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‘香榧’雌配子体发育和原胚形成的组织学观察

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摘 要:**【目的】**明确‘香榧’授粉到越冬前雌配子体的发育、受精和原胚形成过程,丰富和完善榧属植物的生殖发育学。**【方法】**人工授粉后,定期连续采集雌球花,应用常规石蜡切片技术,获得‘香榧’雌球花的纵切片,在光学显微镜下观察。**【结果】**‘香榧’雌球花由4层鳞片包被,由外向内依次为假种皮、珠被和珠心。珠心中下部分化雌配子体。‘香榧’在授粉期尚处于大孢子母细胞时期;授粉后1周内,完成减数分裂形成功能大孢子;6月初到8月底完成雌配子体的发育,包括游离核时期、细胞化阶段和颈卵器时期;‘香榧’有2个颈卵器母细胞,每个颈卵器分裂形成4个颈细胞和1个卵核,腹沟核很快消失。9月中上旬精卵融合形成合子并发育形成原胚,排列成4层,并以原胚越冬。**【结论】**‘香榧’的雌配子体发育过程基本上遵循了红豆杉科植物的发育规律,只在原胚类型和颈卵器数目上与其他种类有一定差异。‘香榧’原胚为松型,2个颈卵器,而红豆杉科其他种原胚为标准型,绝大多数仅1个颈卵器。

关键词:‘香榧’;雌配子体;原胚形成;组织学观察

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Histological studies on megagametophyte and embryogeny development in *Torreya grandis* ‘Merrillii’

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Abstract:【Objective】The purpose of this research was to determine anatomically the megagametophyte development, fertilization process and proembryo forming in *Torreya grandis* ‘Merrillii’ during the period from pollination to overwintering. It is noteworthy that gymnosperm differs from angiosperm is the interval from pollination to fertilization is longer than 3 months. Other characteristics are archegonium and proembryo. This is a basic research aim to observe these characteristics and find its special rules to differ from other gymnosperms. It is also a research for more reproductive biology research, such as cytoplasm inheritance, meiosis relevant research.【Methods】Artificial pollination was done on April 24 of 2014 when pollination drop appears. Ovules were collected every 3 to 4 days from the middle of April to end September in 2014. Length and diameter were measured and scales were removed. They were fixed in FAA (V_{formalin} : $V_{\text{glacial acetic acid}}$: $V_{\text{50\% ethanol}}$=5 : 5 : 90) at least 24 h, then hydrated in grated ethanol series, displaced by xylene and then paraffin. Paraffin filled ovules were sectioned in 8 μm thick and placed in 37 °C about 12 h. Sections were dyed by safranine and fast green and observed with SEM.【Results】Structure of the ovule includes aril, integument, nucellus and megagametophyte. The aril develops its length in May mainly to surround the nucellus for protection and formed micropyle for pollen entering into the ovule. About

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15 to 20 cells filled with tannins located on the inner side of the integument. Sporogenous tissue locates below the middle of the nucellus. One of these cells differentiates the mega gametophyte and other cells nourish it. The megagametophyte of *T. grandis* 'Merrillii' is still at megasporocyte stage during the pollination. One week after pollination, the megasporocyte undergoes meiosis and gives rise to functional megaspore. Other three megaspores are small in volume and degenerated soon. As the first cell of megagametophyte, functional megaspore' forming means the beginning the megagametophyte development. The functional megaspore needs about one month to develop itself in the volume, nuclei size and cytoplasm abundance. From June to August, megagametophyte is fully developed experiencing free nuclear stage, cellularization stage and archegonium stage. The functional megaspore divides about eight times continually. The division needs nutrition from spongy tissue directly. The spongy tissue is consumed as its volume is on the decrease. Cellularization stage means free nuclei turned into cells with cell wall. The outer free nuclei acquire cell wall first, while the inner free nuclei last. Meanwhile, the megagametophyte forms its thick cell wall. The spongy tissue almost disappears. Moreover, *T. grandis* 'Merrillii' possess two archegonial initials, each of which can divide into four neck cells and one egg cell while ventral canal cell disappeares quickly. Archegonium includes four neck cells and one egg cell. Neck cell can guide the pollen tube's direction into the egg cell. Unfortunately, only two neck cells can be seen in the longitudinal cutting direction. The pollen's stored in a tapered place, which located in the end of the integument and the top of the nucellus. More than one pollen germinates and their trace can be seen clearly, as they move fast and ruin nucellus cells. Tip of the pollen tube stays at the top of the megagametophyte waiting for mature egg cell. It inflates much that formed a big cavity. Spermatophyte divides into two sperms and a sterile nucleus, a tube nucleus. Around early to mid-September, a sperm fuses with the egg cell to form a zygote, which develops into proembryo in the form of four layers. When one egg cell combines with one sperm of two, the other one stopped. So we get only a zygote, the other egg cell stopped to develop and degenerated. In this process, protein vacuole has a change in its volume, amount and location. Protein vacuole gives nutrition for fertilization. We also find the change of the cytoplasm. After fertilization, cytoplasm around the zygote turned dense. New cytoplasm means male cytoplasm may participate in the fertilization and cytoplasm inheritance is worthy. Before next spring comes, *T. grandis* 'Merrillii' live through the winter in the condition of proembryo. The proembryo includes 16 cells arranged in four layers, called standard proembryo. The zygote nucleus divides three times into eight nuclei. Eight nuclei acquired their cell walls and all divide one time. Diameter can measure stage of megagametophyte to some extend while length cannot clearly distinguish every stage. Diameter and length increase obviously in the stage of free nuclei and cellularization. And diameter has an obvious increase in the stage of megaspore forming, archegonial initials' division and fertilization, while length increase little compared the previous stage.【Conclusion】The development of megagametophyte in *T. grandis* 'Merrillii' mostly follows the development pattern of Taxaceae, only with certain differences in the proembryo type and the number of the archegonium. Unique characteristics of *T.grandis* 'Merrillii' include interval of four months from pollination to fertilization, two archegonia and standard proembryo.

Key words: *Torreya grandis* 'Merrillii'; Megagametophyte; Proembryo; Histological observation

榧树(*Torreya grandis*)属红豆杉科(Taxaceae)榧属植物(*Torreya*),自然分布于浙江、安徽南部、福建北部及江西东北部[1]。'香榧'(*Torreya grandis* 'Merrillii')是榧树自然变异类型经无性繁殖培育而成的优良品种,经济寿命长,栽培效益居山地经济树种之首,是我国特有的珍稀干果[2]。'香榧'雌花成对生于叶腋,每根枝条有5~8对雌花,待授粉后第2年8月底,每根枝条仅有1~2枚成熟种子,其余雌花因未能

授粉、授粉后败育宿存在成熟种子基部或者第2年膨大期败育而脱落[2]。

同被子植物大孢子即成熟卵细胞不同,裸子植物的大孢子要经历游离核时期、细胞化阶段、颈卵器原始细胞时期和颈卵器时期才形成卵细胞;被子植物的受精卵直接发育成胚,而裸子植物的受精卵在形成胚前要经历原胚阶段。裸子植物生殖生物学研究主要集中在松柏两科[3]、银杏科[4]。榧属生殖生物学的研究最早始于20世纪初,其中Coulter等[5]对佛罗里达榧(*Torreya taxifolia*)的雌配子体发育和原胚形成以绘图的形式详尽表述。国内榧属植物生殖生物学方面的研究始于20世纪90年代,汤仲勋等[6]初步确定了‘香榧’授粉、雌配子体发育各阶段和受精等过程的时间节点,阐明了‘香榧’授粉受精间隔长达4个月。但是,‘香榧’雌配子体不同阶段发育特征和原胚形成过程等还不得而知。笔者以‘香榧’雌球花授粉后的外观变化为依据,通过石蜡切片技术,从组织学角度观察了‘香榧’雌配子体发育和原胚形成,明确‘香榧’果的形成过程,并对比红豆杉科其他植物和其他裸子植物,为榧属植物的系统发育积累基础资料。

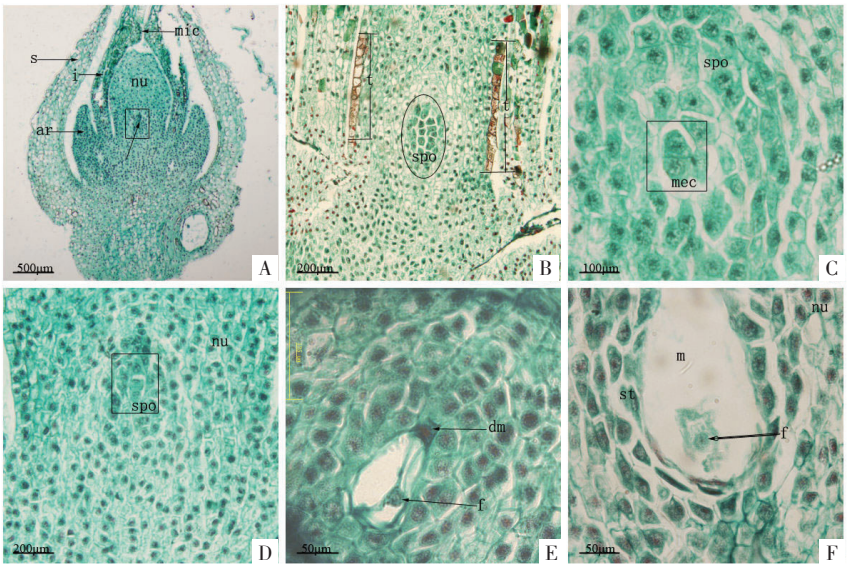
1　材料和方法

材料为浙江农林大学香榧种植基地的‘香榧’。于2014年4月24日对吐露传粉滴的雌球花进行人工授粉。花粉来自同一区域‘香榧’雄株,于4月8日采集,室内阴干待花粉散出,用毛笔将花粉转移到传粉滴上,授粉2 h后对仍有传粉滴吐露的雌球花进行1次补授粉,直至该雌球花无传粉滴吐露。从授粉当日起,每隔5~7 d采集膨大果4~5个,最后采样时间为2015年2月10日,此时膨大果已发育成熟。各时期的膨大果剥去鳞片后测量种子径向和横向的长度,取平均值作为该时期种子的种长、种宽。去鳞片的膨大果用FAA固定至少24 h后,按照常规石蜡切片法制片,切片厚度8 μm,番红-固绿对染,Olympus DP 70观察并拍照。

2　结果与分析

2.1　‘香榧’雌配子体的发育及原胚形成

2.1.1　胚珠的结构　‘香榧’为直生胚珠,由1层珠被包被,厚珠心,珠心下方有细胞质浓厚的造孢组织(图1-A)。珠心的最外一层,有10~15个细胞(图1-



A. 香榧雌球花纵切面,假种皮长度约胚珠的1/3(2014年4月30日);B. 珠被最外一层富含单宁的细胞(2014年5月2日);C. 大孢子母细胞减Ⅰ末期,细胞板形成(2014年4月30日);D. 大孢子母细胞减Ⅱ后期(2014年5月2日);E. 功能大孢子形成,其余3个大孢子退化痕迹(2014年5月2日);F. 发育中的大孢子(2014年5月12日)。s. 鳞片;ar. 假种皮;nu. 珠心;i. 珠被;mic. 珠孔;spo. 造孢组织;t. 单宁细胞;mec. 大孢子母细胞;f. 功能大孢子;dm. 退化的大孢子;st. 海绵组织;m. 雌配子体。

A. Overall structure. Length of aril is about a third of ovule (April 30, 2014); B. The tannins cell out of the integument (May 2, 2014); C. Telophase of the first division of the meiosis, cell plate formed (April 30, 2014); D. Anaphase of the second division of meiotic (May 2, 2014); E. Functional megaspore formed, the trace left of regressive megaspore near micropyle (May 2, 2014); F. The developing megaspore (May 12, 2014). s. Scale; ar. Aril; nu. Nucellus; i. Integument; mic. Micropyle; spo. Sporogenous tissue; t. Taninn cells; mec. Megaspore mother cell; f. Functional megaspore; dm. Degraded megaspore; st. Spongy tissue; m. Magagametophyte.

图1　胚珠结构和大孢子形成

Fig. 1　Structure of the ovule and the megaspore

B)。胚珠外面包被6~20层细胞(图1-A),之后将发育成假种皮,假种皮的生长主要在5月份。

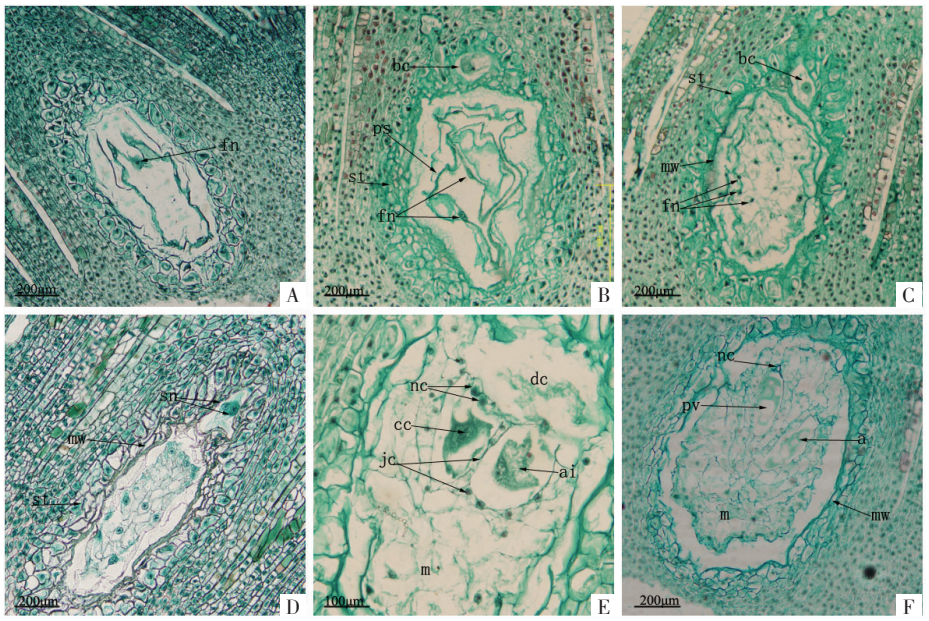
2.1.2 雌配子体发育 主要有以下4个时期。(1)大孢子发生时期:5月初,位于造孢组织近中央的大孢子母细胞进行减数分裂(图1-C~D),形成纵向排列的4个大孢子。合点端的大孢子最终发育成为功能大孢子,近珠孔端的3个退化,退化痕迹可见(图1-E)。大孢子形成以后,体积不断增大,合点端膨大尤其明显,细胞核增大,由一团浓厚的细胞质包围(图1-F)。

(2)游离核时期:经减数分裂形成的大孢子是雌配子体的第1个细胞。6月初,大孢子进行多次有丝分裂,但不形成细胞壁,细胞核呈游离状态(图2-A~C)。随着游离核的分裂,雌配子体不断增大,游离核分布在中央液泡周围的细胞质中。游离核之间有明显的原生质丝连接(图2-B)。

在雌配子体游离核时期,包围在雌配子体周围的几层珠心细胞发育成为海绵组织,通常包含3~4层细胞(图2-A~D)。海绵组织在游离核时期出现,受精后完全消失。

(3)细胞化时期:7月中下旬,游离核完成最后一次分裂后,开始形成细胞壁。细胞壁是在连接游离核的原生质丝的基础上形成的。雌配子体边缘的游离核最先形成细胞壁,然后向心式的形成细胞壁,最后在中央汇合,游离核全部细胞化(图2-D)。该阶段除了游离核形成细胞壁,雌配子也形成了雌配子体壁(图2-D)。

(4)颈卵器时期:7月底,在细胞化的雌配子体中,2个颈卵器母细胞形成于近珠孔端,明显比周围细胞大,核大质浓(图2-E)。2个颈卵器母细胞的分裂不同步,右侧的颈卵器母细胞还未分裂,左侧已经进入颈卵器时期,有颈细胞和中央细胞形成(图2-F)。颈卵器母细胞进行一次不均等分裂,形成一个偏向珠孔端较小的初生颈细胞和偏向合点端一个较大的中央细胞。初生颈细胞进行2次有丝分裂,形成4个颈细胞(纵切面只能看到2个)。颈细胞比周围细胞小,染色比周围细胞深(图2-E)。中央细胞进行一次核的不均等分裂,形成卵核和腹沟核。腹



A. 游离核初期(2014 年 6 月 3 日);B. 游离核由原生质丝连接(2014 年 7 月 13 日);C. 游离核增多(2014 年 7 月 18 日);D. 完全细胞化的雌配子体,精核形成(2014 年 7 月 23 日);E. 香榧的 2 个颈卵器母细胞的分裂不同步。右侧处于颈卵器母细胞时期,左侧颈卵器母细胞分裂为 4 个颈细胞和中央细胞(2014 年 9 月 4 日);F. 蛋白泡出现(2014 年 9 月 6 日)。fn. 游离核;st. 海绵组织;ps. 原生质丝;bc. 体细胞;mw. 大孢子壁;sn. 精核;dc. 溶解腔;nc. 颈细胞;cc. 中央细胞;jc. 套细胞;ai. 颈卵器母细胞;a. 颈卵器;pv. 蛋白泡。

A. Less free nuclei (June 3, 2014); B. Plasma silk (July 13, 2014); C. Free nuclei continue to divide (July 18, 2014); D. Cellularization spongy tissue, sperm nuclear formed (July 23, 2014); E. The two archegonium mother cell do not divide at the same time. When the right one has not divided, the left one has divided into the central cell and four neck cells (September 4, 2014); F. The protein vacuoles appeared (September 6, 2014). fn. Free nuclei; sp. Spongy tissue; ps. Plasma silk; bc. Body cell; mw. Magaspore wall; sn. Sperm nucleus; dc. Dissoved cavity; nc. Neck cell; cc. Central cell; jc. Jacket cell; ai. Archegonial initial; a. Archegonium; pv. Protein vacuole.

图 2 游离核时期、细胞化阶段和颈卵器时期

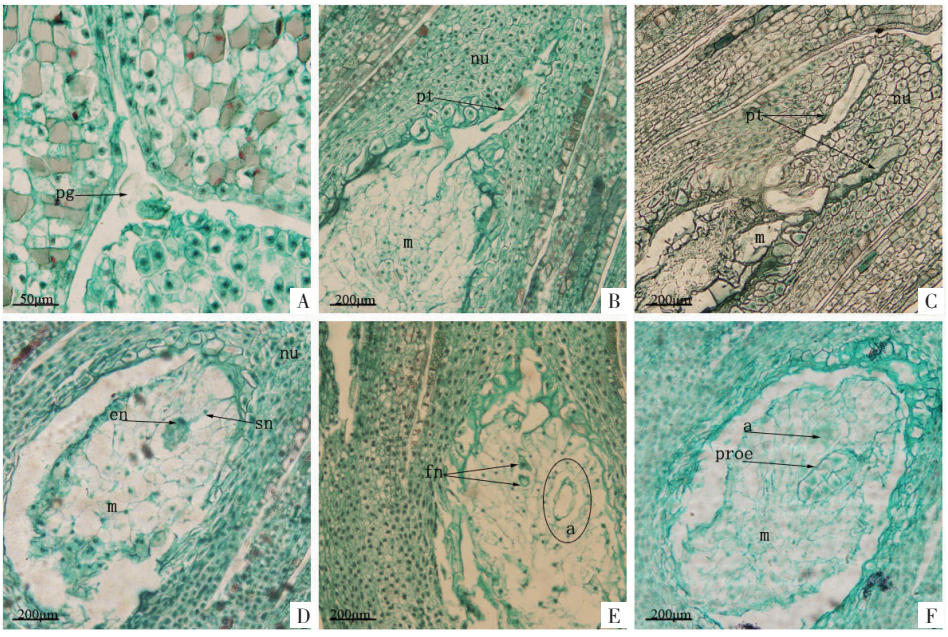
Fig. 2 The stage of free nuclei, cellularization and archegonium forming

沟核很快消失。卵形成后向颈卵器中央移动,同时蛋白泡出现(图2-F)。

颈卵器时期,雌配子体壁仍清晰可见。套细胞紧密排列在颈卵器周围,含一层细胞,体积小,核大(图2-E~F)。

2.1.3　*花粉萌发及精核释放*　4月底,花粉进入胚珠,储藏在珠心组织上方与珠孔交叉的位置——储粉室(图3-A);直到7月初,花粉萌发花粉管,伸入珠心组织(图3-B);萌发的花粉管不止一条(图3-C),花粉管在珠心组织中的生长极快,穿透了珠心组织。7月中下旬,花粉管先端抵达大孢子壁(图2-B~D),此时花粉内含物质集中于先端,体细胞分裂形成2个大小相等的精子(图2-D)。此后一段时间,花粉管生长基本停滞,停留在颈卵器上方,形成一个很大的溶解腔(图2-E)。溶解腔也是花粉管在珠心细胞中穿行速度过快破坏了珠心细胞导致。9月上旬颈细胞形成后,花粉管才继续伸长。花粉管先端到达颈细胞后,释放精细胞等内容物到颈卵器中(图3-D)。

2.1.4　*受精和原胚形成*　9月上旬,精细胞进入颈卵器后,与卵细胞接触,随后融合;受精后合子分裂,形成2个游离核(图3-E);之后2个游离核分裂4次,形成16细胞原胚,排成4层(图3-F)。



A. 花粉进入胚珠后储存在储粉室中(2014 年 5 月 8 日);B. 花粉管伸入珠心组织(2014 年 7 月 23 日);C. 不止一条花粉管进入珠心组织(2014 年 7 月 23 日);D. 受精前精卵两核(2014 年 8 月 24 日);E. 二核原胚形成(2014 年 9 月 4 日);F. 原胚形成(2014 年 9 月 20 日)。pg. 花粉粒;pt. 花粉管;nu. 珠心;m. 雌配子体;en. 卵核;sn. 精核;fn. 游离核;a. 颈卵器;proe. 原胚。

A. Pollen chamber (May 8, 2014); B. The trace left by the movement of pollen tube in nucellus (July 23, 2014); C. One or two more pollen tubes appear in nucellus (July 23, 2014); D. The egg nucleus and the sperm cell (August 24, 2014); E. Fertilization ended with two free nuclei (September 4, 2014); F. Proembryo with sixteen cells (September 20, 2014). pg. Pollen grain; pt. Pollen tube; nu. Nucellus; m. Megagametophyte; en. Egg nucleus; sn. Sperm nucleus; fn. Free nuclei; a. Archegonium; proe. Proembryo.

图 3　花粉管伸长、受精作用与原胚形成

Fig. 3 Elongation of the pollen tube, the fertilization and the proembryo formation

2.2　球果外观与雌配子体发育时期的关系

‘香榧’膨大果去鳞片后,测得径长、径宽,按雌配子体的发育进程统计,得到不同阶段的平均径长、径宽(表1)。因为树龄、林地肥沃性的不同和天气等原因,‘香榧’吐露传粉滴的时间不同,导致授粉的时间不同,进而导致雌配子体发育的时期有差异。根据表1可将球果外观及大小作为确定雌配子体发育时期的一个参考。

从授粉到大孢子形成,种子膨大主要体现在径宽的增加,而长度没有明显变化;游离核时期和细胞化阶段,种子的长宽比之前有了一个大幅的增长;颈卵器阶段径宽进一步增加;受精阶段与游离核时期的种子大小差别明显;原胚时期的宽度达到最大。综合分析,授粉后种子径长的增加主要发生在游离核时期,而径宽在每一时期都有明显增加。

表 1 ‘香榧’有性生殖过程

Table 1 The process of sexual reproduction in *Torreya grandis* ‘Merrillii’

日期 Date	种子径长 Lenth in the diameter	种子径宽 Width in the diameter	花粉管伸长 Growing of the pollen tube	雌配子体发育 Development of female gametophyte
4月24日 April 24	2.64±0.10 a	2.06±0.04 a	授粉 Pollination	造孢组织 Sporogenous tissue
4月30日 April 30	2.70±0.04 a	2.17±0.03 ab		减I中期 Meiotic metaphase
5月2日 May 2	2.87±0.02 a	2.21±0.03 b		功能大孢子 Megaspore
6月3日至7月18日 June 3 to July18	4.60±0.03 b	3.19±0.01 c	花粉管先端到达海绵组织 Pollen tubes near to spongy tissue	游离核时期 Free nuclei
7月18日至7月23日 July 18 to July 23	5.02±0.19 c	3.26±0.04 c	体细胞分裂 2 sperm cells	细胞化阶段 Cellular
7月23日 July 23	5.26±0.02 cd	3.40±0.02 d		颈卵器母细胞 Archegonium mother cell
7月23日至9月4日 July 23 to September 4	5.31±0.01 cd	3.43±0.04 d	释放精核 Near to archegonium	颈卵器阶段 Archegonium
9月4日 September 4	5.50±0.13 de	3.67±0.01 e		受精 Fertilization
9月16日 September 16	5.59±0.20 de	3.80±0.06 f		二核原胚 2 free nuclei of proembryo
9月20日 September 20	5.69±0.03 e	3.81±0.16 f		16细胞原胚 Proembryo

3 讨 论

3.1 授粉受精周期

裸子植物的有性生殖过程与被子植物的重大区别在于前者授粉时雌雄配子体均未形成或成熟。相比大部分被子植物，大部分裸子植物由大孢子母细胞减数分裂形成大孢子，再经历游离核时期、细胞化阶段，进入颈卵器时期到卵核形成需要很长时间；同时，花粉萌发、花粉管伸长到精核形成也需要较长时间。

不同裸子植物完成授粉受精所需时间不同。比如太白红杉(*Larix chinensis*)[7]在5月中旬散粉，受精过程发生在7月中旬，从授粉到受精大约需要45 d；银杏(*Ginkgo biloba*)[8]4月中旬花粉成熟散粉，8月底完成受精，间隔4个多月；白皮松(*Pinus bungeana*)[9]散粉时间发生在5月初，受精过程发生在散粉后第2年5月底。少数被子植物也存在这种现象，木麻黄(*Casuarina equisetifolia*)[10]、杨梅(*Myrica rubra*)[11]和桤木属(*Alnus*)[12]的两个树种从授粉到受精需要1个多月。Sogo等[11]对杨梅(*Myrica rubra*)授粉受精间隔的1个月内花粉管的伸长过程进行了研究，从雄配子体发育的角度研究了授粉受精间隔长的原因是胚囊尚未发育成熟。

‘香榧’的受精作用发生在8月底，与散粉间隔达4个月；期间，花粉萌发和花粉管伸长所需营养都由胚珠供应。推测这种发育方式可能与大部分裸子植物是风媒传粉有关，风力传粉的不确定性使裸子植物形成这种尽可能减少营养消耗的机制，传粉成功后雌雄配子体才进一步发育。

3.2 ‘香榧’雌配子体发育特性

根据雌雄花着生位置[13]、种子蛋白多肽和针叶过氧化物酶种类[14]、胚胎发育[15]差异，榧属被认为是红豆杉科的进化类群。本文研究发现，‘香榧’雌配子体的发育过程、授粉受精过程和原胚类型等方面与其他裸子植物明显不同，而雌配子体的发育过程与其他红豆杉科植物的发育特性基本一致，即中央细胞分裂不伴随细胞壁的形成、以原胚越冬、颈卵器单生等，但在原胚类型和颈卵器数目上有一定差异：‘香榧’原胚为松型，红豆杉科其他种[16-17]的原胚为标准型，‘香榧’有2个颈卵器，白豆杉(*Pseudotaxus chienii*)[18]和穗花杉(*Amentotaxus argotaenia*)[19]只有1个颈卵器，短叶红豆杉(*Taxus brevifolia*)[20]的颈卵器为1~8个。生殖发育过程的简化是演化趋势[21]，标准型原胚被认为比松型原胚更原始[22]。据此验证前人研究，‘香榧’比红豆杉科其他植物更进化。

4 结 论

‘香榧’雌配子体的发育过程从大孢子形成开始，大孢子由大孢子母细胞减数分裂而来。5月初，

大孢子形成,细胞和细胞核经过一系列变化后,于6月初进入游离核时期;游离核时期长达1个半月,细胞化阶段在很短时间内完成,雌配子体进入颈卵器阶段;‘香榧’有2个颈卵器,卵核在颈卵器内形成,与精核完成受精,形成16细胞原胚,以原胚越冬。

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