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Three *Pteris* species (Pteridaceae: Pteridophyta) reproduce by apogamy

Yao-Moan HUANG[1], Sheng-Yuan HSU[2], Tsung-Hsin HSIEH[3], Hsueh-Mei CHOU[4], and Wen-Liang CHIOU[2,*]

[1]*Division of Silviculture, Taiwan Forestry Research Institute, 53 Nanhai Rd., Taipei 10066, Taiwan*

[2]*Division of Botanical Garden, Taiwan Forestry Research Institute, 53 Nanhai Rd., Taipei 10066, Taiwan*

[3]*Department of Biological Sciences and Technology, National University of Tainan, 33 Su-Lin St., Sec. 2, Tainan 70005, Taiwan*

[4]*Department of Biotechnology, Yuanpei University, 306 Yuanpei St., Hsinchu 30015, Taiwan*

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ABSTRACT. The ploidy levels, morphologies of gametophytes and young sporophytes, and reproductive modes of *Pteris cretica*, *P. pellucidifolia*, and *P. wulaiensis* were determined in this study. *Pteris cretica* and *P. wulaiensis* were diploid while *P. pellucidifolia* was triploid. Sporophytes of all three species produced 32-spored sporangia. Spore sizes of the triploid *P. pellucidifolia* were significantly larger than those of the other two diploid species. However, the frequencies of normal spores were lower in *P. pellucidifolia* (73%) than those of the other two species (95%-98%). Gametophytes of *P. cretica* and *P. pellucidifolia* produced normal antheridia but no archegonium. Although *P. wulaiensis* produced archegonia, those archegonia had lost their function. Every antheridium of these three species produced 32 active sperms. All of these three species apogamously produced sporophytes. Young sporophytes were produced directly on the gametophytes without fertilization. The first fronds of young sporophytes predominantly possessed midribs, with only a few of them lacking midribs. This study also suggests that the best way to infer the reproductive mode of ferns is to detect whether the embryo formed from the vegetative cells of gametophytes, although those other reproduction-related traits may provide useful indicators.

Keywords: Apogamy; Chromosome; Gametophyte; Morphology; Ploidy; *Pteris cretica*; *Pteris pellucidifolia*; *Pteris wulaiensis*.

INTRODUCTION

Fertilization is a prominent feature in the sexual life-cycle of ferns. However, a number of fern species exhibit another way to produce sporophytes: they are born out of gametophyte cells without fertilization, in a process known as apogamy. Many characteristics have been used to infer the occurrence of apogamy in ferns, e.g., ploidy levels, spore number per sporangium, spore size, the development of gametangia, and the formation and morphology of young sporophytes.

Apogamous reproduction is strongly associated with ploidy level. More than 75% of apogamous ferns are polyploid (Walker, 1962; Kanamori, 1972; Park and Kato, 2003). Heilbronn (1932) found that opportunities for apogamy increase with the addition of chromosomes. Furthermore, Raghavan (1989) suggested that an important provision for apogamy is an increase in gene dosage. That implies that polyploidy might induce apogamous occurrence. More, it may increase the probability that occasional apogamous sporophytes will be selected because they are the only viable sporophytes that can be produced when unbalanced chromosome combination prevents typical sexual reproduction.

Most advanced, sexual leptosporangiate ferns have 16 spore mother cells per sporangium. After meiosis each spore mother cell will yield 4 spores, and thereby 64 spores are produced per sporangium. On the other hand, most apogamous species have only 8 spore mother cells, instead of 16, and yield 32 spores per sporangium through normal meiosis. A few apogamous ferns may produce 16 spore mother cells but will also yield 32 spores per sporangium due to a failure of meiosis (Manton, 1950; Braithwaite, 1964). Thus, having 32 spores per sporangium is presumptive evidence of apogamous ferns (Knobloch, 1966).

Spore size is commonly used to distinguish different ploidy levels within allied species (Stebbins, 1950; Huang et al., 2006). Pteridophyte spores are usually larger in apogamous, polyploid taxa than in their sexual, diploid counterparts (Moran, 1982; Huang et al., 2006).

*Corresponding author: E-mail: chiou@tfri.gov.tw; Tel: 886-2-23039978; Fax: 886-2-23076220.

One piece of evidence indicating apogamy is the lack of archegonia in the gametophyte (Knobloch, 1966). However, the gametophytes of some apogamous ferns were documented to produce both antheridia and archegonia, such as *Diplazium megaphyllym* and *Cheilanthes hirsuta* (Chiou et al., 2006; Huang et al., 2009). Archegonia of those apogamous ferns develops aberrantly and loses its function.

The origin and development of apogamous sporophytes were described in many ferns (Farlow, 1874; Allen, 1914; Steil, 1919; Mehra, 1938; Patterson, 1942; Duncan, 1943; Nayar and Bajpai, 1964; Kanamori, 1967; 1972). Usually, an apogamous embryo projects directly from the apical notch or on the ventral midrib approaching the apical notch of the gametophyte (Steil, 1939), instead of from the archegonium. The appearance of multicellular hairs or scales in the embryonic area is regarded as a reliable indicator of apogamy, as is the appearance of tracheids within the cushion, or the venation of the first frond of a young sporophyte (Steil, 1939; Wagner, 1952; Chiou et al., 2006; Huang et al., 2006).

Pteris is a large fern genus, containing about 280 species (Copeland, 1947). About one-third of them are apogamous (Walker, 1962; 1992). Apogamy is mostly judged by the location of apogamous embryo formation, e.g., *P. argyraea*, *P. sulcata* (Steil, 1918), *P. biaurita* (Mehra, 1938), *P. cretica* (Farlow, 1874; DeBary, 1878; Kanamori, 1972), *P. kiuschiuensis*, *P. natiensis*, *P. nipponica*, *P. oshimensis*, *P. setulosocostulata* (Kanamori, 1972), and *P. fauriei* (Kanamori, 1972; Huang et al., 2006), with a few of them being indicated by ploidy levels (e.g., Walker, 1962; Chang, 1991). In Taiwan, estimated by ploidy levels, 41% of *Pteris* was reported to reproduce by apogamy (Chang, 1991). However, a gametophyte was directly studied in a few species of this genus (Huang et al., 2006; Chao et al., 2010).

In this study, sporophytes and gametophytes of three *Pteris* species native to Taiwan, i.e., *P. cretica* L, *P. pellucidifolia* Hayata, and *P. wulaiensis* Kuo, were cultured. Their characteristics related to reproductive biology—such as ploidy level, spore number per sporangium, spore size, gametophyte morphology, embryo formation, and morphology of the first fronds—were observed. Based on the observations of these characteristics, we concluded that these three species reproduce by apogamy.

MATERIALS AND METHODS

Pteris cretica, *P. pellucidifolia,* and *P. wulaiensis* were collected from forests in Taiwan (Table 1). They were then transplanted to a fern greenhouse at the Taipei Botanical Garden. After two months in culture, some vigorous root tips were cut, fixed, stained, and squashed to count their chromosome numbers following Sharma's (1982) method. Voucher specimens were deposited in the Taiwan Forestry Research Institute herbarium (TAIF).

To calculate the number of spores per sporangium, fresh fronds with mature sporangia were collected, and ten sporangia from each sporophyte were randomly sampled. To determine the frequency of normal spores, fronds were air-dried for 2 days to release spores, and 400 spores were randomly sampled from each sporophyte. The size (the average length of 3 equatorial axes) of 100 randomly selected normal spores from each plant was measured under a microscope.

To observe the sexual expression of gametophytes, spores were collected from sporophytes and directly sown onto medium (vermiculite: peat: perlite = 4: 4: 2). All cultures were maintained under white fluorescent illumination of PAR 24 μmol m^{-2} s^{-1} for 12 h d^{-1}. The temperature ranged 20-28°C. The sizes (width) and genders of gametophytes were recorded when the gametophytes began to produce young sporophytes and at the end of culturing.

To count the number of sperms in an antheridium, five gametophytes bearing antheridia were sampled from each species and immersed in 95% alcohol for one min. Ten mature antheridia from each of five gametophytes of each species were observed.

To observe the development of gametangia and the position of sporophyte embryos, ten gametophytes of each species were dehydrated, ultrasectioned, and stained (Spurr, 1969).

All traits and development were observed with light microscopy (Leica, Wild M8; Leitz, Dialux 20), and photographs were taken with a digital camera (Coolpix 995, Nikon). Means were compared with ANOVA followed by a Tukey post-hoc test ($p < 0.05$) with the program SPSS. Throughout the text, values are given as mean ±SD.

RESULTS

Chromosome numbers

Assuming a basic chromosome number of 29 (Manton, 1950), *P. cretica* and *P. wulaiensis* were diploid (2n = 2x = 58), and *Pteris pellucidifolia* was triploid (2n = 3x = 87) (Figure 1).

Sporangia and spores

Sporophytes of these three species produced 32-spore sporangia. Mature, normal spores were dark brown and

Table 1. Sources of the three *Pteris* species in Taiwan.

Species	Location and elevation	Voucher
P. cretica	Henglong, Miaoli County (27°09' N, 120°57' E); 1,310 m	*PF Lu 9700* TAIF
P. pellucidifolia	Yenhai, Hualien County (24°10' N, 121°30' E); 1,200 m	*PF Lu s.n.* TAIF
P. wulaiensis	Wulai, Taipei County (24°52' N, 121°32' E); 500 m	*YM Huang s.n.* TAIF

trilateral. Some spores of these three species were abortive and had an irregular shape (Figure 2). Proportions of normal spores were 98% in *P. cretica*, 73% in *P. pellucidifolia*, and 95% in *P. wulaiensis*. The diameters of normal fresh spores of the triploid *P. pellucidifolia* (55.5±3.1 μm) were significantly larger than those of the diploid *P. cretica* (39.7±4.9 μm) and *P. wulaiensis* (42.7±2.4 μm). However, the spore sizes of *P. wulaiensis* and *P. cretica* exhibited no significant difference (Table 2).

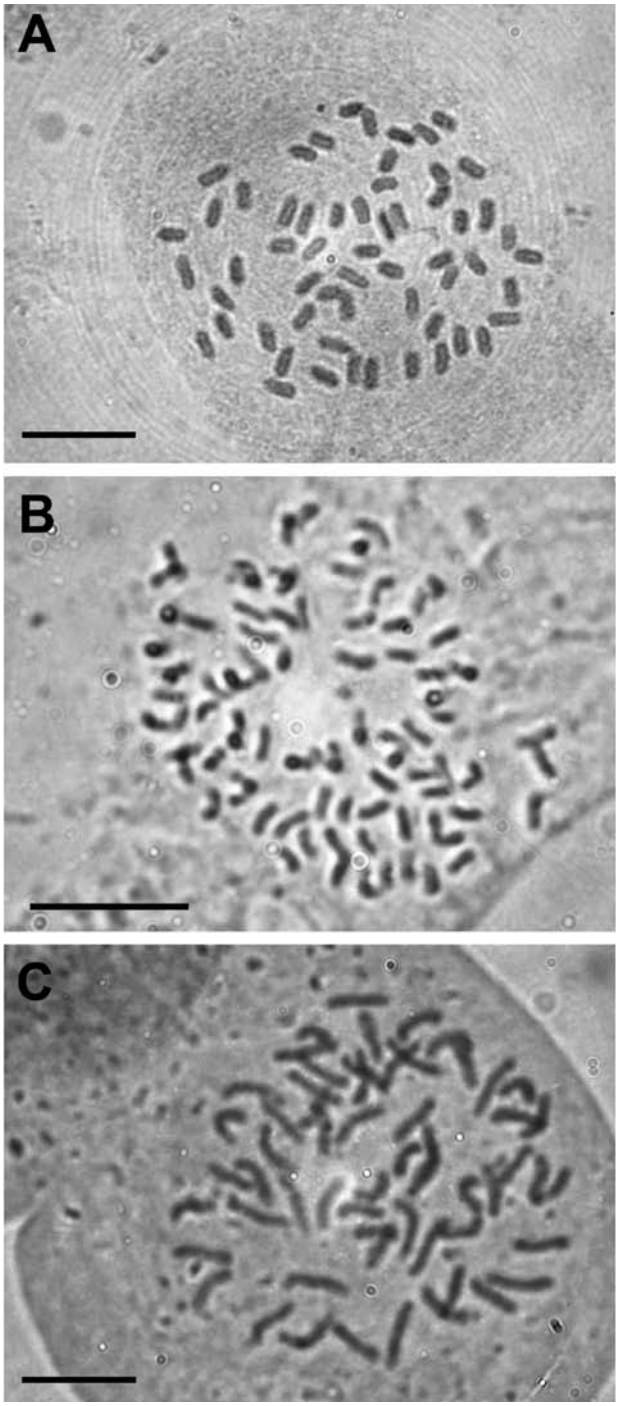

Figure 1. Somatic chromosomes of root cells. A, *Pteris cretica* (2n = 58); B, *Pteris pellucidifolia* (2n = 87); C, *Pteris wulaiensis* (2n = 58). Bar =10 μm.

Table 2. Cytology and spore characteristics of the three *Pteris* species.

Species	Chromosome no. (2n)	Spore number per sporangium	Normal spores (%)	Spore size (μm)[1]
P. cretica	58	32	98	39.7±4.9[b]
P. pellucidifolia	87	32	73	55.5±3.1[a]
P. wulaiensis	58	32	95	42.7±2.4[b]

[1]The same letters indicate the mean sizes of trilateral spores are not significantly different according to ANOVA followed by Tukey post-hoc test (p <0.05).

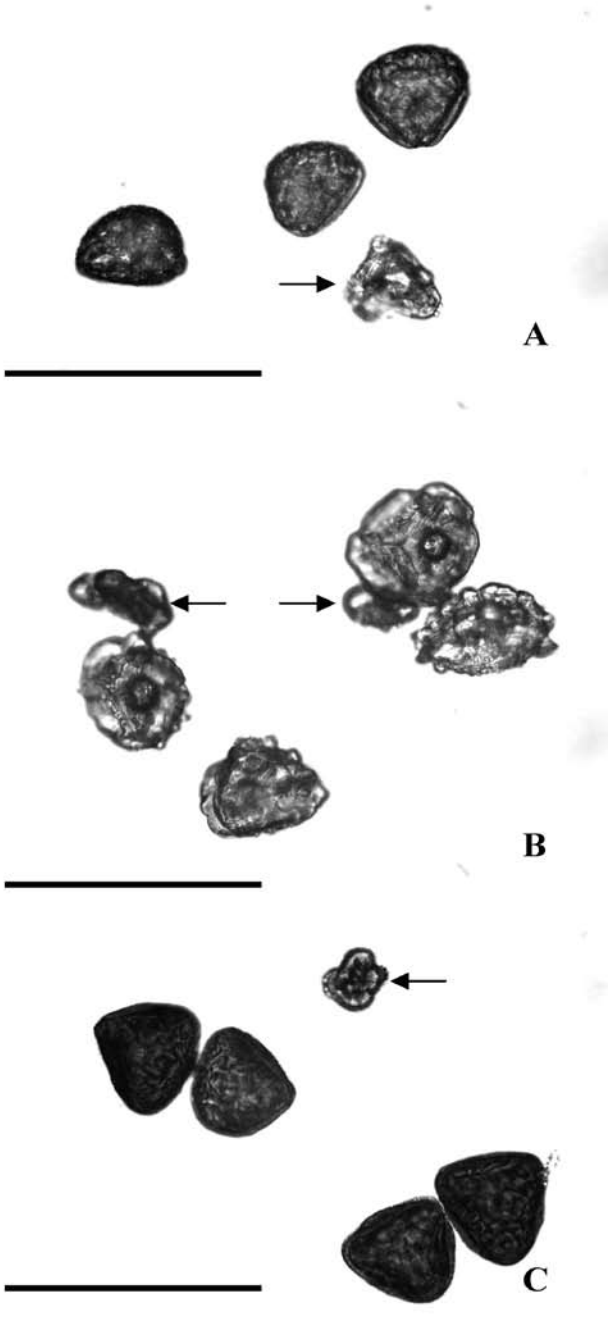

Figure 2. Mature spores of three apogamous species of *Pteris*. A, *P. cretica*; B, *P. pellucidifolia*; C, *P. wulaiensis*. Arrows indicate abnormal abortive spores.

Gametophytes

When gametophytes of *P. cretica*, *P. pellucidifolia*, and *P. wulaiensis* were cultured for 5, 9, and 5 weeks, respectively, sporophytes began to be produced. In *P. cretica*, there were 19% asexual and 81% male gametophytes. Asexual gametophytes were significantly larger than male ones (4.1±1.5 vs. 1.9±1.3 mm). In *P. pellucidifolia*, 46% asexual and 54% male gametophytes were found. Asexual gametophytes were significantly larger than male ones (5.0±1.9 vs. 2.7±1.8 mm). No archegonium was found in these two species. In *P. wulaiensis*, there were 11%, 81%, 3%, and 5% of asexual, male, female, and hermaphroditic gametophytes, respectively. Female gametophytes were the largest (6.5±0.6 mm), and male gametophytes were the smallest (2.3±1.2 mm) (Table 3).

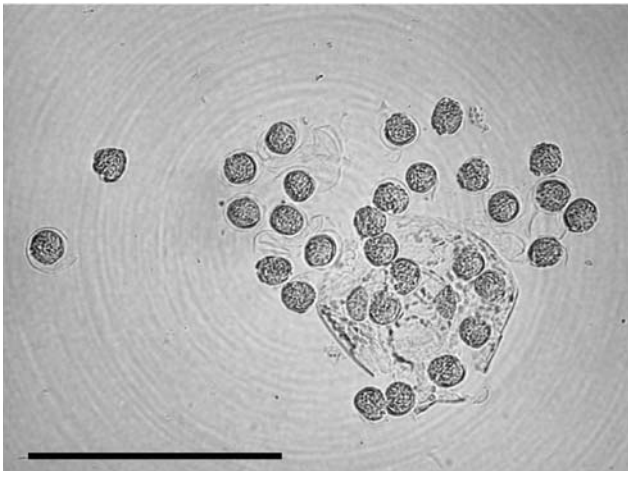

Figure 3. Thirty-two sperm released from an antheridium of *Pteris wulaiensis.* Bar = 100 μm.

There were 32 active sperms enveloped in each of the antheridia of these three species (Figure 3). The diameters of antheridia were significantly larger in *P. pellucidifolia* (63.4±1.8 μm) than in *P. cretica* (45.0±4.9 μm) and *P. wulaiensis* (50.8±3.4 μm). Antheridia had a normal external morphology and released swimming sperm after the cap cell opened. Archegonia were only found on gametophytes of *P. wulaiensis*, and although they had a normal morphological outline, no sporophytes arose from them.

Young sporophytes

Gametophytes of these three species apogamously produced sporophytes (Figure 4). Except for a few (< 1%) apogamous sporophytes that occurred in the apical notch of gametophytes, most apogamous sporophytes were

Table 3. Sexual expression of the three *Pteris* species. Gametophytes of *P. cretica*, *P. pellucidifolia*, and *P. wulaiensis* were cultured for 5, 9, and 5 weeks, respectively, when sporophytes began to be produced.

Species	Gender (%)[1]				Width of gametophytes (mm)[2]			
	A	M	F	H	A	M	F	H
P. cretica	19	81	0	0	4.1±1.5[a]	1.9±1.3[b]	--	--
P. pellucidifolia	46	54	0	0	5.0±1.9[a]	2.7±1.8[b]	--	--
P. wulaiensis	11	81	3	5	4.0±1.3[b]	2.3±1.2[c]	6.5±0.6[a]	4.8±1.2[b]

[1]A, asexual; M, male; F, female; H, hermaphroditic.
[2]Average ± standard deviation; different letters in a given line indicate a significant difference (Turkey post-hoc test, $p<0.05$).

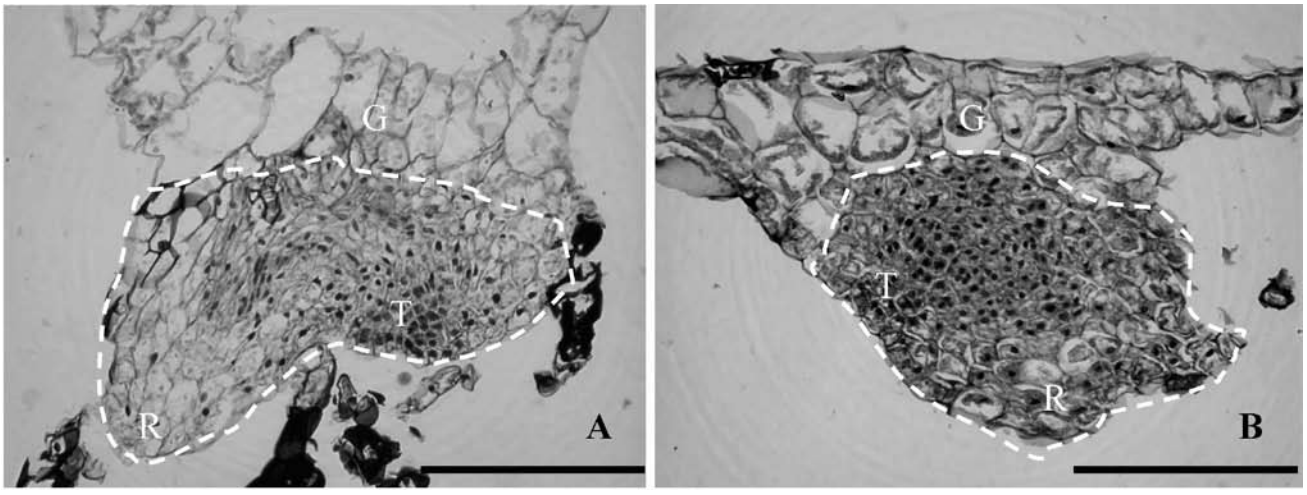

Figure 4. Gametophytes bearing apogamous embryos (dashed line area). A, *Pteris pellucidifolia*; B, *P. wulaiensis*. G, gametophyte; R, root; T, terminal bud. Bars = 100 μm.

Table 4. Venation of the first frond of young sporophyte of three *Pteris* species.

Species	Fronds lacking midribs[1]		Fronds possessing midribs[2]	
	Frequency (%)	Length (mm)	Frequency (%)	Length (mm)
P. cretica	2	5.4±1.2	98	8.8±1.9
P. pellucidifolia	4	5.0±1.3	96	8.1±1.7
P. wulaiensis	2	3.6±1.3	98	8.4±3.3

[1]Five fronds were randomly sampled to measure the length.
[2]Ten fronds were randomly sampled to measure the length.

directly derived from the ventral surface of the anterior cushion near the apical meristem (Figure 5). Scales sparsely covered the base of the petiole (Figure 5). Most first fronds (96%-98%) had midribs (Figure 6) and were larger than those without midrib (Table 4).

At the end of culture, when no more sporophytes were produced, the sizes and genders of all gametophytes bearing young sporophytes were determined. Results showed that the difference in sizes between asexual and male gametophyte was not significant in these three species. However, female gametophytes of *P. wulaiensis* were markedly larger than asexual and male ones (Table 5).

Table 5. Frequencies and sizes of gametophytes bearing sporophytes of the three *Pteris* species when no more sporophytes were produced[1].

Species	Gender (%)[2]				Width of gametophytes (mm)[3]			
	A	M	F	H	A	M	F	H
P. cretica	81	19	0	0	4.8±0.6[a]	4.4±0.7[a]	--	--
P. pellucidifolia	80	20	0	0	5.9±1.0[a]	5.5±0.9[a]	--	--
P. wulaiensis	28	44	12	16	4.5±1.2[a]	4.0±1.1[a]	6.5±0.6[b]	5.1±1.2[ab]

[1]Numbers of gametophyte sampled were 21, 44, and 25 for *P. cretica*, *P. pellucidifolia*, and *P. wulaiensis*, respectively.
[2]A, asexual; M, male; F, female; H, hermaphroditic.
[3]Average ± standard deviation; different letters in a given line indicate a significant difference (Turkey post-hoc test, $p<0.05$).

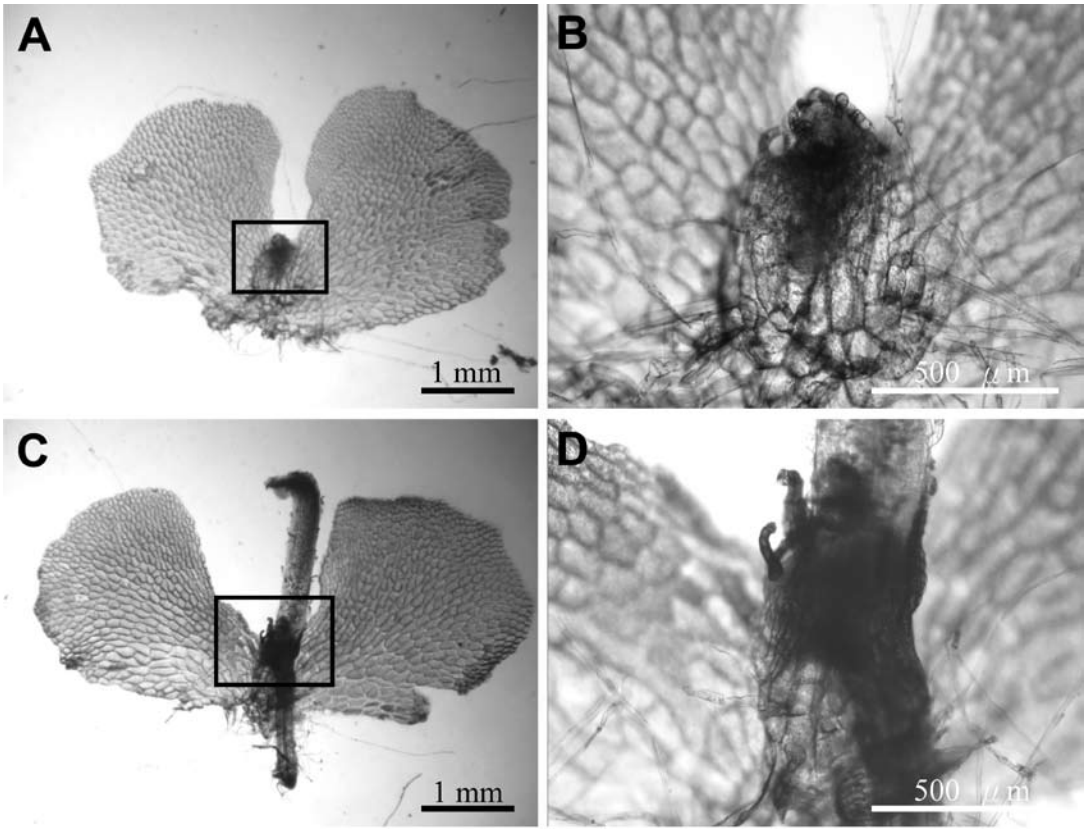

Figure 5. Formation of young sporophytes in *Pteris pellucidifolia*. A, Apogamous embryo at the notch; B, an enlargement of the block of fig. 5A; C, a developing apogamous young sporophyte; D, some scales on the base of the first leaf, an enlargement of the block of fig. 5C.

DISCUSSION

Many species of the genus *Pteris* are apogamous (Walker, 1962; Verma and Khullar, 1965; Iwatsuki et al., 1995). The judgment concerning apogamous reproduction is mainly based on cytological data (e.g., Walker, 1962), or the spore number per sporangium (Wang and Zhang, 1981; Mitui, 1982), or a combination of different related traits (Chiou et al., 2006; Huang et al., 2006). In this study, cytology, spore number, gametogenesis, and embryogenesis data were applied to a judgement on the apogamy of three *Pteris* species.

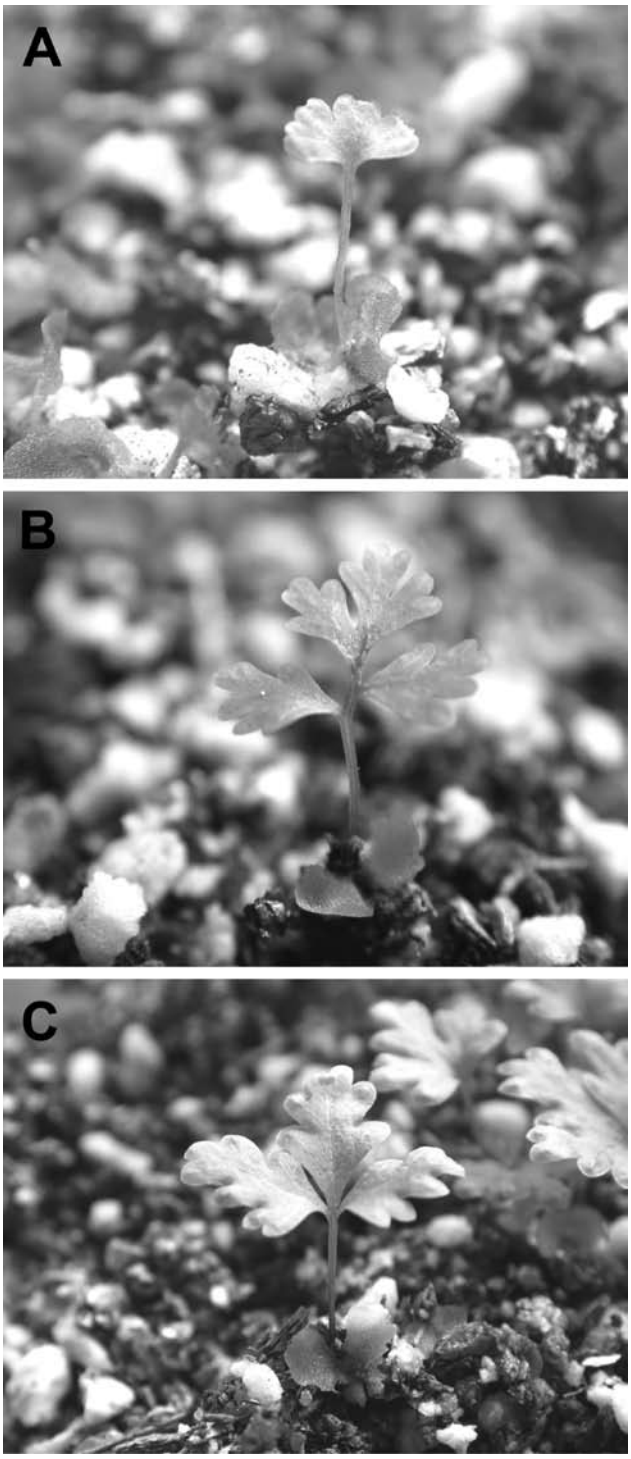

Figure 6. First fronds of young sporophytes mostly possessed midribs (B, C) while a few of them lacked midribs (A) in each of the three *Pteris* species. A, *P. cretica*; B, *P. wulaiensis*; C, *P. pellucidifolia*.

In the genus *Pteris*, most apogamous ferns are polyploid, especially triploid, such as *P. fauriei* var. *fauriei* (Huang et al., 2006), *P. bella*, (Chang, 1991), and *P. pellucidifolia* in this study. Diploid apogamous ferns are not uncommon in the genus *Pteris* (Walker, 1962). In this study, *P. cretica* and *P. wulaiensis* were found to be diploid but apogamous.

In most advanced ferns, apogamous sporophytes usually produce 32 spores per sporangium (Manton, 1950; Braithwaite, 1964). The apogamous triploid *P. pellucidifolia*, diploid *P. cretica*, and diploid *P. wulaiensis* all fit this generality. However, the fact that a species possesses 32 spores per sporangium does not absolutely insure apogamy. For example, some species of the Lindsaceae and *Cheilanthes* (Pteridaceae) with 32 spores per sporangium sexually produce sporophytes (Lin et al., 1990; Huang et al., 2009). On the other hand, apogamous ferns may produce different numbers of spores per sporangium, such as *Cheilanthes hirsuta*, an apogamous fern that produces 16 spores per sporangium (Huang et al., 2009).

The sperm number per antheridium was reported to be larger in basal eusporangiate ferns (usually > 100) than in advanced leptosporangiate ferns (commonly 16-32) (Nayar and Kaur, 1971). In *Pteris fauriei*, 64 and 32 sperms per antheridium were documented in diploid sexual and triploid apogamous varieties, respectively (Huang et al., 2006). Similarly, 32 sperms per antheridum were also found in triploid apogamous *Diplazium megaphyllum* (Chiou et al., 2006). In this study, all three apogamous gametophytes produced 32 sperms in each antheridium regardless of whether they were diploid or triploid. Although those limited data suggest that apogamous ferns produce 32 sperms per antheridium, more studies are needed before generalizing the conclusion.

In ferns, spore sizes are often positively correlated with ploidy levels within a genus (Kanamori, 1975; Huang et al., 2006). In this study, spores of the triploid *P. pelludifolia* were significantly larger than those of the other two diploid species.

Likewise, a positive correlation between antheridium size and ploidy level was also reported (Huang et al., 2006). Antheridium diameters of the triploid *P. pellucidifolia* were significantly larger than those of the diploid *P. cretica* and *P. wulaiensis* in this study. Momose (1967) observed that the diameters of antheridia of *P. cretica* ranged from 65 to 85 μm, much larger than the antheridium size of the apogamous diploid gametophytes in this study (45.0±4.9 μm; range 40-54 μm). This indicates that the ploidy levels of these two materials probably differ. The antheridia examined by Momose (1967) were perhaps sampled from triploid or tetraploid ferns since three cyto-

types (2×, 3×, and 4×) were previously documented for *P. cretica* (Manton, 1950; Verma, 1959; Walker, 1962; Mitui, 1965; Verma and Khullar, 1965; Roy et al., 1971; Chang, 1991).

For apogamous ferns, antheridia might occur, as in these studied species while archegonia are usually absent or abnormal and nonfunctional. Laird and Sheffield (1986) reported that apogamous *P. cretica* had fully functional antheridia which released active sperms, but its archegonia had lost its function due to the failure of the neck canal to open. In this study, it was also suggested that antheridia were functional because they released active sperms. No archegonium was found in the gametophytes of diploid *P. cretica*, as described by Momose (1967), or in the triploid *P. pellucidifolia*. Although *P. wulaiensis* produced "normal-morphological" archegonia, the embryo arose from the gametophyte cells, instead of from the archegouium, indicating apogamous reproduction. A detailed observation and analysis of egg viability and ultrastructure is needed to explore the cause of the failure of its archegonium function.

In this study, the male gametophytes were significantly smaller than the asexual ones when sporophytes began to produce, but they were not much smaller when no more sporophyte was produced. This suggests that apogamous sporophyte production is more dependent on the size rather than on the gender. It supports a general hypothesis regarding apogamous growth that the accumulation of sufficient size and sugar production causes the cushion to become a sugar sink, which presumably activates genes for the expression of sporophyte morphology (Whittier, 1964; Kawakami et al., 1995; Cordle et al., 2007).

The morphology of the first frond is regarded as a reliable characteristic to distinguish apogamy from sexual gametophytes of ferns. For example, the first frond of the sexually diploid *P. fauriei* lacks midribs and is bifurcate while that of the apogamous triploid *P. fauriei* has midribs and is pinnate (Huang et al., 2006). However, despite most first fronds of these three species possessing midribs in this study, 2%-4% of them lacked midribs.

Sporophytes of the three *Pteris* species in this study reproduced apogamously. Many traits related to reproduction, such as, triploidy, 32 spores per sporangium, the nonoccurrence of archegonium, 32 sperms per antheridium, and a midribbed first frond, are inferred to indicate apogamy. However, diploidy in *P. cretica* and *P. wulaiensis*, archegonia in *P. wulaiensis*, and a few first fronds lacking midribs were found in these three apogamous species. Although 32 spores were found in each sporangium of the three species in this study, this character was also reported in other sexual species, and other spore numbers, e.g., 16, per sporangium were found in apogamous species. Thus the best way to infer the reproductive mode of ferns is to detect whether the embryo formed from the vegetative cells of gametophytes although those other traits described above may provide useful hints.

Hybridization following polyploidization is considered an important evolutionary pattern in developing successful sexual reproduction (Soltis and Soltis, 1993; Schwarzbach and Rieseberg, 2002). The apogamous ferns produce offspring without gamete fusion and thus favor their colonization to a new habitat through a single spore dispersal (Lloyd, 1974; Crist and Farrar, 1983). Because most apogamous ferns produce functional antheridium and release active sperms, e.g., the three *Pteris* species in this study, they may engage in sexual reproduction by offering sperm to an egg of another allied species. Polyploidization thereafter may evolve a successful sexual lineage in the new colonized habitat. However, more experiments are needed to test this hypothesis.

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三種鳳尾蕨屬 (鳳尾蕨科：蕨類植物門) 植物行無配生殖

黃曜謀[1] 徐昇圓[2] 謝宗欣[3] 周雪美[4] 邱文良[2]

[1] 林業試驗所 育林組
[2] 林業試驗所 植物園組
[3] 臺南大學 生物技術學系
[4] 元培科技大學 生物技術學系

本研究確認大葉鳳尾蕨、闊葉鳳尾蕨及烏來鳳尾蕨的倍體數、配子體與幼孢子體形態及其生殖模式。大葉鳳尾蕨及烏來鳳尾蕨是二倍體，而闊葉鳳尾蕨則是三倍體。三倍體的闊葉鳳尾蕨孢子顯著大於其它兩種二倍體的孢子，但闊葉鳳尾蕨的正常孢子比例 (73%) 則較其它兩種的正常孢子比例 (95%~98%) 低。大葉鳳尾蕨與闊葉鳳尾蕨的配子體產生正常的藏精器，但沒有藏卵器。雖然烏來鳳尾蕨具藏卵器，但卻失去了功能。這三種鳳尾蕨的每一個藏精器都長有 32 顆精子，都行無配生殖。幼孢子體自配子體上直接產生而沒有受精的過程發生。第一片葉子大多數具中肋，僅少數不具中肋。這些與生殖有關之特徵雖然提供了辨認生殖模式的參考資訊，但最可信賴的生殖模式辨別方法仍爲檢視「胚」是否形成自配子體上的營養細胞。

關鍵詞：無配生殖；染色體；配子體；形態；倍體數；大葉鳳尾蕨；闊葉鳳尾蕨；烏來鳳尾蕨。