helical thickenings of endothecium. Scale bars: 30 μm in **a** and **w**; 20 μm in **c**–**e** and **v**; 10 μm in **b** and **f**–**u**.

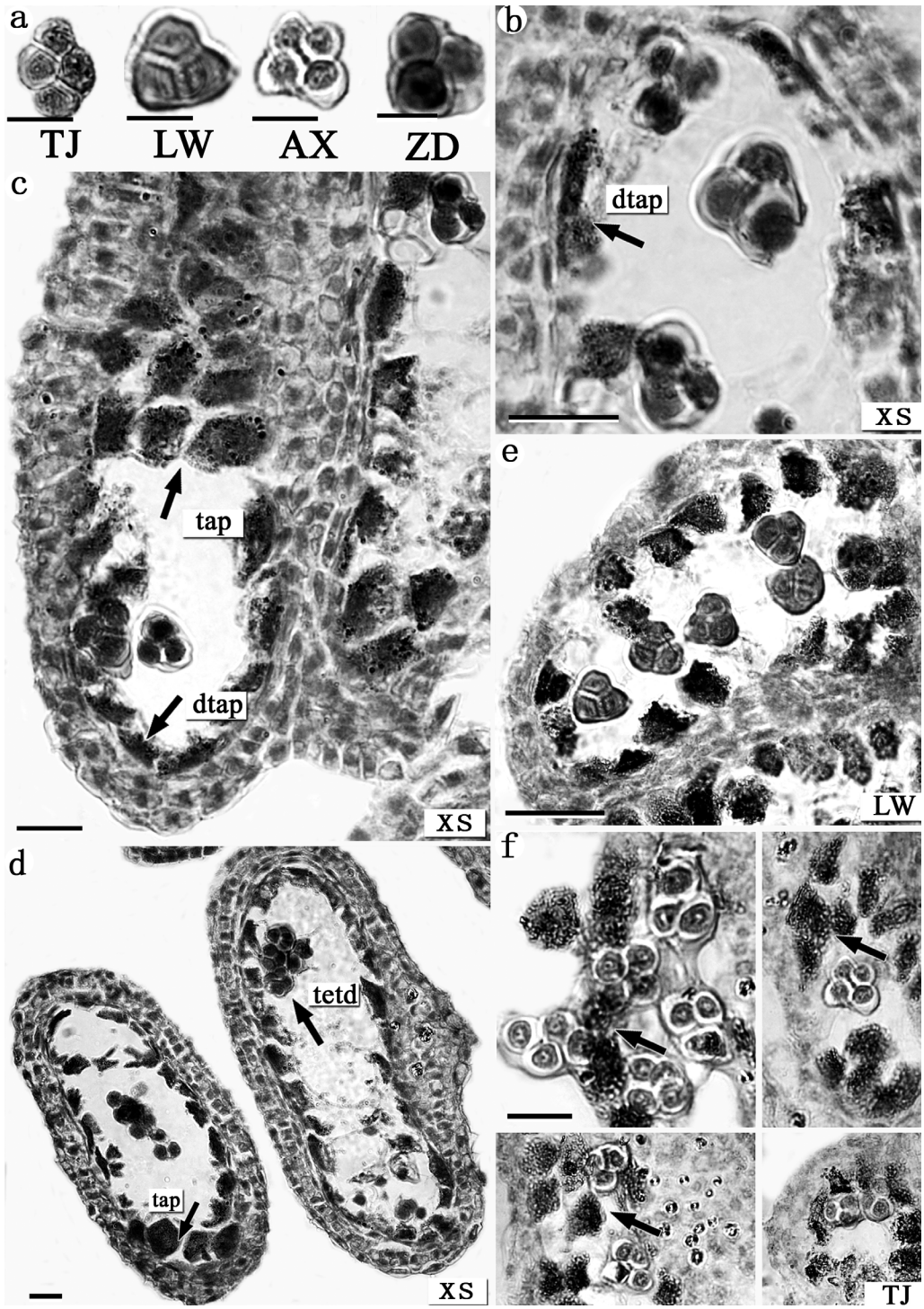

Fig. 5. Tetrads and anther walls, transverse sections. — **a**: Tetrads in populations TJ, LW, AX, ZD (left to right). — **b–d**: Tetrads and anther wall in population XS. — **b**: Thick callose wall surrounding a tetrahedral tetrad (tetd) and early degenatated tapetum (dtap) segments (arrow). — **c**: The 'placentoids' tapetum piling up in the corner of the anther (arrows). — **d**: Tetrads stuck together and failing to separate from each other. — **e** and **f**: Tetrads and dimorphic tapetum (arrows) in populations LW and TJ. Scale bars: 10 μm in **a**; 20 μm in **b–f**.

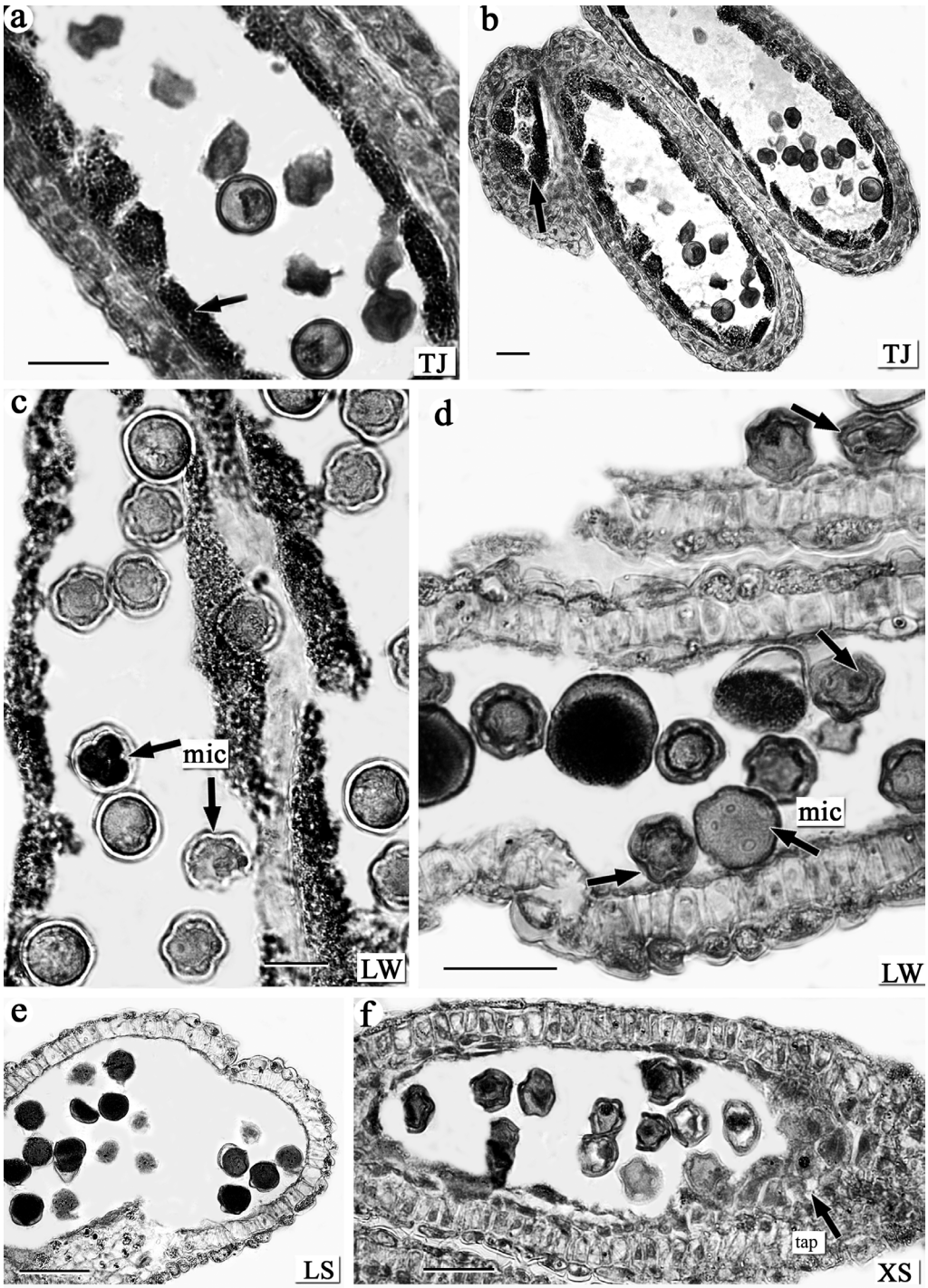

Fig. 6. Abnormal development of tapetum and microspores. — **a**: Undegraded tapetum at the stage of mature pollen grains in population TJ (arrow). — **b**: Vestigial anther in population TJ (arrow). — **c** and **d**: Tetrads failing to separate from each other, empty microspores, and undegraded tapetum remnant in population LW (arrows). — **e**: Normal mature pollens and cell wall when dehiscing in population LS (for comparison). — **f**: Abnormal pollen and undegraded 'placentoids' in tapetum at two-celled pollen period in population XS. Scale bars: 10 μm in **a**; 20 μm in **b**–**f**.

Table 2. Summary of the differences in embryological characters among populations of *Psammosilene tunicoides* during microsporogenesis and male gametogenesis. *See* Table 1 and Fig. 1 for the population codes.

Population code	XS	AX, ZD, LS	TJ, LW
Sample size[a]	60 flower structures per population	60 flower structures per population	60 flower structures per population
Tapetum origin	dual	dual	dual
Tapetum type	typical glandular	typical glandular	dimorphic
The initial of tapetum degeneration	early-stage of microspore mother cells' meiosis	late-stage of microspore mother cells' meiosis	late-stage of tetrad period
The end of tapetum degeneration	early-stage of uni-nucleate microspores	early-stage of two-nucleate pollen grains	mature stage of pollen grains
The way of tapetal cells' degeneration	uneven	even	uneven
Tetrads	most tetrahedral and rarely rhomboidal	all tetrahedral	all tetrahedral
meiotic divisions	synchronous	synchronous	un-synchronized (20% sections)
Pollen abortion (%)	63	12 (AX), 9 (ZD), 10 (LS)	21 (TJ), 23 (LW)

[a] numbers of flower structure examined for each feature.

indication of accumulating rich nutrition for a further development of microspores (Fig. 2e and 3a). At about the time of pollen tetrad formation, the walls of the tapetal cells became indistinct. Meanwhile, the cells of middle layers began to degenerate from inner to outer, prior to the tapetum, the visible partition emerged between the tapetum and the middle layers (Fig. 3a–c). In the period from the end of the tetrads to one-nucleated free microspores, the tapetum cells drew close to each other and the radial thickness was about 7–8 μm on average, having been about 6 μm before the degeneration. In the cross section it looked like a circle (Fig. 3d). The degeneration of tapetal cells reached the peak at the end of one-nucleate free microspore stage. Their radical thickness decreased to about 1 μm. The cytoplasm became less dense and vacuoles were formed in the tapetum cells. The nucleoli began to break into small and visible nuclei (Fig. 4g and l). At the end of the two-nucleated pollen grain stage, the tapetum became fragmented and degenerated completely.

The tapetum's degeneration began and ended early in XS population and late in LW and TJ populations (Table 2).

Microsporogenesis and microgametogenesis

The anther primordium began to form as a teat, composing of meristematic tissue enveloped by an epidermal layer (Fig. 2a–c). It became bilobed (Fig. 2d and e) and then tetralobed after the development of the four sporangia (Fig. 2g). Simultaneously with the changes taking place in the wall of the microsporangia, four groups of hypodermal cells differentiated near each of the four corners of the young anther (Fig. 2d, arrows).

The primary sporogenous cells underwent mitosis to form secondary sporogenous cells surrounded by dense cytoplasm, from which microsporocytes were derived. There were obvious partitions in the cytoplasm. In the development of microsporocytes, the cytoplasm separated by the partition and formed polygonal microsporocytes (Fig. 4a and b).

Microsporocyte underwent meiosis for two to three days after the formation of microspo-

rocyte and the process of meiosis involved double cell divisions. Only the second divisions occurred with the formation of wall between microspores, thus the microsporogenesis was simultaneous (Fig. 4f and g). In all sections, we observed around 300 tetrads for each population. We found a majority of them are tetrahedral tetrads and rarely found rhomboidal tetrads, only in seven sections from different individuals in the TJ population (around 20 rhomboidal tetrads) (Fig. 5a and f).

There was a thick callose wall surrounding a microspore tetrad (Fig. 4e). As the microspore developed, callose walls disappeared. Four uninucleate microspores were released from the tetrad. The microspores were 12–17 μm in diameter. They had a dense cytoplasm, conspicuous wall, inconspicuous vacuoles, and a prominent central nucleus (Fig. 4h). During further development, uninucleate microspores gradually increased their volumes and had more vacuoles. As the central vacuole developed, the nucleus was pushed to the pollen wall in a peripheral position (Fig. 4i). The microspore soon experienced mitosis and divided unequally, forming two nuclei (Fig. 4j and k). Between the two nuclei, there was a cell plate in an arc, beeline or broken line shape (Fig. 4m and n). The two-celled microgametophyte, or pollen grain contained a larger vegetative cell and a smaller generative one (Fig. 4o). Along with the pollen development, the vacuole disappeared and the wall became thickened. The aperture became clear (Fig. 4p and q). The microspores were bigger than at the uninucleate stage, reaching 22–26 μm in diameter.

During pre-anthesis, an equal division of the generative cell produced two sperm cells (Fig. 4o). The sperm cells were rounded at the beginning, then almond-shaped or elliptic in cross section (Fig. 4r), and finally long-elliptic to linear (Fig. 4s). The microspores were the largest, reaching 26–30 μm in diameter. The mature pollen grains were three-celled and had multiple apertures.

From the sections, we found that most meiotic divisions were synchronous within an anther and the cells at the same stage often gathered together, However, in 20% of the sections in TJ and LW populations, there was unsynchronized development among different anthers in the same flower, or three to four stages' lag between different sacs (Fig. 2d and 3a), and even two to three stages' lag between different microspores in the same sac. Especially in the second meiosis, the un-synchronicity was prominent (Fig. 4t–u).

It often took six to seven days from the archesporium to pollen maturation and shedding.

Pollen abortion

Around 1% of the individuals in all populations had an abnormal stamen structure. All or part of their stamens stopped developing and appeared short, atrophic and gray. We also found stagnation during the microspores' development in TJ population. For instance, the region corresponding to the sporogenous tissue completely collapsed and the anther appeared to stop developing and got atrophic, and consequently no pollen grains formed at all (Fig. 6b).

Abnormal phenotypic variations in the process of microsporogenesis and male gametogenesis among populations were also found. The percentage of underdeveloped pollen — determined in transverse sections — was significantly higher in XS populations than in other populations. TJ and LW populations displayed 21% and 23% pollen abortion, respectively (Table 2). The abortion pollen appeared in different shapes, such as lunate and deltoid, or empty of content in later development (Fig. 6c, d and f). Further abnormal development was found on the tapetum. In the XS population, the tapetum segments invaded into the locule and stuck together with the developing spores, or some tetrads stuck together and failed to separate from each other (Fig. 5d). However, in the TJ and LW populations, some tetrads failed to produce four separated microspores and then stopped developing at the tetrad stage (Fig. 6c).

Observations on the breeding system

Bagging experiment proved that *P. tunicoides* is a self-compatible species. We found that after two styles of each flower protruded from the corolla, they generally outspreaded like 'V' at

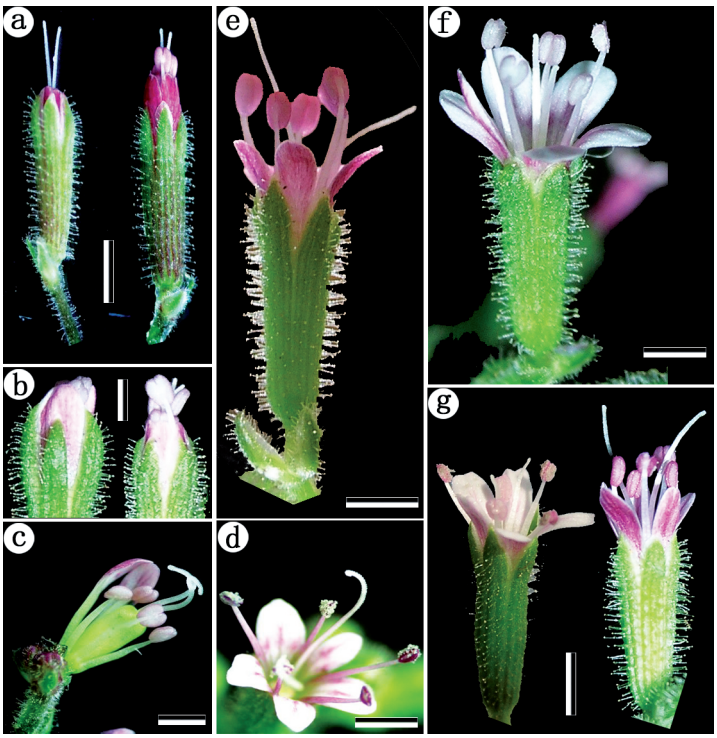

Fig. 7. Development of propagation organs. — **a** and **b**: Two styles protruding from corolla, outspreading like 'V' in population TJ. — **b**: Pistil and stamens protruded from corolla tube almost at the same time in population XS. — **c** and **d**: Stigma bent inward and reflexed to touch the anther of the same flower. — **e**: Posture of pistil and anthers when the anthers began to shed pollen in population XS. — **f**: Posture of two styles and stamens protruding from corolla in population TJ. — **g**: Posture of styles and anthers when the anthers began to shed pollen in population XS (left) and AX (right). Scale bars: 2 mm.

an obtuse angle between them at first (Fig. 7a), which would be helpful to the reception of pollen from other flowers. But once they failed to receive pollen from other sources, the angle between the two styles would became sharper, and they were able to become self-pollinated by reflexing their stigmas in a counter-clockwise direction to contact the anthers (Fig. 7c). This kind of selfing could be regarded as a supplementary mechanism in case cross-pollination fails. After bagging, the XS population got the highest fruit set, reaching 76.2%. Populations AX, TJ, LW and LS ranked next, at 61.8%, 52.1%, 51.0% and 50.3%, respectively. Population ZD got the lowest fruit set, being 40.3%.

In the XS population, the pistil and stamens protruded from the corolla tube almost at the same time (Fig. 7b). Besides, the style was slightly longer than or as long as the stamens when the anthers began to shed pollen grains, so the stigma would easily touch the anther when it reflexes inward or its upper part turns in counter-clockwise direction (Fig. 5f). Furthermore, the population XS had flesh-coloured stamens (Fig. 7g), lacking any aroma and with smaller size of flowers, both of which decreased the out-crossing chance. In the population TJ, the styles protruded from the corolla tube three or four hours earlier than the stamens did, and the stigmata were receptive four or eight hours later. When the anthers began to shed pollen, the styles were generally much higher than the anthers (Fig. 7e), so the stigmata would easily receive pollen from other flowers. If it fails, with the elapse of time the pollen on the same flower might shed and lose vitality, which decreases the selfing possibility (Fig. 7d). Furthermore, the flowers had dark purple stamens (Fig. 7g) with rich aroma, which would enhance cross-pollination.

Discussion

Embryological features

Psammosilene tunicoides is very similar in essential embryological features to the other members of the Caryophyllaceae, such as having tetrasporangiate anthers, fibrous endothecium, glandular tapetum, two nucleate tapetal cells,

simultaneous cytokinesis of pollen mother cells, and three-celled pollen grains (Cook 1909, Davis 1966, Pal & Murthy 1974, Dang & Chinnappa 2007). Pollen grain development in *P. tunicoides* followed the usual angiosperm pattern (McCormick 1993). Because of a lack of sufficient data from other genera in the Caryophyllaceae, we have not compared the embryological characters of *Psammosilene* with those of many other taxa. More intensive studies of genera in Caryophyllaceae are needed to clarify the embryological attributes for systematic study.

Notably, the tapetum was of dual origin, a feature also reported from some plants in the Gentianaceae and Scrophulariaceae (Vijayaraghavan & Sharada 1973, Ho *et al*. 2000, Xue *et al*. 2005, Yang *et al*. 2007) but not so far in the Caryophyllaceae. In addition, in the TJ and LW populations, the tapetum was dimorphic. According to Pacini and Franchi (1993), the movements of tapetal cells in a dimorphic tapetum led to more intimate contact between the tapetum and pollen grains by corresponding rotation and intermixing of microspores in the loculus. In view of its structure and function, this kind of the tapetum has been considered to be more evolutionally advanced than the normal glandular type (Zhu & Shen 1989). Pacini (1997) considered that such variations in the tapetum may result from different types of adaptation of the pollen grains to the most varied habitats and pollinator agents. Therefore, the tapetum structure might be one of the important characters at the population level in *P. tunicoides*.

Male sterility and population differences

Male sterility in plants implied an inability to produce or to release functional pollen and is the result of failure of formation or development of functional stamens, microspores or gametes (Phul *et al*. 1996). There were some instances of failure to develop normal pollen, probably caused by the abnormal development at three levels: the sporogenous tissue, tapetum layer, and microspore. Abnormal development at any level seems to be relevant to the tapetum development (Horner & Palmer 1995). Tapetum's major functions include producing pollen wall components, nutrients for pollen development and enzymes for microspore release from tetrads (Robert 1993). Moreover, all nutrients reaching the sporogenic cells must pass through tapetal cells (Maheshwari 1950).

According to Ciampolini *et al*. (1993), after total loss of its walls in the final stage of tetrad, tapetum secretion cells can begin their main activity: formation and transport of substances passing through them until reaching the microsporangium. The AX, ZD and LS populations reached the peak of the degeneration at the final stage of tetrad, when the development of pollen needed a large amount of nutrition. It assisted the nutrition accumulation and starch reserves. That is probably the reason why pollen grains in the three populations possessed the lowest amounts of aborted pollen. In the XS population, owing to the tapetum degenerating at early stage, the pollen grains were unable to gain enough nutrition through the tapetum, and then produced abnormal pollen grains. Moreover, since the tapetum was partially responsible for the synthesis of exine, its abnormal development could explain the absence or scarcity of exine and therefore the lack of a well developed pollen cell wall (Domínguez *et al*. 1997), thus the presence of pollen grains with the cytoplasm flowing out of the exine (Fig. 6f), and of empty pollen grains, could be caused by their thin exine wall. In contrast, in the TJ and LW populations the tapetum and the middle layer degenerated late. The unseparated abnormal tetrads could be a result of the deficiency of the tapetum to digest the callose wall (Domínguez *et al*. 1997). It might subsequently affect unsynchronized phenomena in the second meiotic phase in the LW and TJ populations, because the cytoplasmic connections between tapetum and meiocytes can be interrupted by callose deposition in the meiocytes (Souza & Pereira 2000). The nuclear independence of meiocytes, causing possible lack of synchrony, generally occurs in the anaphase II (Frankel 1973). Therefore, we inferred that the abnormal development of the tapetum and callose layer might delay or accelerate the meiosis of meiocytes, and then further control pollen shedding time. However, whether the un-synchronized development affect the population reproductive fitness still needs further investigations.

Breeding system

Compared with the TJ population, the XS population displayed some remarkable adaptive variations of the breeding system, particularly the shorter interval between the pistil becoming receptive and the stamen shedding pollen, and the closer proximity of the styles and the stamens. These variations facilitated the flowers to receive pollen from the same genotype and showed a higher potential for self-pollination. That might account for why the XS population got the clearly higher selfing rate. The germination rate of the seeds produced by selfing is 20%, while those by xenogamy reached 80% (Y. Qu unpubl. data). Apparently, seeds' vitality would probably be greatly reduced by selfing depression (Luis & Javier 2002).

Implications for conservation

Observations of both microsporogenesis, male gametogenesis and breeding system indicated that (1) abnormal male structure, early or late degeneration of the tapetum, pollen abortion, and high-selfing rate might cause reproductive failure and incur the species' endangerment. If we can identify the functional gene controlling the abnormal development, especially the abnormal tapetum development, it will be possible to remove the obstacles during the male reproductive process by trans-genetic technology. (2) The abnormal development also varied among populations. The XS population had the highest percentage of pollen abortion and of autogamy rate, while the ZD population had the lowest percentage of both. In order to find out the correlation between pollen abortion and autogamy, we should carry out a more comprehensive study on reproductive processes and embryological traits at population level, especially its megasporogenesis and the development of female gametophytes. In addition, the ZD, LS and AX populations with less potential of pollen abortion should be given top priority in the case of weighing and choosing germ plasm resources for GAP, and (3) in order to reduce the high selfing possibility and avoid subsequently poor seed vitality in the XS population, it will be preferable to conduct *ex situ* conservation and cross breeding with the ZD population by artificial supplementary pollination. This may preserve the rare alleles in the XS population and improve the vitality of F1 hybrid seed production. Besides, artificial emasculation should be combined with cross-pollination among populations due to conspicuous self-compatibility of the species. It will be very interesting to probe the genetic basis of male sterility in the populations and create male sterile lines, which will assist us in sustainable utilization of germ plasm and give rise to heterosis or hybrid vigor. Although the molecular basis of hybrid vigor is little known, hybrids with a heterotic phenotype are more resistant to disease, less susceptible to environmental stress, and have a higher yield than their inbred parents (Robert 1993). Male sterile lines are also free of artificial emasculation that can save intensive labor. It will be helpful to conduct large-scale seed production commercially.

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