

## Development of the male reproductive structures in *Taxus yunnanensis*

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**Abstract** *Taxus yunnanensis* Cheng et L. K. Fu is a rare species endemic to Southeast Asia. The development of the male reproductive structures with emphasis on microsporogenesis and microgametogenesis are described for the first time. Our results showed that *T. yunnanensis* exhibits several features that are very different from other species of *Taxus*, such as pollination occurring in the same year as pollen cone initiation; production of several forms of tetrads including isobilateral, T-shaped or tetrahedron; shorter duration of microsporogenesis; longer duration of microgametogenesis; and production of more microsporophylls per pollen cone suggesting faster growth rate. Reports on the details of male reproductive development in *Taxus* spp. have focused on the activities of generative nuclei, while the activities of the sterile and tube nuclei are rarely mentioned. This paper provides a few more details about the sterile and tube nuclei, particularly, their relative position during the division of the generative nucleus to form two sperm. Furthermore, as our knowledge of the phenology of *Taxus* is confined to species that grow on

high latitude areas, this paper provides information on the phenology of *Taxus* in low latitude areas. Overall, this paper provides several new insights about pollen cone development, microsporogenesis, and microgametogenesis in *Taxus*.

**Keywords** *Taxus yunnanensis* · Conifers · Microsporogenesis · Microgametogenesis · Reproductive biology · Sexual reproduction

### Introduction

*Taxus yunnanensis* was first described by Cheng et al. (1975). Its distribution extends from Burma through southwest China to Bhutan (Cheng and Fu 1978). *T. yunnanensis* became a focus of research because of its high content of taxol, which is an effective anti-cancer drug (Su et al. 2005). Interest on this species is also high because of its endangered state, and so several studies have examined the possible causes of its disappearance (Chen et al. 2001, Wu and Chen 2001, Li et al. 2005). To further our understanding of the biology of this species, basic information on its life history should be available. The development of the male reproductive structures, particularly pollen cones, microspores, and male gametes (sperm) are important components in the reproductive biology of *T. yunnanensis* and information on these processes will contribute to a better understanding of its life history.

A total of 7–12 species are acknowledged in *Taxus* (Wilde 1975; Miller 1977; Price 1990; Cope 1998), which are distributed across the Northern Hemisphere (Chen and Wang 1978). Except *T. canadensis* which is monoecious, other *Taxus* spp. including *T. yunnanensis* are considered functionally dioecious (Allison 1993; Anderson and Owens

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2000). Phenology and development of microspores have been described for *T. baccata*, *T. brevifolia*, and *T. canadensis* (Anderson and Owens 2000; Dupler 1917; Pennell and Bell 1985; 1986a). The morphology of pollen cones has also been described for *T. baccata*, *T. brevifolia*, and *T. canadensis* (Dupler 1919; Wilde 1975; Anderson and Owens 2000). The three species mentioned above occur in high latitude areas. However, the phenology and morphology of the male reproductive structures of any *Taxus* spp. in low latitude areas have not yet been examined.

Conifer pollen tubes are an important but underused experimental system in plant biology (Fernando et al. 2005). Microgametogenesis in Taxaceae and Cephalotaxaceae is distinct among conifers because the male gametes are formed only after three mitotic divisions (Fernando et al. 2005). Furthermore, in these families, there is an overlap between the use of the terms microspores and pollen grains and this is due to the uninucleate microspores being the structures that are dehisced from the pollen cones and reach the ovules. As the terminology on microgametogenesis in conifers varies tremendously in the literature, this paper follows the terminology proposed by Fernando et al. (2005). Microgametogenesis has been described in *T. baccata*, *T. brevifolia*, *T. cuspidata*, and *T. canadensis* (Robertson 1907; Dupler 1917; Sterling 1963; Pennell and Bell 1986b; Anderson and Owens 2000). However, there is a disagreement as to whether the two sperms are of equal sizes or not, and if they are nuclei or cells (Robertson 1907; Dupler 1917; Chen and Wang 1979, 1985; Pennell and Bell 1986b; Anderson and Owens 2000). In addition, the literature has focused on the activities of the generative cell or nucleus, while activities of the sterile and tube cells or nuclei are seldom elaborated upon (Dupler 1917; Pennell and Bell 1986b; Chen and Wang 1979; Anderson and Owens 2000). The objectives of this study are to describe pollen cone development, microsporogenesis, and microgametogenesis in *T. yunnanensis*. The phenology and development of the male reproductive structures in *T. yunnanensis* are discussed in relation to other *Taxus* spp.

## Materials and methods

All materials were collected from *T. yunnanensis* plantation at Yipinglang Forest Farm in Lufeng County, Yunnan Province, China. The plantation, with an area of about 0.5 ha and an elevation of 1980 m, was established in 1994 with three-year old seedlings from Lijiang, Yunnan Province. All the *T. yunnanensis* trees were planted with a 3- and 4-meter spacing. There were 786 individuals including 225 male trees, 409 female trees, 35 bisexual trees, and 117 unidentified trees.

Pollen cone development, microsporogenesis, and microgametogenesis were examined using paraffin-embedded samples. The samples for examining pollen cone development and microsporogenesis were collected from four male trees, with at least ten developing pollen cones per tree. Collection was done every five to eight days from July to November in 2006. Samples for examining microgametogenesis were collected from four female trees, with at least ten developing pollen cones per tree. Collection was done every 11–15 days from December 2006 to April 2007. Branches with buds, pollen cones or ovules were maintained in bottles with water and placed in coolers for transportation to the laboratory. The bud scales were removed and the median sections were fixed in formalin-acetic acid-alcohol. Specimens were embedded in paraffin and stained with safranin and fast green following Xue et al. (2005).

Microsporogenesis was also examined using pollen squashes. From September to December 2006, part of the collected samples were fixed in Carnoy's solution for 2 h and then stored in 70% alcohol at 4°C. These cones were squashed and stained with aceto-carmine. As aceto-carmine stains the chromatin red, it allowed rapid determination of the stages of microsporogenesis. To observe meiosis and structure of mature microspores, stain-clearing technique was used following Li and Huang (2005). All slides were viewed and photographed using a compound microscope.

The mature microspores of *T. yunnanensis* were dusted on aluminum sample stubs and sputter coated with gold. These were viewed under a scanning electron microscope (HITACHI S-3000N) at 18 kV.

To observe the duration of pollen cone shedding, 30 male trees were sampled and investigated every two to three days starting when the pollen cones began to shed and until all the pollen cones had completely shed. A total of 100 mature pollen cones were randomly collected from male trees in the plantation. Sporophylls on each male strobilus and microsporangia on each sporophyll were counted. Diameter of the male strobilus was measured using a vernier caliper. Correlation analysis between the diameter and sporophyll number per strobilus was carried out with Microsoft Excel software.

In vitro microgametogenesis was examined using microspores grown on culture medium prepared according to Anderson and Owens (2000). A total of 30–60 unshed pollen cones were randomly collected from the plantation, surface sterilized, and allowed to shed under sterile conditions following Fernando et al. (1997). Mature microspores were plated on 8% sucrose Brewbaker and Kwack (1963) medium and incubated at 25°C in continuous darkness. Every two to four days, some of the germinating pollen were stained with aceto-carmine and

others with DAPI following Anderson and Owens (2000). Germinated pollen were examined and photographed using a fluorescence microscope.

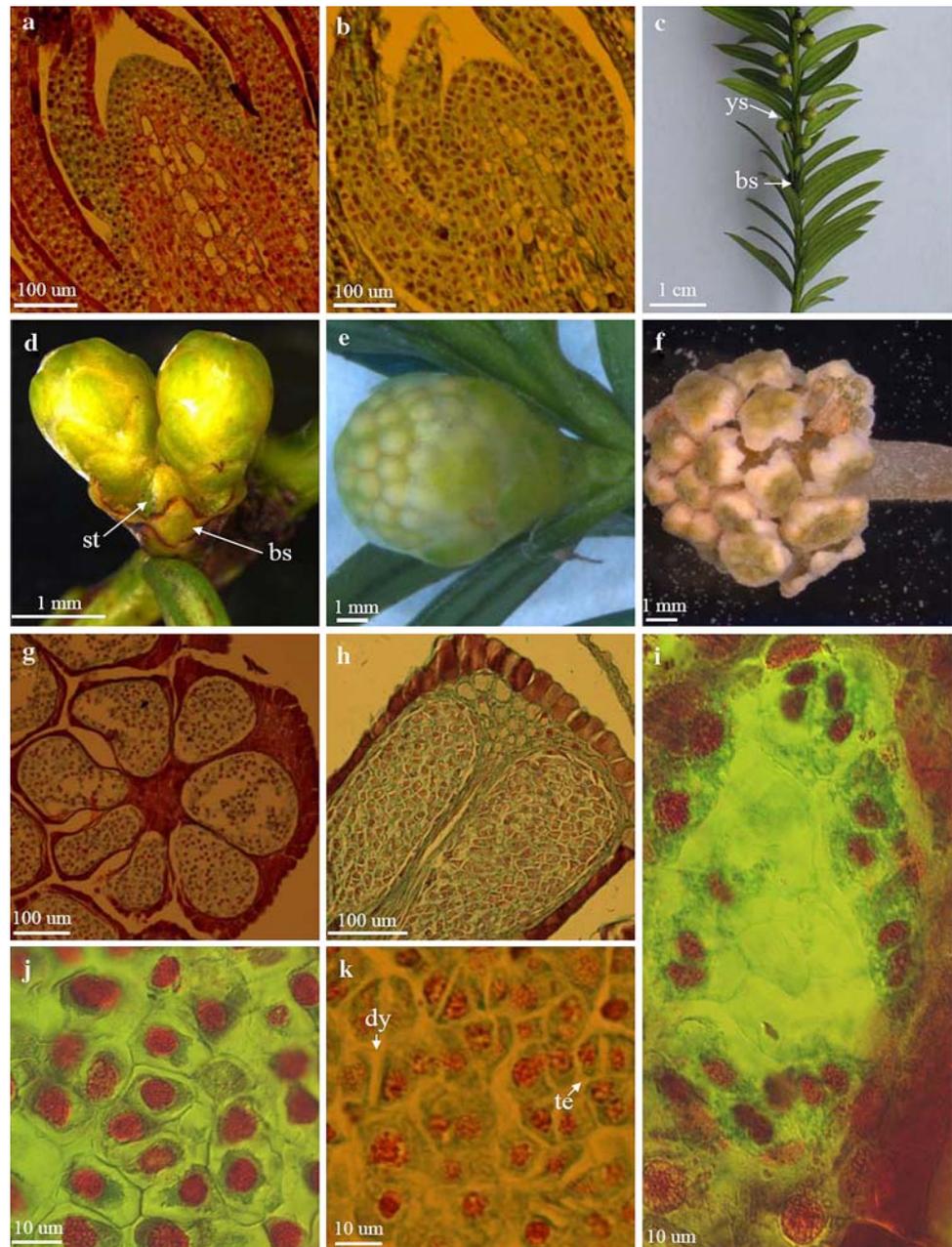
## Results

### Development of the pollen cone

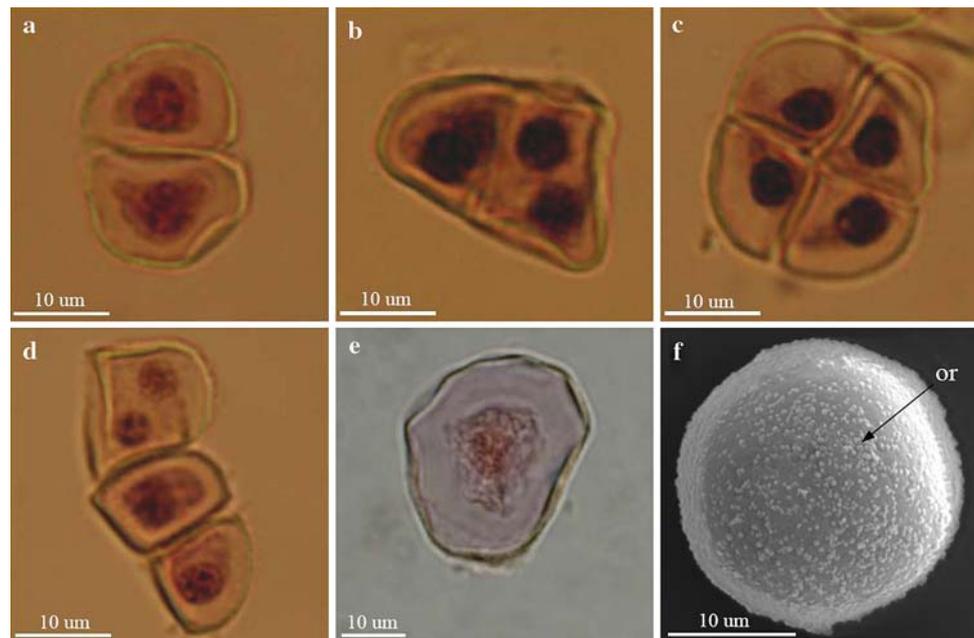
In late June, buds were produced on the axils of the leaves from most male trees. At this stage, these buds could not be distinguished as vegetative or reproductive based on

morphology or anatomy. In middle of August, histological analysis showed that the apical meristems of some of these buds were conical similar to the vegetative shoot tips (Fig. 1a), while the apical meristems of the other buds were broad (Fig. 1b). The former was considered as typical to vegetative buds and the latter to young pollen cones (Dupler 1919). At this stage, these buds were still indistinguishable based on external appearance. After one month, the sporophylls expanded out of the apex of the pollen cone and the pollen cone appeared more globular than the vegetative bud, and therefore, could now be easily distinguished by external appearance (Fig. 1c). All the pollen cones occurred

**Fig. 1** Pollen cone development and microsporogenesis in *Taxus yunnanensis*. **a** Longitudinal section of vegetative bud. **b** Longitudinal section of young pollen cone. **c** Branch with young pollen cone or strobilus (ys) on foliage above the budscale (bs). **d** Pollen cone occurring on a very short one-year-old twig (st). **e** Appearance of mature pollen cone. **f** Structure of mature pollen cone. **g** Cross-section of microsporophyll. **h** Longitudinal section of microsporangia. **i** Origin of microsporocytes. **j** Microsporocytes. **k** Meiosis in microsporangia (dy and te, short for dyads and tetrads)



**Fig. 2** Dyads and tetrads of microspores, structure and appearance of mature microspores. **a** Dyads. **b–d** Tetrads. **e** Structure of mature microspores. **f** SEM of mature microspores (or, short for orbicules)



on one-year-old twigs, but occasionally, the one-year-old twigs on which the pollen cones occurred were very short and were quite difficult to notice (Fig. 1d). In addition, the vegetative and reproductive buds could also be distinguished based on their relative position in the branch, i.e. the reproductive buds occur closer to the shoot tip, while the vegetative buds occur more towards the lower part of the same branch.

The mature pollen cones were round, subtended by several decussate budscales, and enclosed by a thin transparent epidermis (Fig. 1e). At pollination, the axis of the pollen cone elongated and the microsporophylls spread out. The microsporangial walls ruptured and allowed the mature microspores to be released (Fig. 1f). Pollen cones were composed of microsporophylls that bear microsporangia (Fig. 1g). The microsporangia were scutellate in longitudinal section (Fig. 1h). The size of mature pollen cones varied largely and their diameter ranged from 2.2 to 4.0 µm. The number of microsporophylls on each pollen cone varied from 15 to 28, and the number of microsporangia on each microsporophyll ranged from 3 to 12. The correlation between the diameter and number of microsporophylls on each strobilus was found to be weak with a coefficient correlation of 0.084.

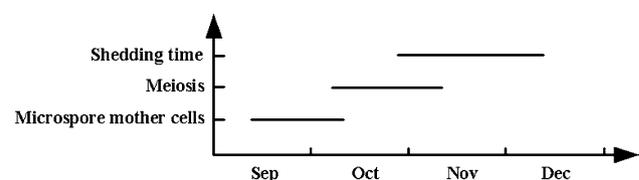
#### Development of the microspores

Microsporogenesis started when the reproductive buds were still morphologically indistinguishable from the vegetative buds. However, anatomical analysis was helpful in distinguishing these two types of buds. The microspore mother cells were positioned mostly along the inner margin of the

microsporangium (Fig. 1i). They enlarged, their chromosomes condensed (Fig. 1j), and meiosis occurred (Fig. 1k). After the first meiotic division, two hemispherical cells were formed (Fig. 2a). The second meiotic division took place in several directions, and thereby, the tetrads were produced in various configurations such as isobilateral, tetrahedron, and T-shaped (Fig. 2b–d). Occasionally, the divisions were not simultaneous between the two daughter cells (Fig. 2d). The newly formed microspores separated from each other, enlarged, and developed thick pollen walls. The mature microspores were uninucleate (Fig. 2e), about 25 µm in diameter, non-saccate, and with numerous orbicules (Fig. 2f). Microsporogenesis was not synchronous within a single microsporangium or entire pollen cone. The phenology of microsporogenesis is presented in Fig. 3.

#### Development of the male gametes

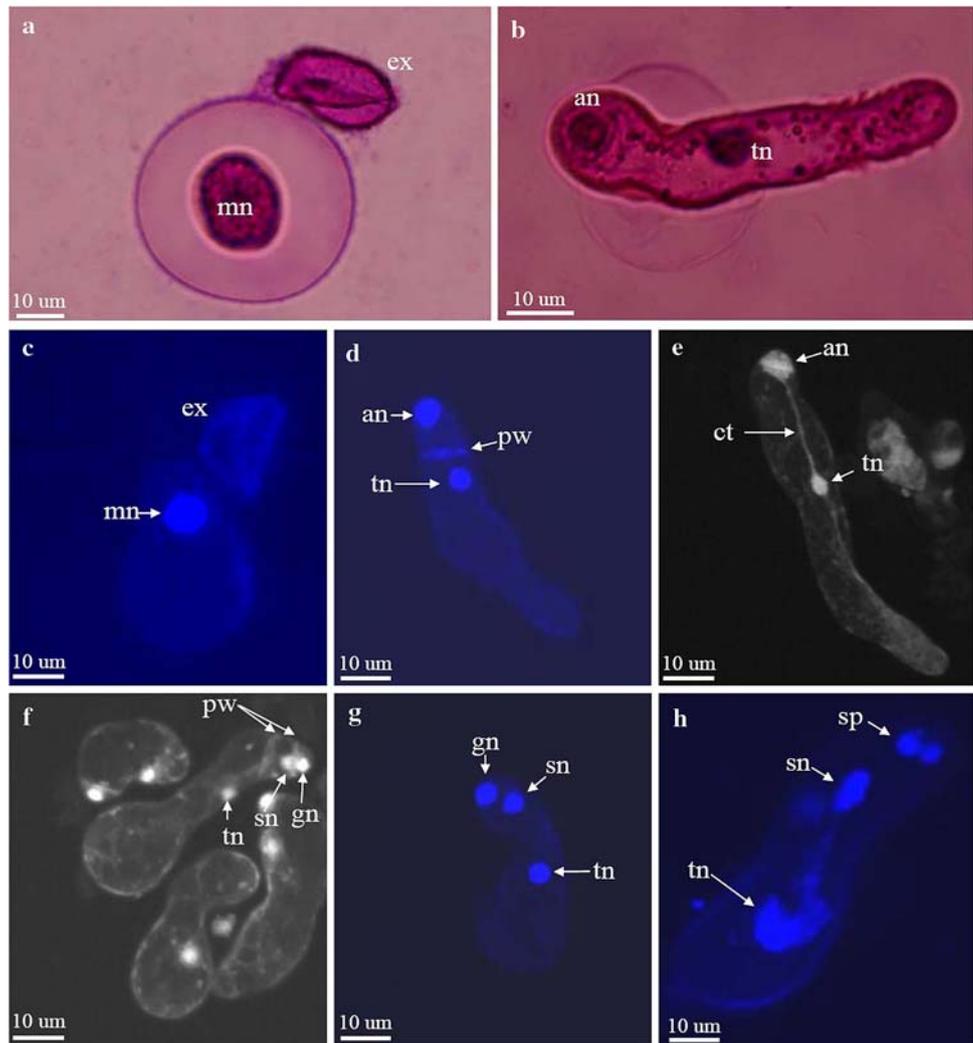
Soon after hydration in vitro, the mature microspores expanded and cast off their exines (Fig. 4a, c). After three days in culture, a pollen tube was produced and the microspore nucleus started to migrate into the pollen tube (Fig. 4c). Within 6 days of germination, the microspore



**Fig. 3** Phenology of microsporogenesis in *T. yunnanensis*

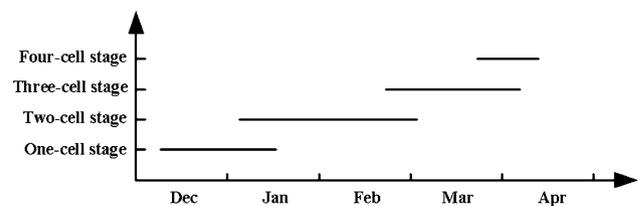
**Fig. 4** Microgametogenesis in vitro, stained with aceto-carmine or DAPI.

**a** Microspores nucleus (*mn*) after hydration (*ex*: exines) (stained with aceto-carmine). **b** Two nuclei (*an* and *tn*, short for antheridial nucleus and tube nucleus) stained with aceto-carmine. **c** Microspores with nucleus (*mn*) in one side before first division (stained with DAPI). **d** Newly formed antheridial nucleus (*an*) and tube nucleus (*tn*) separated by partition wall (*pw*) (stained with DAPI). **e** Antheridial nucleus and tube nucleus are connected by a cytoplasmic thread (*ct*) (stained with DAPI). **f** Three nuclei male gametophyte (*gn*, *sn* and *m*, short for generative, sterile and tube nuclei, respectively) with partition walls (stained with DAPI). **g** Three nuclei male gametophyte without partition wall (stained with DAPI). **h** Formation of two equal-sized male gametes or sperm (*sp*) (stained with DAPI)



nucleus in some pollen tubes divided and formed an antheridial cell and a tube nucleus (Fig. 4d). The wall surrounding the antheridial nucleus rapidly disappeared in most pollen tubes. The resulting antheridial and tube nuclei had dense cytoplasm (Fig. 4b). The antheridial nucleus remained on one end of the pollen tube while the tube nucleus migrated further into the elongating part of the pollen tube, but these two nuclei remained connected by a cytoplasmic thread (Fig. 4e). On the 20th day of culture, the antheridial nucleus divided and formed sterile and generative cells (Fig. 4f). However, their cell walls appeared to be short-lived since no more cell walls were observed in the following stages (Fig. 4g). The third mitotic division was observed beginning at 40 days of culture, i.e., the generative nucleus divided and formed the two male gametes or sperms (Fig. 4h).

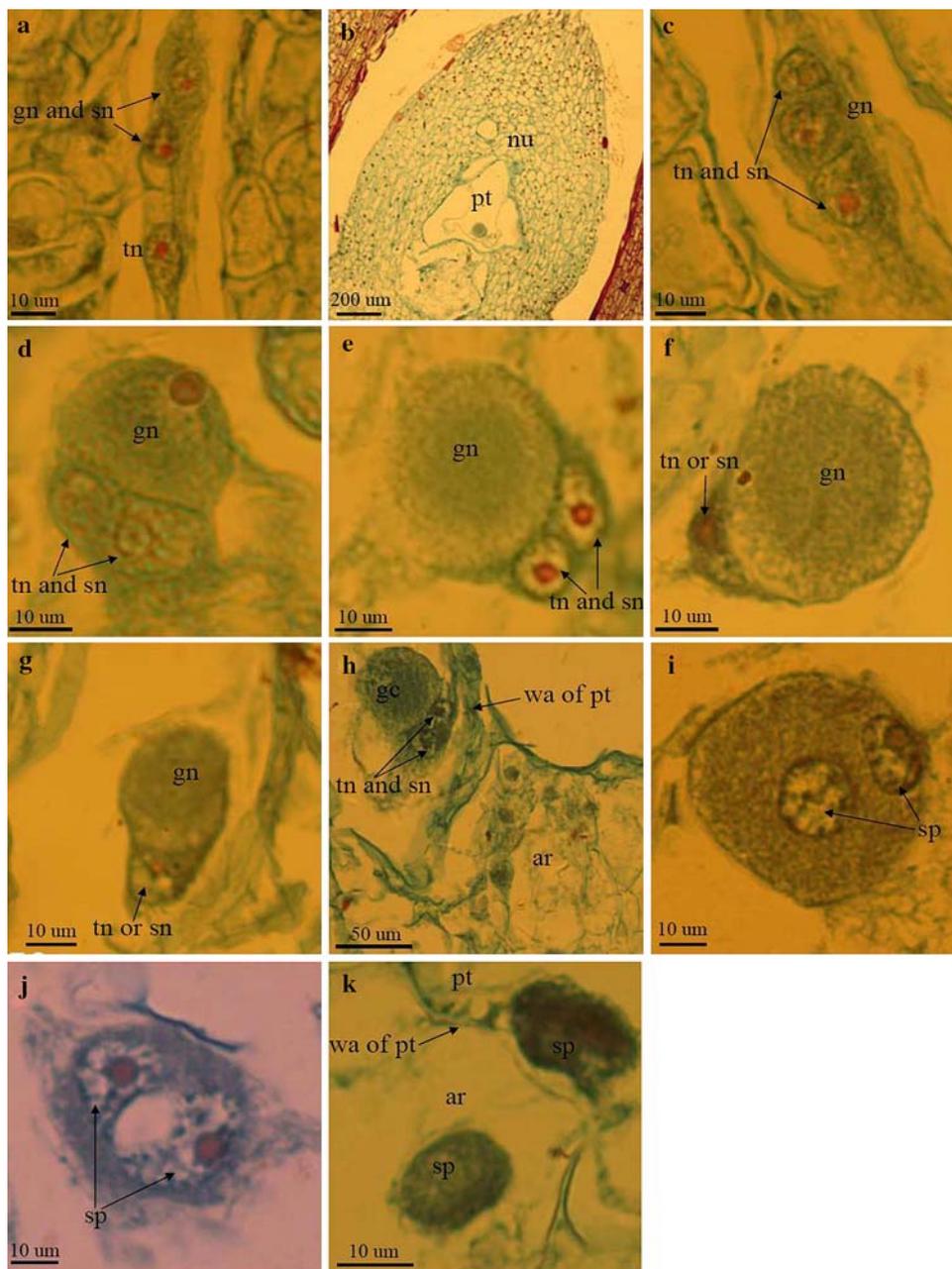
In vivo, pollination was recorded on 5 December 2006. The timing of microgametogenesis in vivo is shown in Fig. 5. The first mitotic division resulted in the formation of an antheridial cell and a tube nucleus, similar to that



**Fig. 5** Phenology of microgametogenesis in *T. yunnanensis*

described above. The second mitotic division formed sterile and generative nuclei with similar diameters of about 20  $\mu\text{m}$  (Fig. 6a). Prior to the third mitotic division, the pollen tube, which in this stage is in the nucellus, swelled rapidly (Fig. 6b). At this stage, the sterile, tube and generative nuclei were positioned close to each other forming a straight line. Furthermore, the generative nucleus had enlarged and occupied the middle position (Fig. 6c). The generative nucleus further enlarged and separated from the rest, while the sterile and tube nuclei became closer to each other and were both situated opposite the generative

**Fig. 6** Microgametogenesis in vitro. **a** Microgametophyte with three nuclei scattered in the pollen tube (*s*, *sn*, and *gn*, short for tube nucleus, sterile nucleus and generative nucleus, respectively). **b** Swollen pollen tube (*pt*) in nucellus (*nu*). **c** Nuclei in a straight line. **d, e** Tube and sterile nuclei below the generative nucleus. **f, g** Connections between generative nucleus and the tube nucleus (or sterile nucleus). **h** Tube and sterile nuclei occur between the generative cell and archegonium (*ar*) (*wa*, short for wall). **i** Generative nucleus dividing in the single hemisphere (*sp*: sperm). **j** Formation of male gametes or sperm (*sp*). **k** Two equal-sized sperm (*sp*), one of them proceeding into the archegonium (*ar*)



nucleus (Fig. 6d). As the generative nucleus continued to enlarge, the sterile and tube nuclei became smaller and smaller. In fact, just prior to the third mitotic division, their diameters were only about 10  $\mu\text{m}$ , while the generative nucleus was about 50  $\mu\text{m}$  in diameter (Fig. 6e). The generative nucleus exhibited a two-layer nucleoplasm, where the inner layer appeared denser than the outer layer (Fig. 6e, f). The cell membrane of the generative nucleus appeared to be connected with the membranes of the sterile and tube nuclei (Fig. 6f). The membranes of the tube and sterile nuclei adjacent to the generative nucleus appeared to have disintegrated, thereby appeared to form a nucleoplasm with three nuclei (Fig. 6g). In addition, it was observed that

when the pollen tube was near the archegonium, the sterile and tube nuclei always preceded the generative nucleus (Fig. 6h). The generative nucleus divided and formed two lens-shaped male gametes (Fig. 6i). The chromosomes became denser and more distinct as the cytoplasm divided to produce two equal-sized male gametes (Fig. 6j). The sterile and tube nuclei have disintegrated at this stage. Before fertilization, the cytoplasm of the male gametes became denser, and one of them migrated into the archegonium (Fig. 6k). No difference was found between the two male gametes. From our samples, the presence of male gametes was observed on 20 March 2007, while fertilization was observed on 3 April 2007.

## Discussion

The phenology of pollen cone development and microsporogenesis varies among *Taxus* spp. In Pennsylvania, Michigan, and Illinois, development of pollen cones in *T. canadensis* begins in spring (Dupler 1919), microspores are formed in October, and pollination occurs in late April of the following year (Dupler 1917). In Victoria, British Columbia, microspores begin to form in October, and pollination occurs from March to April of the following year (Anderson and Owens 2000). Development of pollen cone in *T. baccata* begins in August and meiosis occurs in November (Pennell and Bell 1985). In Illinois, pollination in *T. cuspidata* occurs in the middle of March (Sterling 1948). In our results, development of pollen cone in *T. yunnanensis* begins in July, microsporogenesis during August and November, and pollination from late October to December. As indicated above, the duration of microsporogenesis varies among *Taxus* spp., and that microsporogenesis in *T. yunnanensis* is shorter than those in other *Taxus* spp. reported. Furthermore, other *Taxus* spp. pollinate in spring of the following year; this paper is the first to report that pollination in a species of *Taxus* occurs during the same year as pollen cone initiation. These differences may be caused by the local weather because *T. yunnanensis* is one of the few species in *Taxus* that is located in low latitude areas, while all other species mentioned above are located in high latitude areas. We believe that the timing of pollination in *Taxus* is susceptible to changes in the environment. In fact, in *T. brevifolia*, it varies a lot not only under different sites but also under the same site on different years (Anderson and Owens 2000). Richard (1985) also reported that pollination in *T. baccata* varies between years.

Contrary to microsporogenesis, microgametogenesis in *T. yunnanensis* proceeds longer than those in other *Taxus* spp. The time from pollination to fertilization in other *Taxus* spp. is about two months (Chen and Wang 1985), while as short as one month in *T. canadensis* (Dupler 1917). In our study, the time from pollination to fertilization is more than three months. Using the same in vitro culture conditions as Anderson and Owens (2000), microgametogenesis lasts longer in *T. yunnanensis* than in *T. brevifolia*. In the latter, the first division occurs after three days, second division on the 17th day, and the sperm formation within 24 days (Anderson and Owens 2000). In our study, the first, second, and third divisions of microgametogenesis of *T. yunnanensis* occurred on the 6th, 20th, and 40th days, respectively. We believe that although microgametogenesis in vitro is susceptible to culture conditions, the lag in the timing could still reflect on the nature of microgametogenesis in *T. yunnanensis*. This longer duration of microgametogenesis in *T. yunnanensis* may be an adaptation to the lower temperatures during December to March, of the following year.

Microgametogenesis in other *Taxus* spp. occurs after March, when temperature is higher.

The morphology of pollen cones among *Taxus* spp. is known to be similar, and this is confirmed in our study. However, the number of microsporophylls per pollen cone and number of microsporangia per microsporophyll vary among the species of *Taxus*. In *T. baccata*, each pollen cone has up to 15 microsporophylls and each microsporophyll has three to eight microsporangia (Pennell and Bell 1985). In *T. brevifolia*, each pollen cone has nine to 11 microsporophylls and each microsporophyll has four to six microsporangia (Anderson and Owens 2000). In *T. canadensis*, each microsporophyll has three to eight microsporangia (Dupler 1919). In *T. yunnanensis*, the number of microsporophylls per pollen cone ranges from 15 to 28, and the number of microsporangia per microsporophyll varies from 4 to 12. The number of microsporophylls per pollen cone in *T. yunnanensis* is far greater than those of other *Taxus* spp. reported. As the strobilus is considered as a modified branchlet (Fernando et al. 2005), the number of microsporophylls per pollen cone may reflect the growth that occurs in one year. This suggests that pollen cones of *T. yunnanensis* grow faster than those of the species mentioned above.

The newly formed microspores in *Taxus* are known to be produced in one plane (Dupler 1917; Anderson and Owens 2000; Pennell and Bell 1986a). Our results show that in *T. yunnanensis*, microspores are not only produced in one plane, such as in the formation of T-shaped or isobilateral tetrads, but also in the form of a tetrahedron. These differences may provide some taxonomic or systematic information in *Taxus*. In *T. canadensis*, *T. brevifolia*, and *T. baccata*, microspores are uninucleate when shed, have many orbicules on the surface, and without prothallial cells (Dupler 1917; Anderson and Owens 2000; Pennell and Bell 1986a). These features have been confirmed in *T. yunnanensis*.

Under in vitro culture, a cell wall is formed after the first mitotic division of the microspores in *T. baccata* and *T. brevifolia* (Anderson and Owens 2000; Pennell and Bell 1986a), and this is confirmed in *T. yunnanensis*. However, another cell wall is formed after the second mitotic division in *T. yunnanensis*. We believe that this cell wall has not been reported in other *Taxus* spp. because it exist a very short time, which could easily be missed during observation. Besides their position in the pollen tube, no difference was found between antheridial, tube, and generative nuclei until the beginning of the third mitotic division. In *T. brevifolia*, additional cell division is found in 24-day-old cultures (Anderson and Owens 2000); the same phenomenon was also observed in *Pinus bungeana* (Chen and Wang 1985). Contrary to these reports, we did not observe further cell divisions in *T. yunnanensis* even in 60-day-old cultures.

As the generative nucleus enlarges by increasing its diameter from 20 to 50  $\mu\text{m}$ , the sterile and tube nuclei

decrease in diameter from 20 to 10  $\mu\text{m}$ , and until they are completely disintegrated. Because of the close association of the nuclei and timing of disintegration, it seems that the nuclear materials from the tube and sterile nuclei are utilized by the enlarging generative nucleus. Based on this, it appears that the first two mitotic divisions during microgametogenesis are related to sperm formation. Although direct evidence for this is lacking, it is suggested, based on the position and close relationships of the nuclei, that the sterile and tube nuclei are important in male gamete formation.

Our results showed that two equal-sized nuclei are formed at the end of microgametogenesis. This confirms the report in *T. baccata* (Pennell and Bell 1986b). In *T. baccata* and *T. brevifolia*, only a single male gamete participates in fertilization (Pennell and Bell 1988, Anderson and Owens 1999). This is also the case in *T. yunnanensis*, as well as in other conifers. Since no difference between the two male gametes were observed, Pennell and Bell (1986b) believes that as to which of the two will proceed into the archegonium and fuse with the egg is simply a matter of chance. Although this may be true, an ultrastructural analysis needs to be done to establish the presence or absence of preferential fertilization in *Taxus*.

This paper has provided data on the structure and development of the pollen cone, microspores and male gametes in *T. yunnanensis*. These aspects of the male reproductive system will contribute to a better understanding of sexual reproduction in *T. yunnanensis* and other *Taxus* spp. The phenology of microsporogenesis and microgametogenesis in *T. yunnanensis* differs from those of other *Taxus* spp. This study has raised an interesting issue on the close relationships between the nuclei of the microgametophytes and the possible contribution of the sterile and tube nuclei to sperm formation. A deeper analysis of these phenomena, particularly at the molecular level, will provide a new dimension for the study of conifer sexual reproduction.

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