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Variation in Tree Fern Stipe Length with Canopy Height: Tracking Preferred Habitat Through Morphological Change

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ABSTRACT.—*Cyathea caracasana* is a common open-habitat tree fern in the Andes of Colombia. In full sun, stem growth rates are high (up to 2 cm/month) and individuals regularly produce spores. However, even the fastest growing ferns are overtopped by woody angiosperms after 10 to 15 years of natural forest regeneration. As individuals are overtopped, *C. caracasana* produces nearly vertical fronds with long stipes (commonly over 3 m) apparently to place the photosynthetic surface into the canopy. We compared stipe length and blade length and width among individuals growing in open sites and in the understories of two regenerating forests: one with a canopy of 20–25 m, and one with a canopy of 5–8 m. Stipes and blades were shortest in open habitat and longest in the low-canopy forest. Ferns in the high-canopy forest had intermediate measurements. Despite the change in frond length, the number of primary pinnae per-frond did not differ among the habitats sampled. This suggests that elongation cues are received late in the development of the frond. This conclusion is supported by a positive relationship between stipe length and the distance of the fern meristem below the canopy. Because both understory populations show stipe elongation relative to open-hapitat ferns, the cue to elongate is likely a low red/far-red wavelength ratio of the light received by the apical meristem. Extraordinary elongation is probably made possible by extra carbon resources available to low-canopy plants, which still have leaves in full sun. This sense and response mechanism allows individual plants to produce elongated fronds as their apical meristems are overtopped. Functionally, the long-stiped plants remain in full sun even after they are overtopped, thus they "track" their preferred, open habitat.

Plant populations respond to environmental variation in several ways. When the environment fluctuates infrequently relative to the life span of individuals, adaptive segregation may produce differential response to environmental cues among separate populations. Consider variation in light environment. Sun-adapted populations of *Impatiens capensis* L. showed enhanced growth in response to changes in the ratio of red (600–700 nm) to far-red (700–850 nm) light relative to shade-adapted populations (Dudley and Schmitt, 1995). Similar sun/shade segregation in growth response to red/far-red (R/FR) has been observed in many angiosperm species in open (as compared to understory) communities (Morgan and Smith, 1979). In contrast, when the environment varies frequently relative to the life span of individuals, adaptive segregation cannot occur and individuals must rely on morphological and physiological flexibility (plasticity) (Thompson, 1991; Sultan, 1993; Ackerly and Bazzaz, 1995; Arens, 1997). In such cases, plants may change form or physiology to suit new conditions, for example switching from sun-leaf to shade-leaf anatomy (Arens, 1997). Alternatively, individuals "track" or follow their preferred habitat as it moves. Conventionally, habitat-tracking in plants has been applied to intergenerational migration (Davis et al., 1986; Webb, 1987; Davis and Sa-

FIG. 1. Location of La Reserva Natural La Planada within Colombia, South America.

binski, 1992). Within the life span of a clonal plant, differential growth allows modules to follow resources (Salzman, 1985; Slade and Hutchings, 1987). This form of habitat-tracking may also be applied to differential organ growth in non-clonal plants.

When growing in low-canopy secondary forest, some individuals of the tree fern *Cyathea caracasana* (Klotzsch) Domin produce unusually long, vertically oriented stipes that place the photosynthetic portion of the frond into the canopy, where it experiences full sun. This paper documents our observation as an example of habitat-tracking by means of changing allometric growth of individual plant organs.

STUDY SITE AND METHODS

La Reserva Natural La Planada is located between 1,850 m and 2,300 m above sea level on the Pacific slope of the Andean Cordillera in Nariño, Colombia, 1°09′37″N, 77°59′13″W (Fig. 1). La Planada receives about 4,500 mm

of rainfall annually (cumulative data from 1982 to 1997) with a "dry" season from June to August. Cloud-harvesting approximately doubles the moisture available to plants (C. Ríos, Instituto von Humboldt, Villa de Leyva, Colombia, unpublished data). Average daily temperature is 19°C, with no seasonal variation. Vegetation within the reserve is typical of Pacific slope cloud forest at this elevation, with a natural canopy approximately 25 m in height composed of relatively few tree species. The most common canopy trees include *Alchornea* (Euphorbiaceae), *Clusia* (Guttiferae), *Inga* (Fabaceae), *Miconia* (Melastomataceae), *Myrica* and *Psidium* (Myrtaceae), and *Otoba* (Myristicaceae). Eleven described tree fern species have been reported from the reserve (Arens and Sánchez Baracaldo, 1998). The majority of the reserve's 3,200 hectares are covered with primary forest that has been little-disturbed by human activity.

Within primary forest, canopy gaps are an important part of the habitat dynamic. Primary forest turnover due to gap formation and canopy closure was estimated at 3% annually (Samper K., 1992). This suggests that at a given point in the forest, a canopy gap will open every 30 years, on average—well within the life span of most tree ferns. The reserve also contains areas of recently abandoned pasture and secondary forest, which represent less than 50 years of natural regeneration. Secondary forest was identified by relatively homogeneous size class of canopy trees; land-use records establish the time of abandonment. Regeneration of abandoned pasture is rapid, with canopies of 5 to 6 meters closing within 10 to 20 years after abandonment. Land use history information available at the reserve permits precise estimation of regeneration-time. This makes La Planada a valuable site for studies of successional dynamics in the cloud forest ecosystem.

Field work was initiated in 1992 and completed in 1994. We identified co-occurring individuals of *Cyathea caracasana* whose apical meristems experienced three distinct habitats: (1) open habitat, (2) the understory of 35-year-old secondary forest, and (3) the understory of 10-year-old secondary forest. Open habitat plants grew in recently abandoned pasture and cleared areas, commonly within 1,000 m of reserve buildings. Abandoned pastures were in the earliest stages of forest regeneration. Vegetation was characterized by grasses, herbaceous angiosperms, moss, lycophytes, ferns, and *Miconia* saplings. In open habitat, tree ferns grew in full sun. Understory individuals grew in the shade below the closed canopy of secondary forest (approximately 35 years of regeneration); this sample is referred to as "high canopy" forest in subsequent discussion. The canopy was approximately 25 m in height with no emergent trees. Canopy gaps were uncommon and none were observed greater than 2 m in diameter. The younger secondary forest represented approximately 10 years of regeneration based on land-use history records, and had a multi-layered canopy ranging from 5 m to 8 m (uncommonly up to 10 m) in height; this sample is referred to as "low canopy" forest in subsequent discussion. Most ferns in low-canopy forest placed their blades in the canopy; however, the apical meristems of these individuals resided below canopy. For this study, we chose only those individuals in the low-canopy forest that had placed their photosynthetic surfaces in the canopy, because they maintain open-habitat

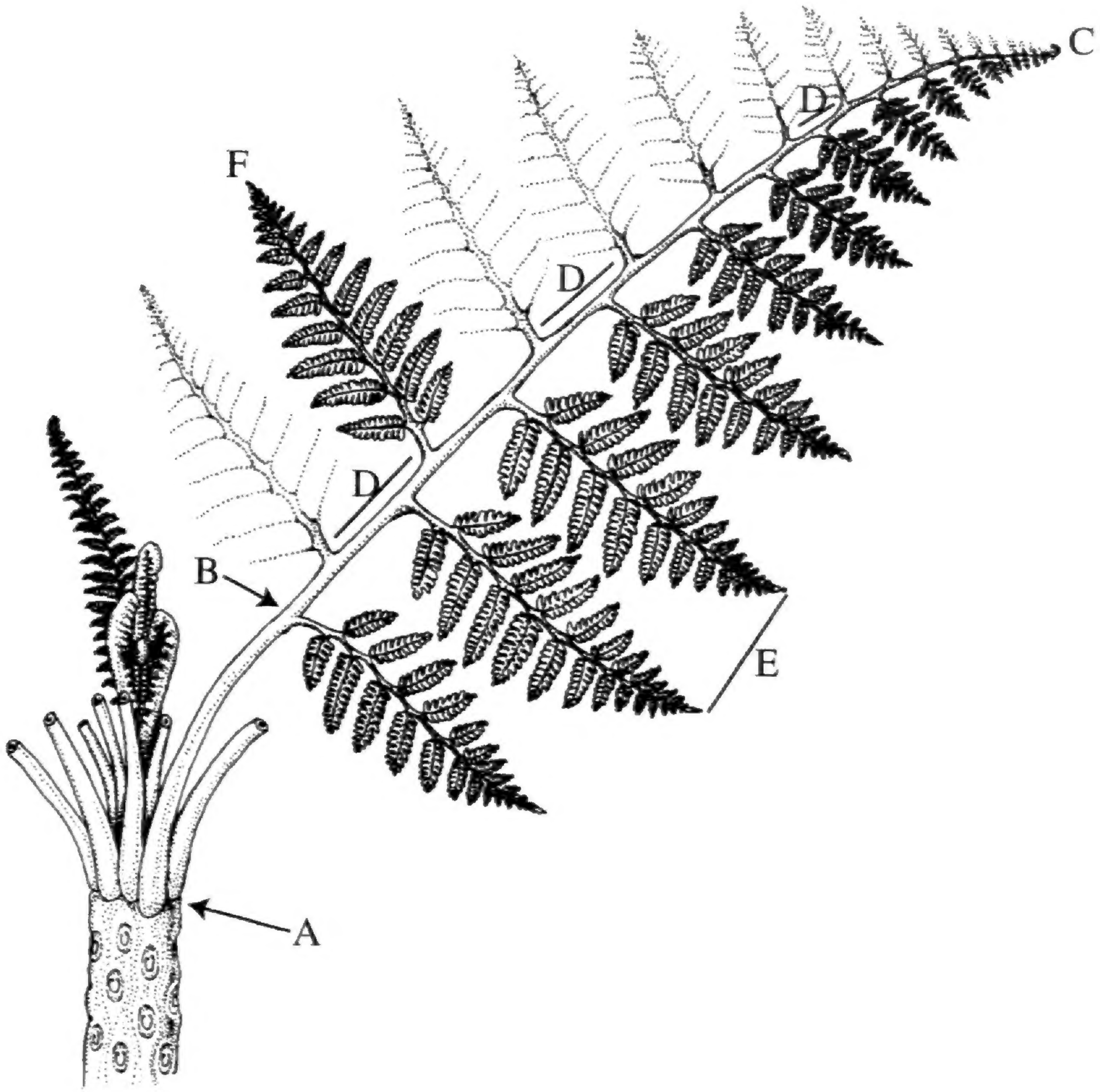

FIG. 2. Frond of *Cyathea caracasana* showing the position of measurements made in this study. Length of stipe was measured from A–B; length of frond blade was measured along the rachis from B–C; distance between first-order pinnae at three standardized positions (D) along the rachis. Blade width was measured at the widest part of the blade E–F. Original drawing by Caroline A.E. Strömberg.

growth rates (Arens and Sánchez Baracaldo, 1998) and are therefore proposed as habitat-trackers. Co-occurring individuals (commonly with trunk heights less than 30 cm) did not place their blades in the canopy and functioned as understory plants (Arens and Sánchez Baracaldo, 1998).

For 20 individuals in each habitat type, we measured trunk height, stipe length, blade length, blade width, number of primary pinnae (*Cyathea caracasana* is twice-pinnate-pinnatifid), and distance between first-order pinnae at three standardized locations along the rachis (Fig. 2). Sample individuals were chosen to represent a range of trunk heights (21 cm to 150 cm); the range of trunk heights sampled in each habitat type was statistically indistinguishable ($p < 0.001$). Since trunk height is commonly used as a rough proxy for age in tree ferns (Conant, 1976; Seiler, 1981; Bittner and Breckle, 1995; Seiler, 1995), this result suggests that each habitat sample included individuals of a com-

TABLE 1. Pearson product-moment correlation coefficients (R) for log-transformed frond measurements. Stem height, stipe length, blade length, and blade width at widest point measured in centimeters. Log (distance) is the logarithmic transformation of average distance along the rachis between primary pinnae. NS = not statistically significant; * significant at $p < 0.01$; ** significant at $p < 0.001$.

	Log (stem height)	Log (stipe length)	Log (blade length)	Log (blade width)	Log (distance)
Log (stem height)	1				
Log (stipe length)	−0.03 NS	1			
Log (blade length)	0.34*	0.44**	1		
Log (blade width)	0.29 NS	0.30 NS	0.19 NS	1	
Log (distance)	−0.02 NS	0.90**	0.53**	0.30 NS	1

mensurate range of age-size classes. Furthermore, there was no statistically-significant correlation between trunk height and stipe length ($R = -0.03$, Table 1). Thus, differences in frond measurements among habitats were not due to different age-size populations sampled, or age- or size-related allometric variation.

Light environment experienced by individual ferns was estimated using photo-paper (Azon non-erasable diazo sepia paper, Proprint) light sensors placed at the apical meristem of each tree fern (Friend, 1961; Kitajima and Augspurger, 1989). Paper sensors were calibrated for a 24 hour interval with a LI-COR 1000 light meter (Lambda Instruments Corp., Lincoln, NE). Photosynthetically active radiation (PAR) was calculated for each paper photo sensor increment using the regression derived from LI-COR calibration data. Although this method is less precise than instantaneous light meter measurements, it does give integrated measures of light environment that can be compared among individuals. From the plant's perspective, integrated measurements are more ecologically meaningful than instantaneous light meter readings, which are subject to the vagaries of cloud cover and time of day. While this method does provide useful information on light quantity, it is insensitive to light quality, which is also ecologically important (Perez-Garcia et al., 1994; Ritchie, 1997; van Hinsberg and van Tienderen, 1997).

Manipulations and analyses were performed in SYSTAT 5.2 (Wilkinson, 1989) and MS Excel 5.0a for Macintosh.

RESULTS

We compared frond measurements in pair-wise fashion between habitats using a t-test assuming unequal variances. Mean stipe length differed among all three habitats (Fig. 3A, $p < 0.001$). This result held ($p < 0.001$) when stipe lengths were logarithmetically transformed to compensate for unequal variances. Open habitat ferns possessed the shortest stipes (66.4 cm ± 15.22 cm, Appendix 1); ferns living in the high-canopy forest had intermediate stipe lengths (102.9 cm ± 21.7 cm); ferns growing in low-canopy forest produced significantly longer and more variable stipes (207.9 cm ± 49.1 cm). Blade length differed between low- and high-canopy forest samples, and low-canopy

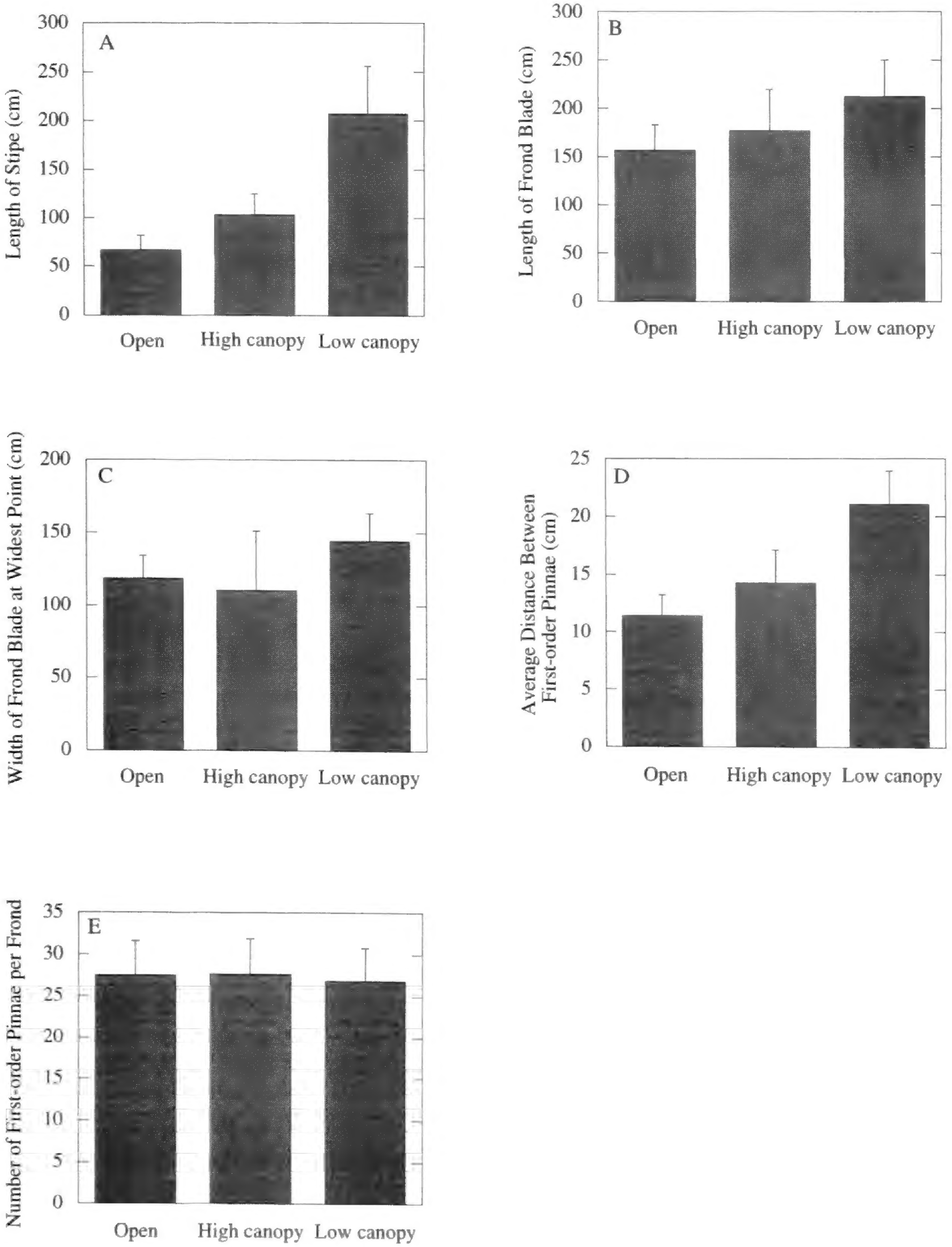

FIG. 3. Average frond measurements compared among habits. Data (Appendix 1) are averages of 20 individuals sampled per habitat; error bars are one standard deviation. A) Length of stipe from trunk to the first primary pinna; B) length of the frond blade measured along the rachis from the first primary pinna to the frond tip; C) width of the frond blade at its widest point; D) average (of three measurements taken at standard positions) distance along the rachis between primary pinnae on the same side of the frond; E) average number of first-order pinnae per frond.

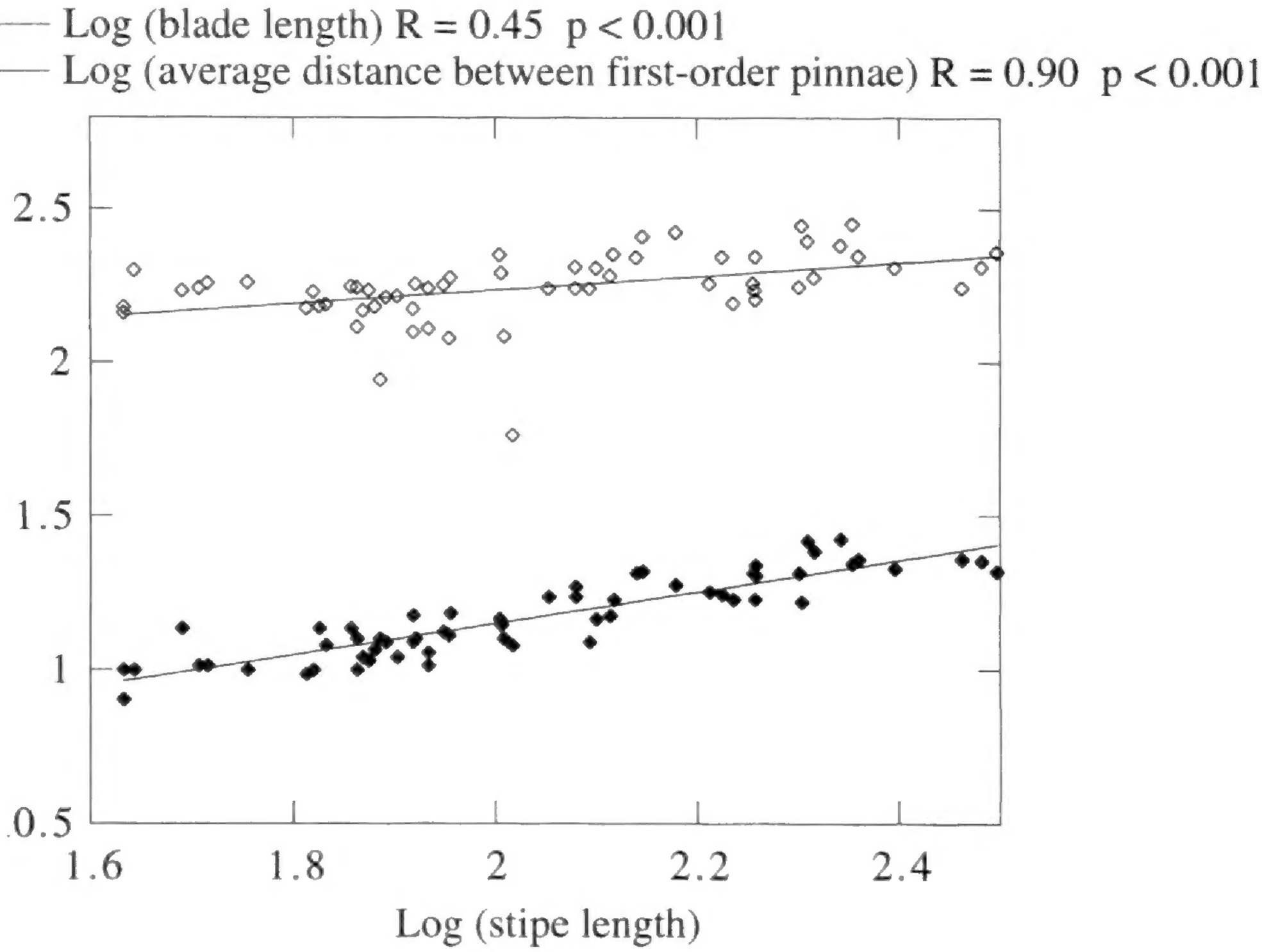

FIG. 4. Logarithmically-transformed data for stipe length, blade length, and the average distance between first-order pinnae. Linear relationships between these parameters suggest an allometric growth relationship between stipe length and both blade length and average distance between first-order pinnae.

forest and open habitat (Fig. 3B, $p < 0.001$). However, there was no significant difference in blade length between open habitat and high-canopy forest. Similarly, blade width differed between low- and high-canopy forest, and low-canopy forests and open habitat (Fig. 3C, $p < 0.001$); blade width did not differ significantly between open habitat and high-canopy forest. The average distance between first-order pinnae also differed among the three habitats (Fig. 3D, $p < 0.001$). However, the number of first-order pinnae per frond did not differ statistically ($p < 0.001$) among any of the three habitats: open habitat mean = 27.5 ± 4.1; high-canopy mean = 27.7 ± 4.2; low-canopy forest mean = 26.9 ± 4.0 (Appendix 1).

Variation in stipe length might be allometrically related to size or age of the plant. To evaluate this hypothesis, we performed a logarithmic transformation on measurement data and calculated Pearson product-moment correlation coefficients (R) for all parameter combinations (Table 1). If an allometric relationship was present, such transformed data would show high correlations. Non-significant correlations between trunk height and all parameters except blade length ($p < 0.01$, Table 1) indicate that differences in frond size were not due primarily to an allometric relationship with size-age class. Moderate correlation between stipe length and blade length (Fig. 4), and between blade

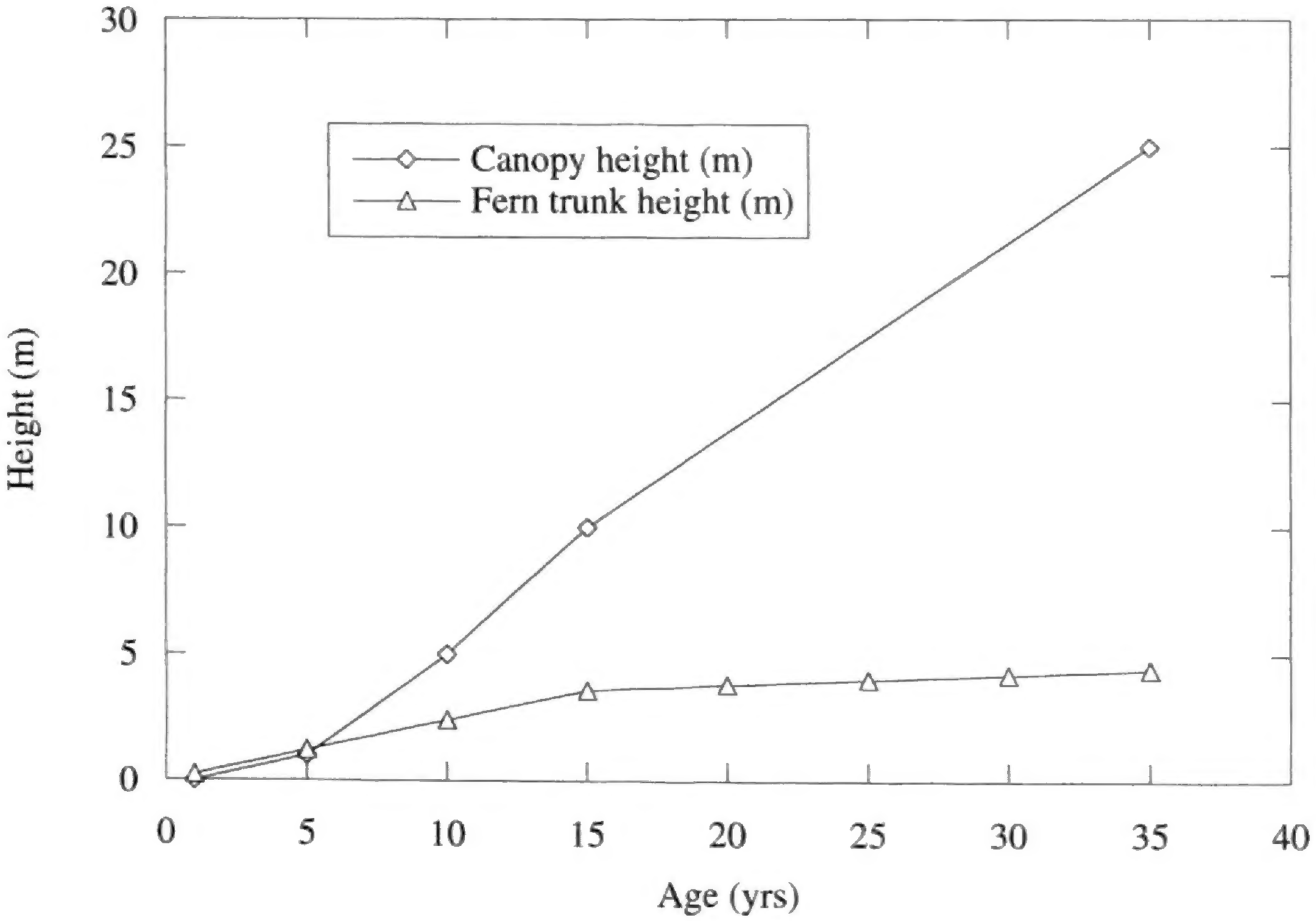

FIG. 5. Canopy height in regenerating secondary forest at La Planada and average stem height of *Cyathea caracasana* plotted through time. Average canopy heights were estimated based on plots of regenerating forest of known age within the reserve. Tree fern stem heights based on average growth rates of plants within open and understory habitats.

length and the distance between first-order pinnae suggests a growth relationship between these parameters. Significant correlations between stipe length, blade length, and the distance between first-order pinnae (Table 1, Fig. 4) show a clear, allometric growth relationship between these parameters.

In contrast to stipe and blade lengths, the number of first-order pinnae per frond does not vary with environment (Fig. 3E). The invariance of this parameter, compared with differences in stipe, blade, and distance between first-order pinnae, suggests that variation in frond dimension arise by elongation of support structures (stipe and rachis) during the elongation phase of frond expansion, rather than early in development when the number of pinnae are set.

DISCUSSION

At La Planada, *Cyathea caracasana* was among the first forest species to colonize abandoned pastures (Arens and Sánchez Baracaldo, 1998). This species was also very common along roadsides and other open habitats. Within mature forest, *C. caracasana* produced spores only when growing in forest gaps, although it persisted beneath the canopy for many years. In full sun, *C. caracasana* trunks had an average growth rate of 2 cm per month (Arens and

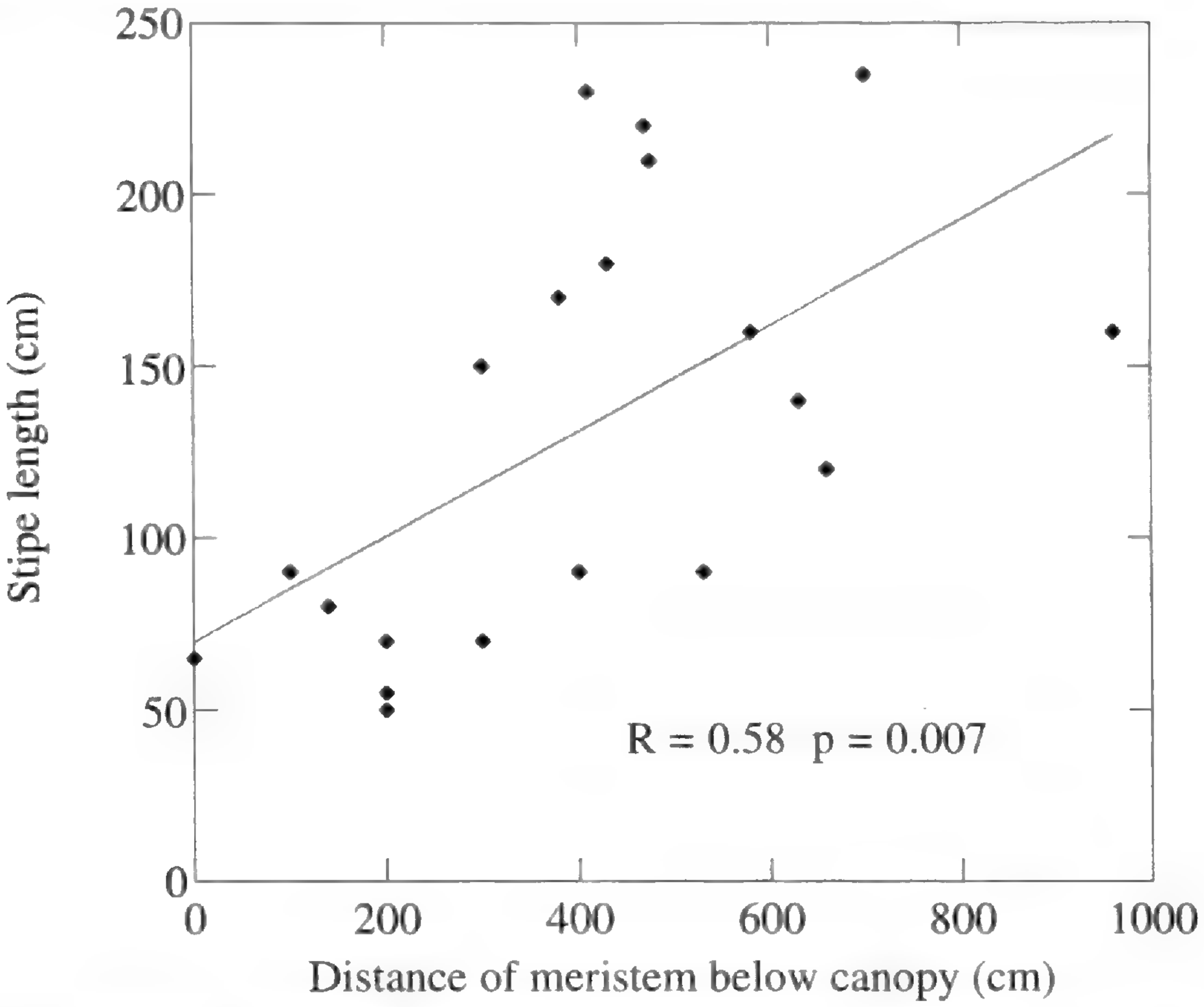

FIG. 6. Untransformed measurements of stipe length plotted against the distance of the plant's meristem below the canopy. The linearity of this relationship suggests a direct gradient response of stipe length to the light gradient below the low canopy.

Sánchez Baracaldo, 1998) and reached a height of up to two meters before woody species formed a canopy. Individuals of *C. caracasana* in sunny habitat produced abundant spores and recruited new sporophytes. In contrast, *C. caracasana* grew slowly in the understory of the closed canopy forest and seldom produced spores (Arens, 1996; Arens and Sánchez Baracaldo, 1998). These data suggest that *C. caracasana* prefers open, sunny environments such as human-disturbed landscapes or forest gaps. Since mature forest canopy turnover is high in this montane forest, long-lived ferns like *C. caracasana* might be expected to show habitat-tracking or plastic responses to such changes in light environment.

Growth rate data for forest ferns showed that individuals placing their photosynthetic blades in the canopy by means of long, vertical stipes have statistically indistinguishable growth rates compared to those in open habitats (Arens and Sánchez Baracaldo, 1998). Similarly, ferns in a low-canopy forest that do not produce stipes sufficiently long to reach the canopy had low growth rates, similar to individuals growing below the closed canopy (Arens and Sánchez Baracaldo, 1998). These observations support the conclusion that stipe elongation in *Cyathea caracasana* allows individual plants to continue growth and spore production as open-habitat plants—to track their preferred habitat by means of a morphological change.

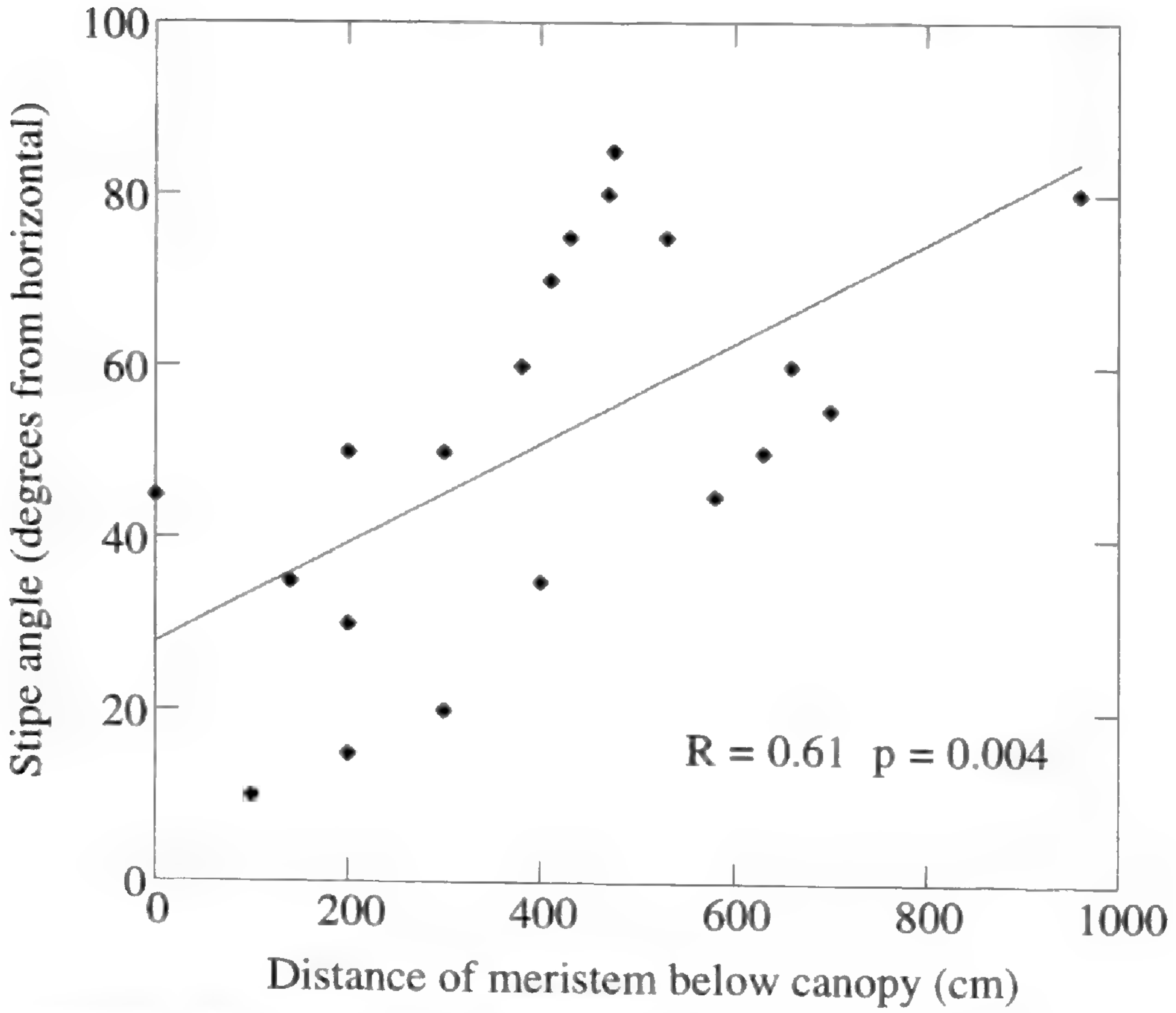

FIG. 7. Untransformed measurements of stipe angle plotted against the distance of the plant's meristem below the canopy. The linearity of this relationship suggests a direct gradient response of stipe length to the light gradient below the low canopy.

For this strategy to work, individual plants must sense and respond to their environment in appropriate ways. How does *Cyathea caracasana* detect a low canopy into which it might extend its frond? Why is extreme stipe elongation observed only in the low-canopy forest?

In angiosperms, changes in light quality (R/FR) can induce internode and petiole elongation. Low R/FR induced significant internode elongation in *Glycine max* (L.) Merr. (Thomas and Raper, 1985), *Phaseolus vulgaris* L. (Beall et al., 1996), *Impatiens capensis* Meerb. (Dudley and Schmitt, 1995), *Teucrium scorodonia* L. (Morgan and Smith, 1979), *Sinapis alba* L., and *Datura ferox* L. (Ballaré et al., 1991). In *Plantago lanceolata* L., leaf blade elongation was stimulated by low R/FR (van Hinsberg and van Tienderen, 1997). Low R/FR produced petiole elongation in *Citrullus lanatus* (Thunb.) Matsum. & Nakai (Graham and Decoteau, 1997), and *Thlaspi arvense* L. (Metzger, 1988). Compared with plants grown in high R/FR light, elongated structures result primarily from lengthening individual cells with a minor component of additional cell division in support structures (Beall et al., 1996). These data are consistent with our observation that support structure (stipe and rachis) length in *Cyathea caracasana* increases to produce fronds that can reach the canopy of young secondary forest; however, the architecture of the frond (number of first-order pinnae) does not change.

Light in the forest understory generally has low R/FR (Chazdon and Fetcher, 1984; Lee, 1987; Lee, 1989; Endler, 1991; Turnbull and Yates, 1993). Because R/FR reliably indicates overtopping, plants have evolved phytochrome-mediated sensory systems that trigger morphological or physiological responses, such as petiole elongation. Although no research has systematically evaluated a R/FR trigger for petiole elongation in non-angiosperms, ferns do possess phytochrome systems similar to those of angiosperms and these systems function as light-environment sensors (Haupt, 1985; Sugai and Furuya, 1990). Light quality signals for spore germination have been explored in some detail (Haupt, 1985; Sugai and Furuya, 1985; Psaras and Haupt, 1989; Sugai and Furuya, 1990; Esteves and Felippe, 1991; Perez-Garcia et al., 1994). It seems reasonable, therefore, that low R/FR experienced by meristems in the understory cues stipe and blade elongation in *Cyathea caracasana*. This is consistent with our data that show both stipe and blade are elongated in all forest plants, relative to open habitat individuals.

As they differentiate from the apical meristem, *Cyathea caracasana* crosiers may use a phytochrome mechanism to detect that they reside in the low R/FR environment of the understory. This cue triggers the stipe and blade elongation observed in all understory plants. In low canopy ferns, having fronds already in the full sun of the canopy provides carbon and energy resources needed for dramatic stipe elongation. In contrast, ferns under the high canopy may simply lack sufficient photosynthetic resources to produce extremely elongated stipes. This conclusion is supported by the lower average trunk growth rates (0.35 cm/month) observed in understory ferns at La Planada (Arens, 1996). Figure 5 shows that overtopping of tree ferns begins at about 10 years of forest regeneration, as the growth rate of angiosperm trees exceeds that of the ferns. It is in the 10-year-old forest that we observe dramatically elongated stipes as ferns attempt to forestall overtopping of their photosynthetic surfaces.

To explore the effect of meristem position in the multi-layered canopy, we selected a second sample of 20 individuals in the low-canopy forest and recorded the distance of each apical meristem below the canopy. Position below the canopy was positively correlated with both stipe length ($R = 0.58$, $p = 0.007$, Fig. 6) and with the angle of the stipe measured from the horizontal ($R = 0.61$, $p = 0.004$, Fig. 7). These data show that meristems well below the canopy produce fronds with longer and more erect stipes, capable of projecting the frond's photosynthetic surface into the sunny canopy. This supports the conclusion that stipe elongation in *Cyathea caracasana* is triggered by low R/FR conditions present at the meristem and terminated when the frond reaches light of high R/FR in the canopy.

From these results, we conclude that at La Planada, *Cyathea caracasana* responds to overtopping of its apical meristem by producing elongated, erect stipes that place blades into the full sun of the canopy. In this way, the plant fine-tunes its morphology to specific conditions in its environment. This allows the plant to continue growth and maintain spore production rates similar to those of individuals growing alone in open habitat, even in the early stages of overtopping by fast-growing angiosperm trees. These morphological chang-

es, likely stimulated by low R/FR, allow the plant to continue growth and reproduction even as forest regeneration relegates it to the understory.

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APPENDIX 1. Frond measurement data analyzed in this study. Open habitat individuals are indicated by "open"; individuals in the understory of high-canopy forest are indicated by "high"; young secondary forest individuals that place their fronds in the low canopy are indicated by "low".

Individual	Trunk height (cm)	Stipe length (cm)	Frond length (cm)	Frond width (cm)	Distance between pinnae (cm) (average)	Number of pinnae
Open 1	59	73	176	135	12.7	30
Open 2	57	90	120	125	13.0	26
Open 3	68	75	173	137	10.7	32
Open 4	76	72	177	154	13.7	28
Open 5	73	49	171	121	13.7	30
Open 6	70	68	155	111	12.0	22
Open 7	54	67	153	111	13.7	24
Open 8	51	65	151	99	9.7	27
Open 9	99	44	200	103	10.0	32
Open 10	55	77	88	109	12.7	26
Open 11	46	73	131	91	10.0	22
Open 12	42	86	129	110	10.3	21
Open 13	71	86	175	126	11.3	30
Open 14	121	83	150	136	15.0	30
Open 15	86	74	148	107	11.0	22
Open 16	107	57	183	133	10.0	28
Open 17	78	52	181	127.5	10.3	32
Open 18	102	51	174	113	10.3	22
Open 19	57	43	145	103	10.0	33
Open 20	85	43	152	121	8.0	32
Average	72.7 ± 21.7	66.4 ± 15.2	156.6 ± 26.0	118.6 ± 15.6	11.4 ± 1.8	27.5 ± 4.1
High 1	90	126	204	141	14.7	33
High 2	91	131	226	143	17.0	32
High 3	150	89	179	123	13.3	30
High 4	21	102	122	99	12.7	23
High 5	107	101	224	128	14.7	34
High 6	103	90.5	190	135	15.3	30
High 7	33	83	126	109	12.3	21
High 8	29	78	163.5	11.5	12.3	24
High 9	88	101.5	195.5	165	14.0	28
High 10	77	130	193	147	15.0	28
High 11	23	76	153	97	11.7	28
High 12	63	120	205	131	17.3	26
High 13	57	140	257	137	21.0	34
High 14	65	104	58	119	12.0	29
High 15	82	113	175	135	17.3	25
High 16	77	83.5	180	11	12.7	31
High 17	59	124	174	105	12.3	28
High 18	34	120	175	79	18.7	22
High 19	55	66	170	81	10.0	20
High 20	70	80	165	110	11.0	28
Average	68.7 ± 32.3	102.9 ± 21.7	176.8 ± 42.3	110.3 ± 40.4	14.3 ± 2.8	27.7 ± 4.2

APPENDIX 1. Continued.

Individual	Trunk height (cm)	Stipe length (cm)	Frond length (cm)	Frond width (cm)	Distance between pinnae (cm) (average)	Number of pinnae
Low 1	44	200	177	132	20.7	26
Low 2	37	229	223	141	23.0	28
Low 3	77	181	161	169	22.0	30
Low 4	96	314	228	149	21.0	28
Low 5	110	226	282	115	22.3	32
Low 6	39	180	182	139	20.7	24
Low 7	101	201	279	171	16.7	32
Low 8	118	151	266	189	19.0	34
Low 9	60	181	222	119	20.3	26
Low 10	44	180.5	173	139	17.0	20
Low 11	148	168	222	161	17.7	30
Low 12	68	249	204	147	21.3	24
Low 13	76	290	176	167	23.0	30
Low 14	42	163	181	129	18.0	22
Low 15	46	207	190	129	24.3	24
Low 16	69	303	205	149	22.7	28
Low 17	73	204	248	141	26.3	26
Low 18	43	220	240	147	26.7	26
Low 19	45	172	157	125	17.0	19
Low 20	61	138	220	129	20.7	28
Average	69.9 ± 30.8	207.9 ± 49.1	211.8 ± 37.8	144.4 ± 19.2	21.0 ± 2.9	26.9 ± 4.0

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Cryopreservation of *In Vitro* Grown Fern Gametophytes

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ABSTRACT.—Two methods of protecting fern gametophyte tissues through exposure to liquid nitrogen (LN) were examined. *In vitro* grown gametophytic tissues from six fern species were exposed to LN after open drying or after encapsulation dehydration, with and without preculture on abscisic acid (ABA). Open drying itself decreased survival with little further effect from LN exposure, although survival was somewhat improved by preculture on ABA. In contrast, encapsulated tissues survived drying and LN exposure at rates comparable to controls (86–100%) irrespective of ABA preculture. Sucrose pretreatment of the encapsulated tissues was important for their subsequent survival through these procedures. Tissues prepared by encapsulation dehydration were successfully regrown after 3.5 years in LN storage. Thus, cryopreservation appears to be a technique which could be used for the stable preservation of *in vitro* cultures of fern gametophytes and for the long-term storage of rare or endangered germplasm of ferns.

Although the more prominent sporophyte and its spores are frequently the focus of study, the small, fragile thallus of the fern gametophyte has also been the subject of much research (Stokey, 1940; Miller, 1968; Dyer, 1979). Fern gametophytes are easily grown in culture, making them good candidates for physiological and developmental studies, but maintaining stock lines or lines from a number of species requires a consistent input of time and labor.

Cryopreservation, or storage in liquid nitrogen (LN) at −196°C, has been used to preserve a variety of living tissues of both vascular and nonvascular plants. Seeds, shoot tips, cell cultures, callus, protoplasts, pollen, and embryos of seed plants (Kartha and Engelmann, 1994; Stanwood, 1985; Pence, 1991), as well as fern spores and the gametophytes of bryophytes (Christianson, 1998; Pence, 1998; and Pence, submitted) have all been successfully maintained in LN.

In this study, the possibility of using cryopreservation to preserve fern gametophytic tissue was explored. Two protocols, which have been used successfully with a variety of other tissues, were tested for preparing gametophyte tissue for cryopreservation: open drying of the tissues before LN exposure and the encapsulation dehydration procedure of Fabre and Dereuddre (1990).

MATERIALS AND METHODS

Gametophyte cultures were initiated from spores of *Davallia fejeensis* Hook., *Drynaria quercifolia* (L.) John Sm., *Cibotium glaucum* (Sm.) Hook. & Arn., *Adiantum trapeziforme* L., *Adiantum tenerum* Swartz., and *Polypodium aureum* L. collected from fronds supplied by Mr. Jeff Kapella, Supervising Florist of the Krohn Conservatory (Cincinnati).

For surface sterilization, spores were wrapped in small packages made by

folding pieces of Whatman No. 1 filter paper. The spores and packages were immersed in a 1:20 dilution of commercial sodium hypochlorite for 5 min, followed by two rinses in sterile distilled water. The packages were then opened, and the spores were blotted onto sterile germination medium, consisting of half-strength Linsmaier and Skoog (LS) (1965) salts and organics, with 1.5% sucrose and 0.22% Phytagel (Sigma Chemical Co.), in 60 × 15 mm disposable plastic petri dishes, approximately 15 ml/dish. The spores were incubated at 26°C under CoolWhite fluorescent lights in a 16/8 hr light/dark cycle. Once germination occurred and gametophytes were formed, the cultures were maintained by subculturing the tissue every 2–3 months onto fresh medium. In some experiments, gametophytes were precultured for one week on this same medium, with and without 10 μM abscisic acid (ABA), which was added to the medium after autoclaving.

For open drying, tissues were cut into pieces, approximately 2–5 mm long, blotted onto sterile filter paper to remove excess moisture and placed in a sterile petri dish under the air flow of the laminar flow hood for 3 hrs.

For encapsulation dehydration, the method of Fabre and Dereuddre (1990) was followed. Tissues were cut into small pieces, approximately 2–3 mm long, and transferred to a solution of 3% alginic acid in calcium-free MS medium plus 0.75 M sucrose. This solution, containing one or more pieces of gametophyte tissue, was then pipeted dropwise into a solution of 100 mM $CaCl_2$, which caused the alginic acid to gel, encasing the tissue in an alginate bead. After 20 min, the beads were removed from the calcium solution and transferred to liquid MS medium containing 0.75 M sucrose, 25 ml in 125 ml flasks, and placed onto a gyratory shaker, 125 rpm, for 18 hr as a pretreatment. In one experiment, different concentrations of sucrose, ranging from 0–30% were tested in the pretreatment step. The pretreated beads were then blotted on sterile filter paper to remove excess moisture and placed on dry filter paper in sterile petri dishes under the air flow of the laminar flow hood to dry for 3–4 hours.

Open dried tissues and dry encapsulated tissues were then placed into sterile 2 ml polypropylene cryovials and immersed directly into LN where they were left either for 1 hr or overnight (no difference was observed between these two LN exposure times). Tissues were thawed by placing the cryovials on the benchtop at ambient temperature for 20 min, after which the tissues or beads were removed and placed onto growth medium for rehydration and recovery. Survival was measured as the recovery of growth from each tissue piece for open dried tissue or the number of beads containing tissues resuming growth. As controls, some tissues were transferred to recovery medium after drying but without LN exposure. With the encapsulation dehydration procedure, tissues which had been pretreated for 18 hours in .75 M sucrose but which had not been dried were cultured as an additional control.

Fern gametophyte tissue from each species was also prepared by the encapsulation dehydration method for long-term cryostorage and banked in LN. After 3.5 years, samples of each were removed from storage and placed onto medium for rehydration and recovery growth.

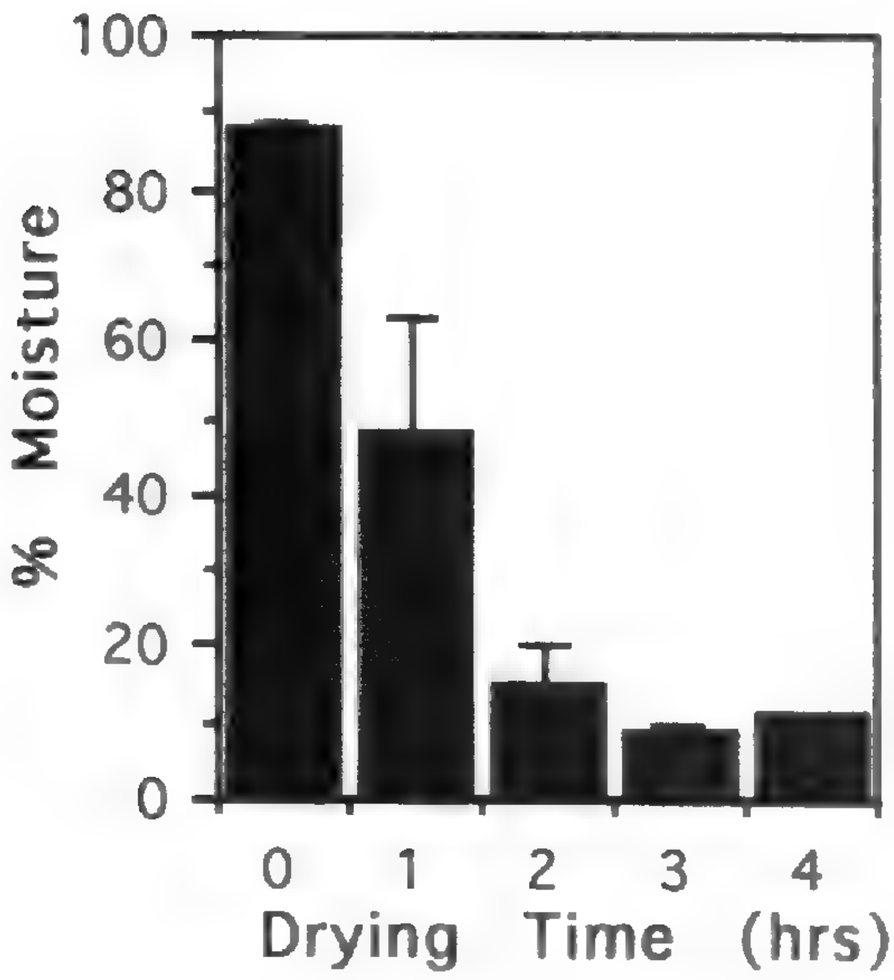

FIG. 1. Moisture loss from open dried gametophytic tissue of *D. fejeensis* during a 4 hour drying period (n = 3).

Moisture determinations were made on tissues of *D. fejeense* harvested at various times during drying under the laminar flow hood. Three samples of groups of small tissue pieces weighing between 0.15 and 0.25 g before drying were used. The percent moisture was calculated on a wet weight basis from the weights of the tissues before and after drying overnight in an oven at 95°C. Samples of tissues encapsulated in alginate beads were also analyzed for moisture after drying in the laminar flow hood.

RESULTS

The moisture level of gametophytes of *D. fejeense* during open drying was reduced to approximately 10% within 3 hours (Figure 1). The gametophytes of the other five species appeared to dry similarly from visual and tactile examination. When tissues and their surrounding alginate beads were dried, moisture levels were somewhat higher, decreasing to only 19–27% during the 3–4 hr drying period.

Survival of gametophyte tissue was reduced after open drying, compared with non-dried controls (Table 1). There was no further decrease in survival with exposure of the dried tissues to LN. *A. trapeziforme, P. aureum* and *C. glaucum* were particularly sensitive to drying, while the other species showed some survival of dried tissues. Preculture on ABA improved survival of the gametophytes through both drying and LN exposure. However, in only one species, *A. tenerum,* was survival of ABA treated tissues equivalent to that of the undried controls.

In contrast, there was excellent survival when gametophytes were encapsulated, pretreated with sucrose and dehydrated in alginate beads prior to LN exposure (Table 2). Tissues showed 100% survival through the 18 hr sucrose pretreatment, with no decrease in viability when the encapsulated tissues were

TABLE 1. Percentage survival and growth of gametophyte tissue pieces of six fern species through 3 hours of open drying followed by LN exposure, with and without preculture on medium containing 10 μM ABA. (n = 8 for controls; n = 10–32 for dried and LN exposed).

Species	Preculture on ABA	% Survival[a]		
		Control	Dried	LN exposed
C. glaucum	−	100	0	10
	+	100	45	88
A. tenerum	−	100	20	60
	+	100	100	100
D. quercifolia	−	100	23	62
	+	100	88	71
D. fejeensis	−	100	58	50
	+	100	92	83
	−	nd	18	30
	+	nd	35	69
P. aureum	−	100	5	0
	+	100	47	33
	−	nd	0	0
	+	nd	6	0
A. trapeziforme	−	100	0	16
	+	100	67	69
	−	nd	0	0
	+	nd	33	39

[a] nd = not determined.

dried. In a few cases, there was a slight decline in survival after LN exposure, but this effect was small (<15%). Because of the high survival rate without ABA, there was no apparent effect of the ABA preculture on survival when encapsulation was used.

Although survival of encapsulated material was good, some damage of the tissues was still evident. Whereas pretreated controls remained consistently green when placed on recovery medium, tissues which were dried or dried and exposed to LN often had some areas which were brownish green in color. Survival came from areas which remained bright green and which eventually grew out and reestablished the culture.

When encapsulated tissues of *D. fejeense* were exposed to different concentrations of sucrose during the 18 hr pretreatment, there was little or no survival through drying and LN exposure when sucrose was omitted completely from the pretreatment medium (Figure 2). However, good survival was observed at all but the highest sucrose concentrations.

Samples of encapsulated gametophyte tissues from these six species showed good survival after 3.5 years in LN storage (Table 3; Figure 3). Survival rates ranged from 50–100%, depending on the species.

DISCUSSION

These results indicate that the encapsulation dehydration procedure can be used successfully to cryopreserve gametophytes of at least six fern species and

TABLE 2. Percentage survival and growth of tissue pieces of six fern gametophytes through encapsulation pretreatment, drying and LN exposure. (n = 5–17). Survival through encapsulation without pretreatment was 100%.

Species	Preculture on ABA	% Survival[a] Sucrose pretrmt	Dried	LN exposed
C. glaucum	−	100	100	100
	+	100	100	100
A. tenerum	−	100	100	94
	−	100	100	100
	+	100	100	93
D. quercifolia	−	100	100	100
	−	100	100	100
	+	100	100	100
D. fejeensis	−	100	100	100
	+	100	100	100
	−	100	100	100
	−	100	100	100
	+	100	100	100
P. aureum	−	100	100	86
	+	nd	100	100
	−	80	82	82
	+	100	100	100
A. trapeziforme	−	100	100	100
	+	nd	100	100
	−	100	100	86
	+	100	100	100

[a] nd = not determined.

suggest that this technique might be broadly applicable to the gametophytes of other fern species, as well. Survival of encapsulated dehydrated gametophytic tissues through LN exposure was generally equivalent to that of controls.

The ability of fern gametophytes to survive drying without encapsulation varied with the species and, in many cases, was very poor. This is not surprising, since these tissues are generally adapted to moist conditions in the wild. ABA has been implicated in increasing stress tolerance in a number of systems (Hartung and Davies, 1991). Preculture on ABA improved the survival of dried fern gametophyte tissues somewhat, but in only one of the six species did survival increase to the level of controls. In a similar study with bryophytes, the effects of ABA on improving tolerance to open drying were also variable, depending on the species (Pence, 1998). However, with these clonal cultures, even a low percentage of survival will regenerate the culture, and open drying with ABA preculture could provide a straightforward method for freezing gametophytes of a number of species.

Survival through the encapsulation dehydration procedure, however, provided a higher level of survival of the fern gametophyte tissue. The presence of sucrose was important to the survival of *D. fejeense* through this method, suggesting that it is an important component in the protection afforded by this

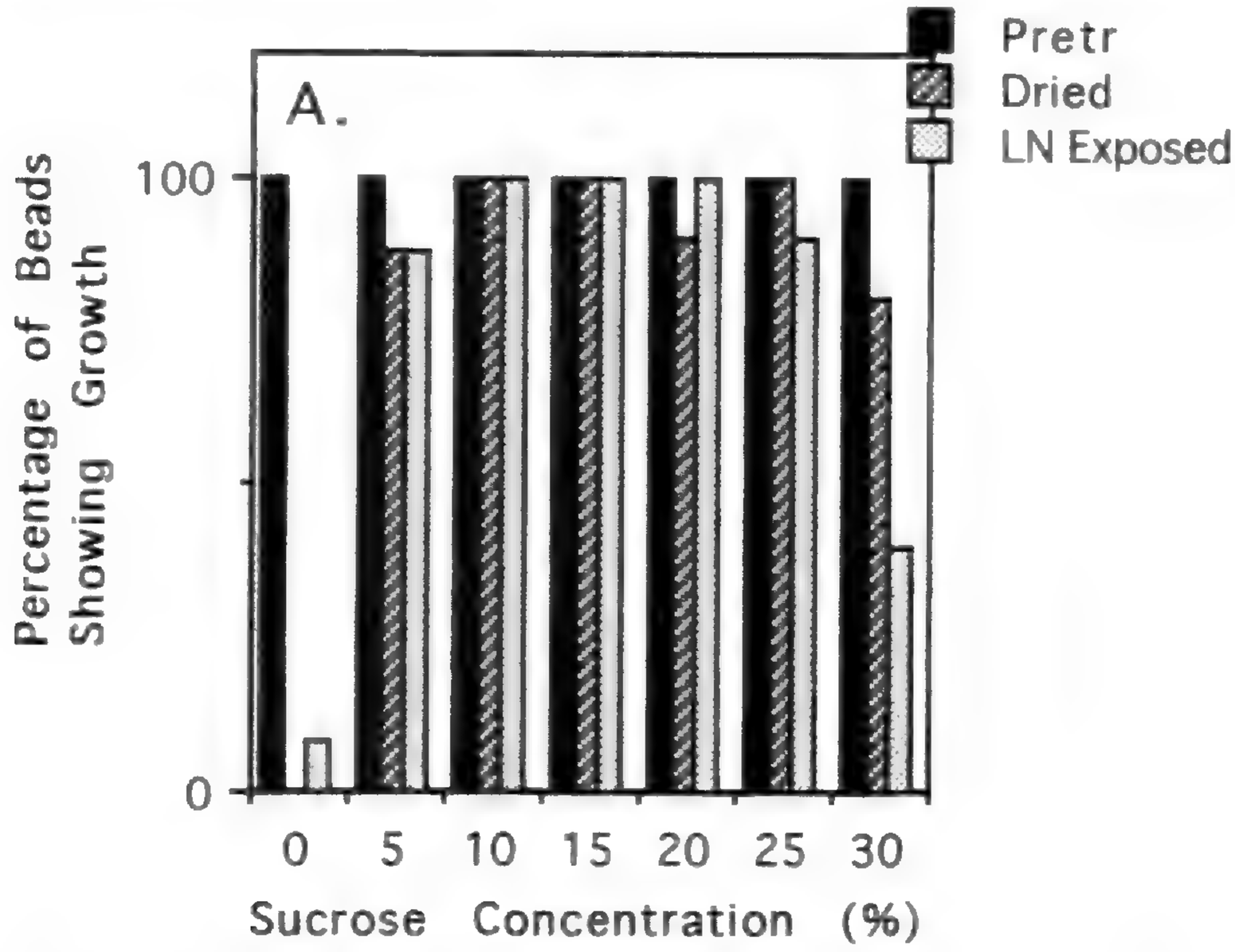

FIG. 2. Growth after drying and LN exposure of gametophytic tissue of *D. fejeensis* pretreated in several concentrations of sucrose using the encapsulation dehydration method (n = 8–10).

procedure. Research in this laboratory with byrophytes has shown that a sucrose pretreatment can improve the survival of open dried tissues (Geiger et al., unpublished). In addition, disaccharides and oligosaccharides have been implicated in the natural desiccation tolerance of several types of plant tissues,

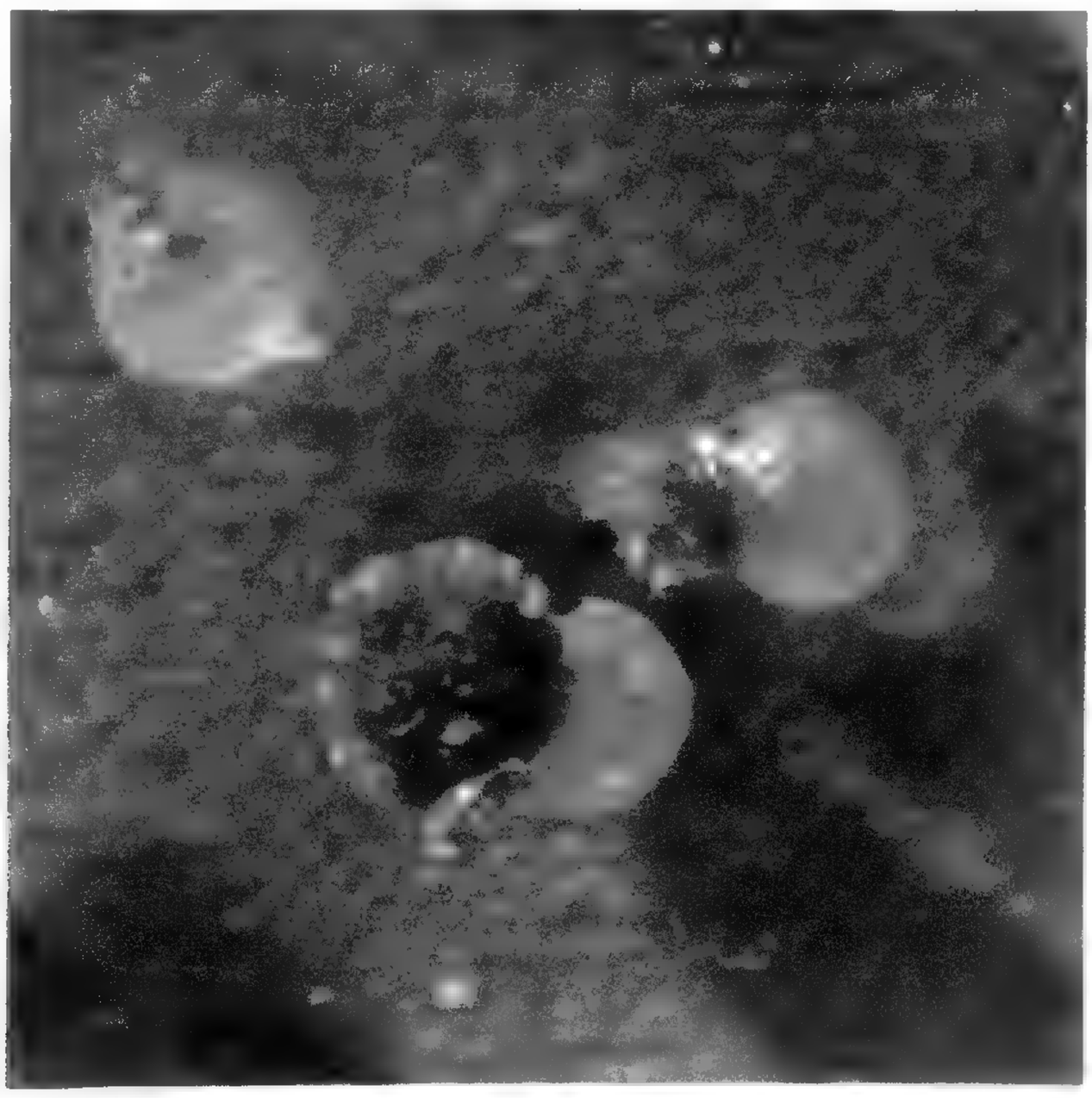

FIG. 3. Gametophyte tissue of *C. glaucum* resuming growth after 3.5 years of storage in LN, prepared for storage with the encapsulation dehydration method. 3.5×

TABLE 3. Regrowth from fern gametophyte pieces which were prepared by the encapsulation dehydration method and stored in LN for 3.5 years.

Species	Number of pieces	Percent of tissue pieces growing
C. glaucum	20	75
A. tenerum	8	88
D. quercifolia	12	67
D. fejeensis	10	100
P. aureum	10	90
A. trapeziforme	6	50

including seeds (Koster and Leopold, 1988), bryophytes (Smirnoff, 1992), and pteridophytes (Adams et al., 1990).

Encapsulation appears to be a technique that should be useful for long-term germplasm storage of fern gametophytes. Tissues resumed growth and appeared normal after 3.5 years of storage in LN, and it is likely that much longer storage times will be achieved. Less than 100% survival was observed with these tissues, in contrast to the experiments done with short-term LN exposure. While the possibility that the longer storage time was detrimental cannot be discounted, it is also possible that other factors, such as the state of the cultures (time since last subculture, etc.), may account for this difference. More tissues will be removed from storage over the next few years in order to determine whether viability does decline with time in storage.

Although survival was less than 100%, each culture was easily regenerated from the stored material. Thus, cryopreservation should be a useful method for preserving research lines and *in vitro* collections of fern gametophyte tissues, decreasing the time and resources necessary to maintain cultures as well as decreasing the opportunity for genetic changes to occur in the cultures.

Gametophytes might also be preserved when spores or sporophytes are not available for germplasm preservation. In the field, some tropical ferns are known only from the gametophyte stage in certain temperate areas (Farrar, 1967), while with other species, gametophytic tissue is more readily available for culture than are spores (Raine and Sheffield, 1997). Studies on soil spore banks and the germination of long-dormant spores can result in gametophytes which may or may not be easily grown into sporophytes (Dyer and Lindsay, 1992; Dyer, 1994). In such situations, cryopreservation could be used for maintaining *in vitro* cultures of such gametophytes for future study.

Nonseed plants have not received as much attention as seed plants with regard to germplasm resources and conservation. However, there are indications that preserving the biodiversity of these organisms could be potentially very important, for example, as sources of useful phytochemicals (Soeder, 1985). Cryopreservation of fern gametophytes is a technique that can supplement *ex situ* spore and sporophyte collections for the long-term storage of fern genetic diversity, providing a stable resource of valuable genetic lines as well as an *ex situ* back-up for species which are rare or endangered in the wild.

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Vessels in Roots and Rhizomes of *Dryopteris crassirhizoma* (Dryopteridaceae) from Heilongjiang Province, China

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ABSTRACT.—In the present study, tracheary elements in roots and rhizomes of *Dryopteris crassirhizoma* were observed with scanning electron microscopy (SEM). SEM observation revealed that all tracheary elements in both organs were vessels. These vessel elements have end-wall perforation plates and lateral-wall perforation plates. End-wall perforation plates in roots are more specialized than in rhizomes; they are all scalariform and obliquely positioned in end walls. Most end-wall perforations, especially in the center portion of end-wall perforation plates, lack pit membrane remnants, but pit membrane remnants are relatively abundant in some end-wall perforations of roots and rhizomes. It is noteworthy that several larger perforations on lateral walls usually are grouped together and form local lateral-wall perforation plates. Wide perforations alternating with narrow perforations characterize vessels of roots and rhizomes. In addition, the majority of perforations in lateral-walls have porose pit membranes or pit membrane remnants, range from intact pit membrane to nearly devoid of pit membrane remnants. Some vessels in rhizomes have several facets in which long scalariform pits have various degrees of porose membranes. These vessels contact several other tracheary elements for transferring materials.

In his classic study on fern tracheary elements, White (1961,1963) not only reported that vessels occur in *Pteridum* and *Marsilea,* but also used such terms "presumptive vessel" or "tracheary elements with end plates" to describe vessel-like tracheary elements in some ferns, such as *Dryopteris thelypteris* and *Woodsia obtusa.* The end plates of those cells are highly specialized in comparison with the lateral walls; scalariform pits on the end walls are wider than these on the lateral walls. Because of limitations of light microscopy, White (1963) was unable to determine the degree of pit membranes on end plates of those vessel-like tracheary elements (i.e. whether entire pit membranes on end plate were present or absent). The nature and presence of vessels are not revealed with light microscopy. Recently, Carlquist and Schneider (1997a,b) have confirmed by means of SEM that vessel-like tracheary elements as described by White (1963) in *Astrolepis* and *Woodsia* are vessels. SEM observations on those ferns showed that so-called pits on end walls are actually perforations with various degrees of pit membrane remnants from minutely to clearly absent, and revealed that there are lateral-wall perforations. It was noted that those genera, which grow in seasonally dry and cold places, have varying differentiated vessel elements in their roots and rhizomes in these authors' studies (Carlquist and Schneider 1997a,b, 1998a,b, 1999; Carlquist, Schneider and Yatskievych, 1997; Schneider and Carlquist 1997, 1998a,b, c, 1999). These studies seem to further support the hypothesis that fluctuation

in water availability appears basic to evolution of vessels in vascular plants (Carlquist 1975). Ferns distributed in Heilongjiang province can be formally divided into four different ecological groups according to plant-water relationships as follows (Ao and Li, 1987):

1. Xerophytes: *Selaginella sibirica, S.tamariscina, Aleuritopteris argentea, Dryopteris fragrans, Lepisorus ussriensis* and *Pyrrosia petiolosa* etc.
2. Mesophytes: *Adiantum pedutum, Athyrium multidentatum, A.yokoscense, Dryopteris crassirhizoma, Equisetum pratense* and *E.silvaticum* etc.
3. Helophytes: *Matteuccia struthipteris, Osmunda cinnamomea* var.*asiatica, Onoclea sensibilis* and *Thelypteris palustris* etc.
4. Hydrophyte: *Equisetum fluviatila, Marsilea quadrifolia, Azolla filicoloides* and *Salvinia natans* etc.

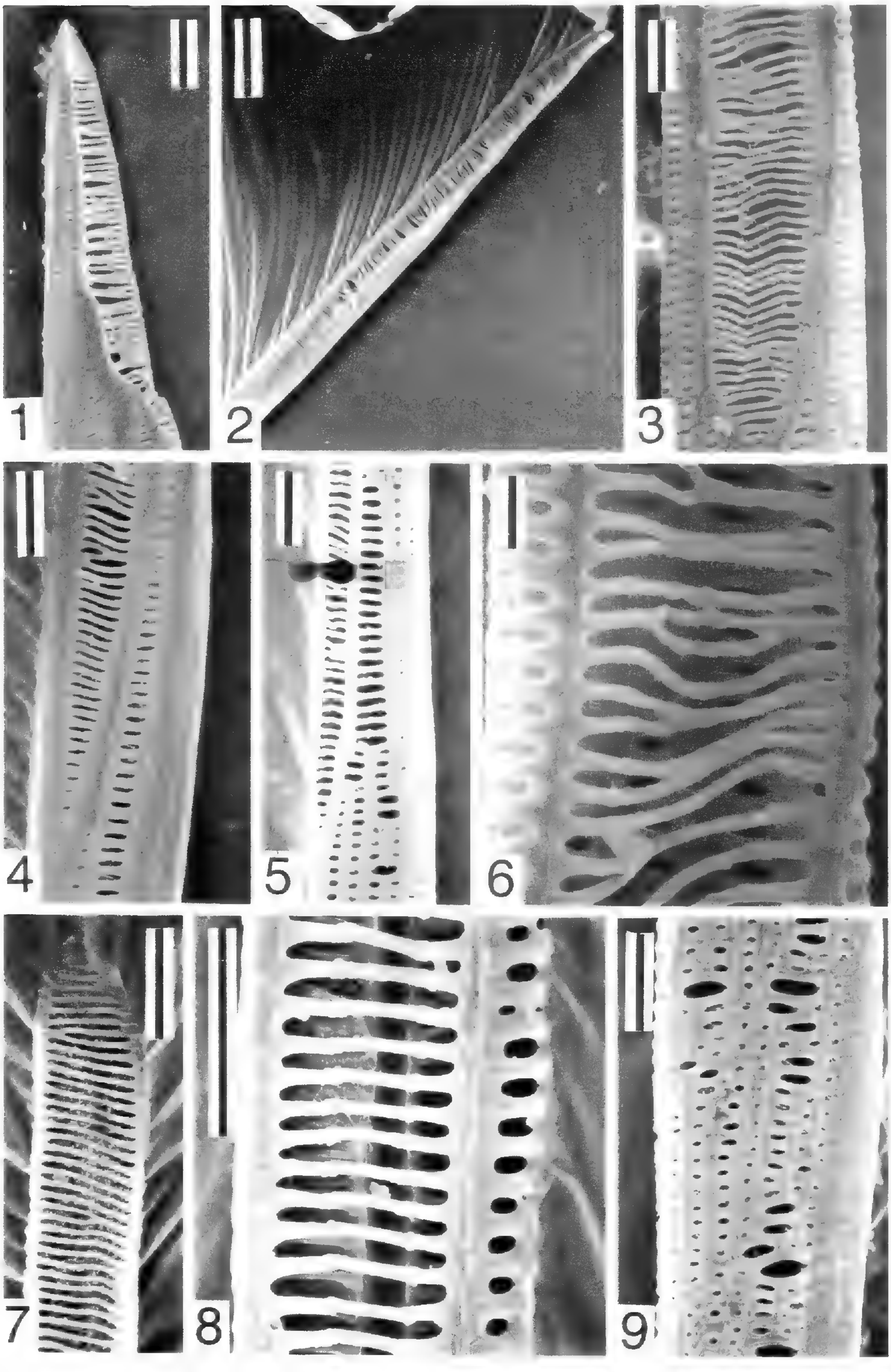
We have observed features of vessel elements in roots and rhizomes from *Matteuccia struthipteris* and *Osmunda cinnamomea* var.*asiatica* and found that even if they belong to the same ecological grouping, their vessel elements show different patterns of specializtion, i.e. primitive *O. cinnamomea* var. *asiatica* had little differentiation between perforation plate on the end wall and lateral wall pitting whilst *M.struthipteris* was markedly differentiated (Li et al., 1999). Thus we choose those different taxa, which are in different systematic positions, in the same ecological group as research materials to observe the microstructures of trachery elements by means of SEM for understanding evolutionary trends of treachery elements of ferns in similar and/or the same environment.

Dryopteris Adans. (Dryopteridaceae) has about 400 species distributed mainly in the North Temperate Zone (Wu & Ching, 1991). Russow (1873) considered that roots of *Nephrodium* (= *Dryopteris*) had true vessels because of the combination of lateral pitting which was most often alternate or opposite with short scalariformly pitted overlapping areas between the tracheary elements. Schneider and Carlquist (1997) have documented presence of vessels in *Polystichum* of the same family by means of SEM.

Dryopteris crassirhizoma Nakai is common in Heilongjiang province, which belongs to the second ecological species group but it also intrudes into other habitats including dry places. We attempt to demonstrate with SEM whether tracheary elements in root and rhizomes of this species are vessels or tracheids and make a comparison of microstructures of trachery elements to these of *Adiantum pedatum* in the same ecological group and *Polystichum acrostichoides* in the same family.

Materials and Methods

Fresh roots and rhizomes of *Dryopteris crassirhizoma* were collected from 3–5 wild plants of the Maoershan population, Shangzhi County, Heilongjiang province, and fixed in 3:1 absolute ethanol-glacial acid for 2–24 hours. Fixed materials were transferred into 70% aqueous alcohol and stored. We used mac-



FIGS. 1–9. SEM photographs from tracheary elements in root of *Dryopteris crassirhizoma*. 1. Lateral view of relative short end wall. 2. Face view of elongate end wall, scale bar = 50μm. 3. The lower part of the end wall, to show wider scalariform perforations in end wall that contrast with

erated and sectioned materials for SEM observation. We use two maceration methods (Flanklin's method: solution consisted of glacial acetic acid and 3% hydrogen peroxygen (1:1) and Jeffrey's method used by Carlquist et al. 1997). Roots and rhizomes were macerated in Franklin solution at 60°C for 4–5 days, while in Jeffery solution at 23°C for 2–3 days. These macerated materials were transferred into 70% alcohol, then spread onto the surface of SEM aluminum stubs. After drying, they were sputtercoated and examined with a Hitachi S-520 SEM. The sectioned materials were made as follows: the materials fixed in FAA were cut longitudinally, and dehydrated by an ethanol series. Then the ethanol was removed by isoamylacetate. The materials were dried in a critical point dryer with carbon dioxide and coated with gold.

RESULTS

In *Dryopteris crassirhizoma* roots, all end walls of tracheary elements examined bear perforations. These perforation plates range widely in morphology. Some perforation plates are relatively short with numerous bars (Fig.1), whereas other perforation plates are very long with numerous bars (Fig. 2). Perforation plates are markedly differentiated from lateral walls; the perforations on end walls are larger and more elongate than lateral-wall pits or perforations arranged in alternate or opposite pattern (Figs. 3–5). In figure 5, the end wall is at the left, but some lateral-wall perforations are similar to the end-wall perforations in absence of pit membrane remnants. In some plates, pit membrane remnants are very few (Fig. 6), but sometimes relatively abundant (Fig. 7). In the end wall shown in figure 8, monomorphic perforations mostly lack pit membranes. Some end walls show dimorphism between wide and narrow perforations in figure 6. In addition to perforation plates on end walls, lateral-wall perforations are also found (Figs. 5,8,9). It is interesting that lateral-wall perforations range from very small to large; several larger perforations (usually two to four perforations) usually group together (Fig. 9).

Most tracheary elements in rhizomes possess end walls with perforations, which are less specialized than those in roots (Fig. 10). Some wider perforations on end walls clearly lack pit membranes in this figure and those narrow perforations that alternate with wide perforations in the same perforation plate usually have various degrees of pit membrane remnants (i.e. from intact pit

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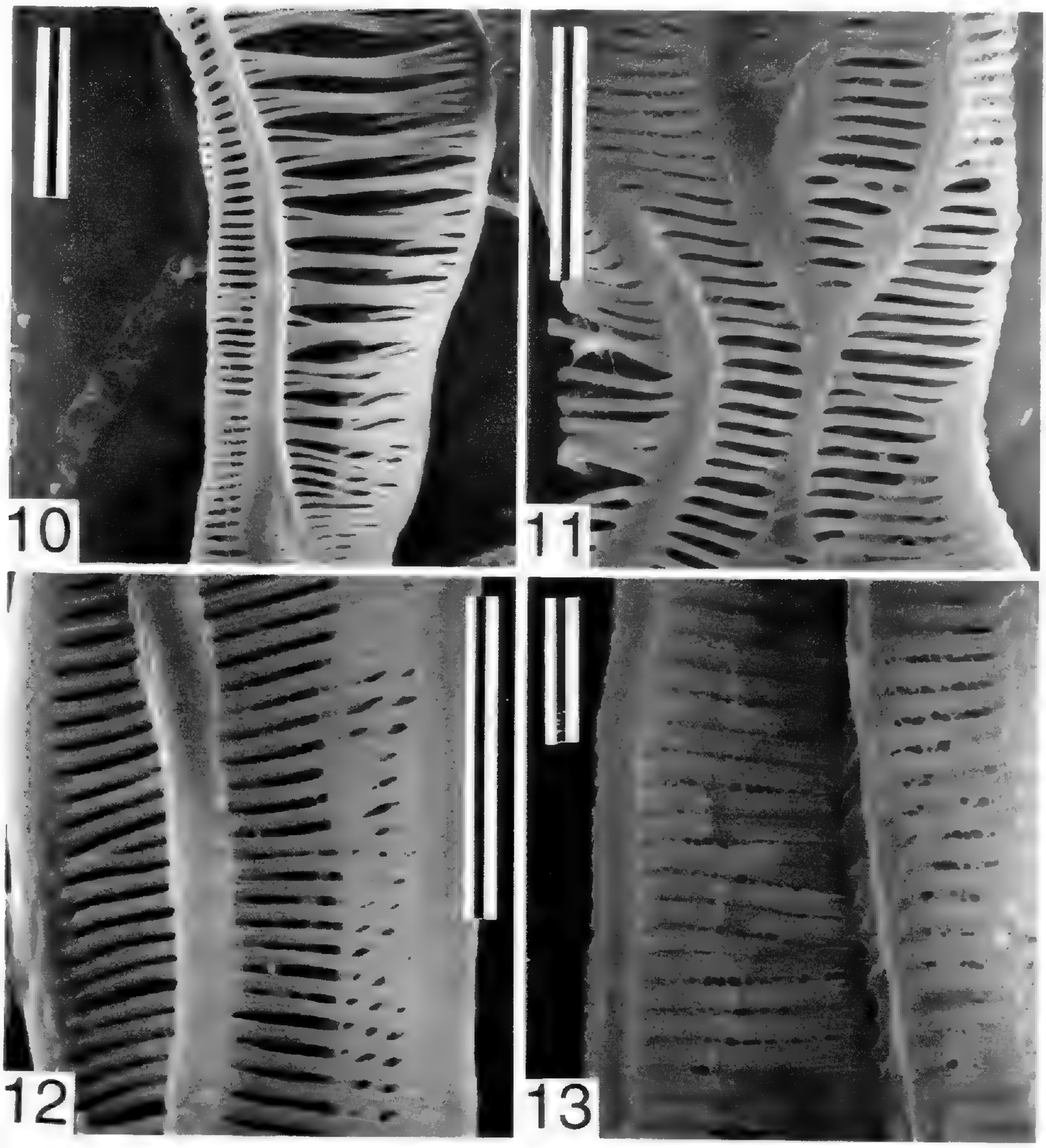
the alternate or opposite pits or narrower perforations with pit membrane remnants in lateral wall. 4. The lower part of end wall, to show those perforations with absence of pit membrane. 5. The lower part of end wall, to show those perforations with absence of pit membranes and lateral-wall perforations in the right of end-wall perforation plate. 6. End wall, to show perforations with few pit membrane remnants and dimorphism in wideness of perforations; grain of unknown materials lodged on perforation plate. 7. End wall, to show perforations with many pit membrane remnants. 8. Central portion of an end-wall perforation plate, to show monomorphic perforations that completely lack pit membrane. 9. Lateral-wall perforation plate, to show larger perforations grouping in lateral wall. Scale bars = 30μm.



FIGS. 10–13. SEM photographs from tracheary elements in rhizomes of *Dryopteris crassirhizoma*. 10. Perforations with absence of pit membrane and the lower perforations with some pit membrane remnants on end wall; 11. Central section of this vessel, to show four perforation plates. 12. Lateral-wall perforations with porose pit membranes of vessel element shown in figure 10. 13. Lateral wall, to show various sizes of pores in pit membrane, scale bar = 10μm. Scale bars in the other figures = 30μm.

membrane to nearly devoid of pit membrane remnants). The majority of lateral walls consist of scalariform perforations in which there are porose pit membranes or pit membrane remnants (Figs. 12, 13), but in some lateral walls, perforations markedly lack pit membranes as is the case for end-wall perforations. One can find many tracheary elements that have several facets in contact with other tracheary elements. The tracheary elements possess as many as eight facets with long, sclariform pits, which have entire pit membranes, porose pit membranes, or degrees of pit membrane remnants (Fig. 11).

Discussion

In the present study, *Dryopteris crassirhizoma* has been shown to possess vessels in both roots and rhizomes, and the same characters of these vessel elements in materials made by three methods are observed by mean of SEM. Our SEM observations not only reveal that pits on oblique end walls as seen in the light microscopy are perforations which lack pit membranes, we also find perforations with degrees of pit membrane remnants on lateral walls. *Dryopteris crassirhizoma* therefore has end-wall perforation plates and lateral-perforation plates. End-wall perforation plates in roots are more specialized than in rhizomes; these perforation plates are obliquely positioned in end walls. End walls in roots are relatively short with wider scalariform perforations that contrasts with the alternate or opposite pits or narrow perforations with pit membrane remnants on lateral walls. Thus end walls are markedly differentiated from the lateral walls. Moreover their perforation plates are more specialized than those of *Adiantum pedatum* belonged into the same ecological group (Li et al., 1999) and *Polystichum acrostichoides* in the same family (Schneider and Carlquist, 1997) based on morphology of perforation plate on the end wall. *Dryopteris crassirhizoma* not only occurs in mesic habitat, but also ranges into other habitats such as dry place and grow normally. In the genus *Woodsia, W. scopulina* and *W. ilvensis,* which occurs in places where winter freezing and summer drought abbreviate the growing season, all had high differentiation in morphology between perforation plate and lateral wall pitting, in contrast, *W. obtusa* growing in a mesic habitat had little differentiation (Carlqusit, Schneider and Yatskievych, 1997; Schneider and Carlquist, 1998a; Carlquist and Schneider, 1998a). This phenomenon was explicated to markedly differentiated vessels as a benefit to increase water supply when ferns occupied area where there was water availability stress in some seasons.

Carlquist and his colleagues observed transverse perforation plates with several bars in vessels of *Pteridium* roots (Carlquist and Schneider, 1997a) and no lateral-wall perforation plates in *Woodsia obtusa* (Carlqusit, Schneider and Yatskievych, 1997). It is evident that vessels of *Dryopteris crassirhizoma* are moderately specialized. In rhizome vessels, narrow scalariform perforations of end walls are in contrast to the scalariform pits and perforations with pit membrane remnants on the lateral walls; there is less differentiation between end walls and lateral walls. This result is observed in several other ferns (Carlquist & Schneider, 1997a, b; Carlquist et al., 1997; Schneider & Carlquist, 1997), which supplies evidence that vessels may have first occurred in fern roots. Dimorphic perforations are common in vessels of roots and rhizomes; Figure 9 shows the end walls of some root vessels which have perforations differentiated into two forms. This dimorphism in width of perforations also occurs in end walls of rhizome vessels, but it is less pronounced.

Lateral-wall perforations are common in *Dryopteris crassirhizoma*. We noted some particularly larger perforations which differ markedly from the other perforations in the same lateral wall shown in figs. 5, 8 and 9, which (two to four) group together to form a local perforation district or plate. In *Astropis,*

Phlebodium, Polystichum, Pteridium and *Woodsia,* this phenomenon is newly reported. Gwynne-Vaughan (1908) considered that the formation of lateral-wall perforations in *Pteridium* was related to abundant tyloses, but further study is needed on the development of this type of perforations in lateral walls of *Dryopteris crassirhizoma.*

The lateral walls of rhizome vessels have porose pit membranes. Perforations of the terminal end wall of vessels in roots and rhizomes may also have this type of membrane. Carlquist and Schneider (1997a) considered that presence of porose pit membrane in *Pteridium* vessels could enhance lateral transport of water without markedly increasing the vulnerability of vessels to transfer of air embolism from one vessel element to another laterally. End walls are less specialized, because numerous pits with porose pit membranes and/or pit membrane remnants occur in lateral wall of rhizomes. We note that some vessels in rhizomes have several facets, in which long scalariform pits have various degrees of porose pit membrane, ranging from smaller pores in pit membranes to absolute absence of pit membranes. These vessel elements contact with several other cells for transferring materials.

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SEM Studies on Vessels in Ferns. 19. *Marsilea*

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ABSTRACT.—Tracheary elements from roots and rhizomes of *M. drummondii*, *M. quadrifolia*, and *M. vestita* supplement the work of other authors by showing features of *Marsilea* vessels not previously reported or not commonly reported. Pit dimorphism (wide pits lacking pit membranes alternating with narrow pits covered with pit membranes) form perforation plates in some vessel elements. Scalariform perforation plates with perforations like lateral wall pitting in size are common, and have not been previously reported in *Marsilea* by workers using light microscopy; further SEM studies may reveal such perforation plates to be more common in the family than presently thought. Circular to oval pits arranged in alternate, opposite, and transition fashion are characteristic of some vessels in the genus, as are tracheary element facets with few or no pits. The occurrence of perforation plates with few bars (found also in xeric and boreal ferns) and simple perforation plates in *Marsilea* may represent a capability for rapid uptake of water in habitats with relatively short periods of water availability.

Marsileaceae have been explored more thoroughly than other fern families for presence and morphology of vessels. Following the initial reports of vessels in roots of *Marsilea* by White (1961, 1962), Mehra and Soni (1971) confirmed presence of vessels in *Marsilea* roots but did not find them in rhizomes of the genus. Tewari (1975) reported vessels in roots of *Regnellidium*. Bhardwaja and Baijal (1977) reported vessels in roots of nine species of *Marsilea*, but in rhizomes of only two *Marsilea* species. Loyal and Singh (1978) observed vessels in roots of seven species of *Marsilea*, and also found vessels in the nodal regions where roots and petioles join the rhizome, but not in internodal regions of the rhizome. Bhardwaja and Takker (1979) observed only tracheids in xylem of *Pilularia*. Details on occurrence of vessels in *Marsilea* were added by Johnson (1986); Sharma (1988) cites the work prior to Johnson's on vessels in Marsileaceae, but does not add original observations.

Note should be taken that all of the studies cited above were performed with light microscopy. Although vessels with well-defined perforation plates consisting of few perforations may be observed with reasonable accuracy with light microscopy, observation of fern vessels with SEM (scanning electron microscopy) seems a procedure that can add more information and is therefore of value. In most of the fern families studied in our papers, we have described vessel elements in which perforation plates are very similar to, or identical to, lateral walls of tracheary elements, and differ in absence of primary walls in the perforations, which are like lateral wall pits in size and shape; the distribution of perforations as seen by SEM is also distinctive and diagnostic, and not at all random. We believe SEM is valuable in identifying such probable perforations. In some carefully observed preparations utilizing particular stains, light microscopy has been used to establish presence or absence of primary walls in possible perforation plates in vessel elements. SEM, however,

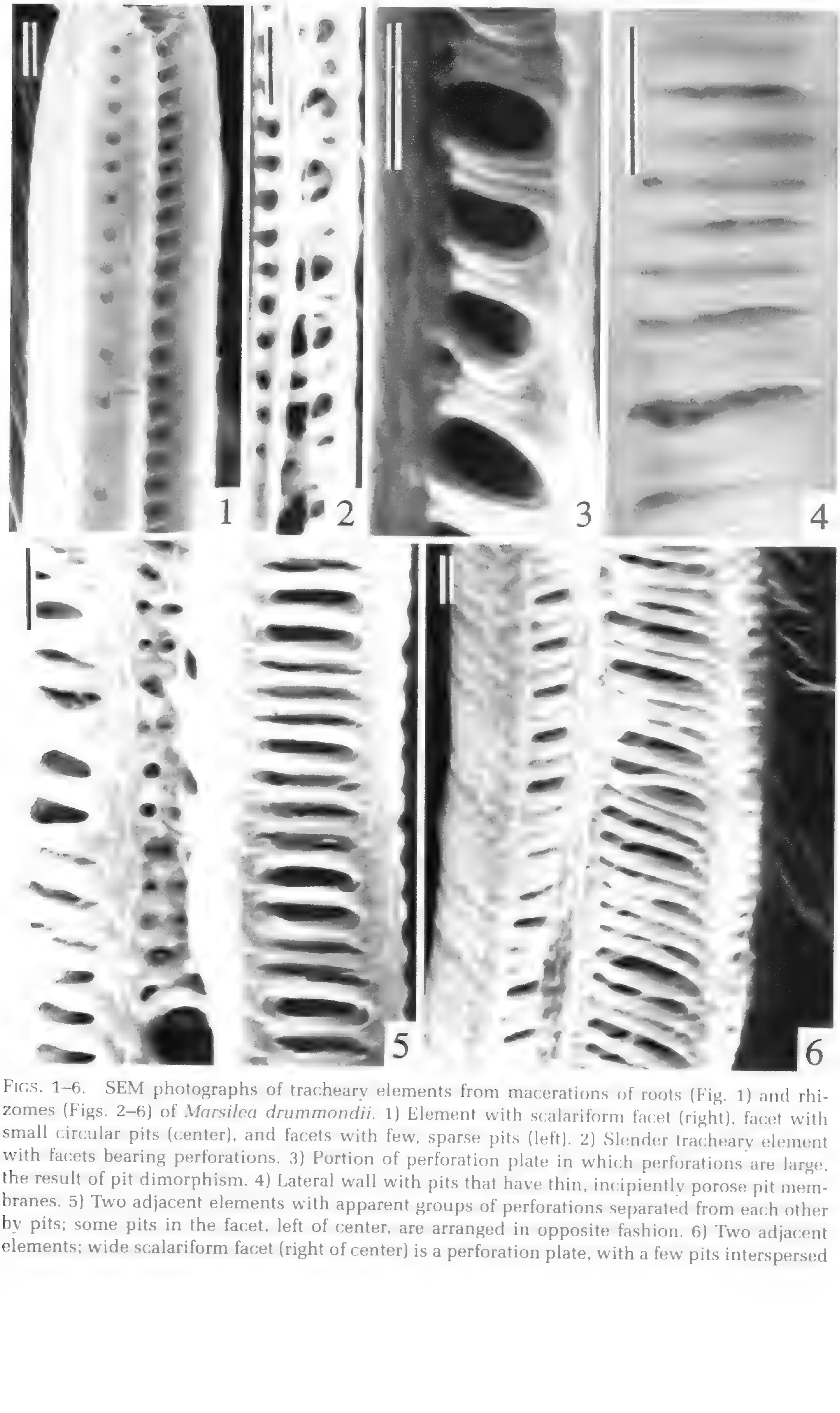
clearly reveals presence of cell walls, which reflect the electron beam. Presence of primary walls and of perforations can be established accurately by means of transmission electron microscopy (TEM). Ultimately, one would wish for observations by all kinds of microscopy, as well as the utilization of particles that can be transmitted through perforations but not through intact pit membranes.

We believe that our results with SEM are accurate indications of vessel presence in ferns because the macerations (and in some instances, sections) in our studies reveal pit membranes with few artifacts. Some pit membranes are ruptured by handling, but removal of all pit membranes on an end wall while pit membranes are intact on adjacent lateral walls cannot, in our opinion, be attributed to the maceration process. Moreover, we frequently find at ends of perforation plates pit membranes that are transitional to perforations by virtue of thinness and presence of porosities or even minimal weblike or strandlike remnants of primary walls. Such incipient perforations can also be found on end walls that have no perforations devoid of pit membranes. By eliminating from consideration all instances we could ascribe to pit membrane absence that might represent artifacts induced by preparation methods, we believe that our reports of perforation plate presence are reliable.

The studies on xylem of Marsileaceae cited above report vessel presence in rhizomes much less frequently than in roots. However, our studies on vessels in ferns have found that in any species in which vessels could be demonstrated in roots, they could also be demonstrated in rhizomes. On account of this finding, we were motivated to search for vessels in rhizomes of *Marsilea*. In fact, the sheaths of fibers around the steles of *Marsilea* roots provided severe difficulties in isolating tracheary elements for observation by SEM, whereas rhizome steles yielded somewhat more easily to the maceration process. These maceration difficulties, greater than those we have encountered in any other fern families in our series of papers, have limited the number of vessel elements we could observe clearly. Consequently, the observations we report here should not be taken as representative of kinds of vessels in these species. Our observations do show the presence of tracheidlike vessel elements in which scalariform perforation plates resemble scalariform lateral wall pitting. These have not been reported by earlier authors and thus we add to the picture of perforation plates in *Marsilea*.

Materials and Methods

Species studied and the sources of collections are as follows: *M. drummondii* A. Braun, cultivated at the University of California Botanic Garden, Berkeley (74.0212); *M. quadrifolia* L., cultivated by San Marcos Growers, Santa Barbara, California; *M. vestita* Hook & Grev., cultivated in the Biological Sciences greenhouses, University of California, Santa Barbara. Voucher specimens prepared from these plants have been deposited in the herbarium of Santa Barbara Botanic Garden. Portions of roots and rhizomes were fixed and stored in 50% aqueous ethanol. Macerations were prepared by means of Jeffrey's Fluid (Jo-



FIGS. 1–6. SEM photographs of tracheary elements from macerations of roots (Fig. 1) and rhizomes (Figs. 2–6) of *Marsilea drummondii*. 1) Element with scalariform facet (right), facet with small circular pits (center), and facets with few, sparse pits (left). 2) Slender tracheary element with facets bearing perforations. 3) Portion of perforation plate in which perforations are large, the result of pit dimorphism. 4) Lateral wall with pits that have thin, incipiently porose pit membranes. 5) Two adjacent elements with apparent groups of perforations separated from each other by pits; some pits in the facet, left of center, are arranged in opposite fashion. 6) Two adjacent elements; wide scalariform facet (right of center) is a perforation plate, with a few pits interspersed

hansen, 1940), stored in 50% ethanol, spread onto aluminum stubs, air-dried, sputter-coated, and observed with a Bausch and Lomb Nanolab 200 SEM at 15 KV.

RESULTS

Marsilea drummondii root xylem proved unusually difficult to isolate by means of maceration. The root tracheary element shown in Fig. 1 illustrates scalariform lateral wall pitting, at right. The facet at the center of the photograph is so narrow that the pits are circular in outline, whereas the facet at left bears pits that are few and small.

Rhizome tracheary elements of *M. drummondii* (Figs. 2–6) were more readily recovered by maceration and proved to bear abundant and diverse perforations. In Fig. 2, pit membranes are absent from all of the facets of a slender tracheary element, and perforations are of various sizes. The phenomenon of dimorphic pits is illustrated in the facet shown in Fig. 3; wide perforations alternate with several pits compressed parallel to the long axis of the tracheary element. The compressed pits are so narrow that one cannot readily observe pit membranes on them. Pit membrane remnants certainly are absent on the wide perforations. Pit membranes are present on the scalariform pits of the lateral wall illustrated in Fig. 4. We cannot certify that pores are present in these pit membranes, but thin areas are discernible in the pit membranes. In a tracheary element shown in Fig. 5 (facet at right), on the other hand, pits that apparently lack pit membranes (and would therefore be perforations) are present in groups, and these groups alternate with groups of several pits that bear intact pit membranes (some membranes torn by handling). If this interpretation is valid and does not represent a condition caused by artifact formation, various facets of the tracheary elements illustrated have what one can regard as intermittent perforation plates. This situation is also present in the pair of adjacent tracheary elements in Fig. 6, although perforations are somewhat more abundant in the tracheary element at right. In the tracheary element at left in Fig. 6, the left facet consists of pits with intact membranes, and the right facet bears, above, narrowed pits and slightly widened perforations alternating with each other, an example of pit dimorphism.

Tracheary elements from roots of *M. quadrifolia* are illustrated in Figs. 7–10. The lateral wall in Fig. 7 bears pits with intact pit membranes, and is noteworthy only in that the pits are irregularly spaced: a few pits appear in close pairs. In Fig. 8, pit membrane remnants of an incipient perforation plate bear striking pores or weblike strands of primary wall material can be seen. Tears due to handling may be seen in the pit membrane remnants, but these can easily be detected and the porose nature of the pit membranes is clear. In

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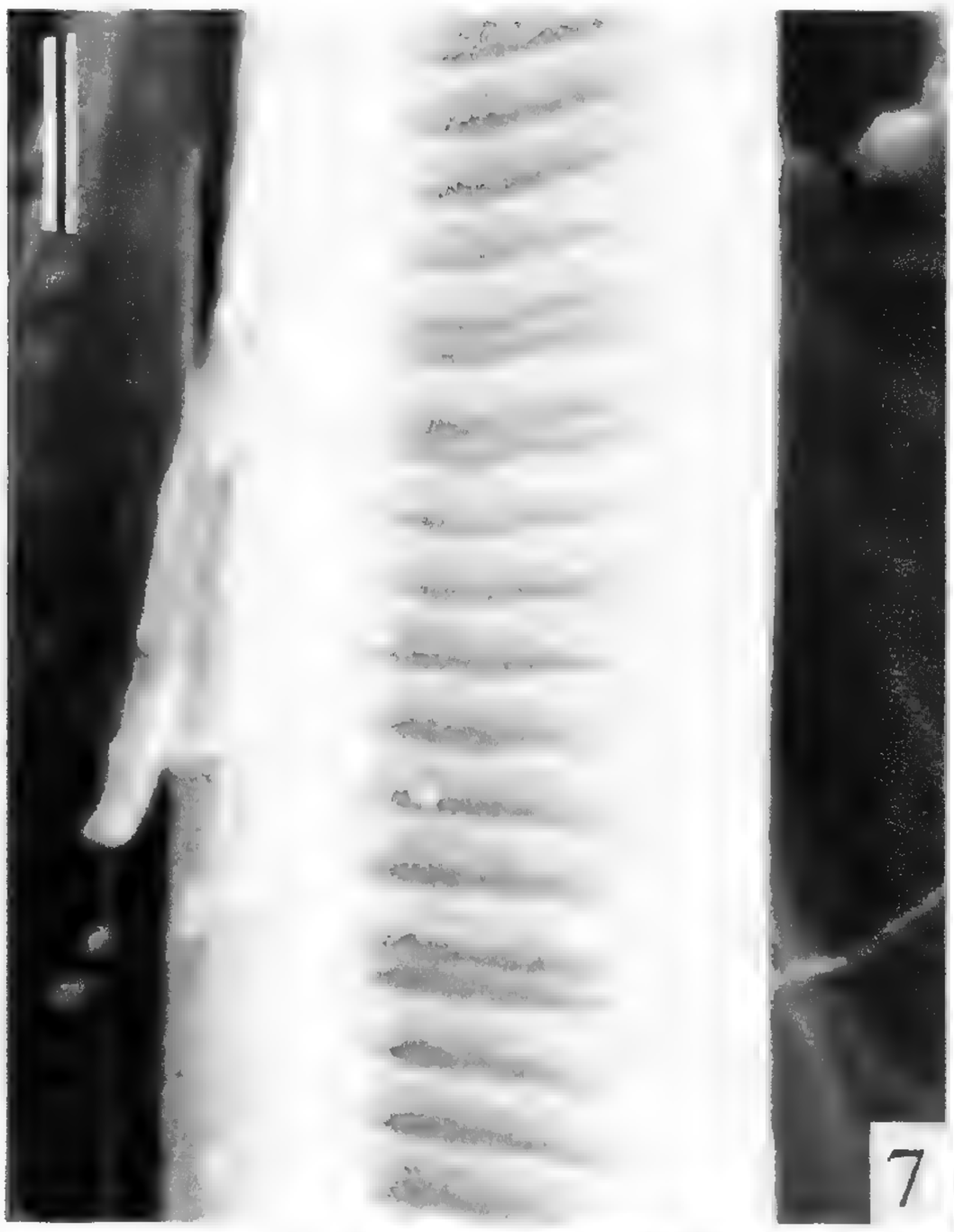
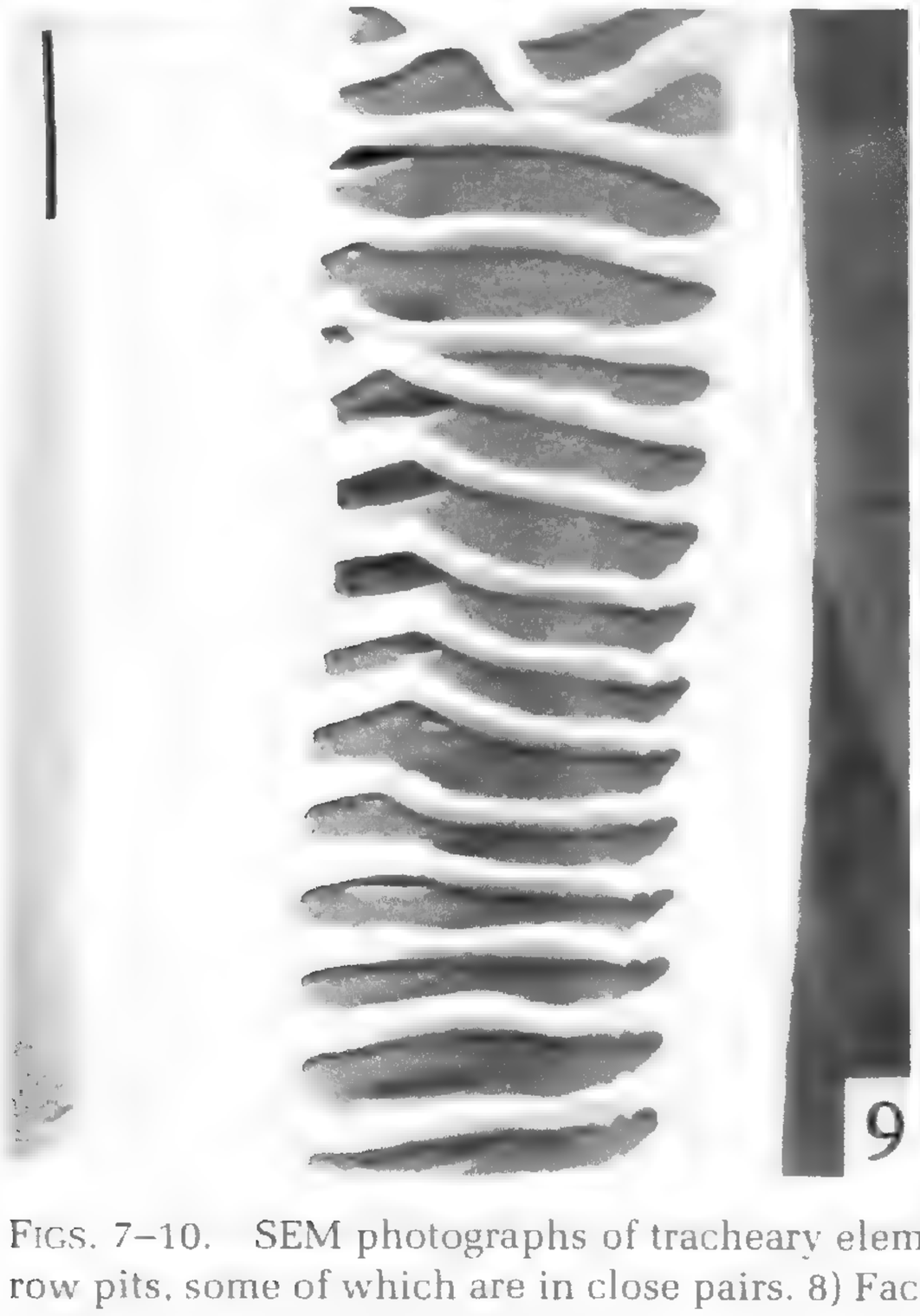
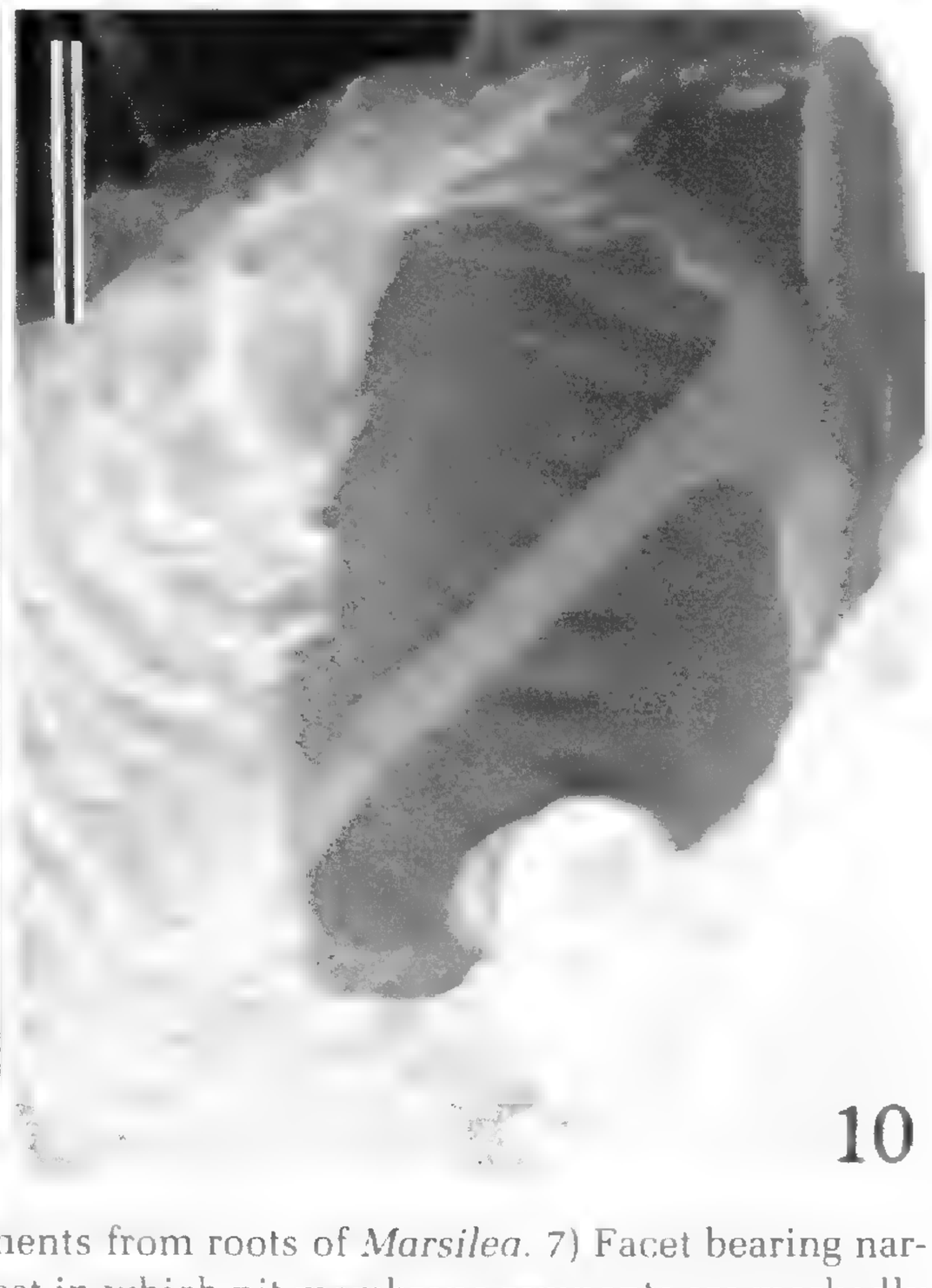
among the perforations; facet left of center shows, above, alternation of perforations and narrow pits (pit dimorphism); facet at left is composed of scalariform pitting. Scales in all figures = 5 μm.







FIGS. 7–10. SEM photographs of tracheary elements from roots of *Marsilea*. 7) Facet bearing narrow pits, some of which are in close pairs. 8) Facet in which pit membrane remnants are markedly porose; some tearing present but most areas are intact. 9) Scalariform perforation plate portion; bars are narrow, perforations wide, and thus the perforations differ in width from lateral wall pits (compared with Fig. 7). 10) Perforation plate bearing a single bar. Scales in all figures = 5 μm.

Fig. 9 a scalariform perforation plate with perforations wider (with respect to the element axis) than pits (compare with Fig. 7) is illustrated. In Fig. 10 an end wall perforation plate traversed by a single bar is shown. We did not with certainty observe any tracheary elements with simple perforation plates.

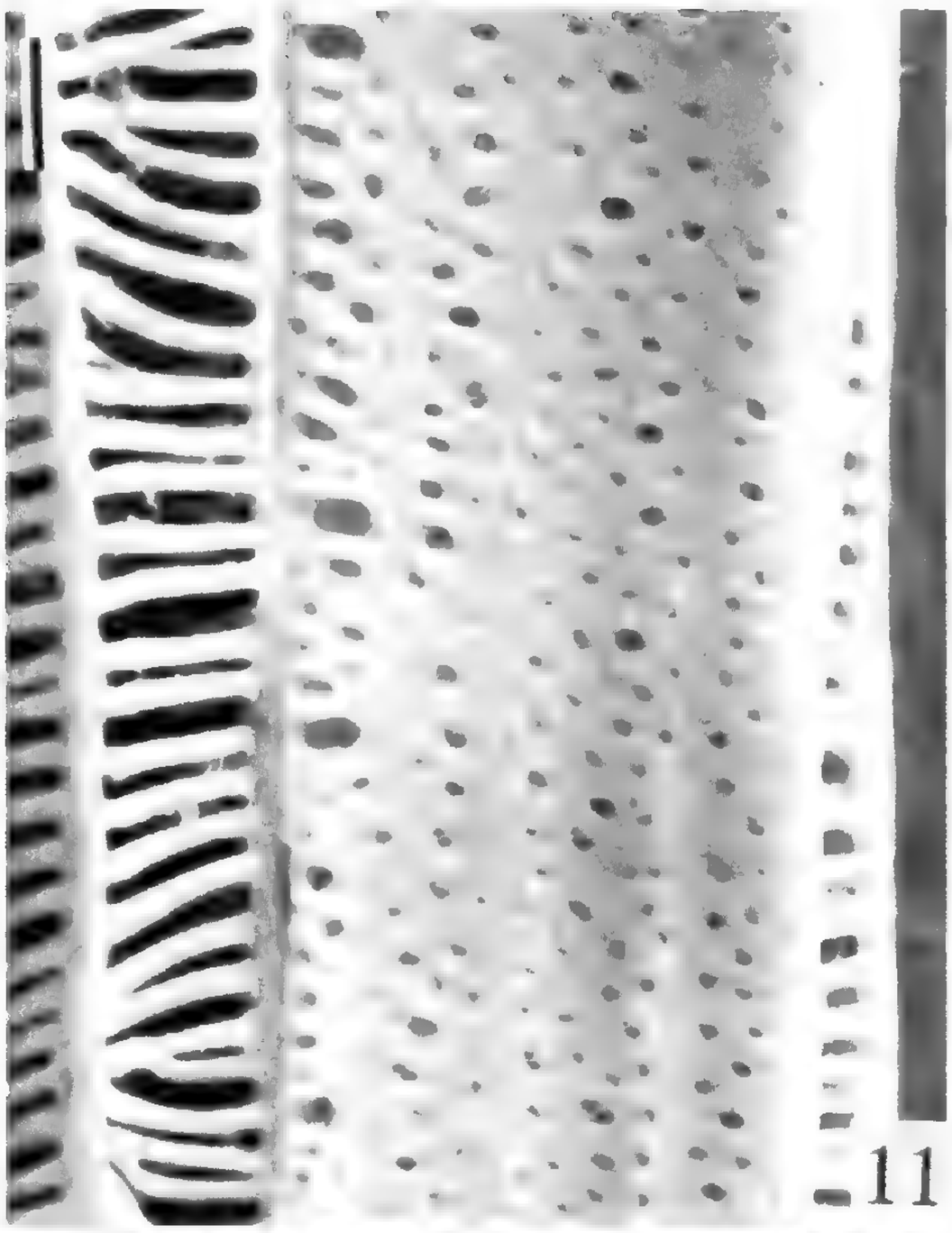
Rhizome tracheary elements of *M. quadrifolia* are relatively wide in diameter, and at least some have well-defined perforation plates (Figs. 11–14). Scalariform perforation plates are illustrated in Fig. 11, left, and Fig. 12, right; the perforations contain pit membrane remnants that do not appear to have resulted from handling. In both Fig. 11 and Fig. 12, alternately arranged circular to oval pits cover other facets. Pitting of this type was reported in drawings of Loyal and Singh (1978, Figs. 35 and 36). However in the tracheary elements in our figures, there is wide diversity in pit size on a single facet not reported in previous studies: a kind of pit dimorphism. The larger pits clearly lack pit membranes, and we have no reason to believe that this membrane absence is an artifact. The scalariform perforation plate in Fig. 13 is noteworthy in that the bars are very slender, the perforations are correspondingly large, and some bars fork or anastomose. The facet at left in Fig. 13 bears intact pit membranes with the exception of two pits, which seem to lack pit membranes, probably a result of handling. The scalariform perforation plate of Fig. 14 has both wide bars and wide perforations and is the kind of perforation plate that could be seen with light microscopy. The other facets of the wide tracheary element in Fig. 14 lack pitting, a condition also shown for *Marsilea* vessels by Loyal and Singh (1978, Figs. 23 and 25). The slender tracheary element at right in Fig. 14 may be a late protoxylem or early metaxylem element; in any case, the facet at left bears intact pit membranes, whereas the facet at right has porose pit membranes like the ones shown in Fig. 8.

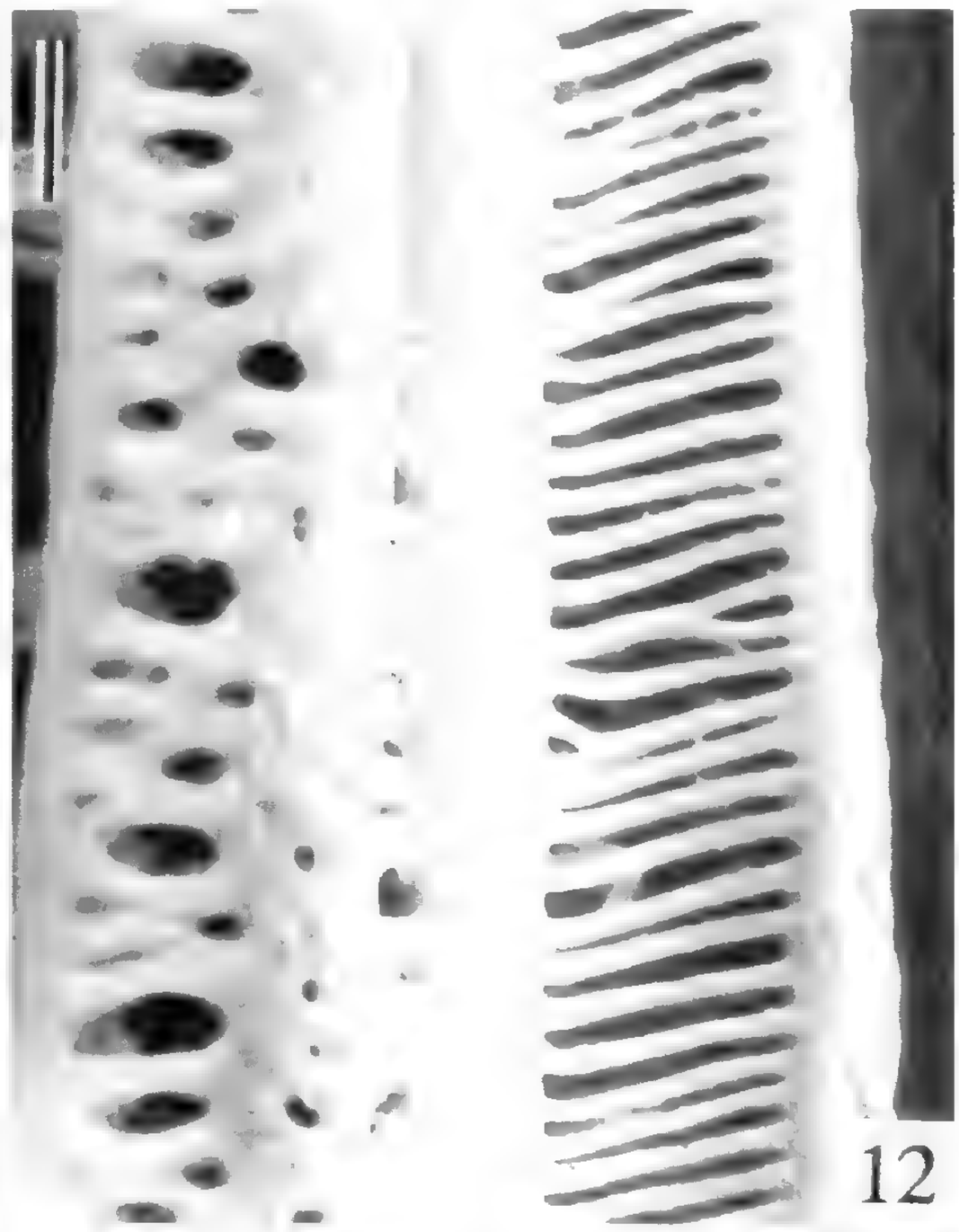
In *M. vestita,* a large tracheary element with very narrow elongate pits (Fig. 15) was found in the rhizome; no perforations were observed on this element. Scalariform perforations were observed on other rhizome tracheary elements (Fig. 16, left), and root tracheary elements (Fig. 17, right; porose pit membranes at bottom of perforation plate and intact pit membranes above perforation plate). The facet at right in Fig. 16 would be termed transitional in dicotyledons; this pitting type has apparently not been previously reported in *Marsilea.*

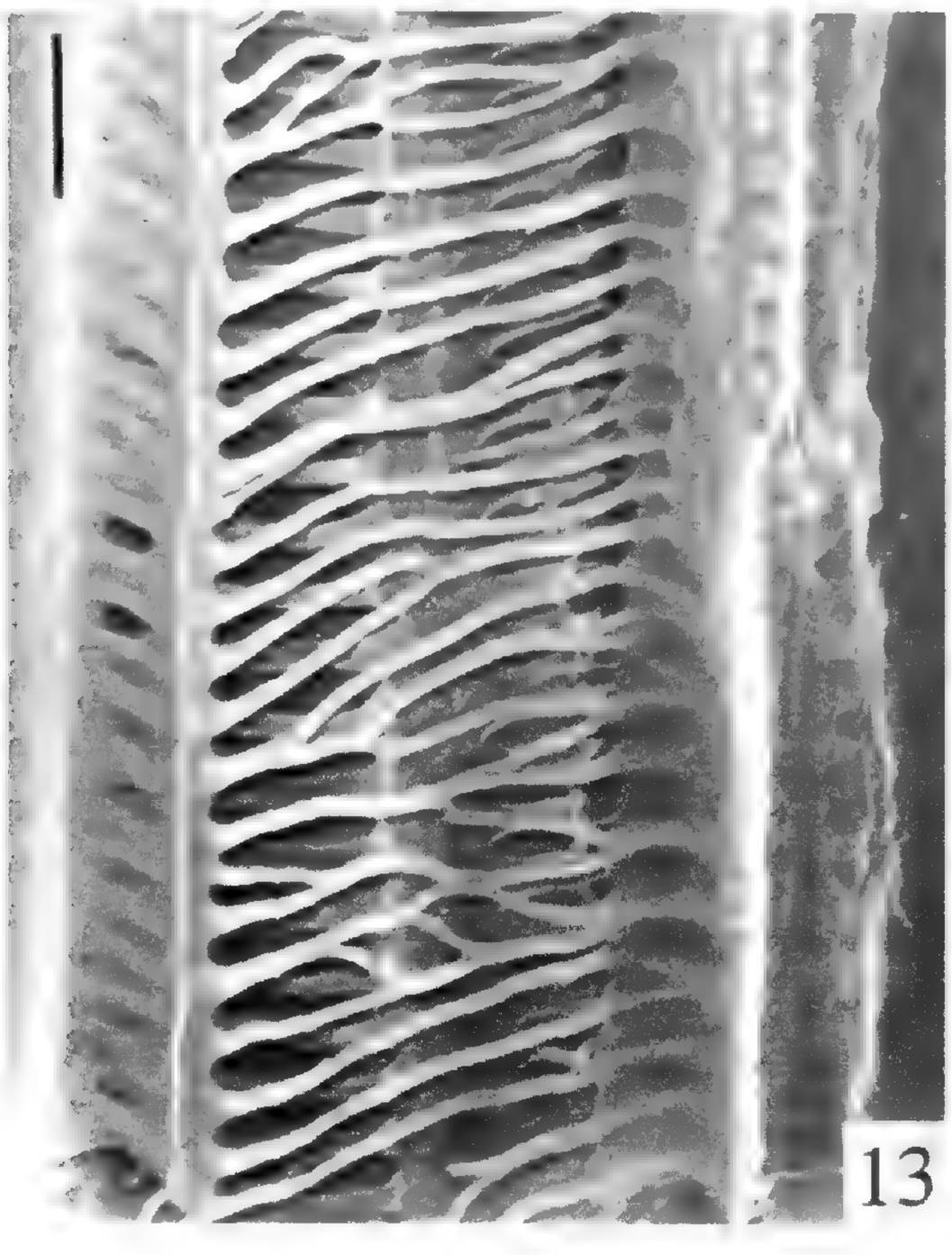
Discussion and Conclusions

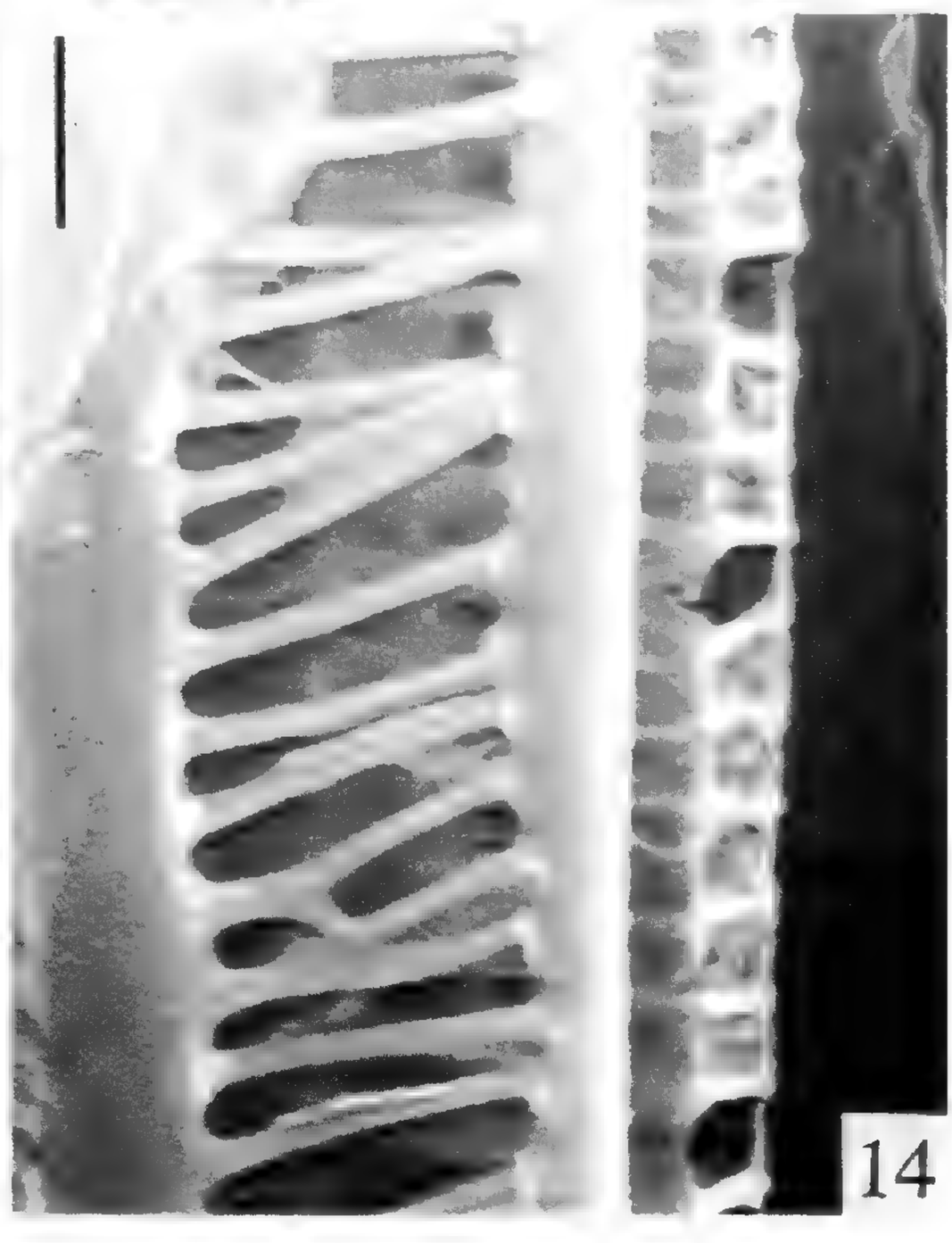
Our investigation revealed features that have not previously been reported in tracheary elements of *Marsilea.* Among the features newly reported are pit dimorphism; transitional pits on lateral walls; and scalariform perforation plates with perforations like lateral wall pits in size and shape.

The pit dimorphism we report consists of wide pits, which lack pit membranes and are therefore perforations, that alternate with one or more extremely narrow pits that have pit membranes. This feature has not been reported to any appreciable extent before SEM studies, although Bierhorst (1960) figures pit dimorphism in *Asplenium.* We have found pit dimorphism (resulting in

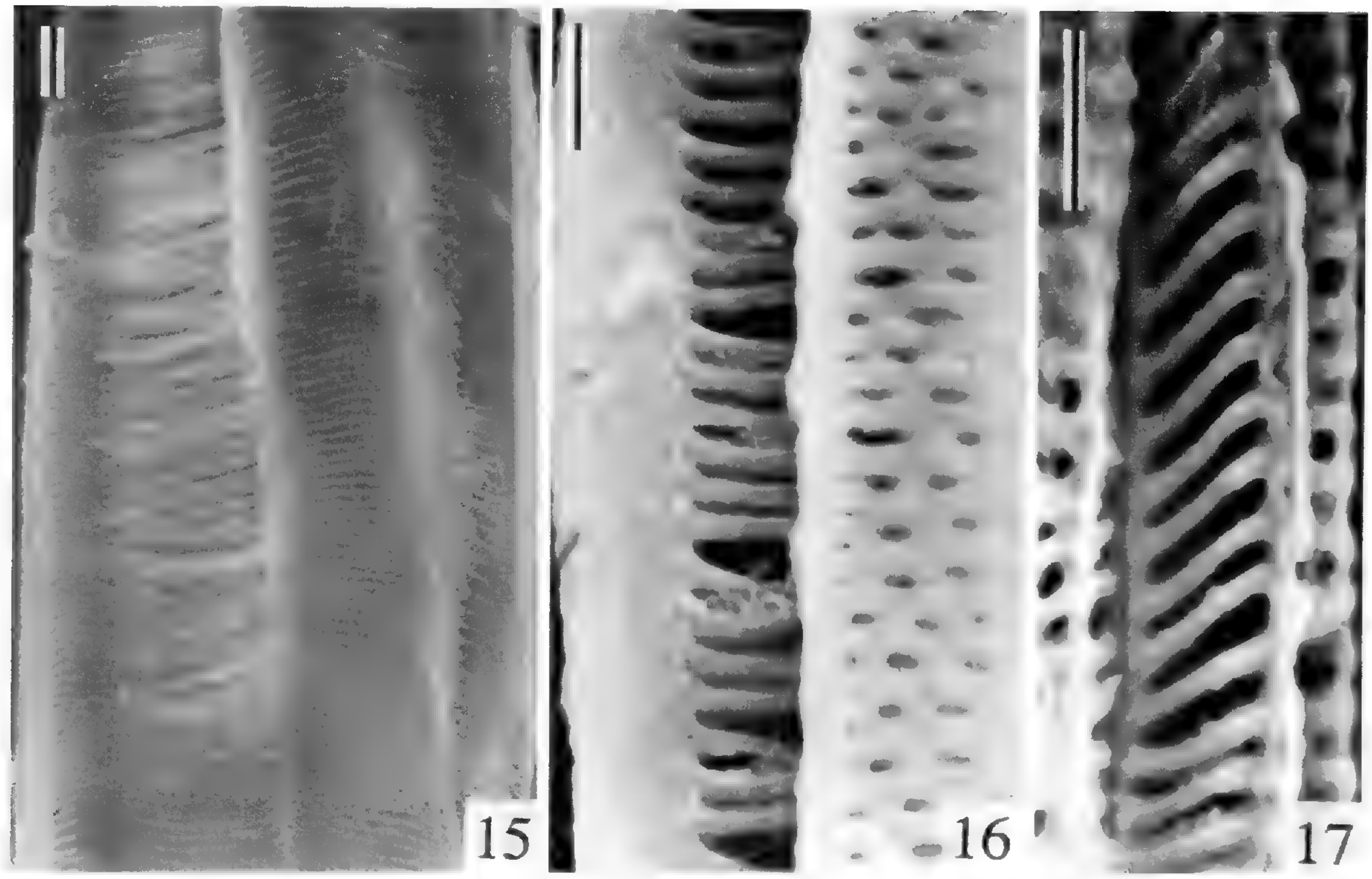








FIGS. 11–14. SEM photographs of tracheary elements from rhizomes of *Marsilea quadrifolia*. 11) Portions of two tracheary elements; element at left bears scalariform perforation plates with minimal pit membrane remnants; element at right bears alternate pitting (some larger perforations appear to be perforations). 12) Portions of two tracheary elements; element at left has pit dimorphism, the larger openings are perforations; element at right has a scalariform perforation plate with pit membrane remnants in some perforations. 13) A well demarcated perforation plate (center) with slender bars, some of which anastomose. 14) Portions of two tracheary elements; perforation plates with wide perforations, left; slender element at right has porosities in pit membranes of right facet, but left facet has intact pit membranes. Scales in all figures = 5 μm.



FIGS. 15–17. SEM photographs of tracheary elements from rhizome (Figs. 15, 16) and root (Fig. 17) of *Marsilea vestita*. 15) Wide tracheary elements with linear scalariform pitting on each of three facets. 16) Scalariform pitting with porose pit membranes in facet at left; facet at right bears transitional pitting. 17) Scalariform perforation plate; several pits with intact membranes at top, several porose pit membranes at bottom of plate. Scales in all figures = 5 μm.

perforation plates) in a number of fern families and genera, such as *Phlebodium* (Schneider and Carlquist, 1997), *Anemia* (Carlquist and Schneider, 1998), *Angiopteris* and *Danaea* (Carlquist and Schneider, 1999).

Loyal and Singh (1978) have figured alternately arranged circular to oval lateral wall pits on *Marsilea* vessels, such as we have illustrated in *M. quadrifolia* rhizomes, but our report of transitional pitting (similar to opposite pitting) in *M. vestita* rhizomes is apparently a new report in ferns. Likewise, we have observed unpitted facets on *Marsilea* vessels, as have Loyal and Singh (1978). Unpitted facets in *Marsilea* vessels may face fibers, which sheathe vascular strands in the genus, but macerations cannot offer a clear interpretation. This matter should be studied, particularly because unpitted facets on tracheary elements have not been reported in ferns other than *Marsilea*.

Simple perforation plates and perforation plates with few bars have been reported for *Marsilea* by several authors (White 1961, 1962; Mehra and Soni, 1971; Bhardwaja and Baijal, 1977; Loyal and Singh, 1978). Our material did not yield vessel elements with simple perforation plates, but we did observe a perforation plate traversed by a single bar. The maceration techniques used for our SEM studies did, however, reveal scalariform perforation plates similar to pitting on the lateral walls of tracheary elements. Loyal and Singh (1978) have figured a few scalariform perforation plates with numerous bars in *Mar-*

silea, and those perforation plates are rather unlike lateral wall pitting in morphology, and are therefore likely to be seen with light microscopy. Our finding of perforation plates similar to scalariform lateral wall pitting is not unexpected in view of the Loyal and Singh (1978) findings, but in addition, perforation plates that are similar in secondary wall framework to lateral wall pits, may have escaped notice by previous workers who worked with light microscopy. Perforation plates that resemble lateral wall pitting in size and shape of the perforations are common in many ferns, according to our studies, so the occurrence of such perforation plates in *Marsilea* is not surprising.

Our material did not yield vessel elements with simple perforation plates. When one views intact stele portions with light microscopy, tracheary elements can be seen even when they are sheathed by fibers. In such preparations, simple perforation plates can be located more readily (e.g., White, 1962). *Marsilea* is unique among ferns in having simple perforation plates. Perforation plates with a few bars do occur in *Marsilea* but also in some other ferns, such as *Pteridium* (Carlquist and Schneider, 1997a) and *Astrolepis* (Carlquist and Schneider, 1997b). The ecological nature of ferns with such vessel elements is illuminating: all grow in areas with marked extremes of moisture availability and/or temperature. Both of these factors make water available for only a short season. Although *Marsilea* is commonly thought to be an aquatic fern, it can grow in temporary ponds in which water is available for only a relatively brief portion of the year. Simple perforation plates and perforation plates with few bars potentially offer the least resistance to rapid flow of a given volume of water per unit time, and the occurrence of such perforation plates in vessels (especially root vessels) of plants (especially monocotyledons) that live in habitats with brief wet seasons (Carlquist, 1975) is understandable.

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Shorter Notes

6-*C*-β-Cellobiosylisoscutellarein-8-methyl ether, a new flavonoid from *Pteris vittata*.—In spite of the fact that analyses of fern flavonoids are of chemotaxonomic interest, data relating to flavonoids of some fern families (e.g. Pteridaceae) are limited. Previous work on the flavonoids of *Pteris vittata* L. has led to the identification of luteolinidin 5-*O*-glucoside by Harborne (Phytochemistry 5: 589–600, 1966) and acid hydrolysis of extracts of this fern led to the identification of kaempferol, quercetin, leucocyanidin and leucodelphinidin by Voirin (Ph. D. thesis, University of Lyon, p. 151, 1970); very recently 3-*C*-(6‴-*O*-acetyl-β-cellobiosyl) apigenin has been isolated from *Pteris vittata* L. by Imperato and Telesca (Amer. Fern J., 89:217–220,1999).

The present paper deals with the isolation of three flavonoid glycosides (I–III) from *Pteris vittata* growing in the Botanic Garden of the University of Naples (Italy). This fern was collected and identified by Dr. R. Nazzaro (University of Naples); a voucher specimen (149.001.001.01) has been deposited in the Herbariun Neapolitanum (NAP) of the University of Naples.

Flavonoids I-III were isolated by preparative paper chromatography in BAW (*n*-butanol-acetic acid-water, 4:1:5, upper phase), 15% HOAc (acetic acid) and BEW (*n*-butanol-ethanol-water, 4:1:2.2) from an ethanolic extract of aerial parts of *Pteris vittata* L.. Further purification was carried out by Sephadex LH-20 column chromatography, eluting with methanol.

R_f values on Whatman No 1 paper (0.33 in BAW; 0.56 in 15% HOAc), color reactions (brown to yellow in UV + NH_3) and ultraviolet spectral analysis in the presence of the customary shift reagents (λ_{max} (nm) (MeOH) 273, 301 (sh), 332; +NaOMe 283, 334, 400 (increase in intensity); +NaOAc 282, 306 (sh), 383; +$AlCl_3$ 279, 304, 348, 385, +$AlCl_3$/HCl 280, 304, 344, 383 were consistent with flavonoid (I) being a flavone glycoside with free hydroxyl groups at position 5 (shifts with $AlCl_3$ and $AlCl_3$/HCl), 7 (shift with NaOAc) and 4′ (shift with NaOMe). Acid hydrolysis (2N HCl/MeOH (1:1); 1 hr at 100°C) gave D-glucose and two flavonoids (IV and V) which showed free hydroxyl groups (detected by UV spectral analysis) at position 5, 7 and 4′ and behaved as flavonoid glycosides in paper chromatography; $FeCl_3$ oxidation of both flavonoids IV and V gave D-glucose. These results suggest that the isolated flavonoid (I) is a *C*-glycosylflavone in which the hydrolyzable D-glucose is not linked to phenolic hydroxyl groups whereas flavonoids IV and V may be a pair of Wessely-Moser isomers. Kuhn methylation (methyl iodide in dimethylformamide in the presence of silver oxide; 18 hr in the dark with stirring) of flavonoid (I) gave a permethyl (PM) ether wihich showed an EI-mass spectrum (MS) with $[M]^{\cdot}$ at m/z (% base peak) 764 (4) and fragment ions at 749 (8), 733 (10), 718 (12), 705 (6), 691 (7), 675 (8), 663 (12), 648 (15), 633 (8), 623 (14), 603 (21), 529 (30), 515 (21), 410 (18), 399 (37), 397 (51), 383 (100), 368 (33), 341 (36), 314 (33), 287 (63). The molecular ion of PM flavonoid (I) (m/z

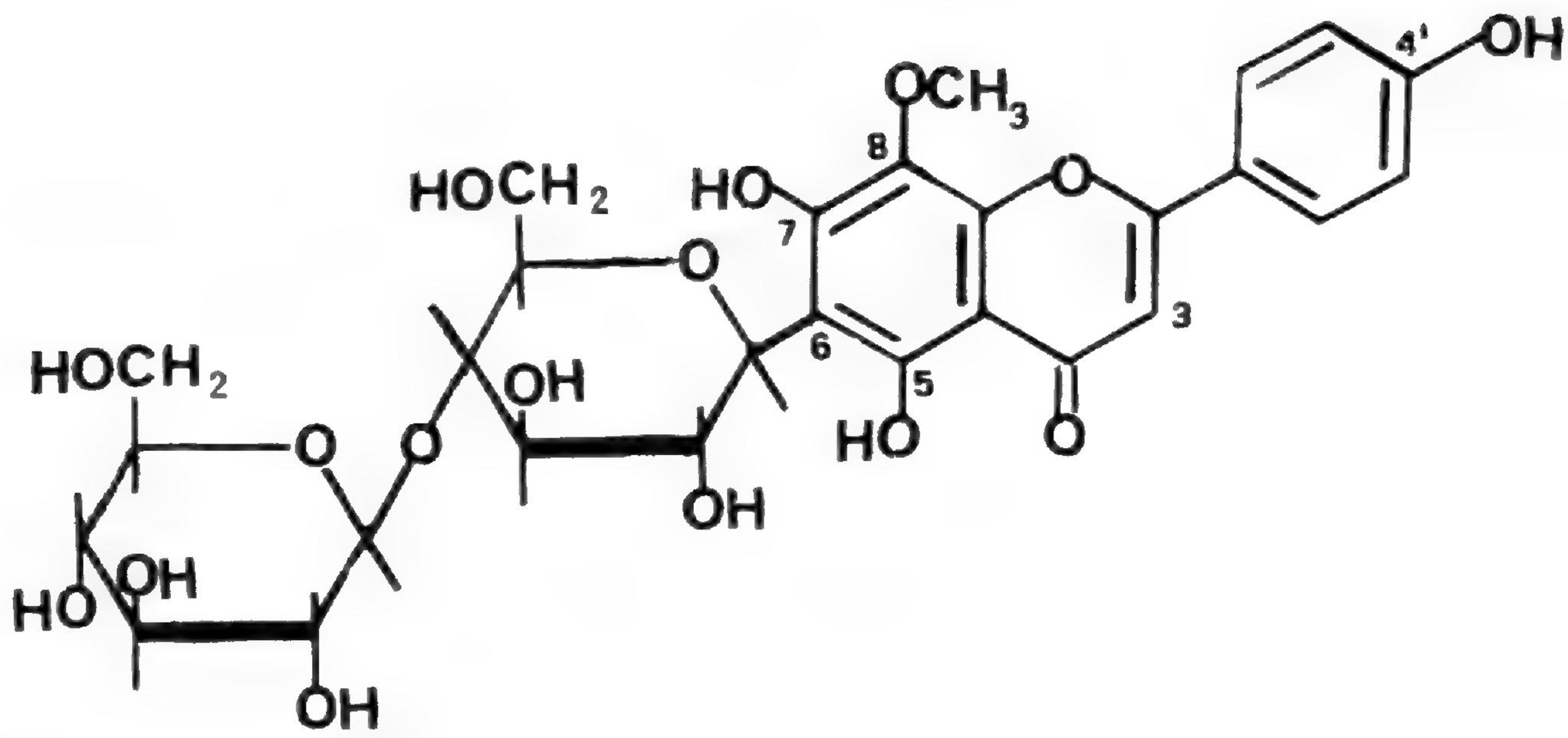

FIG. 1. Structural formula of flavonoid (I). 6-*C*-β-Cellobiosylisoscutellarein-8-methyl ether.

764) and the above UV spectral data suggested that flavonoid (I) may be a flavone *C*-diglucoside based on a flavone moiety with three hydroxyl groups and one methoxyl group. Acid hydrolysis (2N HCl/MeOH (1:1); 1 hr at 100°C) gave 2, 3, 4, 6-tetra-*O*-methyl-D-glucose and a partially methylated *C*-glycosylflavone which gave 2, 3, 6-tri-*O*-methyl-D-glucose by $FeCl_3$ oxidation. Hence a disaccharide (*O*-D-glucosyl- (1 → 4)-D-glucose) is attached to flavone moiety of I. ^{1}H NMR spectrum (DMSO-d_6) of flavonoid (I) showed signals at δ 3.12–3.91 (diglucosyl 12 protons, m), δ 3.84 (3H, s, methoxyl group), δ 4.13 (1H, d, J = 9 Hz, glucosyl anomer), δ 4.79 (1H, d, J = 9 Hz, glucosyl anomer), δ 6.81 (1H, s, H-3), δ 6.96 (2H, d, J = 8.8 Hz, H-3′ and H-5′) and δ 7.91 (2H, d, J = 8.8 Hz, H-2′ and H-6′). Since anomeric protons appeared as doublets with coupling constants J = 9 Hz, the disaccharide of flavonoid (I) is cellobiose (*O*-β-D-glucosyl-(1 → 4)-D-glucose) and this sugar is attached to the flavone moiety by a β-linkage. In addition cellobiose and methoxyl group must be on A-ring of flavone moiety since signals of H-6 and H-8 were absent in ^{1}H NMR spectrum of flavonoid (I) and B-ring protons appeared as an ortho coupled system (A_2B_2). The bathochromic shift with $AlCl_3$/HCl (51nm) of band I in UV spectrum of flavonoid (I) showed the absence of 6-oxygenation according to Markham (pp. 197–235 *in* J.B. Harborne ed., Methods in Plant Biochemistry, Academic Press, London, 1989); hence methoxyl group is at C-8 and cellobiose is attached to C-6 of flavonoid (I) which must be 6-*C*-β-cellobiosylisoscutellarein-8-methyl ether (Fig. 1), a new natural product. The following characteristics of ^{13}C NMR spectrum of flavonoid (I) supported (Table 1) this structure according to a review of Markham and Chari (pp. 19–134 *in* J.B. Harborne and T.J. Mabry eds., The Flavonoids, Advances in Research, Chapman and Hall, London, 1982). A shift (due to *C*-glucosylation) of C-6 to lower field (+9.3 ppm) in comparison with the corresponding carbon of apigenin and a shift of C-8 (due to methoxyl group) to lower field (+30.8 ppm) in comparison with the corresponding carbon of isovitexin were observed. In addition C-4″ showed

TABLE 1. ^{13}C NMR spectral data (DMSO-d_6) of flavonoid I. [a,b]Assignments with the same superscripts may be interchanged.

	Isoscutellarein-8-methyl ether		*C*-Glucosyl
C-2	163.9	C-1″	74.2[a]
C-3	101.9	C-2″	70.8
C-4	182.2	C-3″	77.1
C-5	155.6	C-4″	80.8
C-6	108.1	C-5″	79.3
C-7	153.9	C-6″	61.5
C-8	124.8		
C-9	144.1	*O*-Glucosyl	
C-10	103.1		
C-1′	121.1	C-1‴	104.4
C-2′	128.8	C-2‴	74.1[a]
C-3′	115.8	C-3‴	75.9[b]
C-4′	161.4	C-4‴	69.6
C-5′	115.8	C-5‴	76.1[b]
C-6′	128.8	C-6‴	60.8
OCH_3	59.9		

a shift to lower field (+10.1 ppm) whereas C-3″ and C-5″ showed small upfield shifts (−1.9 ppm and −2.0 pppm respectively) in comparison with the corresponding carbons of isovitexin; these shifts are due to *O*-glucosylation at C-4″. The signals of *O*-glucosyl moiety of flavonoid (I) were similar to those of the hydrolyzable D-glucose of 6-*C*-sophorosylapigenin-7-methyl ether. The following characteristic features of EI-MS of PM flavonoid (I) corroborated this structure as shown by Chopin et al. (pp. 487–490 *in* J.B. Harborne and T.J. Mabry eds., The Flavonoids, Advances in Research, Chapman an Hall, London, 1982) as well as by Bouillant et al. (Phytochemistry 17: 527–533, 1978). The presence of fragment ions at m/z 749 (M-CH_3) and m/z 733 (M-$^{+}OCH_3$) showed the absence of 1 → 2 interglucosidic linkage because these fragment ions are generally absent in EI-MS of 2″-*O*-glycosyl-6-*C*-glycosylflavones. The presence of a fragment ion at m/z 529 ($[S]^{+}$, derived from the loss of PM *O*-glucosyl moiety whitout oxygen of glucosidic bond) higher than the fragment ion at m/z 515 ($[S-14]^{+}$, due to elimination of C-5″ PM glucosylloxymethyl side chain) showed the absence of 1 → 6 interglucosidic link since EI-MS of PM 6″-*O*-glycosyl-6-*C*-glycosylflavone show $[S-14]^{+} > [S]^{+}$. The base peak at m/z 383 (PM aglycone-CH = $^{+}OCH_3$) showed the absence of 1 → 3 integlucosidic link since EI-MS of 3″-*O*-glycosyl-6-*C*-glycosylflavones show PM aglycone-CH = ^{+}OH as base peak. The absence of fragment ion at m/z 545 ($[SO]^{+}$, derived from the loss of PM *O*-glucosyl moiety with the oxygen of glucosidic bond) as well as the absence of fragment ions at m/z 589, 575 and 559 (in which fragments of PM *O*-glucosyl moiety (-CH = $^{+}$OMe, -CH = ^{+}OH, -$^{+}CH_2$) are bound to $[SO]^{+}$) confirmed the proposed structure since these fragment ions are very weak or absent in EI-MS of PM 4″-*O*-glycosyl-6-*C*-glycosylflavones.

Flavonoid cellobiosides are rare plant constituents; in addition cellobiose

has been reported for the first time in association with fern flavonoids only recently as shown by Imperato and Telesca (above reference). According to Swain (pp. 1097–1129 *in* J.B. Harborne, T.J. Mabry and H. Mabry eds., The Flavonoids, Chapman and Hall, London, 1975) flavonoid (I) may be considered an "advanced" biochemical character from the phylogenetic point of view since a methoxyl group is present at C-8. A large number of flavonoid aglycones has been found on the outside of fronds of gymnogrammoid ferns as shown in a review of Markham (pp. 427–468 *in* J.B. Harborne ed., The Flavonoids, Advances in Research since 1980, Chapmann and Hall, 1988); some of these "external" flavonoids have an hydroxyl group (often acylated) or a methoxyl group at C-8. However hydroxyl and methoxyl groups at C-8 are near absent from "internal" vacuolar flavonoids of ferns since there is only the report of 3-*O*-glucosides of herbacetin 8-*O*-methyl ether and gossypetin 8-*O*-methyl ether from one fern species, *Humata pectinata* (Sm) Desv. (Davalliaceae) by Wu and Furukawa (Phytochemistry 22: 1061–1065, 1983). The isolation of flavonoid (I) from *Pteris vittata* represents the first occurrence in ferns of a *C*-glycosylflavone with hydroxyl or methoxyl group at C-8.

Flavonoid (II) has been identified as quercetin 3-*O*-β-glucuronide by UV spectral analysis with the customary shift reagents, total acid hydrolysis (which gave quercetin, glucuronic acid and glucuronolactone), ^{1}H NMR spectrum, ^{13}C NMR spectrum and DEPT experiments. Quercetin 3-O-glucuronide has been found previously in *Adiantum capillus-veneris* L. (Pteridaceae) by Akabori and Hasegawa (Bot. Mag., Tokyo 82: 294–299. 1969); glucuronic acid has previously been found in association with fern flavonoids only in the genus *Adiantum* as shown in the above review of Markham (1988).

Flavonoid (III) has been identified as rutin by UV spectral analysis in the presence of usual shift reagents, total acid hydrolysis (which gave quercetin, D-glucose and L-rhamnose), controlled acid hydrolysis (which gave rutinose in addition to the products of total acid hydrolysis) and co-chromatography with an authentic sample (four solvent systems); this identification was confirmed by Kuhn methylation followed by acid hydrolysis which gave 2, 3, 4-tri-*O*-methyl-L-rhamnose, 2, 3, 4, 6 tetra-*O*-methyl-D-glucose and quercetin 5, 7, 3′, 4′-tetra-*O*-methyl ether. Rutin is here reported for the first time in the genus *Pteris*; as shown in the above review of Markham (1988), rutin has previously been identified in ten species of *Adiantum* (Adiantaceae), five species of *Gymnopteris* (Sinopteridaceae), all four species of *Bommeria* (Sinopteridaceae), four species of *Hemionitis* (Sinopteridaceae), the genus *Trachypteris* (Sinopteridaceae), *Paesia anfractuosa* (Dennstaedtiaceae), *Pteridium aquilinum* (Dennstaedtiaceae) and *Loxsoma cunninghamii* (Loxsomaceae); recently rutin has been identified in *Polypodium decumanum* Wild (Polypodiaceae) by *Vasänge* et al. (Planta Medica 63: 511–517, 1997).

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***Ophioglossum pendulum* L. Naturalized in Miami, Dade County, Florida.**—*Ophioglossum pendulum* L. has been discovered separately by Adrian Tejedor and Craig Allen, growing on cultivated palm trees in Miami, Florida. *Ophioglossum pendulum* is an Old World epiphyte, which grows from Madagascar through tropical Asia and into Polynesia. It has been infrequently cultivated in tropical fern collections in Miami since the mid-1970s. This is the first report of established plants growing outside of strictly man-made, horticultural conditions in the New World. This is quite a surprising discovery due to the relative difficulty of maintaining this exotic species in cultivation.

Two populations are known in two separate sites in Coral Gables, a community located in southeast Miami. One population (discovered by Adrian Tejedor, in April, l998) is growing in the persistent leaf bases of Canary Island date palms (*Phoenix canariensis*) along a public street. In this location, three colonies are on adjacent palms and a fourth is some distance away, in the same row of planted palms. A second, small population, is growing on a sugar palm (*Arenga pinnata*), inside of Fairchild Tropical Gardens. It was discovered during the summer of 1995, by Craig Allen. In both locations, mature, sporulating plants are growing among the old persistent leaf bases on the palm trunks. Two of the date palm colonies were relatively large and vigorous in 1998, the other two were smaller. The largest colony covered 1.5 square meters of the palm trunk, about ten feet from the ground, with an estimated number of 60 fronds. Considering the slow growth typical of *O. pendulum,* this largest colony is estimated to be in excess of 15 years old, and may be much older. In March 1999, only 12 fronds were observed. The majority of the fronds observed the previous year had died and remained in place, completely dried and shriveled. Only the largest fronds bore sporangia. One fertile appendage is borne on the undersurface of the large fronds, which are from 45 to 90 cm. long. Most of the fronds in all the colonies are small, infertile and average 45 cm. in length. Specimens have been taken from this population to document its occurrence and are on deposit at the Fairchild Tropical Garden Herbarium (A. Tejedor, Fairchild Herbarium #81775).

In *Phoenix* and other palm genera, a compact and spongy mass of old leaf bases remains attached to the palm's upper trunk for many years after the leaves are shed. This is where *O. pendulum* and other epiphytes become established. In the case of *Ophioglossum,* the dangling fronds are the only visible part of the plant. The rhizome and root system are hidden under the substrate of old leaf bases of the palm. Adventitious shoot buds that develop on the root system eventually produce a colony of plants on the south-east side of the trunk, sheltered by the leaf crown. The dead leaf bases have an ability to remain remarkably wet for days after a rain. The palm leaf-base habitat seems favorable for these ferns, which otherwise may not survive to South Florida's long, late winter and spring, dry season. During the dry winter of 1999 the colonies seemed to have suffered and appeared decidedly smaller. Other epiphytes that coexist with *O. pendulum* in this habitat are the Boston fern (*Nephrolepis cordifolia*) and young individuals of *Ficus aurea* (strangler fig) and

Brassaia actinophylla (schefflera). A young staghorn fern (*Platycerium* sp.) was observed among the other epiphytes.

The *Ophioglossum* population growing on a sugar palm inside Fairchild Tropical Garden was known to Craig Allen, the gardener in the Rare Plant House, since 1995. He told B. McAlpin, in June, 1998, about the location of this plant, and said that it has grown approximately five times larger, since he first discovered it. However, Fairchild Tropical Garden has never accessioned this plant into its collection. Nonetheless, plants of *O. pendulum* from private collections have been exhibited many times over the last fifteen years at the annual Fern Show sponsored by the South Florida Fern Society, on the premises of the Fairchild gardens. Innoculation by wind blown spores from mature, sporulating plants could have occurred during movement of plants into the fern shows, or during the fern show itself, which occurs for a full weekend. Established horticultural plants, growing in open-air, screened shade houses, could also release spores into the general environment of South Florida. It is still a mystery when these exotic colonies first became established, and, if in fact, the spores, and hence the adventative plants are from cultivated sources.

O. pendulum is grown in very few South Florida fern collections. Snails and poor watering practices usually are responsible for the demise of cultivated plants of this taxon. Successful growers use long-fiber *Sphagnum* moss, mounted on plaques, tied with wire or mono-filament fishing line, in which to grow this fern. Most successful growers also employ automated irrigation systems in shade houses to provide protection from drying winds and to maintain high humidity. In cultivation plants may achieve impressive size, having up to 100 fronds that may reach two meters in length. Plants in cultivation are relatively slow growing. They are seldom divided because sections could easily decay, leading to the death of the division and/or the parent plant. Less than six growers in the Miami area are presently known to have cultivated plants in their possession.—ADRIAN TEJEDOR, Biology Department, University of Miami, Miami, Florida, 33124 and BRUCE W. MCALPIN, Biology Department, Miami-Dade Community College, 11011 SW 104 Street, Miami, Fl. 33176.

American Fern Journal 90(1):48–50 (2000)

REVIEWS

Flora Malesiana, Series II–Ferns and Fern Allies, Volume 3, edited by the Flora Malesiana Editorial Committee. 1998. Rijksherbarium/Hortus Botanicus. Publications Department, P.O. Box 9514, 2300 RA Leiden, The Netherlands. iv, 334 pp. Softcover (ISBN 90-71236-39-0). 100 Dfl. [available in the U.S. from Balogh Scientific Books, www.balogh.com, for $60 + shipping].

The present volume is the seventh installment of the Malesian pteridoflora and includes treatments of the Polypodiaceae (by P. H. Hovencamp and five collaborators), Davalliaceae (by H. P. Nooteboom), Azollaceae (by R. M. K. Saunders), Cheiropleuriaceae (by J. E. Laferrière), Equisetaceae (by J. E. Laferrière), Matoniaceae (by M. Kato), and Plagiogyriaceae (by X. C. Zhang and H. P. Nooteboom). Those familiar with this long-running series (the first pteridophyte fascicle was published in 1959) will find the format quite similar to that of previous parts, with the exception that the species entries are now in a single rather than double column and have been set in a slightly larger typeface, making the work easier to read. As with previous installments, the text is "dense" with discussions and listing of synonyms, typification, and taxonomic interpretations, as well as literature citations, all quite valuable as few other detailed sources of information such as this presently exist for paleotropical regions. There are also lengthy discussions of economic uses, phytochemistry, cytology, spore morphology, and other topics as pertinent. Genera and species are treated alphabetically within families. The descriptions are relatively complete, although (as in previous parts), the distributional and ecological data are relatively brief. There are a number of excellent drawings and photographs, which are numbered as figures independently within each family treatment.

Most of the volume is devoted to the Polypodiaceae, with 18 genera and 183 species in the region. The remaining 6 families account for only 9 total genera and 45 species. Interestingly, the treatments of the two small paleotropical relict families Cheiropleuriaceae and Matoniaceae cover all of the known species and amount to small monographs of these groups. The treatment of Azollaceae (including only a single Malesian species, *Azolla pinnata*) also has five pages of thorough and interesting summary of the symbiotic relationship with cyanobacteria and the concommitant economic importance of the plant in the region.

The family Davalliaceae is of particular horticultural interest. Fern growers interested in reading about *Humata* species will find these submerged in a broadly circumscribed *Davallia,* in keeping with recent systematic studies. The SEM photos of enlarged segments with sori are of particular help in determining the 23 species treated in this genus, and there are two keys to species, so if a given specimen doesn't seem to key out well the first time an alternative set of characters is available.

In the Polypodiaceae, another family of considerable horticultural interest to North American growers, the generic classification generally follows that of Hennipman et al. in the "Families and Genera of Vascular Plants" volume. An exception is the inclusion of *Phymatosorus* in *Microsorum.* For this genus and *Selliguea,* there are secondary keys to species in different geographic subsets of the Malesian region.

In total, this is another outstanding contribution to the Flora Malesiana. In addition to the inclusion of some groups of relatively great horticultural and economic importance, this particular installment will be of value to anyone seeking to understand the modern generic classification of the taxonomically complex Polypodiaceae, which, except for the aforementioned very expensive "Families and Genera" volume, previously has not been summarized in detail in an accessible form for the Old World species.—GEORGE YATSKIEVYCH, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.

Bibliografía sobre Gametofitos de Helechos y Plantas Afines, 1699–1996, by Blanca Pérez-García and Ramón Riba. Monographs in Systematic Botany from the Missouri Botanical Garden, volume 70. 1998. Missouri Botanical Garden Press, 4344 Shaw Blvd., St. Louis, MO 63110. 98 pp. Softcover (ISBN 0-915279-61-4, ISSN 0161-1542). $20.00 plus $4.00 shipping/handling.

This useful bibliography covers nearly 300 years of publications on various aspects of pteridophyte gametophytes. The brief introduction is in Spanish and might have been printed in English as well. However, most non-Spanish speaking pteridologists will be able to understand the gist, if not the details, of the half page that this covers, and the introduction is not necessary to the use of the remaining matter. The main 75 pages of the volume contain the lengthy bibliography itself, arranged in a single alphabetical sequence of 2195 entries. Each entry is followed by one or more numerical codes in parentheses, referring to numbered headings in a subject index that follows. Similarly, a taxonomic index containing a single alphabetical sequence of genera and families has these numerical codes following each author/date citation.

A key to the contents of the subject index appears on p. 2, between the introduction and the main text, again in Spanish. The technical terms are sufficiently similar to their English equivalents as to be usable without translation. The subject index has two main subject headings, spores and gametophytes. The Spores heading is further broken into five subheadings ranging from factors affecting germination to ultrastructure. The factors affecting germination are further subdivided into eight subject areas, ranging from methodological concerns to environmental stimuli like temperature, light, and chemicals. The Gametophytes heading is similarly broken into a number of subject headings. As with any attempt to organize a large body of diverse literature into discrete subject headings, there are inevitable problems of selection of headings and overlapping subject areas in a given paper. The authors have done a creditable job of balancing the tendency to divide the subject

headings ever more finely at one extreme with the loss of utility in headings that are too broad at the other. Nevertheless, individual readers probably will have minor quibbles here. For example, I would have preferred a discrete section on studies dealing with antheridiogens, but these are immersed in a more general subject heading entitled Metabolism, Biochemistry, Molecular Biology, and Chemical Components.

Anyone who develops an interest in fern reproduction will find this bibliography a convenient starting point for delving into the surprisingly large body of literature on various aspects of spore and gametophyte structure, physiology, ecology, biochemistry, and genetics. Even more seasoned prothalliists will gain a more complete historical perspective on their field. It is hoped that the authors will periodically issue updates to this work.—GEORGE YATSKIEVYCH, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166.

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American Fern Journal 90(2):51–59 (2000)

The Chloroplast Genome Structure of the Vascular Plant *Isoëtes* is Similar to That of the Liverwort *Marchantia*

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ABSTRACT.—Restriction site mapping was used to characterize the chloroplast genome of the lycophyte, *Isoëtes melanopoda*. The *Isoëtes* chloroplast genome is approximately 139–145 kb in size with an inverted repeat of 12–13 kb. The gene content and consensus gene order are similar to that of *Marchantia*. A distinctive feature of the *Isoëtes* genome is an increased size of the small single copy (SSC) region possibly due to the insertion of a piece of DNA (3–8 kb) of unknown composition. The inferred insertion, along with a slightly larger inverted repeat, are responsible for the apparent size difference in the total genome relative to *Marchantia*. Patterns of restriction fragments were also consistent with the presence of a small inversion (2–3 kb) in the large single copy (LSC) region.

Structural differences in chloroplast genomes have proven to be powerful characters in understanding relationships among land plants because of their rarity and corresponding apparent low levels of homoplasy (Palmer, 1985a, b, 1987, 1991; Palmer and Stein, 1986; Palmer et al., 1988; Manhart and Palmer, 1990; Raubeson and Jansen, 1992; Downie and Palmer, 1992; Doyle, 1992; Lew and Manhart, 1993; Raubeson and Stein, 1995). The chloroplast genome exhibits a remarkably consistent structure and gene order and content among a wide range of members of the plant kingdom (reviewed in: Stein et al., 1986; Palmer et al., 1988; Olmstead and Palmer, 1994). Among photosynthetic plants this circular molecule is usually 120–160 kilobases (kb) in length and has a capacity to code for approximately 120 genes.

Our understanding of the chloroplast genome comes primarily from angiosperms, but little has been reported about the size, overall structure, or variation of the chloroplast genomes of lower vascular plants (Lycophyta, Psilotophyta, and Sphenopsida) or most of the bryophytes. Among the lower vascular plants only the presence or absence of a 30 kb inversion (Raubeson and Jansen, 1992) has been demonstrated. Among the pteridophytes Stein et al. (1992) and Conant, et al. (1994) have shown that multiple structural rearrangements exist among the diverse groups of ferns and that these structural features may be useful phylogenetic markers at the familial and higher levels. More information about the genomes of the primitive land plants will be needed to attain a comprehensive understanding of the chloroplast genome.

In this study we provide the first complete restriction site maps of the chloroplast genome of a lycophyte, the quillwort *Isoëtes melanopoda*. We find that the *Isoëtes* chloroplast genome shares significant features with those of two

non-vascular plants, the bryophytes *Marchantia* (liverwort) and *Physcomitrella* (moss). The *Isoëtes* chloroplast genome has the following features: (1) its inverted repeat (IR) is several kb larger than the IR of either of the bryophytes but is significantly smaller than the IR of most ferns and seed plants, (2) the small single copy region (SSC) has a large (3–8 kb) region of extra genetic material of unknown origin, and (3) the large single copy region (LSC) appears to have several small inversions (2–4 kb).

Materials and Methods

Leaves of *Isoëtes melanopoda* Gay & Durieu (Isoetaceae) were collected from a single population in Alabama and a voucher specimen (*Duff 9201*) was deposited at the University of Tennessee Herbarium. Total DNA was isolated using the procedure of Doyle and Doyle (1987). Single digests of sixteen restriction endonucleases (*Bam*HI, *Ban*I, *Ban*II, *Dra*I, *Eco*RI, *Eco*RV, *Hae*II, *Hind*III, *Nci*I, *Nco*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Stu*I, and *Xho*I) and selected double digests (*Ban*I/*Ban*II, *Eco*RI/*Eco*RV, *Pst*I/*Sal*I, *Sac*I/*Pvu*II, and *Stu*I/*Xho*I) were made and the fragments separated on 0.9% agarose gels run out 15 cms and transferred by dry blotting to Amersham (Hybond N+) nylon membranes. Cloned cpDNA fragments from lettuce (Jansen and Palmer, 1987) and tobacco (Olmstead and Palmer, 1992; Shinozaki et al., 1986) were used as probes for physical mapping and gene localization. Membrane-bound DNAs were hybridized to ^{32}P-dCTP labeled probes using random primer oligolabeling (Feinberg and Vogelstein, 1983, 1984; Gibco BRL labeling kit) for 24 hr at 55° C. Hybridization buffers and conditions were used as described by the manufacturer (Gibco BRL) except that hybridizations were done at 55° C. Tobacco DNA cut with *Bam*HI provided a control lane to verify the identity of probes. Fragment sizes were estimated by comparison with fragments from *Hind*III digestion of Lambda Phage. Mapping followed the general strategies outlined in Palmer (1982, 1986) and Jansen and Palmer (1987).

Results

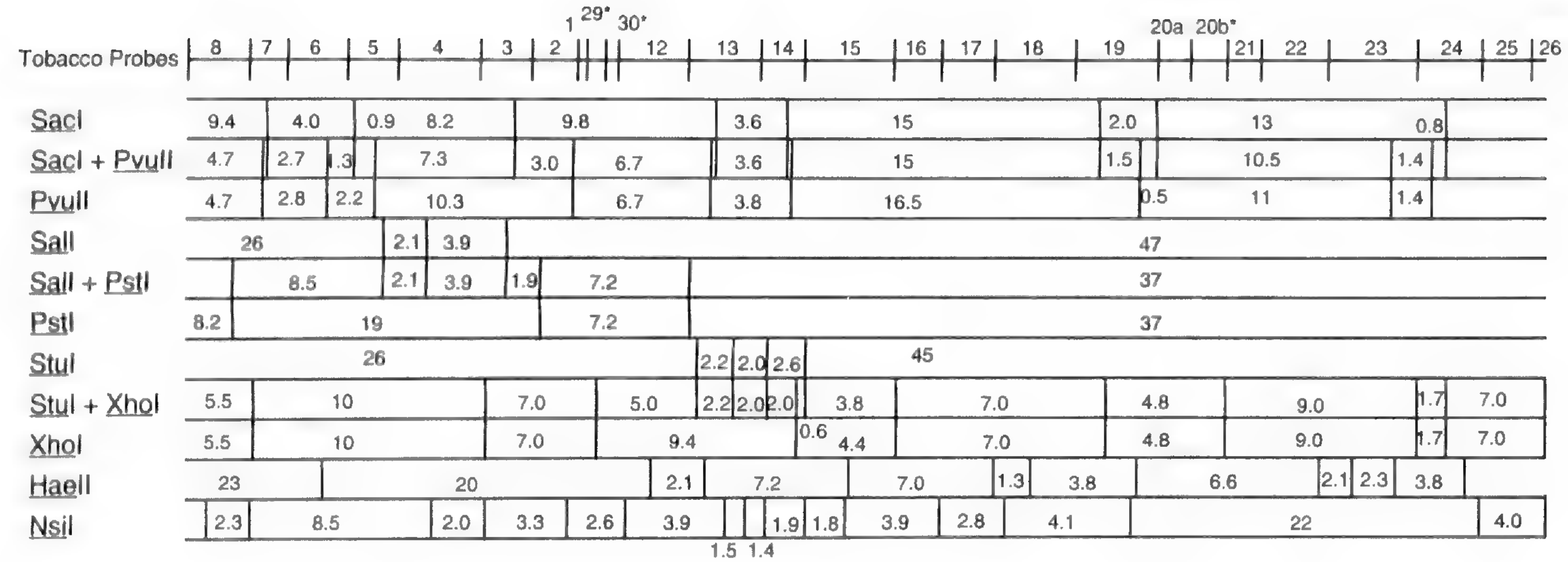
Hybridization to both lettuce and tobacco probes generally gave good results although fine scale mapping of several regions of the *Isoëtes* cpDNA was difficult because there was only very limited hybridization to several tobacco probes. As a result it was possible to generate complete maps only for restriction enzymes that cut the DNA into large fragments spanning these regions. The problematic regions are those covered by tobacco probes 3, 20a, 21, 29, and 30, each of which contain at least some gene sequences not found in the genome of *Marchantia* (Ohyama et al. 1986). Stein et al. (1992) in studies of *Adiantum* also reported a lack of hybridization with tobacco probe 3, which contains the gene *rps*16 that is not found in *Marchantia*. Probes 20b, 21, 29, and 30 account for several open reading frames (ORFs) that are of comparable sizes in *Marchantia*.

Complete restriction site maps for eight single restriction digests and three

double digests of *Isoëtes melanopoda* were successfully generated (Fig. 1). Partial maps for seven additional enzymes were obtained for a great majority of the genome including the inverted repeat and small single copy region, and were utilized in determining portions of the genome structure. Completed restriction site maps gave total chloroplast genome size estimates for *Isoëtes* of 139–145 kb with an average of 141 kb. The estimates for the minimum size of the inverted repeat varied from 11.8 (*Eco*RI + *Eco*RV) to 13.2 kb (*Nsi*I). The size of the large single copy region (LSC) was approximately 85 kb and for the small single copy region (SSC) was 24–29 kb. Consistent with the results of Raubeson and Jansen (1992), the *Isoetes* genome exhibits the presumed ancestral gene order exhibited in bryophytes and lycophtes. Evidence of this genome architecture came from the fact that two non-adjacent pairs of tobacco probes, 31–11 and 2–12, respectively, consistently hybridized to overlapping fragments (Fig. 1). A large insertion in the SSC, relative to both *Marchantia* and tobacco, was inferred from mapping studies and appears to be a feature unique to *Isoëtes*. An additional feature of the *Isoëtes* genome was the apparent presence of a small inversion (1.5–3.0 kb) found in the large single copy region (LSC) as well as several other smaller inversions which may be postulated from individual restriction site maps but cannot be characterized more completely due to the resolution of the current data set.

INVERTED REPEAT.—The size of the inverted repeat in *Isoëtes* (11.8–13.2 kb) is larger than that of *Marchantia* (10 kb) and *Physcomitrella* (9.4 kb). Based on hybridization data, this appears to be due to the inclusion in the IR of *Isoëtes* of regions homologous to those of tobacco fragment 32 (*rps*'12, *rps*7) and a very small portion of probe 31, which are found in the IR of tobacco but are restricted to the large single copy region adjacent to IR_A in *Marchantia* (Ohyama et al., 1986). Just as in *Marchantia,* regions of cpDNA homologous to tobacco probes 28, 29, 30, 36 and 1, all of which are part of the IR of tobacco, were mapped to the large single copy region of the *Isoëtes* chloroplast genome.

SMALL SINGLE COPY REGION.—The single copy region was estimated to be 24–29 kb in size. The variation in this estimate was due to lack of hybridization and presence of genetic material in *Isoëtes* not represented in the tobacco probes resulting in difficulties in resolving the boundaries of the region. The lack of hybridization to several probes combined with the size of spanning fragments made precise estimations of the size of the SSC and the entire genome difficult. Figure 1 shows that the amount of DNA in the area adjacent to the edge of IR_B was more than could be accounted for from the sizes of the tobacco probes used and than its expected content compared with the *Marchantia* genome (Ohyama et al., 1986). For example, a 17 kb chloroplast DNA fragment, the result of digestion by *Pvu*II, only hybridized to probes 36, 37, and very weakly to 35 which account for a maximum of 10 kb of DNA in tobacco. The best estimate of total size of the SSC came from the map of *Sac*I. This enzyme yielded only two fragments that span the SSC; a 23–27 kb fragment and a 1.8 kb fragment each of which hybridized to probe 35. For the same enzyme two 5.4 kb fragments hybridized strongly to probe 35 and very


Tobacco Probes
1 29* 30*
20a 20b*
8 7 6 5 4 3 2 12 13 14 15 16 17 18 19 21 22 23 24 25 26
SacI
SacI + PvuII
PvuII
SalI
SalI + PstI
PstI
StuI
StuI + XhoI
XhoI
HaeII
NsiI
Inverted Repeat B
Inverted Repeat A
31
27 28 1 32 33 34 35 ? 36 37 38 39 40 35 34 33 32 31 11 10 9b 9a
Tobacco Probes
SacI
SacI + PvuII
PvuII
SalI
SalI + PstI
PstI
StuI
StuI + XhoI
XhoI
HaeII
NsiI

weakly to probe 34 indicating that the 5.4 kb fragment accounts for the majority of the 4.7 kb tobacco probe 35. The approximately 27 kb and 1.8 kb fragments that weakly hybridize to probe 35 must lie primarily within the SSC. This gives an upper estimate of the SSC of approximately 29 kb. Considering maps of more than 10 enzymes completed for the SSC, an estimate of 24–29 kb can be made. Compared with *Marchantia* (20 kb SSC), *Physcomitrella* (21 kb SSC, Calie and Hughes, 1986), and *Adiantum* (20–22 kb SSC, Hasebe and Iwatsuki, 1990), this would place the amount of extra DNA in the *Isoëtes* SSC at 3–8 kb.

ABSENCE OF 30 KB INVERSION.—Based on the presence of fragments from *Bam*HI and *Hind*III digests that show overlap to tobacco probes 31 and 10, and 12 and 2, Raubeson and Jansen (1992) report the absence of a 30 kb inversion found in ferns and seed plants. Our data supported the absence of this inversion in these higher plants. Figure 1 shows that genetic material homologous to tobacco probes 29, 30 and a portion of probe 1 were detected between fragments homologous to probes 2 and 12. Tobacco probe 1 hybridized to fragments in two areas: between probes 2 and 29 and between probes 28 and 32 at the edge of IR_B. This is what would be expected were the genome of *Isoëtes* to have the same arrangement as *Marchantia*. In *Marchantia* the transfer RNA, H(GUG), found in probe 1 of tobacco, is present next to probe 2 but the remaining portion of probe 1; *trn*I(CAU), *rpl*23, *rpl*2, can be found in the LSC at the edge of IR_B in *Marchantia*. The presence of very weak hybridization to probe 1 adjacent to probe 2 suggested that the arrangement of genes in *Isoëtes*, in the region of this 30 kb inversion in ferns and seed plants, is identical to that in *Marchantia*.

ADDITIONAL INVERSIONS.—A small (1–3 kb) inversion was detected in the LSC but its precise size was difficult to determine. The restriction enzymes used produced DNA fragments that were too large to allow localization of the endpoints of the inversion. The presence of the inversion was supported by the detection of multiple fragments that hybridized identically to both tobacco fragments 14 and 15. Additional, smaller inversions may be hypothesized from several individual restriction maps. These inversions could not be accurately characterized because they are located in a portion of the genome for which there was poor hybridization signal. This region corresponds to the position of a large open reading frame (ORF2136 = tobacco fragments 29,30). Only

←

FIG. 1. Linearized restriction site maps of the chloroplast genome of *Isoëtes melanopoda*. Enzymes used in single or double digestions to map the genome are indicated at each end of the map. The top line represents the tobacco probe set (Olmstead and Palmer, 1992) in positions relative to their hybridization with *Isoëtes* cpDNA. Large solid bars indicate relative position and boundaries of the inverted repeat in *Isoëtes*. Asterisks denote tobacco probes for which very week hybridization was observed to *Isoëtes* fragments. Fragments less than 0.5 kb were not consistently observed and therefore are postulated based on data from double digests. Fragment sizes larger than 18 kb could not be accurately calculated and so reflect estimations based on additive sizes of fragments detected by double digests.

fragments spanning the entire region were utilized in the mapping data presented here. Raubeson and Jansen (1992) also report the probable occurrence of up to two small inversions in this region as well, but did not characterize them further.

Discussion

Although *Isoëtes* is a highly distinctive genus, whose evolutionary lineage is separated from the majority of vascular plants by up to 300 million years and from the non-vascular plants for as long or longer, it exhibits a chloroplast genome remarkably similar to the liverwort *Marchantia*. Most vascular plants appear to have significantly larger chloroplast genomes than the bryophytes because the inverted repeat is larger. The *Isoëtes* cpDNA genome is also larger than the bryophyte genome in size but the difference is the result of an increased size of the SSC relative to other characterized cpDNAs and to a lesser extent an increase in the size of the IR. The overall size, including the IR, and structure of the chloroplast genome of *Selaginella* (Duff, unpublished data) also appears to be very similar to that of *Marchantia*.

One possible explanation for the remarkable similarity in size and structure of the genomes of *Marchantia, Physcomitrella,* and *Isoëtes* is that the absence of the 30 kb inversion confers some structural integrity to these molecules. The 30 kb inversion that took place in the ancestor of the ferns and seed plants appears to be correlated with a relaxation of physical constraints on the size of the inverted repeat region. Hence the IR subsequently underwent rapidly expand to incorporate genetic material from the LSC in the vast majority of ferns and seed plants (Palmer and Thompson 1982; Jansen and Palmer 1987; Howe et al. 1988). Stein et al. (1992) has proposed that the region around the *psb*A gene is especially prone to recombination. In *Marchantia, Physcomitrella,* and *Isoëtes* this gene is found far from the IR, unlike those plants lacking the inversion, and thus one would expect its potential effects on the structural integrity of the genome not to affect the IR. These taxa are by all accounts highly divergent and yet the sizes of their respective inverted repeats are not significantly different nor have they been demonstrated to have undergone inversion events to the extent observed in plants which contain the 30 kb inversion relative to *Marchantia*. The implication, although a correlation only at this point, is that the similarity in the chloroplast genomes of *Marchantia, Physcomitrella,* and *Isoëtes* is directly related to their gene order.

The variation in the estimated size of SSC (24–29 kb) in *Isoëtes* was unexpected and exemplifies the difficulty in resolving the boundaries of the SSC with the inverted repeats and the lack of probe homologies in this region. For example, it has been shown that the *chl*L gene has been lost from angiosperm cpDNAs although it is present in *Isoëtes* as well as all bryophytes, lycophytes, ferns except *Psilotum,* and gymnosperms except *Welwitschia* (Burke et al., 1993). This gene is typically located in the SSC adjacent to the IR_A though the restriction map generated for *Isoëtes* does not clearly define this region due to the present of several large fragments spanning this region. Furthermore, the

tobacco probes used in constructing the map lack this gene region and thus it is possible that several small fragments generated by *Nsi*I, *Sac*I, and *Pvu*II could have escaped detection in this region between probes 40 and 35. Even accounting for these sources of uncertainty the total size of the SSC appears to be increased over that of all other previously characterized land plant chloroplast genomes. Furthermore this increase in DNA content is most apparent in the fragments that hybridized to tobacco probe 35, found in the IR of both tobacco and *Isoëtes,* and tobacco probe 36. The latter represents genetic material found in the IR of tobacco but whose homologous content can be found in the SSC of *Marchantia* adjacent to IR_B. This extra DNA had insufficient sequence similarity with any portion of the tobacco or lettuce genomes to produce hybridization signals and its presence was only determined by the presence of fragments that spanned the entire region (see *Stu*I, *Sal*I, *Pst*I in Fig. 1). This DNA found in the *Isoëtes* SSC may be the result of either an increased size of spacer regions between the genes or may be due to an insertion of foreign DNA. Very few definitive cases of extra, non-homologous DNA found in the chloroplast genome are known. Conant et al. (1994) report the presence of about 0.9 kb of DNA in the IR of *Cyathea furfuracea* (Cyatheaceae) for which there is no counterpart in the *Adiantum* genome and thus cannot be explained by duplication or inversion events. A more dramatic example can be seen in the unusual choloroplast genome of *Trachelium* (Campanulaceae) in which as many as seven insertions of foreign DNA have been postulated (Cosner et al. 1997). In any case, more detailed analyses will need to be undertaken to confirm the existence and to determine the nature of any additional sequences in the *Isoëtes* chloroplast genome.

In summary, we have established that the genome of *Isoëtes* has the overall structure of the bryophytes, *Marchantia* and *Physcomitrella*. The only significant difference between the bryophyte genome and that of *Isoëtes* is the possible increase in DNA content of the SSC region of the *Isoëtes* genome and increased IR content. Further work to characterize the insertion in *Isoëtes* and to map the genomes of *Lycopodium* sens. lat., *Equisetum,* and *Psilotum* will most likely result in a better understanding of the evolution of the chloroplast genome and possibly provide clues regarding the relationships of the lower vascular plants.

ACKNOWLEDGMENTS

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Patterns of Growth and Reproduction in a Natural Population of the Fern *Polystichum acrostichoides*

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ABSTRACT.—Patterns of growth and reproduction were documented in a natural population of *Polystichum acrostichoides* in southeastern Ohio during the 1994 and 1995 growing seasons. The proportion of biomass allocated to fronds increased with plant biomass, indicating fronds are an increasingly dominant component of the body of *P. acrostichoides*. Regression analysis indicated a minimum size threshold exists at which this species first becomes reproductive. Both reproductive status and frequency of reproduction were positively associated with greater plant biomass and above-ground growth rates. A cost of reproduction to growth was apparent; above-ground growth rates increased during non-reproductive years among individuals that reproduced in only 1994. Minor increases in reproductive effort were associated with increasing plant biomass; ranging from approximately 0.01% to 2.11%. Nevertheless, reproductive effort may be plastic in *P. acrostichoides*; the frequency of reproduction correlated negatively with cation concentrations and positively with phosphorous concentrations, and reproductive effort increased with decreasing canopy cover. Together, these observations suggest reproduction in *P. acrostichoides* only occurs when resources are sufficient to offset it's cost to future growth; a life history that may optimize the advantages of early reproduction and life-time fecundity in a species whose colonizing phases (i.e., gametophyte and juvenile sporophyte) have high risks of mortality.

Patterns of vegetative growth and reproduction are major components of plant life history evolution. They are the basis for investigating the relationships between age, size, growth, and reproduction, as well as the roles of genotypic and allometric constraints versus plasticity in allocation patterns (Harper, 1977; Stearns, 1992). According to resource allocation theory, reproduction and growth compete for the same pool of resources and, therefore, occur at the expense of the other (Schaffer and Rosenzweig, 1977; Harper, 1977; Watson, 1984). The size of an individual's resource pool is a function of its capacities for storage and acquisition balanced against its current and past expenditures. Life history theory predicts that, within the boundaries set by genetic and allometric constraints, a species allocation pattern will evolve to maximize it's contribution to the gene pool (Harper, 1977; Stearns, 1992). In populations where juvenile mortality rates are lower than adult mortality rates, selection will favor the demographic advantage conferred by early reproduction. Conversely, in populations where juvenile mortality rates are higher than adult mortality rates, life time fecundity will be favored.

Among angiosperms and gymnosperms, the challenge of studying patterns of resource allocation is compounded by a number of factors, including the existence of structures that serve both reproductive and vegetative functions (e.g., petals, pedicels, bracts, and ovary walls), loss of meristems to reproduc-

tion, production of nectar, and other floral and fruit characters that effect, or are affected by, pollinator visitation and behavior, pollination success, fertilization, and seed set (Harper, 1977; Willson, 1983; Riska, 1986). Because pteridophytes do not produce seeds or flowers, they represent a major group of vascular plants which lack most of the complications listed above. Despite their advantageous simplicity, very little attention has been given to patterns of allocation to growth versus reproduction among pteridophytes, at developmental or ecological levels (but see Raghavan, 1989, and citations therein, regarding development in gametophytes). Moreover, ephemeral, annual, and perennial life histories occur among pteridophytes, permitting study of a variety of life history categories.

This study describes patterns of vegetative and reproductive performance in a natural population of the homosporous fern, *Polystichum acrostichoides* from which hypotheses regarding the life history of this species were developed.

METHODS

Polystichum acrostichoides is an iteroparous perennial that possesses a short-creeping rhizome (i.e., rarely longer than 40 cm) and semi-evergreen fronds that fully senesce as new fronds emerge in the spring. It is a species common to forests of eastern North America and a major component of the understory at the study site, Watterloo Wildlife Research Station (WWRS), Ohio. WWRS is located in Athens County, Southeastern Ohio. The forest at the study site is a mixed hardwood assemblage dominated by species of *Acer, Quercus,* and *Carya*.

To survey the population of *P. acrostichoides* at WWRS, we established two 200 m transects (streambank and upper-slope) in each of three ravines. Upper-slope transects were initiated at points perpendicular in elevation to the beginning of each streambank transect, and were defined as 70% of the elevational distance from streambank to ridgetop. Elevations were determined using a 7.5 minute USGS topographic map (Athens, OH 1985) and an American Paulin Systems model M1-6 altimeter.

An individual plant was identified every 20 m along each transect using the nearest individual method, for a total of 120 plants. To facilitate unobtrusive observation of growth and reproduction during consecutive growing seasons, above-ground morphological measurements (number of fronds, number of fertile fronds, frond area, and fertile area) of each plant were made between mid-June and mid-July, the approximate month of spore release, in 1994 and 1995. Because sporangial maturation is mixed in *P. acrostichoides,* and the inherent difficulty in efficiently collecting spores in the wild, an indirect measure of spore output (i.e., area of the fertile tip of a frond) was used. Fertile pinnae of *P. acrostichoides* occur at frond tips and are densely covered with sori on their lower surface. Separation between fertile and non-fertile pinnae is typically abruptly dimorphic, accompanied by a reduction in size (Fig. 1). Occasionally, the lower-most fertile pinnae are not reduced in size and possess only a few

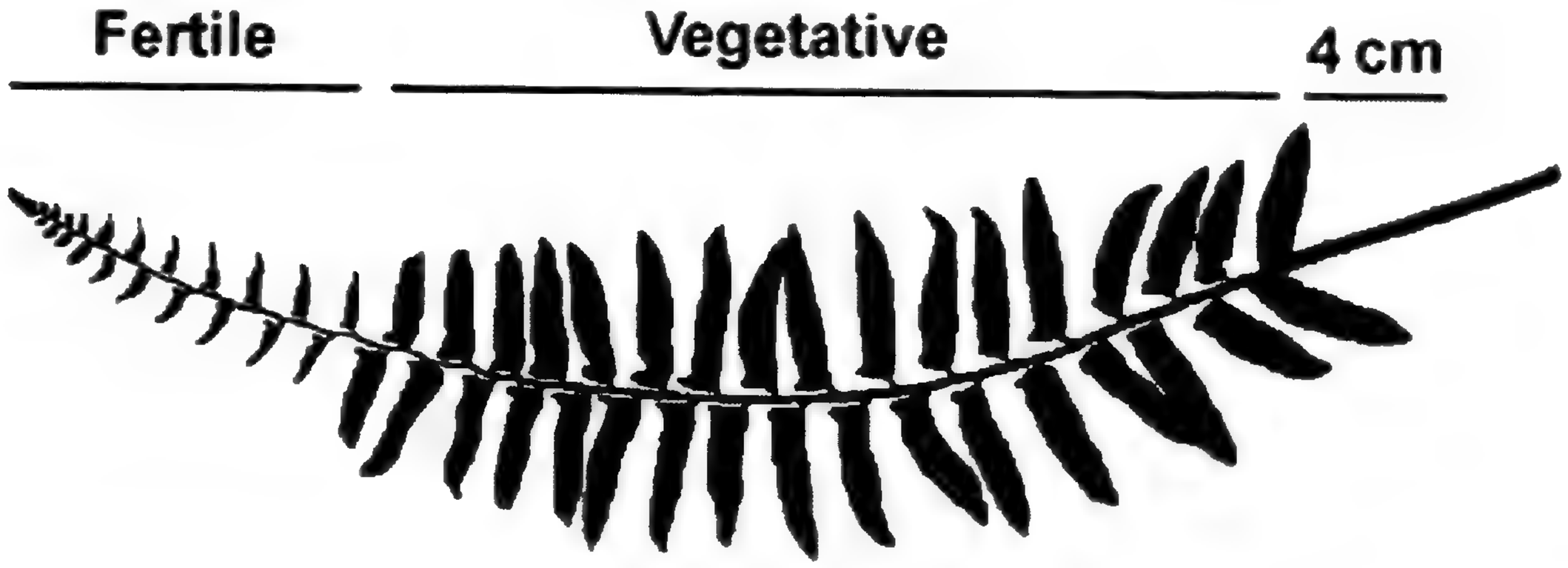

FIG. 1. Silhouette of a fertile frond of *P. acrostichoides* showing the abrupt change in pinnae size between vegetative and fertile pinnae.

scattered sori. To minimize error, these pinnae were not included in the measurement of fertile area. These methods assume the metabolic cost of producing vegetative tissues, sporangia, and spores is relatively constant from year to year (e.g., 1.0 g of spores requires the same absolute metabolic expenditure in 1995 as it did in 1994).

At the end of the 1995 growing season, each plant was collected, stored in a paper bag, dried at 70°C for 72 h, and total frond and rhizome-root mass dry weight determined. Dead rhizomatous tissues and persistent frond bases were removed from the rhizome prior to drying. Eight environmental variables were measured for each plant: canopy cover, moisture status, soil nitrate-nitrogen, phosphorous, pH, and available calcium, magnesium, and potassium. Canopy cover and moisture status for each plant were measured during the summer of 1994. Canopy cover was determined using a Lemmon model-C spherical densiometer. Moisture status was determined using a modified version of the Topographic Moisture Index (Parker, 1982; see Greer et al., 1997 for modification details). Soil was collected in summer 1995 from the rhizome-root mass mantle of each plant, stored in a paper bag, and shipped to the Ohio Agricultural Research Center, Wooster, Ohio, for chemical analysis.

DATA ANALYSES

Spearman's rank (rs) correlations and Mann-Whitney U-tests were used to investigate morphological and environmental differences between non-reproductive and reproductive plants, and between non-sequential and sequential reproducers; i.e., those that reproduced in only 1994 or 1995 versus those that reproduced in both years. Comparisons of plant size and morphology used estimates of 1994 + 1995 plant biomass (total frond + rhizome + root biomass) and mean frond: rhizome-root biomass ratio, respectively, to minimize variance between years. Relative growth rate (reffered to hereon as "growth") was estimated using the equation "(1995 total frond area − 1994 total frond area)/1994 frond area." Environmental variables were represented by loading scores

from Principle Components Analysis (PCA). PCA axes representing more than 10% of total environmental variance, and environmental variables with factor scores greater than 0.50, were interpreted. PCA is sensitive to highly correlated variables within each matrix. Among the environmental variables surveyed, soil pH, available calcium, magnesium, and potassium, were closely correlated, however, removal of all cations except pH did not substantially effect the outcome of the PCA and were therefore retained in both analyses.

Simple linear regressions were used to: 1) explore the relationship between frond and rhizome-root mass biomass throughout development, 2) describe the relationship between plant size and allocation to reproduction versus vegetative growth (i.e., reproductive effort, RE 1), and 3) investigate the cost of reproduction to growth in the following year. RE 1 as well as estimates of above-ground growth, used total fertile area and total frond area to minimize error that would result from conversion to estimates of biomass.

A second measure of reproductive effort (RE 2), based on Willson's (1983) suggestion that a more appropriate measure of reproductive effort for perennials may be yearly allocation to reproductive versus vegetative structures, was used for comparison. RE 2 was estimated for each plant by dividing its 1995 fertile biomass by its change (1995–1994) in plant biomass. Spearman's rank correlations were used to investigate the relationship between reproductive effort and PCA axes representing environmental gradients; mean reproductive effort values were used for each individual (e.g., mean RE 1 = (1994 + 1995 RE 1/ 2)).

The relationship between plant biomass and above-ground growth rate was investigated using linear regression model fitting (SPSS PC+, v. 6.1). Interpretation of regression used to investigate the relationship between reproductive effort and plant size followed Samson and Werk (1986). Their method, which eliminates the autocorrelation that may occur between reproductive biomass and total plant biomass, but also requires untransformed data, permits simple and direct interpretation of the slope and y-intercept. Here, we regressed 1994 + 1995 total fertile biomass against 1994 + 1995 vegetative biomass. To investigate the relationship between plant size and reproductive effort, as defined by Willson (1983) for perennials, we regressed 1995 RE 2 against 1994 plant biomass. Although the potential for autocorrelation exists, total fertile biomass was included within plant biomass for this analysis. Fertile tips typically remain photosynthetic after spore-release (i.e., mid June through early July) and are not reproductive organs. Therefore, deletion of total fertile biomass from estimates of plant biomass would result in under-representation of vegetative biomass.

The D'Agostino-Pearson omnibus test statistic (D'Agostino et al., 1990) was used to determine whether data met requirements of normality. Homoscedascity was tested using the F-max test (Dowdy and Wearden, 1985). All statistics were analyzed using SPSS PC+ v.6.1 (Norusis, 1994). A number of data transformations were used to meet statistical assumptions, including natural log, and power transformations. A critical value of $P = 0.05$ was used in all analyses. All reported r^2 values were adjusted for degrees of freedom.

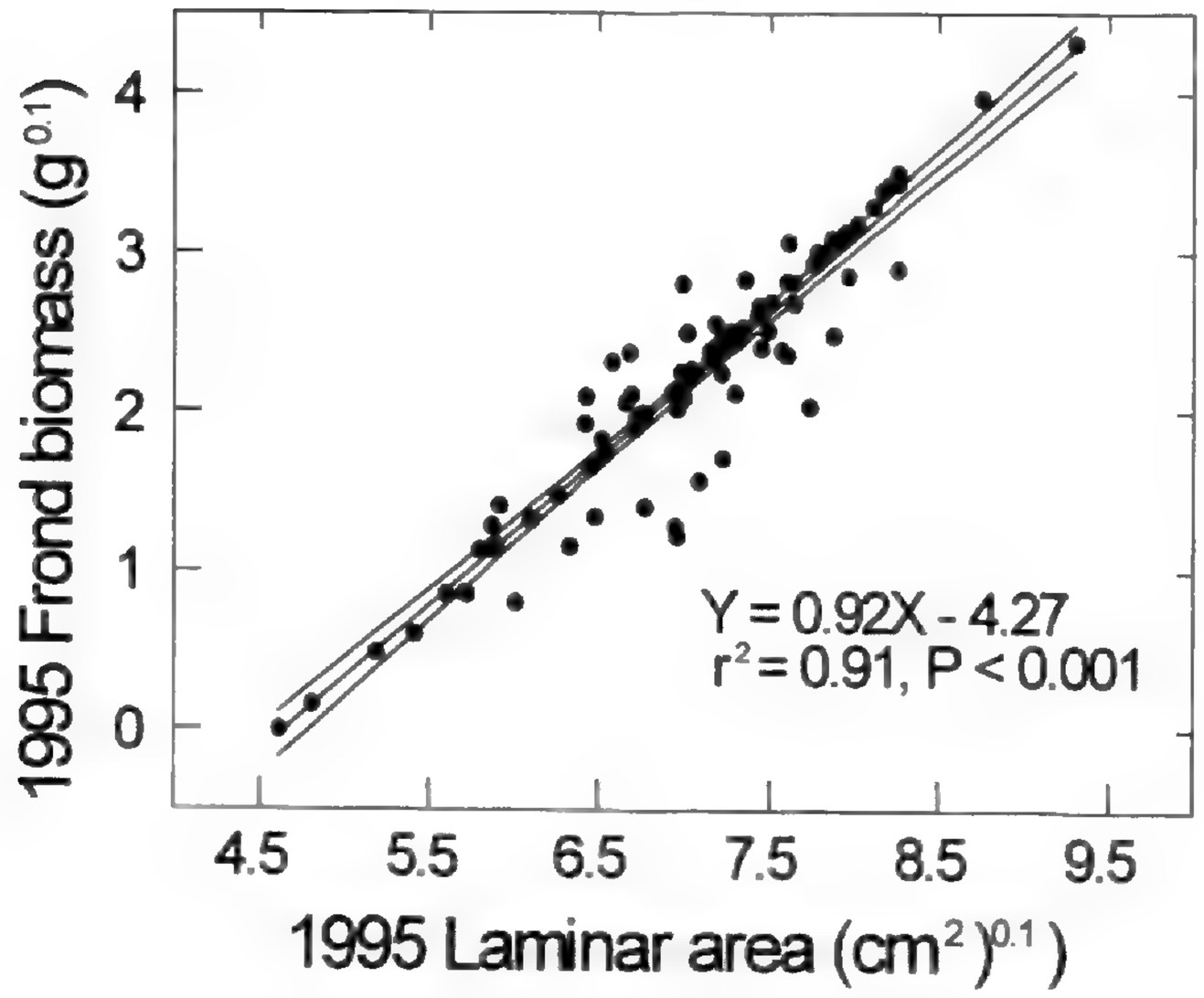

FIG. 2. Regression analysis of 1995 total laminar area (LA) prediction of 1995 total frond biomass (FrB).

RESULTS

Linear regressions indicated frond area and the area of the fertile tip are robust predictors of frond biomass (Fig. 2) and reproductive biomass (Fig. 3), respectively. Consequently, the linear equation "frond biomass = (0.007902 * frond area) − 0.071802" was used to estimate 1994 total frond biomass from 1994 total frond area. Likewise, the linear equation "fertile biomass = 1.85X − 7.66" was used to estimate fertile biomass from fertile area for both 1994 and 1995.

The first four axes from PCA of environmental factors explained 42.2%, 15.3%, 12.7%, and 12.0% of the variance, respectively, for a cumulative 82.2%. The first two PCA axes represented complex environmental gradients; PCA 1 represented calcium, potassium, magnesium, and pH concentrations, whereas, PCA 2 represented moisture and nitrate concentrations (Fig. 4a). PCA 3 and PCA 4 represented canopy cover and phosphorous concentrations, respectively (Fig. 4b).

1994 + 1995 plant biomass correlated negatively with PCA 1 and positively with PCA 4, indicating plant biomass was adversely affected by soil cation concentrations and favorably affected by phosphorous concentrations. Mean frond: rhizome-root biomass ratio did not correlate with any PCA axes (Table 1). Therefore, differences in plant biomass associated with environmental gradients were not associated with differences in biomass allocation to aboveground versus below-ground organs.

Regression modeling indicates growth rate increased with plant biomass and best fit a cubic model (Fig. 5). Gowth rate was associated with changes in frond size as well as frond number. Of 35 individuals that exhibited a decrease in total frond area, 32 (91.4%) exhibited a decrease in frond number. Likewise,

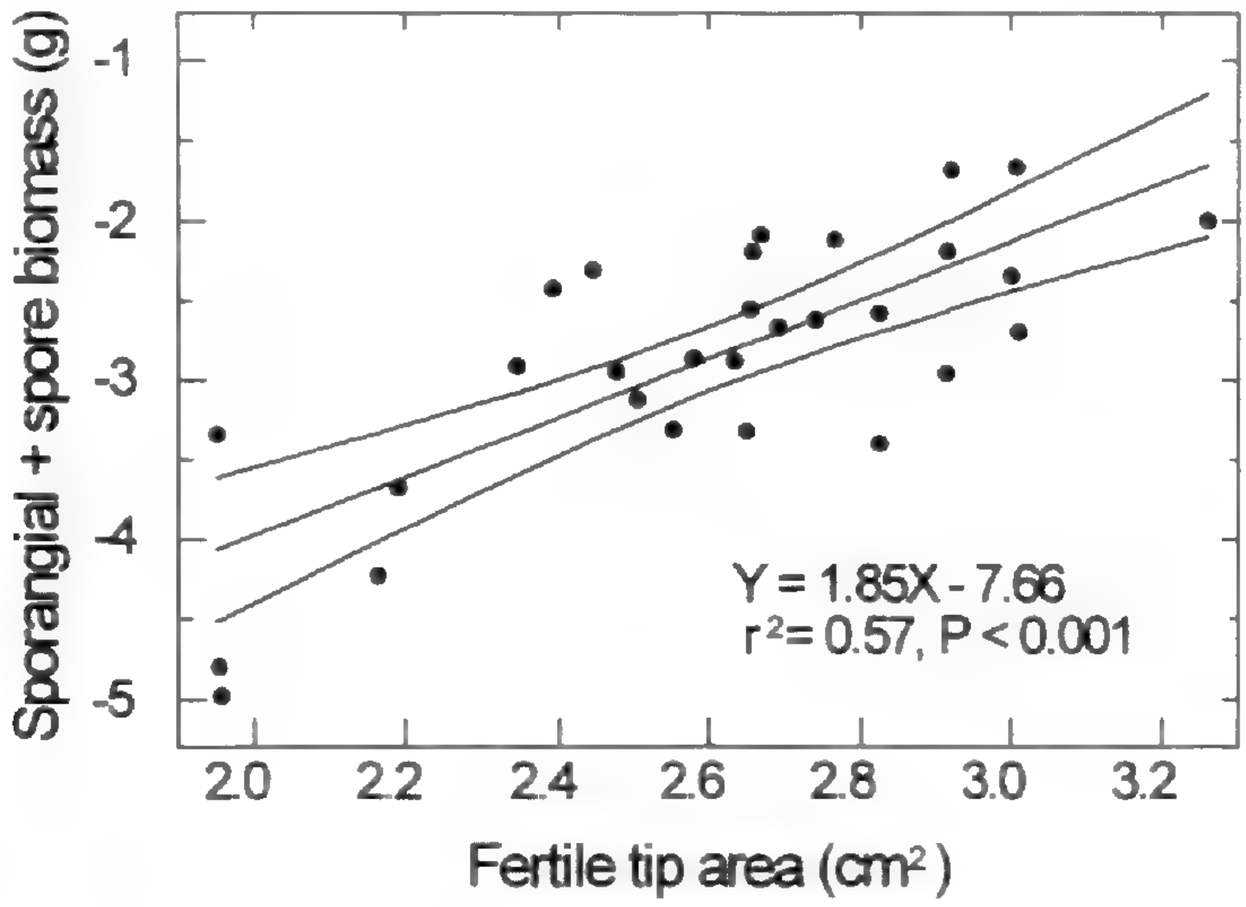

FIG. 3. Regression analysis of total fertile area prediction of sporangial + spore biomass.

54 of 55 individuals (98.2%) exhibited an increase in total frond number. Growth rate correlated significantly and positively with PCA 2 (Table 1), indicating growth increased with moisture and soil nitrate concentrations. However, the low r^2-value indicates considerable variability exists over the range of observations.

The proportion of biomass allocated to fronds increased significantly with plant biomass ($r^2 = 0.31$, $P < 0.001$, $Y = 1.01X - 0.69$). Mean frond: rhizome-root biomass ratio for the entire population was 1.3 ± 1.2, indicating that fronds constituted 56.5% ± 51.7% and rhizome-root mass biomass 43.5% ± 40.2% of plant biomass, respectively. Thus, as plant size increased, allocation shifted from favoring the rhizome-root mass to fronds. As a result, rhizome-root biomass was a poor predictor of frond biomass ($r^2 = 0.19$, $P < 0.001$, $Y = 0.09 + 1.12$).

Of the 120 plants surveyed, 41 (34.2%) were non-reproductive and 79 (65.8%) were reproductive in one or both years. Mann-Whitney U Rank Sum Tests indicate that reproductive individuals were larger (1994 + 1995 plant biomass) and fronds composed a greater proportion of their biomass (mean frond: rhizome-root biomass ratio) than non-reproductives (Table 2). Nevertheless, substantial overlap in plant biomass between reproducers and non-reproducers (Fig. 6) indicates reproduction was not entirely size dependent. Reproducers also exhibited significantly greater growth rates than non-reproducers (Table 2). No significant differences were observed between reproducers and non-reproducers for PCA axes representing environmental gradients (Table 2), indicating their distributions were not influenced by current environmental conditions.

Regression of 1994 + 1995 total fertile biomass versus 1994 + 1995 plant biomass for the entire reproductive population was significant; both slope and y-intercept differed significantly from zero (Fig. 7). The negative y-intercept (−0.29) indicates a minimum size at first reproduction exists in *P. acrostichoides*. Allocation of biomass to reproduction ranged from approximately

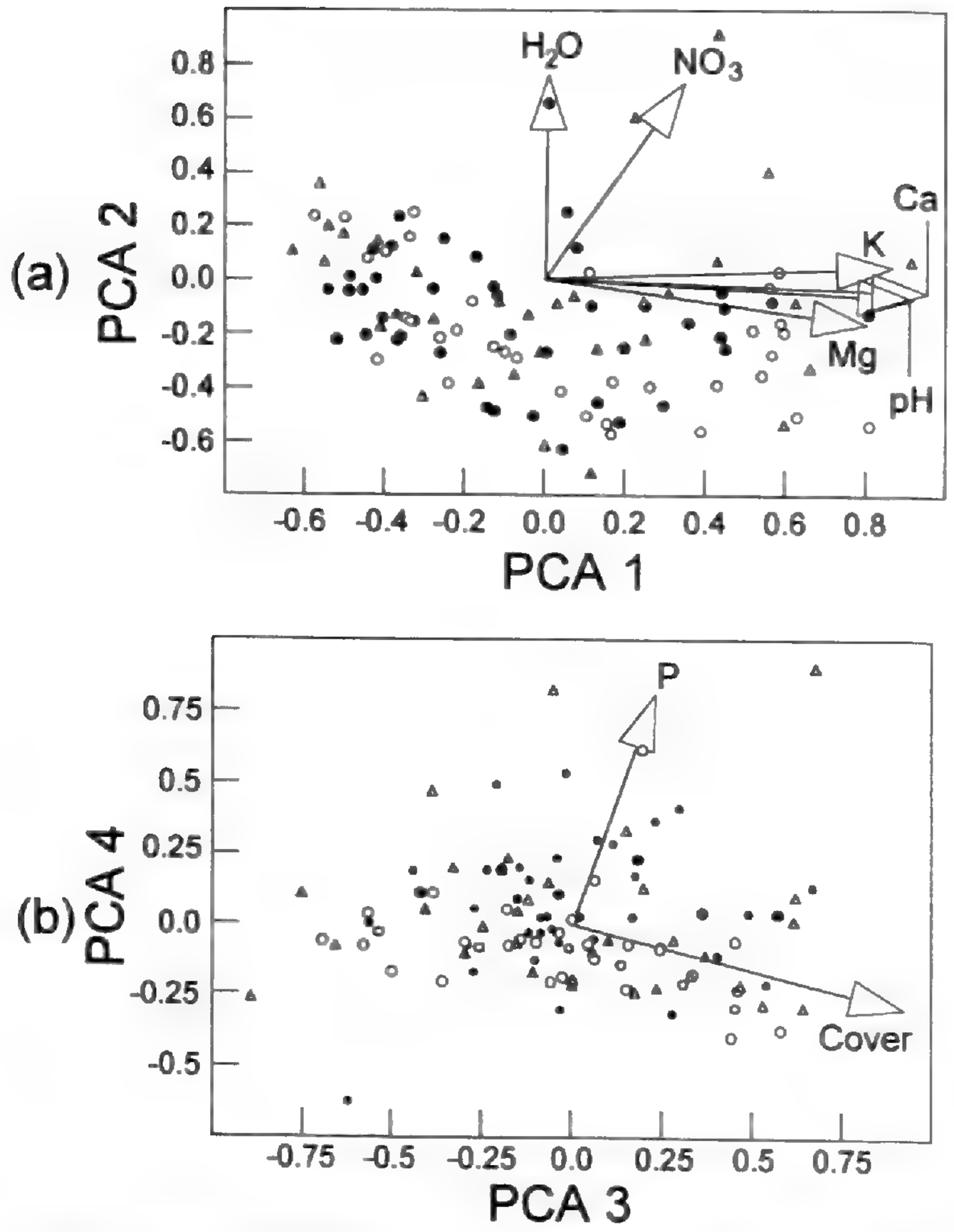

FIG. 4. Ordination from principle components analysis (PCA) of edited data matrix. Arrows indicate strength and direction of environmental gradients based on Spearman's rank correlations between ordination scores and observed environmental values; correlation coefficients can be determined from values on each axis. Symbols represent reproductive status of individual plants as follows: open triangles represent non-reproductives; open circles represent non-sequential reproductives, and closed circles represent sequential reproductives.

0.01% among the smallest individuals to 2.11% among the largest individuals (Fig. 7). The absolute value of the slope was small (0.03) compared to the y-intercept (−0.29), therefore, only a minor increase in the proportion of carbon allocated to reproduction was associated with increasing plant size.

Mean RE 1 and RE 2 correlated negatively with PCA 3 (Table 1). A marginally significant, negative, correlation was also observed between mean RE 1 and PCA 1. These observations suggest allocation to reproduction versus growth decreased with increasing canopy cover, and that soil cation concentrations may have had a small negative effect on reproductive effort.

Of the 79 reproductives, 31 (39.2%) reproduced in only one growing season (i.e., non-sequential reproducers), whereas, 48 (60.8%) reproduced in both growing seasons (i.e., sequential reproducers). Mann-Whitney U Rank Sum Tests indicate sequential reproducers were larger and allocated a greater proportion of their biomass to fronds than non-sequential reproducers; however,

TABLE 1. Spearman's rank correlation coefficients and associated probabilities for morphological traits, measures of above-ground growth, reproductive effort, and PCA axes representing environmental gradients.

TRAIT	PCA 1 (Ca, K, Mg)	PCA 2 (H_2O, NO_3)	PCA 3 (Canopy)	PCA 4 (P)
Plant Biomass (1994 + 1995)	r s^2 = −0.23 P = 0.01	r s^2 = −0.04 P = 0.34	r s^2 = −0.08 P = 0.24	r s^2 = 0.18 P = 0.04
Mean Frond : Rhizome-root Biomass	r s^2 = −0.04 P = 0.36	r s^2 = 0.08 P = 0.22	r s^2 = 0.03 P = 0.39	r s^2 = 0.09 P = 0.21
Growth rate	r s^2 = −0.02 P = 0.49	r s^2 = 0.19 P = 0.04	r s^2 = 0.11 P = 0.16	r s^2 = −0.08 P = 0.23
Reproductive Effort Mean RE 1	r s^2 = −0.17 P = 0.09	r s^2 = 0.16 P = 0.10	r s^2 = −0.28 P = 0.01	r s^2 = 0.09 P = 0.24
1995 RE 2	r s^2 = 0.01 P = 0.46	r s^2s = 0.08 P = 0.29	r s^2 = −0.24 P = 0.04	r s^2 = −0.04 P = 0.39

a substantial overlap in 1994 + 1995 plant biomass indicates that frequency of reproduction was not entirely a function of plant size (Figure 6). Sequential reproducers also grew more rapidly than non-sequential reproducers (Table 3). Moreover, the mean size of sequential reproducers (Figure 6) corresponded with the beginning of an asymptotic increase in growth rate (Figure 5), indicating the ability to reproduce in consecutive years was associated with a high rate of growth.

Sequential reproducers exhibited greater 1994 + 1995 RE 1 and greater RE 2 than non-sequential reproducers (Table 3), indicating, non-sequential reproducers did not compensate for non-reproductive years by increasing allocation to reproduction in reproductive years. Sequential reproducers also exhibited greater growth / 1994 RE 1 than non-sequential reproducers (Table 3), indicating sequential reproducers suffered a smaller cost of reproduction than non-sequential reproducers. Among non-sequential reproducers, individuals that reproduced during only 1994 exhibited greater growth rates than those that reproduced during only 1995 (Table 4). Thus, reproduction was associated with a cost to growth and, subsequently, the probability of reproducing in the following year.

Sequential reproducers possessed lower PCA 1 and greater PCA 4 scores (Table 3) than non-sequential reproducers, indicating environments lower in soil cation concentrations and higher in phosphorus were conducive to reproduction (Figure 4a–b).

DISCUSSION

VEGETATIVE ALLOMETRY.—As sporophytes of *P. acrostichoides* develop beyond an establishment phase, biomass allocation appears to switch from fa-

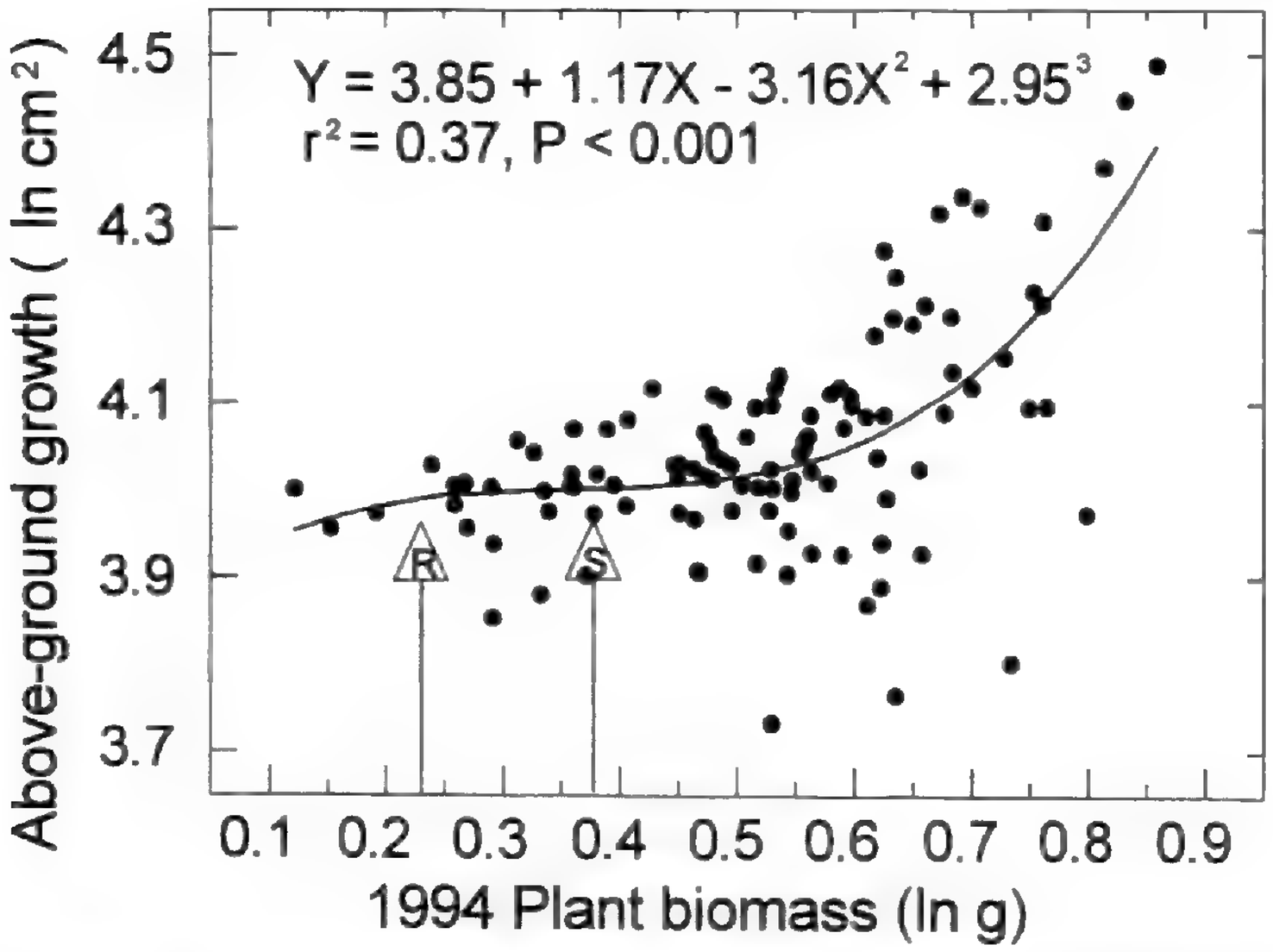

FIG. 5. Cubic model of 1994 plant biomass prediction of above-ground growth rate (i.e., Δ Laminar area). The arrow labeled "R" marks the size at first reproduction determined in Figure 3, whereas the arrow labeled "S" marks the mean size of sequential reproductives (see Figure 6). Growth rates initially increase with plant size during establishment. Reproduction among smaller individuals and/or those in less favorable habitats, may drain resources sufficiently to affect growth, resulting in sporadic (i.e., non-sequential) reproduction and a plateau in growth rates. At some point determined by genotype × environment interactions, resource acquisition and storage may become sufficient to consistently overcome the cost of reproduction to growth.

voring the rhizome-root mass to fronds. The proportion of plant biomass composed of fronds increased with plant biomass; ranging from a 1:4 ratio among the smallest plants to above a 6:1 ratio among the largest plants. The percentage of plant biomass composed of rhizome-root mass reported here (43.5% ± 40.2%) is considerably lower than the 19.0% ± 5.4% root and 60.8% ± 5.8% rhizome reported for this species by Minoletti and Boerner (1993). These differences may have resulted from differences in plant sizes and/or because we removed necrotic tissues from the distal end of the rhizome as well as the relatively large, persistent, frond bases. Early investment to the rhizome-root mass is very likely a trait that has evolved in response to factors that limit survival during establishment. These might include limitation of below-ground resources due to competition, herbivory, environmental composition, fire, and a higher likelihood of above-ground versus below-ground injury.

If biomass also reflects the distribution of most nutrients, then as a *P. acrostichoides* sporophyte develops, the rhizome is superseded by fronds as the primary storage organs. The fronds of *P. acrostichoides* are evergreen, photosynthetically active throughout the year, and a source of metabolites and nutrients throughout the autumn, winter, and spring (Minoletti and Boerner, 1993). Evergreen foliage is important in reducing rates of nutrient loss and in maintaining carbon balance (Aerts, 1995; Jonasson, 1995). The transient nature of fronds, including those that are semi-evergreen, may provide a means for optimizing metabolic expenditures and response(s) to environmental condi-

TABLE 2. Mann-Whitney U rank sum tests of morphological traits and PCA axes representing environmental gradients (see text) between reproductives (R = 79) and non-reproductives (NR = 41). Ranks are ascending.

TRAIT/PCA AXIS	STATUS	MEAN RANK	SUM OF RANKS	U, P-VALUE
Plant Biomass (1994 + 1995)	NR	34.3	1339	U = 559
	R	71.3	5564	P < 0.001
Mean Frond : Rhizome-root Biomass	NR	39.4	1536	U = 756
	R	68.8	5367	P < 0.001
Growth Rate	NR	52.6	2053	U = 1273
	R	62.2	4850	P = 0.08
Reproductive Effort	NR	47.2	1274	U = 832
PCA 1 (Ca, K, Mg)	R	45.5	2912	P = 0.39
PCA 2 (H_2O, NO_3)	NR	51	1377	U = 842
	R	43.9	2809	P = 0.12
PCA 3 (Canopy)	NR	46.8	1264	U = 842
	R	45.6	2922	P = 0.43
PCA 4 (P)	NR	44.0	1189	U = 811
	R	46.8	2997	P = 0.65

tions, and may explain why rhizome-root biomass was a poor predictor of frond biomass. If fronds are the primary storage component in larger plants, then the rhizome may function primarily as a genitor of new fronds and roots. This architectural strategy may be common among fern species with evergreen fronds, particularly those with short-creeping or erect rhizomes.

RELATIONSHIPS BETWEEN PLANT SIZE, GROWTH RATE, AND REPRODUCTION.—Reproduction in *P. acrostichoides* appears to be size dependent. A minimum size at reproduction was apparent; reproducers and sequential reproducers were significantly larger than their respective counterparts, and reproductive effort increased with size. A general relationship between plant size and reproduction has been well established for a variety of species (Sohn, 1977; Pitelka et al., 1980; Young, 1981; Lee and Hamrick, 1983; Willson, 1983; Lacey, 1986; Samson and Werk, 1986; Lotz, 1990; Primack and Hall, 1990; Thompson et al., 1990; Hanzawa and Kalisz, 1993; Linvelle, 1995), and evidence for a minimum size at first reproduction and a relationship between size and frequency of reproduction have been reported for a few iteroparous perennials (Young, 1981; Samson and Werk, 1986; Hanzawa and Kalisz, 1990; Primack and Hall, 1990). Nevertheless, the overlap in plant biomass observed between non-reproducers and reproducers, and between non-sequential and sequential reproducers, indicate reproduction in *P. acrostichoides* is not determined entirely by size.

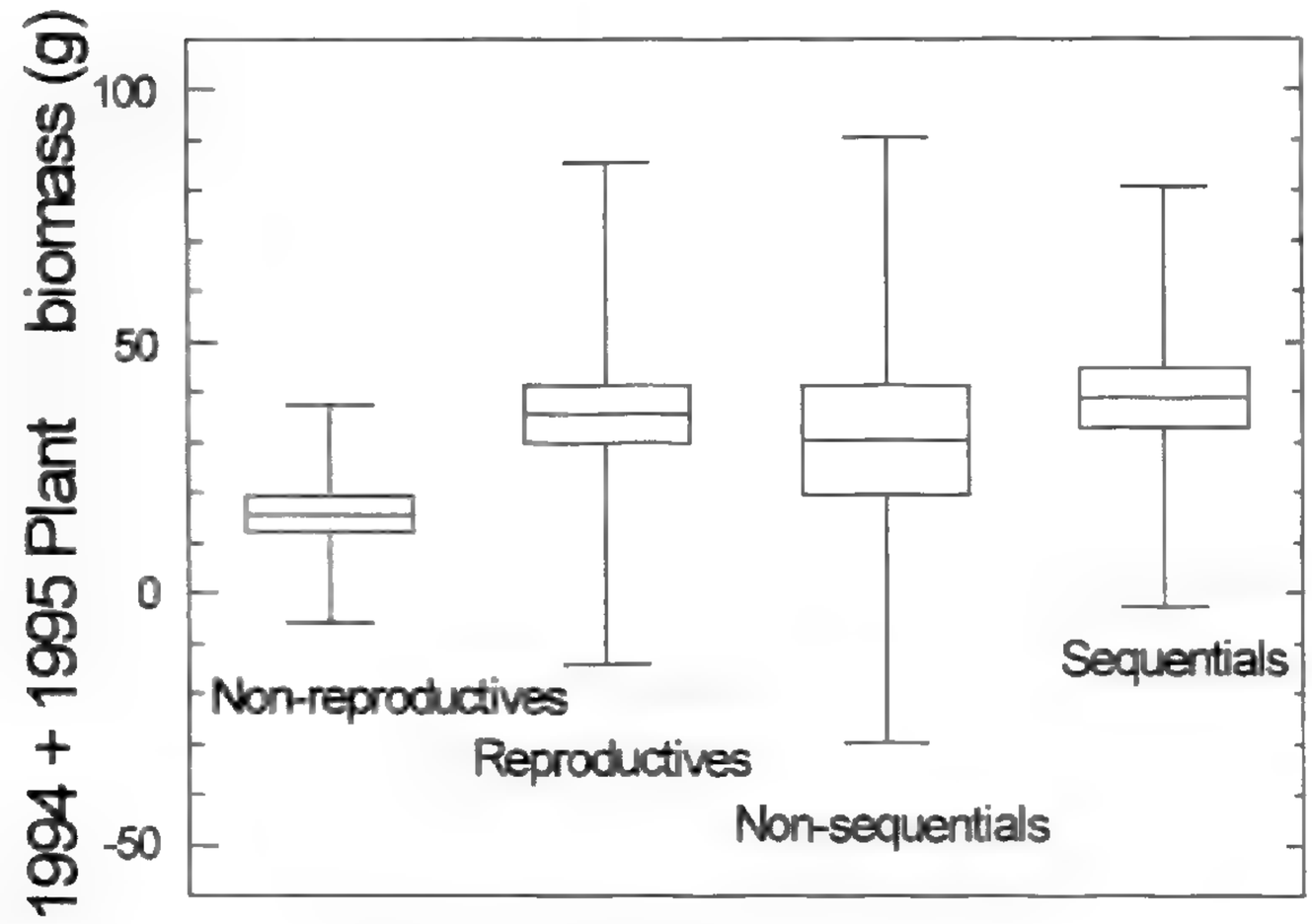

FIG. 6. Box plots of 1994 + 1995 plant biomass (PB) for non-reproductives, reproductives, non-sequential reproductives, and sequential reproductives. Horizontal lines mark the following in increasing order, mean-2*standard deviation, mean-2*standard error, mean, mean+2*standard error, mean+2*standard deviation.

Three observations indicate that reproduction in *P. acrostichoides* occurs only when stored resources exceed that necessary to maintain a threshold rate of growth. First, reproducers possessed higher growth rates than non-reproducers. Second, sequential reproducers possessed higher growth rates and suffered lower costs of reproduction to growth than non-sequential reproducers. Third, a cost of reproduction to growth was evident among non-sequential reproducers; i.e., those that reproduced in 1994 exhibited significantly greater growth rates than those that reproduced in 1995. Similar models of growth-limited reproduction have been suggested for iteroparous perennials by Reekie and Bazzaz (1987c), Saulnier and Reekie (1995), Kozlowski (1992), and Galen and Stanton (1993). According to these models, the curvilinear relationship between growth and plant size may be a function of the threshold of resources necessary for both growth and reproduction.

The value of reproduction is expected to increase with age in iteroparous plants (Kozlowski, 1992; Stearns, 1992). Consequently, iteroparous plants are expected to gradually increase the proportion of resources that are diverted to reproduction with age, or to reach a maximum size, beyond which all resources are diverted to reproduction (Kozlowski and Uchmanski, 1987; Pugilese and Kozlowski, 1990; Kozlowski, 1992; Stearns, 1992; Worley and Harder, 1996). Assuming that size corresponds with age, our observations do not support to these models. We observed no evidence of a maximum size and growth rates increased with size, particularly among the largest individuals. Furthermore, reproduction was sporadic among even the largest individuals, and assuming carbon allocation reflects relative metabolic expenditures (Bazzaz, 1997), only minor increases in reproductive allocation were associated with increases in plant size (i.e., 0.01% to 2.11% of total biomass). Similar patterns of sporadic reproduction and low and/or constant reproductive allocation were reported

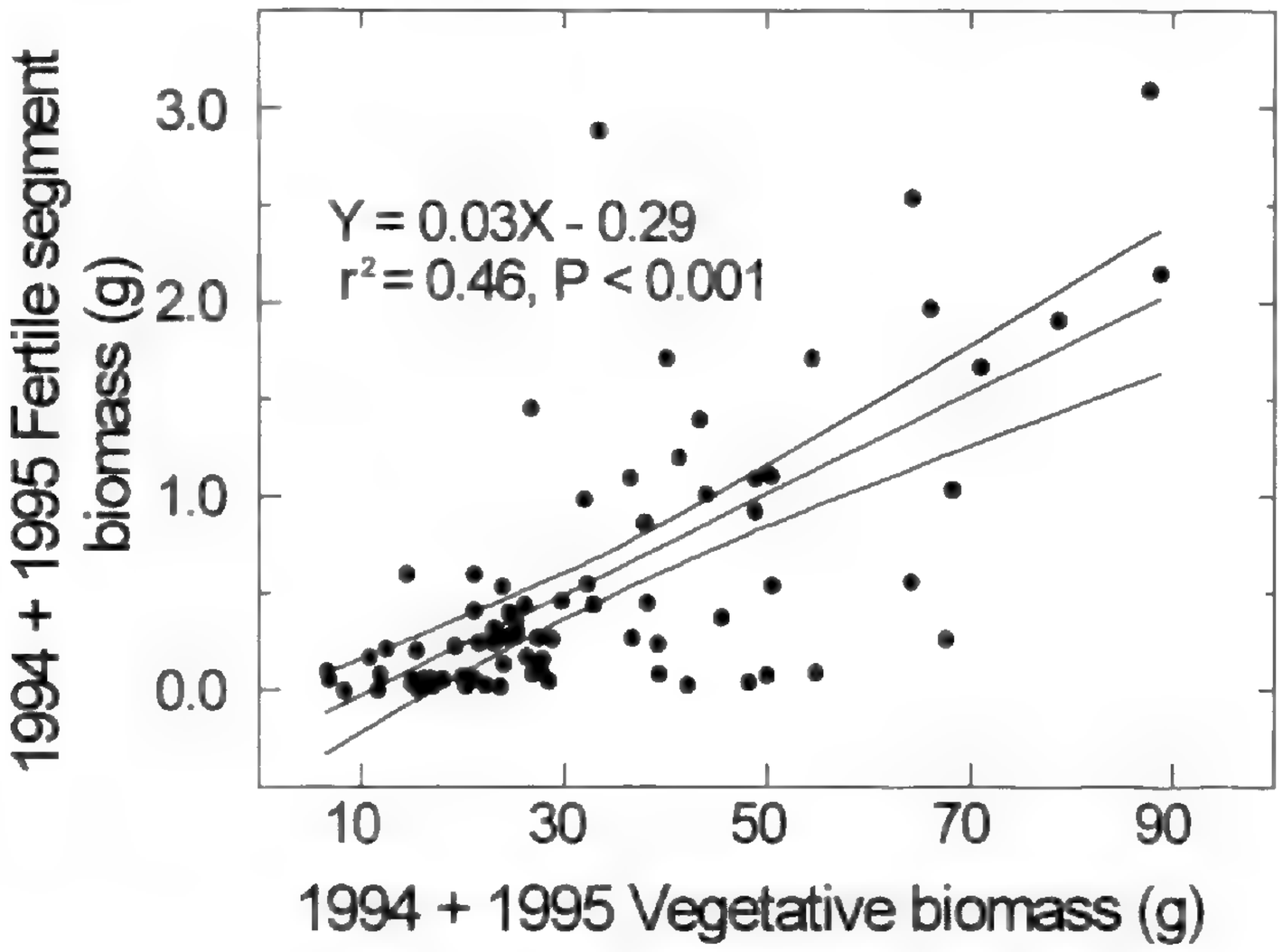

FIG. 7. Regression analysis of 1994 + 1995 vegetative biomass prediction of 1994 + 1995 fertile segment biomass.

for *Chamaenerion angustifolium* (Van Andel and Vera, 1977) and *Cypripedium acaule* (Primack et al., 1994).

Relative construction costs between vegetative and reproductive tissues probably remain constant in most ferns, since they lack the ancillary structures that complicate this relationship (e.g., peduncles, petals, or ovary walls); whether they selectively abort or provision spores is less clear. This is particularly true for species with monomorphic fronds. Nevertheless, life history and demographic traits such as growth, reproduction, and survival, may be more useful measures of the cost of reproduction than limiting metabolic currencies, which are difficult to identify and vary among organs, individuals, populations, and environments (Ricklifs and Miles, 1994; Muir, 1995; Bazzaz, 1997). Assessing the life history and demographic costs of reproduction is also more straightforward for most ferns than seed plants, because, reproduction does not result in the loss of a meristem that could otherwise assist vegetative activity (with the exception of ferns with indeterminate and clone-forming fronds). However, phenological patterns of development may complicate the analysis of resource allocation. Species that reproduce early in the growing season often initiate new vegetative reproductive structures a year or more in advance of emergence (Gerber et al., 1997).

Costs of reproduction were evident among both sequential and non-sequential reproducers as reductions in growth rates and the likelihood of reproducing in the following year. A number of studies have also reported costs of reproduction to survival, growth, the amount of resources allocated to reproduction, and the likelihood of reproducing in the following year (Sohn, 1977; Reekie and Bazzaz, 1987c; Pugilese and Kozlowski, 1990; Fox and Stevens, 1991; Muir, 1995; Worley and Harder, 1996; Gerber et al., 1997). Sporadic

TABLE 3. Mann-Whitney U rank sum tests of morphological traits and PCA axes representing environmental gradients between non-sequential (NS = 31) and sequential (S = 48) reproducers. Ranks are ascending.

TRAIT/PCA AXIS	STATUS	MEAN RANK	SUM OF RANKS	U, P-VALUE
Plant Biomass (1994 + 1995)	NS	30.5	915	U = 450
	S	45.1	2166	P < 0.002
Mean Frond : Rhizome-root Biomass	NS	34.1	1023	U = 558
	S	42.8	2058	P = 0.05
Growth Rate	NS	31.4	942	U = 477
	S	44.6	2139	P = 0.01
Reproductive Effort	NS	31.9	956	U = 491
1994 + 1995 RE 1	S	44.3	2125	P = 0.01
1995 RE 2	NS	27.4	822	U = 357
	S	36.2	1194	P = 0.03
Cost of Reproduction (Growth rate/RE 1)	NS*	17.5	245	U = 140
	S	35.0	1646	P < 0.001
Environment				
PCA 1	NS	37.2	1005	U = 372
	S	29.1	1075	P = 0.04
PCA 2	NS	30.3	819	U = 441
	S	34.1	1261	P = 0.22
PCA 3	NS	33.0	889	U = 488
	S	32.2	1191	P = 0.44
PCA 4	NS	27.6	746	U = 368
	S	36.1	1334	P = 0.04

* 1994 non-sequentials only; N = 15.

reproduction may be common among plants where reproduction is governed by the maintenance of a threshold rate of growth.

ENVIRONMENTAL EFFECTS ON ALLOCATION PATTERNS.—No patterns in mean frond: rhizome-root biomass ratio were observed relative to environmental gradients. These observations suggest that the developmental relationship between fronds and rhizome-root mass, and therefore investment in to aboveground versus below-ground foraging, may be fairly rigid in *P. acrostichoides*.

In contrast, allocation to reproduction may be plastic in *P. acrostichoides*; reproductive effort correlated negatively with canopy cover, and sequential reproducers were more frequent in habitats low in cations and high in phosphorous. These observations are consistent with the classification of *P. acrostichoides* as a weakly acidophilic understory species (Graves and Monk, 1982;

TABLE 4. Mann-Whitney U rank sum tests of morphological traits between 1994 and 1995 non-sequential reproducers (NS). Ranks are ascending

TRAIT	STATUS	MEAN RANK	SUM OF RANKS	U, P-VALUE
Plant Biomass (1994 + 1995)	1994 NS	15.3	230	U = 110, P = 0.92
	1995 NS	15.7	235	
Mean Frond : Rhizome-root Biomass	1994 NS	16.7	251	U = 94, P = 0.44
	1995 NS	14.3	214	
Growth Rate	1994 NS	19.1	286	U = 59, P = 0.01
	1995 NS	11.9	179	

Greer, et al., 1997) and the hypothesis that reproduction in this species occurs only when a threshold growth rate can be maintained. Given the above, growth rate is predicted to be greatest in habitats characterized by sparse canopy, low cation concentrations, and high phosphorous concentrations. However, no relationship between growth rate and any of these environmental gradients was evident. Increased allocation to reproduction in favorable habitats may have maintained growth rates near a minimum threshold. Plasticity of reproductive effort, resulting in increased sexual and asexual reproduction in favorable habitats, was also reported for *Dennstaedtia punctilobula* (Hammen, 1993).

In contrast to the positive correlation between growth and reprodutive effort among sporophytes, the gametophytes of *P. acrostichoides* increase reproductive effort in habitats that are unfavorable to rapid growth (Greer and McCarthy, 1999). This opposing pattern in plasticity of reproductive effort may be common among species of ferns where the gametophyte is ephemeral and the sporophyte long-lived (Greer and McCarthy, 1999).

A MODEL OF LIFE HISTORY EVOLUTION IN *POLYSTICHUM ACROSTICHOIDES*.—Like many iteroparous plants, the colonizing stages of ferns (i.e., gametophyte and juvenile sporophyte) have much greater risks of mortality than mature sporophytes (Peck et al., 1990). Under these circumstances, fitness is highly correlated with life time fecundity, and the value of reproduction is expected to increase with age (Harper, 1977; Willson, 1983; Stearns, 1992). Nevertheless, rates of reproductive success may be sufficiently low in *P. acrostichoides* and many other pteridophytes, that life time fecundity, and therefore fitness, is maximized through essentially "immortal" life histories that maintain growth across all age classes. Indeed, low reproductive output is favored when environmental heterogeneity affects reproductive success more than adult survival (Murphy, 1968; Pianka, 1972; Schaffer, 1974; Goodman, 1979)—a likely condition among most pteridophytes.

Environmentally induced heterogeneity in recruitment favors plasticity in reproductive allocation (Hirshfield and Tinkle, 1975). Even when conditions that confer a fitness advantage to early or delayed reproduction are infrequent, the onset of reproduction may be optimized through phenotypic plasticity

(Travis, 1994). Thus, plasticity rather than simple allometric relationships may account for much of the variation in age and size at first reproduction (Stearns, 1992), and variation in size at first reproduction may account for much of the subsequent variation in allocation (Samson and Werk, 1986; Herndon, 1987). Physiological and reproductive plasticity that facilitate growth and longevity may explain the wide distribution and abundance of *P. acrostichoides* in Eastern North America.

Although observations from unmanipulated studies of natural populations are ambiguous, this study provides essential baseline data for experimental studies of allometry, resource allocation, and phenotypic plasticity in *P. acrostichoides*.

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The Effects of Rhizome Severing and Nutrient Addition on Growth and Biomass Allocation in *Diphasiastrum digitatum*

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ABSTRACT.—The effects of severing and fertilization on ramets of *Diphasiastrum digitatum* in an Ohio hardwood forest were examined to determine the extent of young ramet integration. The extent of integration was measured by overall vigor, and by architectural biomass, and growth variables. Vigor decreased with any plant manipulation. Likewise, all measured architectural and biomass variables were smaller in manipulated treatments. Similar negative effects in growth and biomass were seen with severing or fertilization, while a combination of both showed a further decrease in growth variables compared to either of the individual treatments. Severing reduced growth and biomass fifty percent compared to the control, indicating that integration is important in young multi-module ramet development. The results are consistent with other studies suggesting clonal lycopods are highly integrated and emphasizes the importance of integration in young ramets *of D. digitatum* for survival and growth.

Clonal plants are constructed of basic units, called modules, that are iterated throughout the development of a clone and can potentially function as independent members (ramets) of a genetic individual (genet) (Harper, 1977). The existence of connections between ramets or modules of a clone is inherent to its architecture. These underlying vascular connections facilitate the movement and sharing of plant substances (Pitelka and Ashmun, 1985), and thereby permit the clone to function as a physiologically integrated system (Watson and Casper, 1984). As long as connections persist among modules, there is a potential for resource movement (Marshall, 1990; Pitelka and Ashmun, 1985). However, a great deal of variation in the degree of integration within and among clonal plant species has been demonstrated (Jönsdöttir and Watson, 1997). Specifically, clonal plants may vary in their longevity and/or functionality of connections (months to decades), the substances that are moved (minerals, nutrients, carbohydrates, photoassimilates, hormones), and the direction of flow, whether acropetal, basipetal, or bidirectional (see references in Hutchings and Bradbury, 1986; Jönsdöttir and Watson, 1997; Klimeš et al., 1997; Marshall, 1990; Pitelka and Ashmun, 1985).

Such physiological integration can be a major factor influencing survival of clonal plants that retain ramet connections for any period of time (Eriksson and Jerling, 1990; Jönsdöttir and Watson, 1997; Pitelka and Ashmun, 1985). Adaptive advantages incurred by ramets participating in such integration include successful establishment and survival, accelerated maturity, and localized stress recovery (Caraco and Kelly, 1991; Hutchings and Bradbury, 1986; Jönsdöttir and Watson, 1997; Pitelka and Ashmun, 1985). The significance of these adaptive features are seen in cases where disintegration, or fragmenta-

tion, occur. Fragmentation of the genet into smaller ramets or modules may occur naturally through senescence and stress response, or through environmental disturbances such as herbivory, grazing and trampling (Pitelka and Ashmun, 1985). However this fragmentation or disintegration occurs, it has consequences on the growth, biomass and overall survival of the plant in question, most of which remain relatively unexplored in a number of clonal plant species (Charpentier et al., 1998).

Although physiological integration in clonal plants has attracted a great deal of attention in recent years (see references in Marshall and Price, 1997; Jönsdöttir and Watson, 1997; Pitelka and Ashmun, 1985), most studies have focused on angiosperms rather than lower plants such as pteridophytes. Examining the clonal patterns and processes of integration in pteridophytes offers not only a complete view of the plant kingdom, but also offers an evolutionary view of integration in lineages of clonal plants that have existed in many habitats and geologic time periods (Dyer, 1979). In particular, few studies have investigated the extent of physiological integration in lower plants such as lycopods (Callaghan, 1980; Carlsson et al., 1990; Headley et al., 1985; 1988a; 1988b) and those that do concern lycopods involve relatively few, common species that do not reside in North America. *Diphasiastrum digitatum* (Dillenius ex. A. Braun) Holub is a North American member of the Lycopodiaceae that is relatively common in its native habitat but has been little studied in light of its clonality and physiological integration (Jönsdöttir and Watson, 1997; Lau and Young, 1988). Previous examination of this species' physiological integration has focused on the survival of older modules (Lau and Young, 1988), whereas generalized studies of clonal plants indicate that younger ramets of clonal plants are usually most dependent on connections to the parent ramet (Hutchings and Bradbury, 1986).

In this study, we examine the effects of severing and nutrient addition on newly developing ramet growth in *Diphasiastrum digitatum* in order to determine the general extent of its physiological integration and to determine how it directly responds to disintegration from the parent clone and to a localized increase in nutrients. We hypothesize that during early development, artificially severing rhizome connections to the parent will significantly decrease the vigor, growth, and biomass of young ramets. Furthermore, we hypothesize that adding nutrients to the sterile edaphic environment characteristic of *D. digitatum* will increase the vigor, growth, and biomass of younger ramets.

Methods and Materials

Study Organism.—*Diphasiastrum digitatum* is a common understory herb in the northeastern United States (Wagner and Beitel, 1996) that forms dense clonal patches through vegetative growth. This evergreen perennial displays a rhizomatous growth form, whereby horizontal creeping stems bear distinctive fan-like, dimorphic (vegetative and reproductive) vertical shoots and adventitious roots. *Diphasiastrum digitatum* is the most common species of its genus in North America (Wagner and Beitel, 1996), and is found in a number of

habitats, including hardwood and conifer forests as well as open shrubby areas (Cody and Britton, 1989; Wagner and Beitel, 1996). Many members of this and other closely related genera in the temperate region are characterized as weedy and are believed to grow in relatively infertile soil conditions (Page, 1979; Tryon and Tryon, 1982).

The life span of most vertical shoots is approximately four to six years (Primack, 1973; Railing and McCarthy, unpublished data). Rhizome connections may persist for some time before decaying and thereby separating previously connected ramets. This type of vegetative reproduction has been noted as more common than sexual reproduction (Primack, 1973) in *Diphasiastrum digitatum,* most likely reasoned so because of the unsuccessful or poor spore germination witnessed in gametophyte cultures, rather than in nature (Roberts and Herty, 1934; Whittier, 1981; 1998).

STUDY AREA.—Strouds Run State Park (SRSP) is located in Athens County, Ohio at 39°20′N (latitude) and 82°5′W (longitude), in the southeastern foothills of the state. In the early twentieth century, the area was disturbed by grazing and timbering activities. As such, most of the forests are considered to be even-aged second-growth. What is now known as SRSP was acquired for conservation purposes in the late 1940's and early 1950's by the State of Ohio and eventually became a state park in 1959. The park spans over 1100 ha on Appalachian Ohio's unglaciated Allegheny plateau, and is centered around a man made watershed, Dow Lake. The topography consists of a number of steep hills and ravines believed to be remnants of glacial meltways. Soils in this region of Ohio are classified as Inceptisols (Brady, 1984) and soil conditions throughout the park are characterized as being moderately deep to deep, well-drained, and mostly loamy (typic hapludalfs), originating from sandstone, siltstone, shale, and limestone parent material (National Cooperative Soil Survey, 1985). Precipitation is relatively evenly distributed throughout the year and ranges from 5–10 cm per month, with July being the wettest month and October the driest. Mean temperatures (high/low) are 29/18 C for July and 2/−8 C for January (Midwestern Climate Center, 1999).

The vegetation of SRSP is primarily deciduous forest cover and can be classified as mixed mesophytic (*Acer* spp., *Aesculus octandra, Fagus grandifolia,* and *Liriodendron tulipifera*) in the lowlands and mixed oak or oak-hickory in the uplands (*Quercus alba, Q. rubra, Q. velutina* and *Carya glabra, C. ovata*). A number of white pine plantations (*Pinus strobus*) are also present in the park (<10% of vegetation), having been planted by conservation corps for watershed protection. There are also sporadic old-fields, grassy meadows, and maintained turf grass habitats. The herbaceous layer of the woodlands is largely dependent upon the overstory vegetation and there is considerable diversity of both microhabitats and species (Payne, 1957; Greer et al., 1997).

FIELD METHODS.—Ten *Diphasiastrum digitatum* patches were located within SRSP. In June 1998, 4 apical rhizome tips, approximately 1–2 m apart, were haphazardly located around the perimeter of each patch. Rectangular quadrats (0.25 × 2 m) were established around each of the four rhizome tips such that

the rhizome was situated 0.5 m into the quadrat along the longest dimension. Quadrats within each patch were randomly assigned to one of four treatment groups. The first treatment group (C) served as the control, and remained unmanipulated throughout the duration of the experiment. Rhizomes in the second treatment group (S) were severed at their entrance into the rectangular quadrat. Quadrats assigned to the third treatment group (F) were treated with a standard 10-10-10 inorganic fertilizer (Dragon All Purpose Plant Food) at a prescribed monthly rate (ca. 100 g fertilizer per m^2) for perennials. Rhizomes in the fourth treatment group (SF) were subject to both severing and fertilization treatments. Other above ground vegetation within quadrats was clipped and treatments were applied after quadrat establishment in June 1998.

In August 1998 vigor observations were made in situ before excavating all rhizomes. Rhizomes were assigned to one of three vigor categories that were based on health and color. High vigor (HI) indicated a rhizome or ramet with high health and a green to bright green color, similar to the rest of its original patch. Moderate vigor (MD) and low vigor (LO) referred to a ramet that displayed moderate health and green to yellow color and poor health with yellow to brown color, respectively. Excavated ramets were subsequently measured, oven dried at 80 °C for ca. 48 hours and weighed. Data were collected to reflect growth and biomass patterns since the initial treatment application. Variables examined upon ramet collection include total growth length (cm), number of new shoots and roots initiated, mean internode and shoot length (cm), and aboveground (internode and shoot) biomass (g) for each ramet.

ANALYTICAL METHODS.—A G-test (Chi-Square Goodness-of-Fit Test) was used to analyze the homogeneity of vigor categories among treatment groups (Sokal and Rohlf, 1995) with the null hypothesis that there are no differences in ramet frequency among the twelve possible classes (i.e., treatment/vigor combinations). In order for G to better approximate the chi-square statistic, the Williams correction factor was applied as recommended by Sokal and Rohlf (1995).

A Multivariate Analysis of Variance (MANOVA) was used to test for overall significant treatment effects across all growth and biomass parameters measured using the Wilk's Lambda test statistic (Hintze, 1997). A randomized complete block design MANOVA was employed using treatment group as a fixed effect and patch as a random effect. Data sets were checked for normality, equal variance, and equality of covariance using the D'Agostino Omnibus test (D'Agostino et al., 1990), Modified-Levene Equal-Variance test, and Box's M test, respectively, to meet the assumptions of MANOVA. Those failing the normality assumption were corrected using either $\log_{10}$ or square-root transformations.

Planned (a priori) comparisons (Dowdy and Wearden, 1991) were used to test for significant differences among treatment groups according to the three hypotheses. We used the first hypothesis (H_1) to test for significant differences between the control and all other treatments (C vs. S,F,SF), the second (H_2) to test for differences between treatment types (S vs. F) and the third (H_3) to test

TABLE 1. Observed frequency of ramets within each treatment group according to vigor category, including G-test (Chi-Square Goodness-of-Fit) results. Note that when the cell element marked with an asterisk is removed, G = 14.8 and P > 0.1.

Treatment Group	Vigor Category		
	HI	MD	LO
Control (C)	9*	1	0
Sever (S)	5	5	0
Fertilize (F)	3	6	1
Sever and Fertilize (SF)	0	4	6
G = 24.9, P < 0.01			

for an additive effect of treatment types (SF vs. S,F). The above planned comparisons were only used on those variables significantly contributing to treatment effect within the MANOVA. All statistical analyses mentioned above were conducted using NCSS statistical software (Hintze, 1997) with an alpha of 0.05 used to test hypotheses.

RESULTS

The results of the G-test used for the vigor study indicated that significant differences (P < 0.01) among vigor-treatment combinations existed (Table 1), thereby rejecting the overall null hypothesis and indicating that *Diphasiastrum digitatum* ramets within vigor categories did reflect a treatment effect. However, upon closer examination, one class (Treatment C—HI vigor) may be driving the significance in this analysis (Table 1). In an exploratory procedure, the aforementioned class was removed from the analysis to test for significant differences among the remaining other classes. The resultant G-Test indicated that the classes were then homogeneous (Table 1). Hence, the control group displayed the highest vigor, and any perturbation (treatments S, F, SF) of the ramets affected their health and coloring, reducing vigor to either the moderate (MD) or poor (LO) categories.

Although there are no significant (P = 0.347) differences among patches for the measured variables (Table 2), there are significant differences among treatment groups (P = 0.044). Individual one-way ANOVAs for each of the growth and biomass variables in the MANOVA analysis indicate that shoot biomass, total growth, new shoots initiated and new roots initiated are the specific variables measured that reflect significant differences among treatment groups with high power (Table 3). In addition, relatively little to no significant differences in mean internode and shoot height among treatment groups were witnessed (Table 3). However, confidence in this finding is low due to the minimal statistical power in the respective F-tests. In order to meet the multicollinearity assumption of MANOVA, both above ground biomass and internode biomass variables were dropped from the analysis and examined seperately using one-way ANOVAs. Above ground biomass (P = 0.004) and internode biomass (P = 0.002) did differ significantly among treatment groups (Table 3).

TABLE 2. Multivariate Analysis of Variance (MANOVA) test for differences among treatments and patches across all variables measured. Above-ground biomass and internode biomass variables were removed to meet multicollinearity assumption.

Source	Wilk's Lambda	df1	df2	F-ratio	P-value
Treatment	0.306	18	63	1.81	0.044
Patch	0.125	54	117	1.09	0.347

Overall, the plants in any manipulated group (S, F, or SF) displayed a 50% or more decrease in growth and biomass (Fig. 1). The first planned comparison (H_1: C vs. S,F,SF) was significant across all variables examined ($P < 0.01$) with any manipulation of the rhizomes leading to a decrease in the measured variables (Fig. 1). There was no significant difference between treatment types for all variables as indicated by the second planned comparison (H_2: S vs. F). Results of the third planned comparison (H_3: SF vs. S,F) indicate that only the new shoots initiated ($P = 0.03$) and new roots initiated ($P = 0.08$) showed an additive treatment type effect.

DISCUSSION

The fact that severing showed a decrease in growth and biomass compared to the control group supports our hypothesis that *D. digitatum* ramets should show dependency upon parental connections and are therefore highly integrated at the studied hierarchical level of clone structure. A number of other studies on clonal angiosperms have revealed similar decreases in growth and biomass in severed versus non-severed ramets (Ashmun et al., 1982; Charpentier et al., 1998; Hartnett and Bazazz, 1983; Jönsdöttir and Watson, 1997). However, the severity of such disintegration often depends upon the size and age of the ramet severed from the parent clone—the youngest and smallest ramet segments severed often suffer the most damage to vigor (Hartnett and Bazzaz, 1983; Jönsdöttir and Watson, 1997; Pitelka and Ashmun, 1985). However, when comparing the current study using younger, interconnected ramets to those based on older, independent ramets of *D. digitatum* (Lau and Young, 1988) the effects of severing are essentially the same. This suggests that the increased hardship likely incurred by younger ramets is not seen when those ramets are part of interconnected multi-module units.

Jönsdöttir and Watson (1997) classify *Diphasiastrum digitatum* as displaying full integration in large clonal fragments, and in turn place other pteridophytes and angiosperms within this category. Bracken (*Pteridium aquilinum* (L.) Kuhn) is a widely studied economic pest that displays a number of growth characteristics similar to those of *D. digitatum,* although the former is much more aggressive and has a different growth habit. Both are clonal pteridophytes that grow by means of a perennating, indeterminate rhizome bearing fronds or shoots, sometimes occupying large patches of the landscape (Parks and Werth, 1993). Experiments originally aimed at finding a method of controlling bracken

TABLE 3. Results of one-way ANOVA tests for variables significantly contributing to treatment effect in previous MANOVA. Asterisks denote one-way ANOVAs for two variables originally omitted from MANOVA.

Variable Measured	df	MS	F-ratio	P	Power
Above-Ground Biomass (g)*	3	0.28	5.71	0.004	0.82
Internode Biomass (g)*	3	0.57	6.33	0.002	0.86
Shoot Biomass (g)	3	1.69	6.92	0.001	0.89
Total Growth (cm)	3	0.37	4.69	0.009	0.73
Mean Internode Length (cm)	3	0.51	0.10	0.959	0.06
Mean Shoot Height (cm)	3	0.50	2.69	0.066	0.47
Number New Shoots	3	9.90	8.36	0.001	0.95
Number New Roots	3	0.34	5.67	0.004	0.82

have revealed that severing bracken rhizomes does not elicit the same severe effect on plant growth and biomass witnessed in the present and other studies concerning *D. digitatum* (Lau and Young, 1988). In fact, seasonally-timed, repetitive cutting of above and below ground rhizomes, as well as above ground fronds of bracken is needed to significantly affect respiration and assimilate translocation and ultimately reducing the plant's spread within an area (Lowday, 1986; Lowday et al., 1983). This reaffirms the findings that *D. digitatum* is extensively integrated, particularly in comparison to another clonal pteridophyte.

Comparisons between the physiological integration of the mayapple (*Podophyllum peltatum,* L.) and *D. digitatum* are also useful as these two herbs often occupy similar habitats in the eastern deciduous forest and are frequently found growing near each other. Although the *P. peltatum* tends to have a greater degree of lateral spread than *D. digitatum,* both are extensive integrators (Jönsdöttir and Watson, 1997) that reproduce mainly through vegetative processes, and thereby are severely affected by severing treatments that interfere with acropetal transport to younger ramet fragments in particular. However, the severity of severing in mayapple depends upon the size of that ramet fragment severed (Jönsdöttir and Watson, 1997), unlike the current and other studies (Lau and Young, 1988) concerning *D.digitatum* ramets, which display the same reduction in growth and biomass, regardless of ramet size or age. This comparison suggests that *Diphasiastrum digitatum* is dependent upon integration at any scale within a ramet, indicating that integration is an adaptive trait, possibly because of the resource-poor environments it inhabits (Jönsdöttir and Watson, 1997).

Diphasiastrum digitatum ramets in the fertilized treatment group (F) showed declines in biomass and growth patterns (Figure 1) equivalent to the severed treatment group (S) compared to the control group (C). This result does not support our original hypothesis that fertilization will increase the growth and biomass of *Diphasiastrum digitatum*. Upon closer examination of the experimental methods, the nitrogen source within the fertilizer may be a possible explanation. The fertilizer used within the current experiment delivered nitrogen in two forms: ammonium nitrogen (3.91%) and urea nitrogen (6.09%).

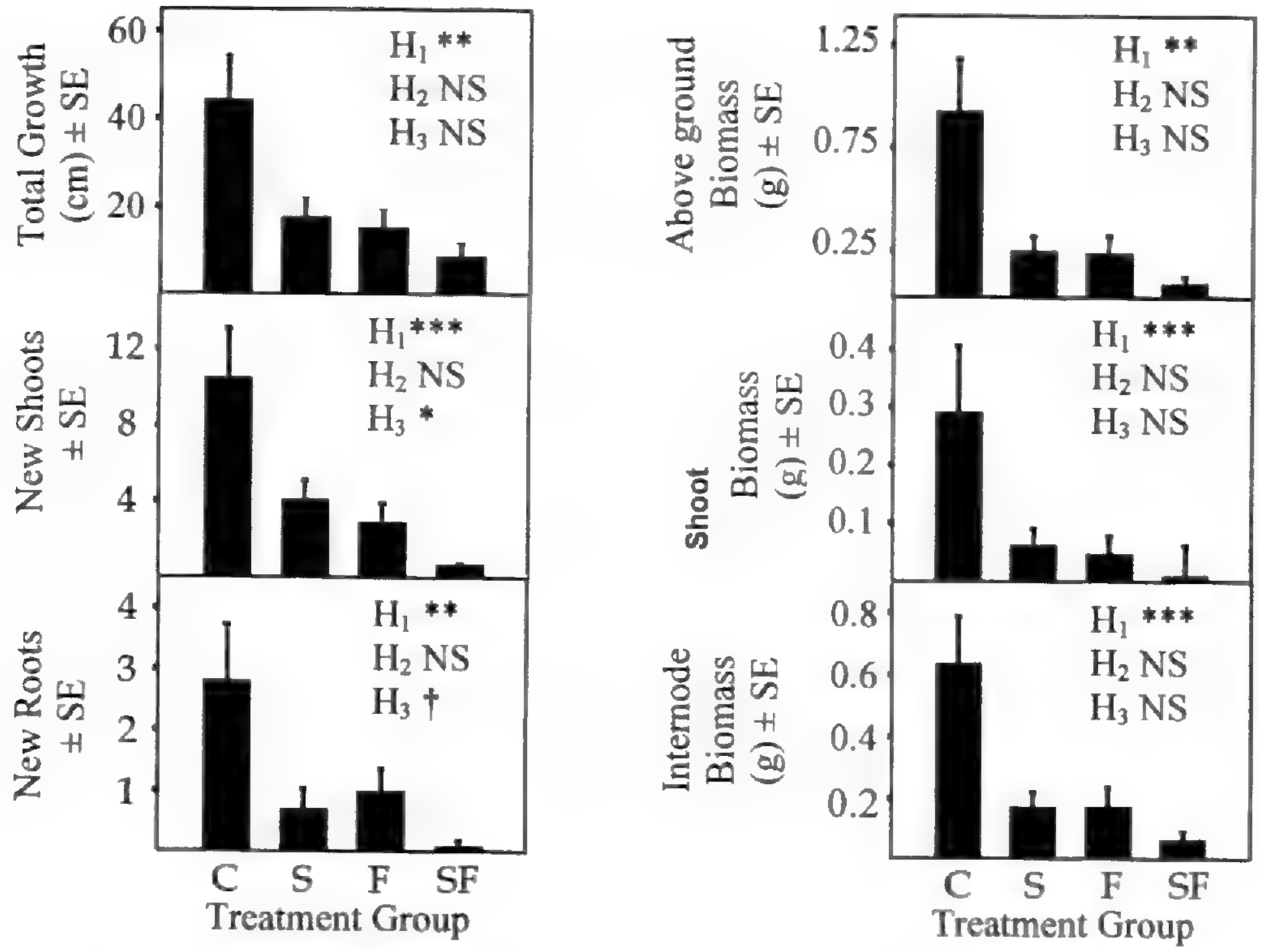

FIG. 1. Mean values (± 1 standard error) of growth and biomass variables under each of four treatment conditions (A = control, B = severed, C = fertilized, D = severed and fertilized). Significance levels for each of the three planned comparisons, (H_1, H_2, H_3), are denoted as such: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, † = $P < 0.1$.

A number of studies suggest that nitrate is the ideal deliverable form of nitrogen in fertilizers, whereas ammonium and urea forms can negatively affect health of some plants, particularly in combination with lower edaphic pH (Feng and Barker, 1992; Magalhäes and Huber, 1989). Such investigations of nitrogen form on crop plant performance have supported that ammonium and urea nutrition stimulate further acidification of the immediate environment, and the foliar production of ethylene and accumulation of ammonium in shoots (Feng and Barker, 1992). The proximate effect of ammonium toxicity witnessed on crop plants includes a decrease in both growth and biomass accompanied by plant wilting, chlorosis, and necrosis of above-ground parts (Cao and Tibbitts, 1998; Kpodar et al., 1992). Similar symptoms of ammonium toxicity were observed in this study for the two treatment groups receiving fertilizer (F and SF).

For the most part, studies concerning physiological integration in lycopod species have focused strictly on documenting water and/or nutrient movement and storage throughout the organism (Callaghan, 1980; Headley et al., 1985; 1988a; 1988b). Consequently, further study is needed to determine the type(s) of ramet transport (water, nutrients, carbohydrates) that is the most crucial to lycopod or clonal plant survival in addition to determining short and long-term plant architectural and physiological responses to a dis-integration event affecting the crucial transport. Such examinations would lend greatly to the

understanding of the presence of clonal plants throughout evolutionary and ecological history.

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Obituary: Joseph A. Ewan (1909–1999)

The American Fern Society lost a longtime member and supporter on the morning of Sunday, December 5, 1999, when Joseph Ewan passed away peacefully at the age of 90. Joe was a fine botanist and plant taxonomist, but also was reknowned as one of the leading botanical historians of our time and a skilled commentator on the literature of natural history and exploration.

Born on October 24, 1909, in Philadelphia, Joseph Andorfer Ewan, lived briefly in Pennsylvania and New Jersey, before moving to California in 1912. In high school, he was influenced strongly by a librarian and teachers in biology and Latin, which in 1928 led him to enroll as an undergraduate at the University of California, Los Angeles and later the University of California, Berkeley, from which he graduated in 1934. He worked at the UCLA library and Los Angeles County Public Library during these years to help fund his studies. Continuing graduate studies at Berkeley allowed him to work as a research assistant under Willis Linn Jepson, but a variety of difficulties, including the birth of the first of three daughters and other financial hardships, forced him to seek more lucrative employment elsewhere. In 1937, Joe accepted his first professional appointment as a lecturer in the biology department at the University of Colorado, in Boulder, where he remained until 1944. There followed a series of appointments, including a year in Colombia with

the U.S. government's World War II *Cinchona* project to develop quinine sources for the fight against malaria, as well as subsequent appointments at the Smithsonian Institution and the U.S. Department of Agriculture. In 1947, Ewan accepted a faculty appointment at Tulane University, in New Orleans, where he remained until his retirement in 1977. In 1972, Tulane awarded him the Ida Richardson professorship in biology, in which he continued in an emeritus capacity following retirement.

During his more than three decades at Tulane, Ewan spent several terms as visiting professor at other schools, such as the University of Hawaii, University of Oregon, and Ohio State University. He also was the recipient of a Guggenheim Fellowship in 1954 to allow him to complete research at the British Museum, as well as receiving a Regent's Fellowship at the Smithsonian Institution in 1984. Although he was unable to realize his earlier dream of obtaining a graduate degree in botany, Ewan was awarded two honorary doctorates, one from The College of William and Mary (1972) and the other from Tulane University (1980). He was a fellow of the Linnaean Society of London and received its Founder's Medal for his contributions to historical studies of natural history. Among his other awards, in 1994 the Missouri Botanical Garden bestowed upon him its highest honor, the Henry Shaw Medal.

While taking a botany course as an undergraduate at UCLA, Joe Ewan met a student of Canadian descent who would change his life immeasurably, Nesta Dunn (1908–) . The two made a perfect couple and were married upon Joe's graduation, in August 1935. Nesta became Joe's partner in all things, working with him on botanical and historical investigations and helping to organize their ever-growing library over the years. Their final project, a bibliographic and biographical commentary on the works of the pioneering American naturalist, Benjamin Smith Barton, will be edited and published in the future by the Missouri Botanical Garden Press.

Joe and Nesta Ewan's interest in the history of natural history led them to assemble huge quantities of correspondence and biographical files. Their fascination with history and naturalists also led to a keen interest in the books themselves, and they acquired a large personal library. These became well known and were frequently consulted by scholars from around the world, much to their delight. The book collection is impressive not only for its size, but also the "special" nature of some of its contents. For example, the copy of William Jackson Hooker's *Genera Filicum* (an 1858 printing, handsomely leatherbound) is one that was presented by the author to his father-in-law, Dawson Turner, and a letter from Hooker and a youthful portrait of him were tipped in by Turner.

After Joe retired from Tulane, there came a time when the future of these collections was in doubt, particularly as there appeared no ideological successor at the university. After investigating various options, the entire collection was sold to the Missouri Botanical Garden in 1986. Arrangements were made for the Ewans to join their treasures in St. Louis and continue research projects with them. At that time the library contained some 4,600 volumes and had to be removed from the facility New Orleans through an upper story win-

dow using a large fork lift. Since arriving in St. Louis, another 600 titles have been added to the library, which continues to be stored as a separate special collection. The Ewans' papers, correspondence, and other biographical files have been inventoried and indexed (Holland, D., M. Riley, and M. Stiffler, eds., *Guide to the Ewan papers,* Missouri Botanical Garden Press, 1997), and were stated by the compilers to occupy some 88 linear feet of space. In 1997, following Joe's stroke, the Ewans decided to lay down their books and moved to a retirement home in Mandeville, Louisiana, near their daughter's family.

Joe Ewan's voluminous writings included more than 425 varied publications. Those prior to 1990 were indexed by Keith Crotz (*Ewaniana: the writings of Joe and Nesta Ewan,* The American Botanist, Chillicothe, IL, 1989) His reviews and commentary of other publications are treasured as much for their eloquent language as for the inciteful opinions, and are treasure troves of reference information pertinent to the covered topics. Ewan's historical works include several books, including the much-cited "Rocky Mountain Naturalists" (University of Denver Press, 1950) and his masterful biography of John Banister (University of Illinois Press, Urbana, 1970). He also edited "A Short History of Botany in the United States" (Hafner Publishing Company, New York, 1969; reprinted 1981), which was published in conjunction with the Eleventh International Botanical Congress, in Seattle. His taxonomic studies focused primarily on the neotropical Gentianaceae and *Delphinium* (Ranunculaceae), but he published on other groups as well. He was also active in floristic work in Arizona, California, and Colorado.

Joe's love of ferns began rather early. He joined the American Fern Society in 1930 and published his first paper in the American Fern Journal in 1931 (21:106–109), entitled "Recent Fern Notes from "Southern California." By our count, Ewan's publications relating directly to ferns total to some 15 titles, but he also included fern species in some of his more general floristic writings. These contributions spanned a breadth of subjects equal to his other writings, including taxonomic, floristic, and historical topics. A good example of Ewan's masterful writing style is his moving biographical tribute upon the death of the famed pteridologist, Conrad V. Morton (Taxon 22:271–274, 1973), another former student of W. L. Jepson's and a longtime professional acquaintance. The Californian species of *Pellaea* and *Polystichum* were among his favorite subjects for taxonomic study, although he made contributions to our knowledge of a number of other groups. Joe was an officer in the American Fern Society for more than a decade, serving as vice president 1940–1947 and as president 1947–1951.

In his long and fruitful career, Joe Ewan witnessed the growth and evolution of the American Fern Society for nearly seven decades, even as he observed the growth and changes in botany and other natural history as a whole. He dedicated his life to the preservation and interpretation of botany and botanists of the past, but his sage council, eloquent writings, and gentle civility have inspired numerous individuals that are shaping botany's present and future. He will be missed.—GEORGE YATSKIEVYCH and DOUG HOLLAND, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.

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American Fern Journal 90(3):91–99 (2000)

The Effect of Spore Density on Germination and Development in *Pteridium*, Monitored using a Novel Culture Technique

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ABSTRACT.—Percentage germination and percentage transition to two-dimensional growth in *Pteridium* (bracken fern) were recorded for spores sown on a solid Phytagel™-based growth medium at known densities of between 3 and 9883 spores mm $^{-2}$. The maximum germination recorded occurred at sowing densities higher than those at which maximum transition occurred. Percentage germination was greatest (43 to 52% of spores sown) at intermediate densities (187 to 2114 spores mm $^{-2}$), being highest (52% of spores sown) at 360 spores mm $^{-2}$. Percentage transition was highest at the lowest densities used. It was concluded that germination and transition have different density optima and that investigations of these two phenomena in ferns should take account of this.

Fern gametophytes are accepted as excellent models for the study of many biological systems. Their utility in the studies of both pure and applied developmental biology and genetics was described by Hickok *et al.* (1987), see also Miller (1968). Their usefulness for the study of developmental selection was illustrated by Klekowski (e.g. 1982). Fern gametophytes have also been used in studies of herbicide tolerance and herbicide mode of action, (Hickok *et al.*, 1987; Keary *et al.*, 2000). However, the density of fern spores on their substrate affects both percentage germination and gametophyte development (see Dyer, 1979 for review). The findings reviewed by Dyer which related to the sowing of spores at known densities on artificial media indicated that percentage germination is inhibited at both high and low densities, yet few fern researchers before or since have specified the spore densities used in their experiments. It follows that fern gametophyte studies would benefit from being conducted at optimal densities for the phenomenon under study; there is a need for quantification.

Two problems arise in attempting to quantify the effects of spore sowing density on germination and transition. Transition is a term used to describe the progression from (1D) filamentous growth to (2D) growth involving divisions in more than one plane, which generates the thallus in most fern gametophytes. The first problem is obtaining an even distribution of spores across the culture medium. Carboxy-methyl cellulose (CMC, manufactured by BDH) has been used to help spread spores evenly over media surfaces (e.g.

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Sheffield *et al.,* 1997). CMC does aid spore dispersal in liquid but does not ensure even distribution across the entire surface of solidified growth media. Even spore distribution can be achieved by spraying using a diffuser (see Dyer, 1979). However, this method only works well on a large scale and is wasteful, an important consideration in work with rare or irregularly fertile species. Given the toxicity of fern spores (Povey *et al.,* 1995; Simán *et al.* 1999; Simán *et al.* 2000), the generation of aerosols containing spores raises health and safety considerations. A second problem concerns counting spores and scoring germination and transition in cultures sown at high densities. At high densities, spores and gametophytes cover one another and make germination difficult to detect.

The aim of this work was to quantify the effect of spore sowing density on germination and transition to two-dimensional growth in *Pteridium.* A new culture technique was developed to allow this.

Methods

Two experiments were conducted under identical conditions, unless otherwise stated: Experiment 1 and Experiment 2. Both experiments used spores of *Pteridium aquilinum* collected in 1990 from northern Majorca, Balearic Islands (Spain). These spores are E.S. Collection Number 196. At the time of collection, rhizome material was also taken. This was planted at the University of Manchester Botanical Grounds where the specimen now forms part of the living collection. Spores were stored in sealed plastic vials at 4 °C after collection and until surface sterilisation in 1999.

Moore's medium (after Moore, 1903), pH 6.5, was used as the growth medium, with the addition of Phytagel™ (manufactured by Sigma) gelling agent (0.2 % weight/volume). Phytagel™ is a gelling agent that forms a solid similar in form to that of conventional agar media. Phytagel™ is distinct from traditional gelling agents in that when agitated, it changes phase from a solid to a liquid. The Phytagel™-based growth medium was autoclaved then allowed to cool to approximately 40°C. In the first experiment (Experiment 1), 100 µl of Phytagel™-based medium was dispensed into each 2.35 mm diameter well microtitre plate (approximate well volume 175 µl). A microtitre plate is a transparent plastic tray, with 96 individual wells). In the second experiment (Experiment 2), 200 µl of Phytagel™-based medium was dispensed into each 6.65 mm diameter well microtitre plate (approximate well volume 370 µl). Microtitre plates were left open until the surface of the medium was dry. Lids were then sealed in place with Parafilm (manufactured by NESCO). All equipment and solutions were autoclaved or purchased sterile. Procedures were conducted in an Envar laminar flow cabinet at room temperature.

Spores were prepared by soaking overnight at room temperature in 15 ml of dH_2O, to which had been added 2 drops of Tween 80 detergent (BDH). Tween 80 acts as a wetting agent and serves to disaggregate clumps of spores. Spore suspensions were then poured into a Sartorius vacuum filter unit into which had been fitted a 0.45µm nitro-cellulose membrane (manufactured by What-

TABLE 1. The spore sowing densities used (spores mm^{-2} of medium surface).

Treatment	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13
Experiment	1	1	2	2	1	2	2	2	2	2	1	2	2
Density	3	35	187	360	538	618	1137	2414	4445	5671	7813	7859	9883

man). The Tween 80 solution was then filtered off from the spores. The spores were then surface sterilised by re-suspending in 50 ml of 5% (vol/vol) aq. sodium hypochlorite (approximately 10–14% available chlorine, BDH) for three minutes. The sodium hypochlorite solution was filtered off and the spores rinsed three times each with 50 ml of sterile dH_2O.

In order to facilitate the inoculation of the growth medium, surface sterilised spores were suspended in a solution of 0.5% (w/vol) CMC (BDH) in sterile dH_2O. This was done by removing the nitro-cellulose membrane from the filter unit and eluting the spores from the membrane using a 1 ml Gilson pipette. In the case of the highest density treatments, inoculation was conducted using a fixed volume of undiluted stock spore suspension. In the case of other density treatments, aliquots were taken from this stock spore suspension and made up to the same fixed volume of inoculum that was used to inoculate at the highest densities. The 2.35 mm diameter microtitre plate wells used in Experiment 1 were inoculated with 10 µl of inoculum. The 6.65 mm diameter microtitre plate wells used in Experiment 2 were inoculated with 20 µl of inoculum. The spore suspension flowed across the surface of the growth medium, distributing the spores evenly.

In Experiment 1, spores were sown at four densities (Table 1). In Experiment 2, spores were sown at nine densities (Table 1). A total of thirteen densities were sown, d1 to d13. For each experiment, four replicate blocks were sown on the same microtitre plate. The experiment was designed so that there was an overlap in the ranges of spores sown in the two experiments. To illustrate the repeatability of the culture technique, one spore density was repeated in both experiments; d11 from Experiment 1 was sown at approximately the same density as d12 from Experiment 2 (see Table 2).

Spores were grown in sealed microtitre plates maintained at 20 ± 2 °C with 12 h light, 12 h dark cycles of illumination for 14 days. Approximate photon flux density was 120 $\mu mol.m^{-2}s^{-1}$.

At the end of the growth period spores were re-suspended using gradual additions of known volumes of 0.5% (w/vol) CMC (BDH) in sterile dH_2O to each treatment until all spores were re-suspended. Gentle fluxing back and forth using a 1 ml Gilson pipette caused the Phytagel™ to change phase from a solid to a liquid, thereby re-suspending the spores and gametophytes that had previously been evenly spread across surface of the solid medium. Each final spore suspension was rendered sufficiently dilute to enable convenient scoring using a Sedgwick Rafter counting chamber and viewed under a Leitz Dialux 20EB binocular compound microscope at ×400 magnification. Final volumes of spore suspension (including the volume of Phytagel™-based

TABLE 2. Mann-Whitney U test of d11 versus d12 for density, germination and transition.

	U-Value	Tied Z-Value	Tied P-Value
Density	51222	0.079	0.9369
Germination	51120	1.271	0.2037
Transition	48861	1.086	0.2775

growth medium) ranged from the equivalent of 0.2 ml to 32.4 ml, depending on spore density.

Following re-suspension, sampling was such that two or three 1 ml aliquots were taken from each treatment final spore suspension, with the exception of treatments with final suspension volumes of less than 2 ml. In such cases, the whole final spore suspension was sampled. Between 266 and 431 one-microlitre counting chamber cells were scored for each treatment sowing density. A total of 4386 one-microlitre counting chamber cells were examined and 7228 spores or gametophytes were recorded. Germination was considered to have occurred with the emergence of a rhizoid.

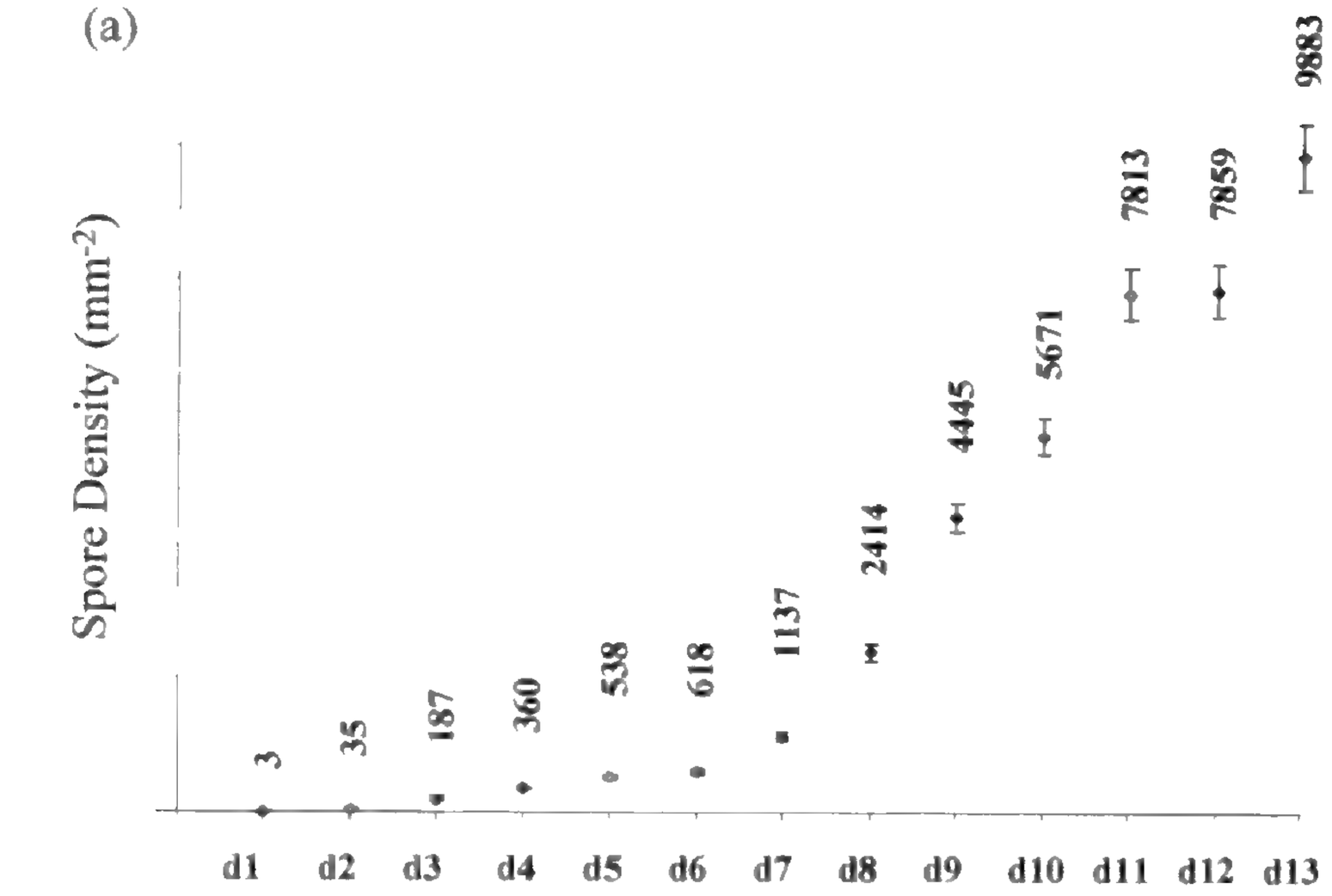
RESULTS

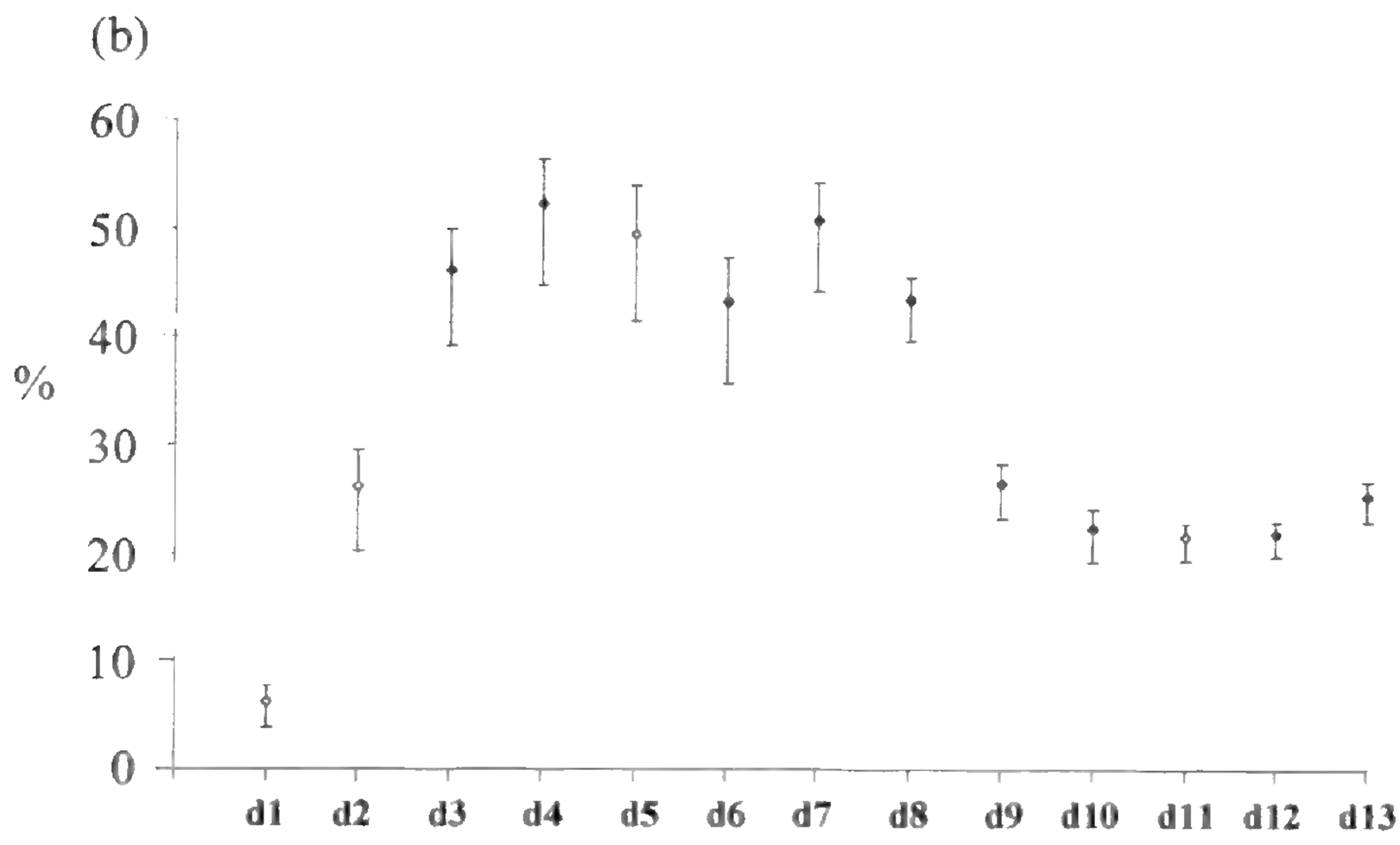
Preliminary analysis of both experiments, using Mann-Whitney U-tests, showed there to be no difference between replicate treatments ($p < 0.01$) and also that there was no significant difference between replicate 1 ml aliquots taken from a given treatment spore suspension ($p < 0.01$). Consequently, values recorded from each counting chamber cell were treated as individual estimates of spore density, germination and transition. Means and confidence intervals were calculated using these estimates.

The data were not normally distributed. Accordingly, spore density estimates were log-transformed and percentage germination and percentage transition data were arc-sin transformed. Ninety five percent confidence intervals were calculated using transformed data according to Wheater & Cook (1999). All statistics were conducted using StatView and graphs produced using Excel.

The spore densities tested are shown in Figure 1 and listed in Table 1. Percentage germination was greatest (43 to 52% of spores sown) at intermediate densities, 187 to 2114 spores mm^{-2} (d3 to d8), being highest (52% of spores sown) at 360 spores mm^{-2} (d4). Percentage transition was greatest (between 0.5 and 1.4% of spores sown) at lower densities (3 to 538 spores mm^{-2}). At densities of 4445 spores mm^{-2} and above, percentage transition did not exceed 0.00005%. Percentage transition was zero at densities of 7813 and 9883 spores mm^{-2}. Far greater variance was observed for transition than for germination.

The two treatments sown at the same density in different experiments, d11 and d12, showed the same proportions of germination and transition, see Table 2.





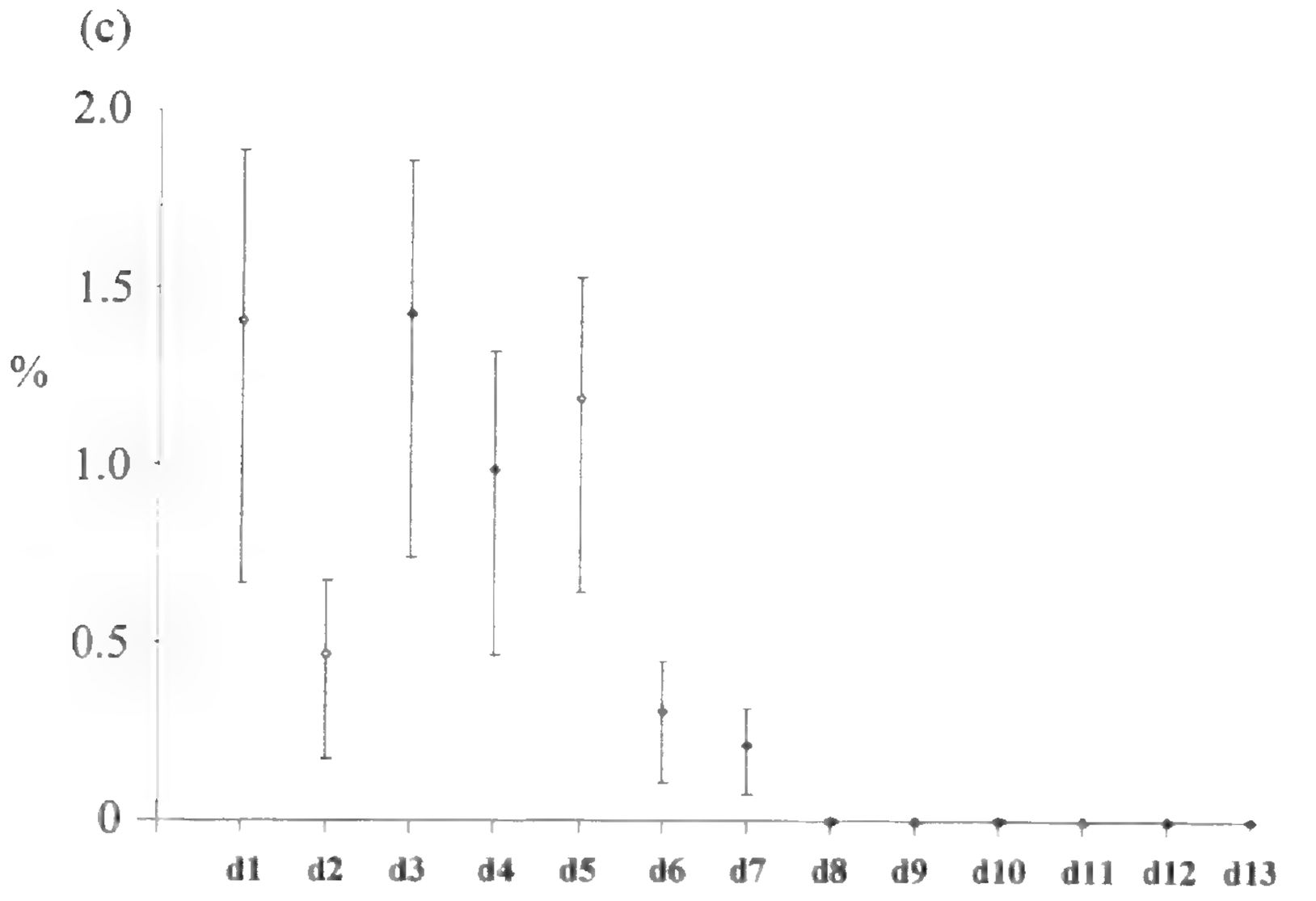


FIG. 1. Sowing density (a), percentage germination (b) and percentage transition (c) for *Pteridium* spores at 13 densities (d1-d13). Open symbols = Experiment 1, closed symbols = Experiment 2. Error bars = 95% confidence intervals.

Discussion

The period of time for which *Pteridium* spores remain viable varies. *Pteridium* spores do not remain viable for long in soil under field conditions (Conway, 1953; Lindsay *et al.*, 1994). In contrast, it seems that spores stored in sealed containers at 4°C may remain viable for many years (Simpson & Dyer, 1999). Our findings show that germination percentage as high as 52% may occur in spores stored in this way for 9 years, this is higher than the percentage germination obtained from 9 year old spores in the study of Lindsay *et al.* (1994). It should be noted that subsequently, transition was low, less than 1.5% in all cases, but the reasons for this were not sought or identified. Sheffield (1996) reported that *Pteridium* spores collected from the same location in different years can differ considerably in percentage germination. It may be that the environmental conditions to which a frond is exposed have an effect on the viability of spores produced by that frond. One environmental variable that is known to influence germination is temperature. Pangua *et al.* (1994), for example, presented evidence temperature influences germination in three species of *Asplenium*.

Percentage germination of *Pteridium* spores collected in the same year from different localities also varies, as does germination in spores stored for different lengths of time, but there is no direct relationship between *Pteridium* spore age and viability (Lindsay *et al.*, 1994; Sheffield, 1996). Given effects of hydration (Lindsay, Williams and Dyer, 1992), growing temperature and storage time on percentage germination, it is clear that storage conditions have an important influence on fern spore percentage germination. It would be valuable if standardised spore conditions could be agreed, or at least that future-authors were encouraged to include a statement describing spore storage conditions. Although germination of many species might be higher following fully hydrated storage (Lindsay *et al.*, 1992) this method is space and time consuming and inappropriate for species, such as *Pteridium*, capable of dark germination. We therefore suggest that pteridophytes spores should routinely be stored dry, in sealed containers at 4°C, in the dark.

Not only are spores inherently variable in terms of germination, but our experiments show that they are also sensitive to growing conditions. The density at which *Pteridium* spores are sown on their substrate affects germination and development. Percentage germination was highest at densities intermediate in the range tested herein. This is in keeping with earlier findings, such as those reviewed by Dyer (1979). Smith & Rogan (1970) found an inhibition of development by gametophytes of *Polypodium vulgare* to increase with spore sowing density. Smith & Robinson (1971) identified three growth factors responsible for the inhibition of cell division in high densities of *Polypodium vulgare*. They concluded that other factors, such as competition for nutrients and low light intensity through mutual shading, may be involved, but were probably important at later stages of development and at extremely high densities. Light is not required for germination of *Pteridium* spores (Lindsay *et al.*, 1994) but is required for photosynthesis to drive gametophyte development.

Smith & Robinson's (1971) investigations into the effect of high spore density were done in liquid growth media. We have expressed our densities on the basis of numbers on the surface of the medium, and undoubtedly depth of medium and speed of diffusion of nutrients into, and exudates from, gametophytes influences results in different vessels or types of solidified medium. We attribute impeded development of *Pteridium* at extremely high sowing densities to both resource limitations and inhibition due to gametophyte exudates. As for storage conditions, discussed above, it is clear that density does have an important influence on germination and growth, and we recommend that future authors standardise, or at least state, their spore sowing densities.

There are ecological implications to density-dependent germination rates. The majority of fern spores probably fall within a few metres of their sporangia but a few travel great distances, see Sheffield (1996) and Simán *et al.* (1999). Our results imply that spores landing close to the parental sporophyte could accumulate to such a high density that germination and gametophyte development are inhibited; the reverse would apply to spores dispersed over great distances. Ecological implications are that lone or sparse spores are unlikely to germinate but if they do, they are likely to undergo transition and therefore go on to produce female gametophytes. This will mean that these female gametophytes will secrete antheridiogens and promote germination in any other spores in the area, and cause those neighbouring spores germinate develop into male gametophytes (Näf, 1958).

The new culture technique employed in this work provides an economical and straightforward means of determining spore density and quantifying germination and transition. The repeatability of the technique was illustrated by the identical germination and transition percentages observed in d11 from Experiment 1 and d12 from Experiment 2, two treatments sown at identical densities in separate experiments.

Laboratory conditions were optimised during the course of preliminary work. In particular, the volume of inoculum used is critical. The volumes presented here were optimised in a laminar flow cabinet at room temperature and are intended only for guidance. Other laboratories may have slightly different environmental conditions, e.g. room temperature, and it may be necessary to fine-tune procedures for local conditions. Preliminary trial and error optimisation is recommended. It was observed during our preliminary experiments that sub-optimal conditions lead to shrinkage of inoculum upon drying, resulting in a distribution of spores which did not extend to the full circumference of the microtitre plate well. In such situations it may still be possible to quantify spore density by estimating the diameter of dried spore inoculum and re-calculating accordingly, using this diameter, rather than that of the microtitre plate well.

In conclusion, *Pteridium* spores stored for nine years at 4°C and sown at 360 spores mm^{-2} had a mean maximum germination rate of 52%. Lower mean maximum germination rates were observed above and below this density. Inhibition of germination at high densities may be due to resource limitation, physical impediment or biochemical inhibition caused by the exudation of

growth inhibitors. Inhibition of germination at low densities may have a biochemical basis; germination may be stimulated by the exudation of growth promoters from other spores. At higher spore densities, the effects of growth inhibitors, physical impediment or resource limitation overcome the effects of growth promoters. The low percentage transition observed could be a consequence of the age of the spores used. Inhibition of transition at high densities may be due to resource limitation, but further work is required to test these last two hypotheses.

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On the Lectotypification of *Danaea elliptica*

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ABSTRACT.—Prior lectotypifications of *Danaea elliptica* are rejected because they did not take into account the one adequate specimen cited by the author of the species. This specimen is chosen here as lectotype, which enables the name *D. elliptica* to continue to be used in its usual sense.

J. E. Smith in Rees (1808), in his treatment of *Danaea,* included *D. alata* J. Smith, *D. nodosa* (L.) J. Smith, *D. simplicifolia* Rudge, and one new species, *D. elliptica* J. E. Smith in Rees. The latter three species are closely related, although *D. simplicifolia* is obviously different in having simple, rather than pinnate laminae. J. E. Smith was careful to distinguish his new species from the related species *D. nodosa,* as his descriptions and excerpts from his notes illustrate:

1. D. nodosa: "Stalk scarcely winged; leaflets linear-oblong, sessile, pointed, nearly entire, covered with capsules to the edge. Radical scales acute.

"Each frond is about four feet high . . . Leaflets . . . six or eight inches long, oblong, almost linear, entire, wavy, with a taper point . . . Capsules . . . [having] each row extending from the main rib very nearly to the margin . . ."

2. D. elliptica: "Stalk scarcely winged; leaflets elliptic-oblong, stalked, pointed, nearly entire, bare of fructification near the margin."

"The fronds are but half as tall as in the former [*D. nodosa*], and their leaflets half as long, though somewhat broader and elliptical. The latter, moreover, stand on short partial footstalks. The rows of capsules scarcely extend to near the edge of the leaflet on which they grow, but are more remarkably separated from each other, at least in a half-ripe state, by a double prominent undulated membrane."

It is clear from the foregoing that J. E. Smith understood the differences between these two species, which have been maintained as separate taxa by virtually all authors since the time of his publication. *Danaea elliptica* is a smaller plant with nodose stipes and fewer, shorter, more elliptical pinnae (Lellinger, 1989, p. 85). Rolleri (pers. comm.) has found additional differences in lamina shape, venation, and perispore surface.

What is less clear is J. E. Smith's citation of material and, consequently, the typification of *D. elliptica.* He cited the pre-Linnaean phrase name given by Sloane (1707, p. 85) and the reference "Sloane Jamaic. v. 1. 85. t. 41, fig. 1." Regarding specimens, he only commented, "Observed by Sloane in Jamaica, from whence the younger Linnaeus obtained a specimen." He did not directly cite any specimens as if he had seen them, which seems strange by modern standards, but was not unusual in his day. According to W. T. Stearn (1988, p. 201), J. E. Smith was very familiar with both the Sloane and Linnean Her-

baria. As it turns out, two specimens are involved, both collected by Sloane in Jamaica.

The specimen from Mt. Diablo, Jamaica (cat. no. 1183, BM-SL) consists of three sterile fronds of *D. nodosa*. One is large and mature, the other two smaller and juvenile. The juvenile fronds, which were drawn for Sloane's illustration t. 41, f. 1, bear some resemblance to *D. elliptica* in size and outline. However, the laminae have more lateral pinna pairs for their size, the pinnae are sessile or nearly so, and the pinna bases are distinctly acute and almost symmetrical, all characters of *D. nodosa*. This specimen may be viewed at http://www.nhm.ac.uk/botany/, in the Sloane database.

Although it seems unlikely, the Mt. Diablo specimen could be a mixed collection, for both *D. nodosa* and *D. elliptica* occur in Jamaica (Proctor, 1985, pp. 61–62). The veins of the juvenile fronds are ca. 1 mm distant with slightly diverging veins in *D. elliptica,* but ca. 0.75 mm distant with strictly parallel veins in *D. nodosa*. These differences can be seen in all but the most juvenile fronds and can be used to make a positive identification of the juvenile fronds.

The specimen from an unknown locality in Jamaica ("1245 Acrostichum *nodosum,*" LINN) was added to the Linnean Herbarium after 1767 (Jackson, 1912, pp. 26, 28), which is consistent with J. E. Smith's statement about it in his description. By 1945, this specimen was not present in LINN (Savage, 1945 p. 186), nor was it on the IDC microfiche of the Linnean Herbarium made a few years later. Fortunately, the specimen is in the J. E. Smith collection (cat. no. 1645.7, BM). Although the specimen lacks a rhizome, it does have one sterile frond with a nodose stipe and three pairs of lateral, ascending pinnae. The pinnae are elliptic-oblanceolate, relatively wide, and taper rather abruptly to an acuminate, entire to subcrenate apex. No centimeter scale is present on the microfiche; I estimate that the pinnae are ca. 13–15 cm long and 3.4 cm wide at their widest point distal to the middle of the pinna. A much smaller, fertile lamina has a partial stipe broken off, presumably above the most distal node, and also three pinna pairs; the middle pair is broken off. The pinnae are oblong-oblanceolate and are estimated to be ca. 3.7–4.8 cm long and 1.1–1.4 cm wide at their widest point distal to the middle of the pinna. According to Savage's handwritten and unpublished catalogue accompanying the IDC microfiche of the J. E. Smith collection, it is labelled "Ind. Occ. H[erb] L. fil.," which can be seen on the microfiche itself. I suppose the specimen was moved from the Linnean Herbarium to the J. E. Smith collection between 1912 and 1945 because it was type material of one of J. E. Smith's own species.

It is clear that J. E. Smith saw both Sloane specimens, as he was familiar with both collections (Stearn, 1988, p. 201). Because his description mentioned a difference in the position of the synangia and because the Sloane specimen then in the Linnean Herbarium was mature and fertile, his description of *D. elliptica* must have been based principally or entirely on that specimen, and the information about the fertile frond exclusively so. The role that the Mt. Diablo specimen played is problematical. On it, an annotation "Asplenium nodosum β" was written between the two juvenile fronds and the larger frond by Solander, Linnaeus' amanuensis. The position of the annotation

might signal Solander's differentiating between the two, or J. E. Smith might have thought that it did. He may have included the reference to t. 41, f. 1 because the drawing somewhat resembles *D. elliptica* or because he thought the two fronds on which the drawing was based were *D. elliptica*. In either case, citing the figure may have been only an attempt to identify it with his species, which was based on the LINN specimen.

Underwood (1902, p. 672), who was the first to choose a lectotype of *D. elliptica,* stated "Type from Jamaica, Sloane, pl. 41, f. 1." This is clearly intended as the illustration, rather than the Sloane specimen from which it was drawn, which he did not cite. According to Curtis (1908, p. 10), Underwood "made repeated visits to the herbaria of Europe for comparison and study of material." Because his monograph (Underwood, 1902, p. 671) cited material from B, K and P, but none from BM, presumably he saw neither Sloane specimen. Underwood probably chose t. 41, f. 1 because J. E. Smith did not directly cite either Sloane specimen or, more likely, because it was the only part of the protologue that he himself had seen. His choice may have even been mechanical, the illustration being the first element in the protologue mentioned by J. E. Smith. Because the specimen underlying the illustration is *D. nodosa,* because the specimen and/or illustration were misidentified, and because the selection may have been mechanical, I believe that Underwood's lectotype should be set aside.

Proctor (1985, p. 62) concluded that the lectotype was Sloane no. 1183, without comment or mentioning Underwood's prior choice. Baksh-Comeau (2000, p. 25) misstated Underwood and also cited the same lectotype. Because Proctor's later lectotype is *D. nodosa* and because he did not show cause to reject Underwood's prior lectotype, I believe this lectotype also should be set aside.

In my opinion, a more logical lectotype is available and should be chosen, which I do here: Locality unknown, Jamaica, *Sloane* (BM-Hb. J. E. Smith cat. no. 1645.7 ex LINN) This choice of lectotype has the advantage of fixing the name *D. elliptica* J. E. Smith in Rees on a specimen inferentially cited by and undoubtedly seen by J. E. Smith, and the additional advantage of allowing the name to be used in its usual sense, rather than becoming a synonym of *D. nodosa.*

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SHORTER NOTE(S)

New Records For The Pteridoflora Of The State Of Chiapas, Mexico.—The State of Chiapas, México, is the second richest State of the country in regard to the diversity of pteridophytes, only surpassed by Oaxaca. Smith (Fl. Chiapas 2:1–370, 1981) treated 609 taxa, Breedlove (*Listados florísticos de México, IV, flora de Chiapas,* Instituto de Biología, UNAM, 1986) cited 675, and Riba et al. (Amer. Fern J. 77:69–71, 1987) added 16 new records for the State, totaling 691 taxa. As a result of intensive field work in the western part of the State, two more species are added, whose presence in Chiapas was already anticipated by Smith (Fl. Chiapas 2:1–370, 1981). They are *Hemionitis levyi* E. Fourn., previously collected in México (Oaxaca) and Central America, and Doryopteris concolor (Langsd. et Fisch) Kuhn var. *concolor,* otherwise known from southern México (Veracruz, Oaxaca) and widely distributed in Central and South America, Antilles, Asia, Africa, Australia, and Pacific Islands.

Hemionitis levyi (*Pérez Farrera 670,* CHIP, UAMIZ) was collected in the Municipality of Jiquipilas, El Campanario, 2.5 km N from Ejido Andrés Quintana Roo, north of the Biosphere Reserve La Sepultura, in tropical deciduous forest with species of Bursera, Mimosa, Plumeria, together with *Cheilanthes, Selaginella, Lygodium,* and several cacti, at 650 m altitude (16°37′N; 93°34′W) in the Central Depression de Chiapas. The specimens were found in small caves in the sandstone, with shallow soil. This species differs from *H. pinnatifida* Baker and *H. palmata* L. in its smaller size (leaves 2.5–16 cm long, 1.7–5 cm wide;

FIG. 1. Pressed voucher specimens of *Hemionitis levyi* (left) and *Doryopteris concolor* var. *concolor* (right), showing general frond morphology. Scale bars = 1 cm.

Fig. 1), simple blade, orbicular to shallowly 3–5 lobed, without proliferous buds, and with short laminar hairs.

Doryopteris concolor var. *concolor* (*Pérez Farrera 442,* CHIP, UAMIZ) was collected in the Municipality of Jiquipilas, Cerro Hojas Moradas, 6 km W Rancho Alpes, in the Biosphere Reserve La Sepultura, in tropical deciduous forest, 1300 m altitude (16°20′30″N; 93°42′30″W), in the Sierra Madre of Chiapas. This taxon (Fig. 1) differs from the other species of *Doryopteris* growing in Mexico, *D. palmata* (Willd.) J. Sm., in its free venation, lack of proliferous buds at the base of the blade, and the basically glabrous petiole.

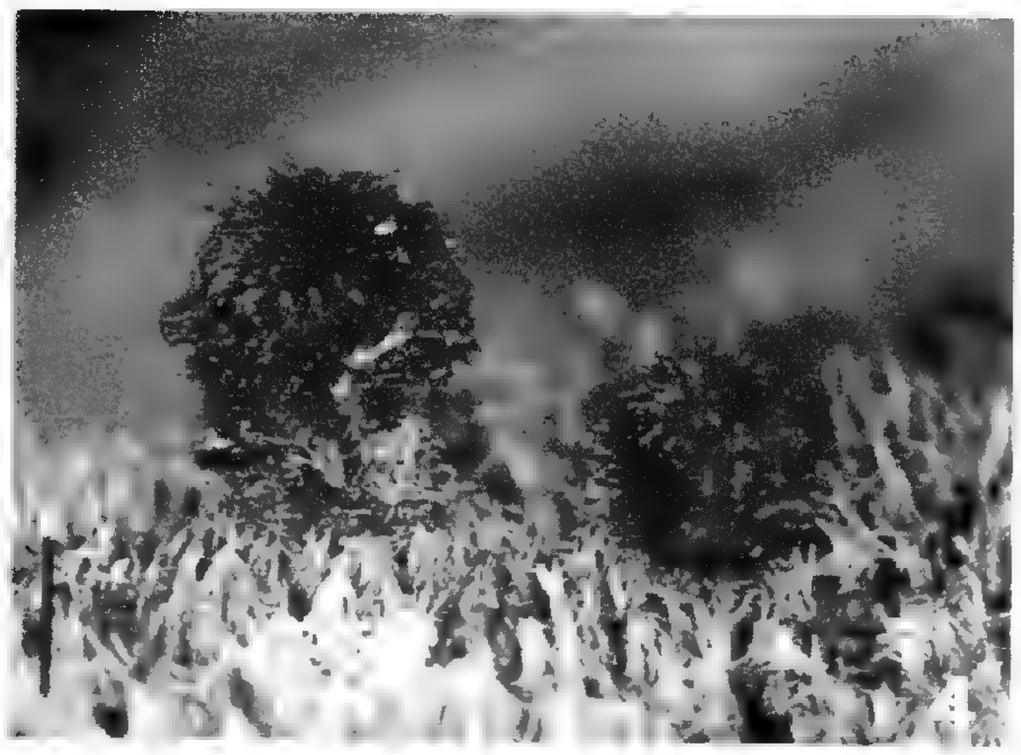
A complete list of the plant species found in the Reserve is in preparation by the junior author.—RAMÓN RIBA (deceased), Universidad Autónoma Metropolitana-Iztapalapa, Ap. Postal 55-535. México, D. F. 09340, and MIGUEL ÁNGEL PÉREZ FARRERA, Instituto de Historia Natural, Depto. de Botánica, Calzada de los Hombres Ilustres s/n, Tuxtla Gutiérrez, Chiapas, México 29000.

Production of Adventitious Buds on the Leaves in *Dicksonia sellowiana*.—The genus *Dicksonia* L'Her. has been successfully propagated through the culture of spores and gametophytes (Constantino et al., *Memorias del I Congreso Nacional sobre Biodiversidad, Cali, Colombia, Dic. 4-7 1994,* p. 303–308, 1995). No secondary or adventitious budding is known for this genus to date. Evidence is presented here for the first time on the production of foliar (petiolar) adventitious buds in *Dicksonia sellowiana* Hook., and on the feasibility of small scale propagation of the species using these buds.

I have been able to propagate *Dicksonia sellowiana* through the culture of adventitious buds that are naturally produced on the basal parts of the petioles, and which become especially noticeable on the old leaves (Figs. 1–2). Such buds are spontaneously produced in some populations inhabiting the West Cordillera of Colombia, at altitudes between 1900 and 2200 m, between Farallones de Cali and La Cumbre (in Depto. Valle). The species has been considered locally threatened for Colombia, according to Constantino et al. (1995) and Instituto Humboldt-Colombia (*Informe Nacional sobre el Estado de la Biodiversidad,* 1998), mainly due to over-exploitation of stems (for construction) and roots (as a substrate for orchid cultivation), but also as a consequence of land clearing.

Two subpopulations have been carefully observed in Depto. Valle: One at Reserva El Refugio-Torremolinos (Mpio. Dagua, 2000 m alt.), the other one at Reserva Himalaya (Mpio. Bitaco, 2050 m alt.). Both localities are currently being protected by private landowners and are located on the continental divide of the Colombian West Cordillera, slightly towards the Pacific side. Voucher material from El Refugio-Torremolinos has been deposited at FMB (*Calderón-Sáenz 103*).

The buds are produced on the adaxial side of the leaf bases, e.g. on the proximal and widest parts of the petioles. Although barely noticeable on young or even on mature leaves (they are covered by a dense layer of hairs), buds



FIGS. 1–5. Adventitios buds in *Dicksonia sellowiana* and *Alsophila erinacea*. 1) Mature *D. sellowiana* at Reserva El Refugio-Torremolinos showing incipient, petiolar buds. 2) Base of an old, detached leaf of *D. sellowiana* depicting thickened, adventitious buds, prior to germination. 3) Mature *D. sellowiana* at Reserva Himalaya, with sprouting petiolar buds; leaves on the right belong to (petiolar) buds that have adhered to the stem. 4) Incipient adventitious stem buds of *Alsophila erinacea*, in a garden specimen at Reserva El Refugio-Torremolinos. 5) Two year-old *D. sellowiana* obtained through bud propagation and planted at Reserva El Refugio-Torremolinos. Arrows point to petiolar buds in *Dicksonia sellowiana*. Scale bars = 5 cm (Figs. 1–3 and 5) or 1 cm (Fig. 4).

become larger in overmature ones and are able to keep growing, independently, on the old, decaying leaves. In some cases, when decaying leaves remain hanging from the stem, adventitious buds tend to adhere to the latter, giving the impression of stem buds, but actually being petiolar buds (Fig. 3). Cultivation of the buds is relatively simple under nursery conditions if humidity is kept high and constant.

Foliar adventitious buds can be produced at the edges of the lamina in a number of unrelated fern genera (see Tryon & Tryon, *Ferns and allied plants with special reference to tropical America,* 1982). Adventitious buds on the latero-adaxial surface of the leaf bases are already known for a tree fern species, namely *Alsophila polystichoides* H. Christ [also known as *Nephelea polystichoides* (Christ) Tryon], as cited by Gastony (*A revision of the fern genus Nephelea,* Contr. Gray Herb., No. 203, p. 86, 1973).

The position of such buds on the petiole suggests that they represent the first one or two pairs of pinnae, which remain undeveloped or dormant while the leaf is still young. Ontogenically, petiolar buds also resemble the aphlebia of some fern species, like those of *Alsophila capensis* (L.f.) J. Sm. (see Tryon and Tryon, 1982).

Initial observations on the spatial distribution of individuals of *Dicksonia sellowiana* in the cloud forest at Reserva El Refugio-Torremolinos suggested that these buds can play a role in the natural propagation of the species, at least in some local subpopulations living near the continental divide, on top of the West Cordillera of Colombia, between 1900 and 2200 m alt. Other populations of this species inhabiting the cooler, montane belt at 2500–3200 m in Colombia (for example at Laguna de la Cocha, Depto. Nariño, and at the East Cordillera in Depto. Cundinamarca) apparently do not produce any adventitious buds at all.

By comparison, adventitious buds in *Alsophila* are usually formed on the stem, near its base or on the upper parts (Fig. 4). Such stem buds can grow to "diageotropic rhizomes", and might also play a role in the natural propagation of some populations of *Alsophila.* This is suggested by the ease with which they detach from the mother plant and by the retained capacity to develop into new plants, as exemplified by cultivated specimens of *Alsophila erinacea* (H. Karst.) D.S. Conant at Reserva El Refugio-Torremolinos (unpublished observation).

Is the capability of producing petiolar buds in *Dicksonia sellowiana* genetically determined or is it triggered by envionmental conditions? More studies are needed for an adequate answer and for a better understanding of the physiological, reproductive-ecological and phylogenetical implications. In the meantime, several two-year old specimens of *Dicksonia sellowiana* obtained from petiolar buds are growing well at Reserva El Refugio-Torremolinos (Fig. 5). Clearly, bud cultivation is another option for the propagation and conservation of this locally threatened and horticulturally desirable species.

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On the Itineraries of Alfred and Alexander Curt Brade in Costa Rica.—Alexander Curt Brade stayed in Costa Rica with his brother Alfred, between 1908 and 1910, commercially collecting orchids but also preparing herbarium specimens, specially pteridophytes, which later constituted the "Herbarium Costaricense" of A. C. Brade. Duplicate series were initially distributed by Goldschmidt and later (but still in 1908) by Rosenstock as "Filices Costaricenses Exsiccate", indicating "*A. & A. C. Brade*" as collectors. Alexander kept intensive correspondence with Rosenstock at least up to 1932. His costarican herbarium had 912 pteridophyte specimens representing 502 species of which 60 were described as new, as well as 27 new varieties and 5 forms. The collection is now part of the Herbarium Bradeanum—HB (Rio de Janeiro). Markgraf (Bot. Jahrb. Syst. 93(1): 1–8. 1973) in his biography of A. C. Brade included some facts about his stay in Costa Rica and a general itinerary map. Complementing this information, their field trips during that period and some major events are here presented in more detail.

1908: February 22, A. C. Brade arrives at Puerto Limón.
February 23, train trip to San José.
March 4, Tablazo; 17, La Palma; 26, idem.
April 10, Carpintera; 25, idem.
June 10–14, Farm of Mr. Zent (11, farm in Chiripó; 12, Barmouth Farm—Atlantic coastal region).
July 1, Tablazo; s.d., Guadalupe (Finca de los Padres); 25, Cartago.
August 1, La Palma; 4, Candelaria; 11, Granadilla; 28, Tablazo.
September 8, Irazú; 17, Tablazo; s.d., Carpintera; s.d., Candelaria.
October s.d., La Palma.
November s.d., Tablazo; 25, Carpintera.
December 2, La Palma; 9, San Domingo; 14, La Verbena; 21–23, Tablazo (Finca Haberl.).
1909: January 14, Rio Grande; 21–23, Volcán Barba.
February 1–27, trip to Guanacaste (Orotina, Finca Schild-Burgdorf, Esparta, rio Baranca, Puntarenas, Pitahaja, Isla de Chira, rio Tempisque, Bebedero, Mojica, Miravalles, Aguas Calientes, Mocote).
March 5, La Palma; 12, Candelaria; 20, La Palma.
April 7, Candelaria; s.d., Barba-Poas; May s.d., Orotina; s.d., Finca Schild—Burgdorf (rio Surubres); s.d., Cartago.
June 16–22, Carrillo (La Palma, Hondura).
July 20, Tablazo.
August 5–8, Turrialba; 18, La Palma; 28, Tablazo.

September 2–22, Finca of the brothers Hundriesser in the Atlantic coastal region, between the rivers Reventazon and Parasmina).
October and November: sick.
December 4, Carpintera; 17, Candelaria.
1910: January s.d., Turrialba; 20, Finca Schild-Burgdorf (rio Surubres).
February 9, Finca Schild-Burgdorf (rio Surubres); 10–13, Cerro Turubales; 26, Granadilla.
March 5, La Verbena; 11, Candelaria; 17, La Palma; 28–31, Juan Viñas, region of the rio Chis; April s.d., Turrialba [14? eruption of Volcán Poas]; 16, Cartago; 22, San Jerónimo de Grecia (region of Zisma, Poas); 25–May 7, trip to Llanuras de San Carlos (Naranjo, Zarcero, Buena Vista, Finca Koschny).
April (see above).
May 1–7, conclusion of trip to Llanuras de San Carlos [7, catastrophic earthquake destroys Cartago]; 10, Cartago, Aguacaliente.
June 2, Tablazo; 12, Turrialba; 22, La Palma.
July s.d., Quassimo; s.d., Finca de los Padres.
August 21, departure from Puerto Limón.

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***Asplenium* ×*alternifolium* in the Black Hills of South Dakota.**—On September 19, 1998, we discovered one individual of *Asplenium* ×*alternifolium* Wulfen on the north side of the Iron Creek drainage ca. 14 air km northeast of the town of Custer, Custer County, South Dakota. This area has numerous large outcrops of the Precambrian Harney Peak granite. The plant was found on the south face of a large rock massif ca. 100 meters in height, on a shaded microsite in a deep crack 30 meters above the ground. The elevation of the site is 1023 m (5400 ft.). Many fronds with sori were present. No other individuals were found in a survey of the massif and other nearby outcrops. The individual was growing vigorously when revisited on July 5, 1999. That year, two additional individuals were found in similar habitat at a second site ca. 6.5 km southwest of the original discovery. Material was sent to Dr. Robbin Moran (NY) for identification; vouchers (Marriott 11782, 11786) with photographs have been deposited at NY and RM.

Asplenium ×*alternifolium* is a sterile hybrid between *Asplenium septentrionale* and *Asplenium trichomanes,* both of which occur at the sites. *Asplenium septentrionale* is common throughout the granitic Central Core region of the Black Hills. *A. trichomanes* is uncommon in the area, and is sufficiently rare to be tracked by the South Dakota Natural Heritage Program. The Black Hills populations most likely are the diploid cytotype, which occurs primarily on acidic rocks, including granites (Moran, Amer. Fern J. 72: 5–11. 1982). The hybrid was first collected in North America in West Virginia (Wagner *et al.*,

Castanea 56: 128–134. 1991). It is considered common in Europe and rare in the eastern United States. The South Dakota locations are disjunct by more than 2000 km from the sites reported by Wagner *et al.*—HOLLIS MARRIOTT, 655 N. Cedar St., Laramie, WY 82072, and JAN AND HERB CONN, HCR 83 Box 93, Custer, SD 57730.

***Dryopteris goldiana* and its Hybrid with *D. celsa* New to Arkansas.**—Wood Ferns (*Dryopteris*) were studied intensively in Arkansas for twenty years (Peck et al., Amer. Fern J. 75:71, 1985; Peck and Peck, Proc. Arkansas Acad. Sci. 42: 74–78, 1988; Peck and Taylor, Proc. Arkansas Acad. Sci. 49:130–137,1995), yet surprises remain to be found in a state with no less than 16 previous lists of its pteridophyte flora. *Dryopteris celsa* (W. Palmer) Knowlton, Palmer & Pollard was discovered in the Ozark National Forest, Sylamore District, in 1992 by Phil Hyatt [*4947.03* (UARK)]. J. H. Peck's efforts in 1997 and 1998 to relocate and inventory that population were unsuccessful. Unfortunately, the specimen's label coordinates were incorrect, being off by six miles to the west. Earl Hendrix relocated the population and told Peck that "more than *D. celsa* is present." A brief visit by Hendrix and Peck on 20 July 199 confirmed that it was a most exceptional woodfern community for Arkansas. We noted three species plus three hybrids of *Dryopteris,* including a species and a hybrid new to Arkansas. Additional specimens, locality data, and habitat observations were gathered by Theo Witsell on 27 November 1999. All specimens are deposited at LRU.

The locality (Merrill Ridge Road Blowing Cave, Baxter Co., AR, T17N, R12W, S30, Norfolk SE Quad.), is located in a remote portion of north-central Arkansas within the Sylamore District, Ozark National Forest. The locality has three special microhabitats for Arkansas, including limestone bed-rock, a breakdown strewn and partially blocked entrance of a blowing-cave, and a hillside seep and stream located on a north-facing toe-slope. These microhabitats occur at the bottom of a forested ravine with 100 m of vertical relief. The associated vegetation is composed of canopy trees such as *Carya* spp., *Nyssa sylvatica, Quercus alba,* and *Quercus rubra,* with understory plants of *Cornus florida, Rhamnus caroliniana, Vaccinium pallidum,* and *Vitis rotundifolia.* The adjacent Stewart Fork stream, cave-cooled spring-seepage water, and cool-blowing air from the cave provide a cool, moist, and moderated local environment.

This is a very protective habitat for ferns of a more northerly habitat and range. The humus-rich soils on the toe-slope supports three species of *Dryopteris,* including five plants of *D. goldiana* (Hooker *ex* Goldie) A. Gray [*Peck 99417* (LRU)]. Adjacent to the seepage area and stream are several dozen plants of *D. celsa* (Palmer) Knowlton, Palmer, & Pollard. *Dryopteris marginalis* (L.) A. Gray occurs throughout the valley at various elevations on or near rock outcrops. This population of *D. goldiana* occurs at the extreme southwestern edge of its range, modestly disjunct about 200 km from outliers in Missouri,

an isolation distance far less than that evident in western Missouri or in northern Minnesota (see Fig. 2, Werth, Syst. Bot. 16:446–461, 1993).

The habitat is moist enough to promote the occasional formation of hybrids. A few plants of each of three hybrids were found: *D.* ×*australis* Small (*D. celsa* × *ludoviciana*) [*Peck 99701* (LRU)], *D.* ×*leedsii* Wherry (*D. celsa* × *marginalis*) [*Peck 99704* (LRU)], and *D. celsa* × *goldiana* [*Peck 99425* (LRU)]. *Dryopteris celsa* × *goldiana* was discovered for the first time in Arkansas. *D* ×*leedsii* Wherry was noted at its second Arkansas location. The Arkansas range *D.* ×*australis* Small, was expanded from its previously known occurrence in the Ouachita Mountain Region to include the Ozark Mountain Region as well. Although not found at this locality, another hybrid might be present in the area, *D.* ×*neowherryi* W. H. Wagner (*D. goldiana* × *marginalis*). All previous reports of *D.* ×*neowherryi* in Arkansas (J. D. Montgomery and E. T. Paulton, Fiddlehead Forum 8:25–31,1981; J. D. Montgomery and D. E. Fairbrothers, New Jersey Ferns and Fern Allies, Rutgers Press, 1992) actually refer to *D.* ×*leedsii*.

With this report, five *Dryopteris* species [*D. carthusiana* (three localities in three counties; *D. celsa* (23 localities in five counties), D. *goldiana* (one locality in one county), *D. ludoviciana* (one locality in one county), and *D. marginalis* (numerous localities in 38 counties)] plus three *Dryopteris* hybrids [*D.* ×*australis* (nine localities in four counties), *D.* ×*leedsii* (two localities in two counties), and *D. celsa* × *goldiana* (one locality in one county)] are known from Arkansas.—JAMES H. PECK, Department of Biology, University of Arkansas at Little Rock, 2801 S. University Ave., Little Rock, AR 72204; C. Theo Witsell, Arkansas Dept. Natural Heritage, Little Rock, AR 72201; EARL HENDRIX, Sylamore District, Ozark National Forest, Mt. View, AR 72560.

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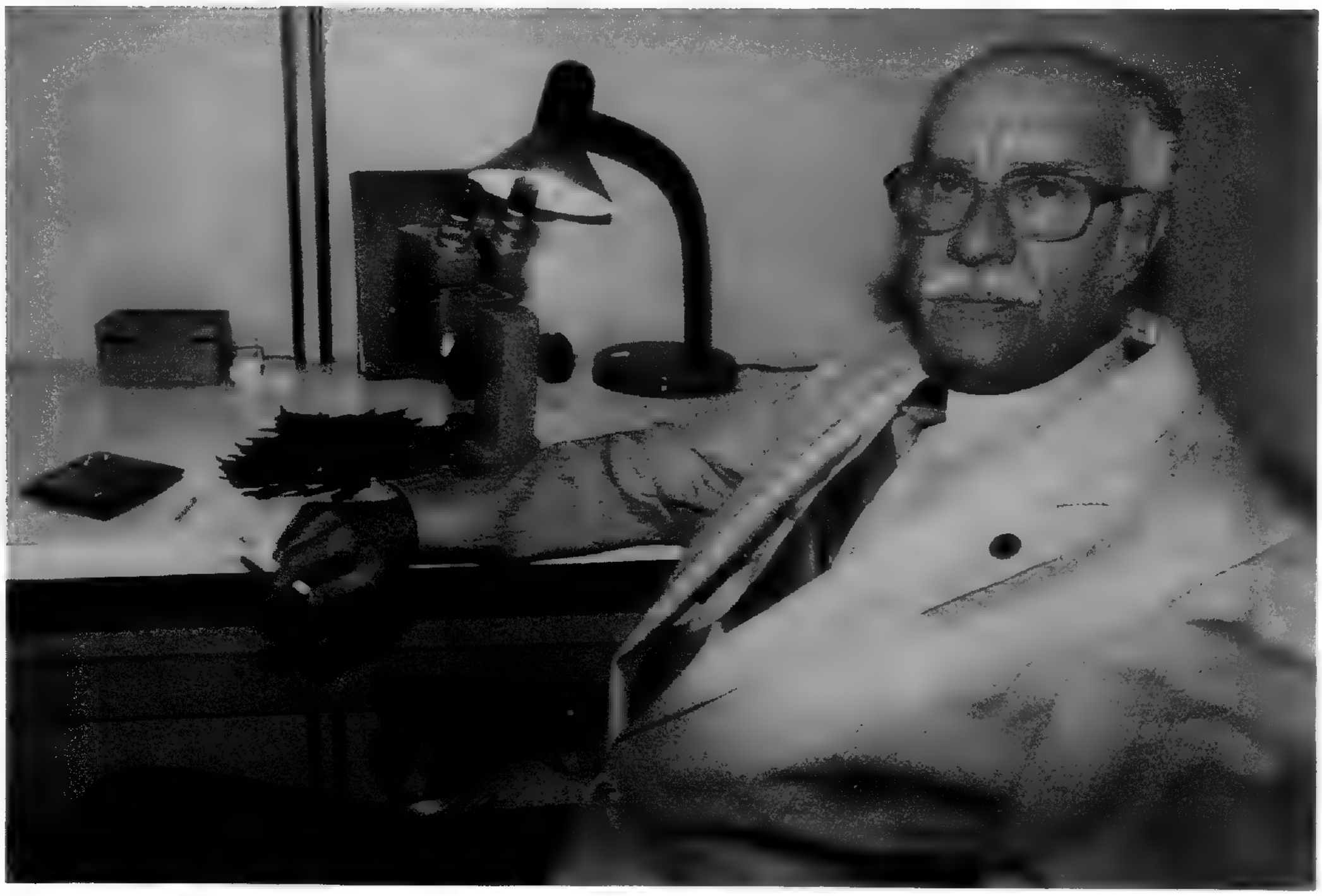
Obituary: Ramón Riba y Nava Esparza (1934–1999)



Ramon Riba was born on April 24, 1934, in Mexico, D. F.; he was Professor of ecology, scientific drawing, scientific photography and botany at the Universidad Nacional Autonóma de México (1955–1991) and Universidad Autónoma Metropolitana-Iztapalapa (1974–1999). His interests in botany included the taxonomy, floristic, conservation, spore banks, bibliography and morphogenesis of ferns.

He earned a John Guggenheim fellowship to work his Ph. D. thesis at Harvard University, for which he wrote a dissertation on "The *Alsophila swartziana* complex (Cyatheaceae)" under the direction of Rolla M. Tryon. He received his Ph. D. in Biology from Universidad Nacional Autónoma de México in 1967.

At the Universidad Autónoma Metropolitana Iztapalapa he served as Chairman of the Department of Biology; he was a foundation member of this University and the Herbario Metropolitano (UAMIZ). He served as President of the Botanical Society of Mexico (1982–1984) and Latin American Association of Pteridology (1998–1999). Dr. Riba was co-editor of Volume 1. Pteridophytes of the Flora Mesoamericana and author of several monographs. He held memberships in the American Fern Society, International Association of Pteridologists, Latin American Association of Pteridology, Mexican Academy of Sci-

ences, Botanical Society of Mexico and National Council for the Teaching of the Biology.

He was very active in research and earned an international reputation as a distinguished pteridologist, having been one of the first people to study this group of plants in Mexico. For these efforts he was named by the Botanical Society of Mexico and by the Universidad Autónoma Metropolitana, as one of their distinguished professors and by the Sistema Nacional de Investigadores in Mexico as a member. His field notebooks and the principal set of his fern collections, are deposited in the herbarium of the Universidad Autónoma Metropolitana Iztapalapa (UAMIZ) and the National Herbarium of Mexico (MEXU).

Ramon Riba also excelled as a teacher and was awarded by the Universidad Nacional Autónoma de Mexico and Universidad Autónoma Metropolitana Iztapalapa for teaching excellence. He has co-authored five books on teaching. Ramon's enthusiasm for teaching in the field was remarkable and he was fun to be in the field with. He could drive several hours nonstop to Oaxaca or Veracruz, and when he arrived, he always knew the best place to eat. He spent a lot of time in the field teaching his students about ferns, and he enjoyed it tremendously when someone discovered the beauty of ferns for the first time.

He was patient with all his students and his office was open to them all day-no appointment necessary. Whether the topic was academic or personal, he always had an encouraging word for everybody, and he would listen carefully to what everyone had to say, usually while drinking a warm black coffee and enjoying a cigarette.

Ramon was highly disciplined, demanding in his work, in the presentation of his papers and correspondence. He wrote and spoke well and frequently liked to explain the meaning of a term or to improve the accuracy of the manuscripts that his students presented to him. He hated the lack of commitment and determination in other people, and he was persistent and tenacious in what he wanted to get accomplished. He motivated co-workers to do the same thing. He would frequently come to our offices unexpectedly to "check our discipline," to make sure we were working. Actually, he just wanted to see how things were going, whether our research and classes were going well.

Ramon had a well-developed sense of friendship and solidarity with his collaborators and pupils, and he did not tolerate any kind of dishonesty on the part of those associated with him. As a man, he was intelligent, talented, creative, imaginative, humble, generous, and a gentile person with all those that he worked with. He was a religious man, faithful to his beliefs, marriage, and family, always giving love to his wife, children, and grandsons. He never forgot to call his wife every afternoon before leaving work. He was an international scientist but he always remembered his roots, the country necessities; he encouraged his students and colleagues to follow up on and conclude their work. He was a man with a lot of character, his professional charisma and leadership converted him into a successful man. He loved Latin American and romantic music and he transmitted this love to his children. He also had a

beautiful voice that we enjoyed on field trips. He was a very special person who liked to make jokes and laugh at them himself.

Ramon Riba passed away on December 13, 1999. That day Ramon insisted that we accompany him to eat, and once there, he began as always to tell jokes and amusing anecdotes. He commented that he did not have mobility in his left arm but that we should not worry about his health. We asked him to go to the doctor immediately, but he refused, saying that he had already made an appointment with his doctor for the following day. Later during the meal we insisted that he go to the doctor, but he insisted that we were worrying about nothing. At about 7:30 that evening, he died in his house of a heart attack. He is survived by his wife María Estela Ramírez de Riba, their sons, Ramón Miguel, Arturo and Roberto Carlos, their daughters, Laura Estela and Alejandra, three grandchildren, and a sister María Eugenia. We were privileged to count ourselves among his doctoral students (at Universidad Nacional Autónoma de México) and we have been "in touch with the divine's ray" as he said to all who loved and worked with ferns.

Ramon is commemorated by the species *Callipteris ribae* Pacheco & R. C. Moran and *Bursera ribana* Rzed. & Calderón.

Ramon has not left; people that knew him and had the privilege to know him and to live next to him for more than eight hours of work a day all knew what he told us:

Everything is all right . . .
The death is not anything, it does not matter
I have only slipped to the next room . . .
There is an absolute and intact continuity
What is death but an accident without importance?
Why must I remain outside of your mind
Only for not being visible?
I am not but waiting for you, for an interval,
In some place, very close, to the turn of the corner,
Everything is all right . . .

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Survival of Chlorophyllous and Nonchlorophyllous Fern Spores Through Exposure to Liquid Nitrogen

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ABSTRACT.—Thirty-three species of ferns with nonchlorophyllous spores and five species with chlorophyllous spores were studied in regard to their ability to survive exposure to liquid nitrogen (LN). Air dried spores showed no inhibition of germination after LN exposure when planted on soil or growth medium. Spores of three species that were stored for 75 months either at 4°C, −20°C, or in LN showed no decrease in viability over that time, and spores of four other species were maintained successfully for 52 months in LN. Fresh chlorophyllous spores that were air dried, dried over silica gel, or prepared with the encapsulation dehydration procedure also showed good survival through desiccation and LN exposure. Spores of *Osmunda regalis* germinated well after 18 months of LN storage. These results indicate that both nonchlorophyllous and chlorophyllous spores are candidates for long-term germplasm storage at low temperatures, including storage in LN.

Ex situ preservation of spores, particularly of rare species from threatened habitats, can be an important supplement to the maintenance of pteridophyte species in the wild. Many species of homosporous ferns produce nonchlorophyllous spores that can be stored for relatively long periods of time, although they can eventually lose viability in storage (Beri and Bir, 1993; Camloh, 1999). It has also been shown that hydrated spores of some of these species can remain viable in the soil spore bank for a number of years, and moist storage of spores ex situ has also been effective (Lindsay *et al.*, 1992; Dyer, 1994). Other species of ferns and fern allies produce chlorophyllous spores, which generally have limited viability, even though they may survive desiccation (Lloyd and Klekowski, 1970).

Low temperature storage has been shown to improve the longevity of dry seeds (Dickie *et al.*, 1990), and lower temperatures improved the survival of spores of the tree fern, *Cyathea delgadii* during two years of storage (Simabukuro *et al.*, 1998). Cryopreservation, or storage at −196°C in liquid nitrogen (LN), has the potential for maintaining viability in living tissues over long periods of time. Many seeds can survive direct exposure to LN (Stanwood, 1985; Pence, 1991), and spores of the endangered tree fern, *Cyathea spinulosa* have been successfully germinated after cryopreservation (Agrawal *et al.*, 1993).

In order to examine the possibility of extending cryopreservation to other species of ferns, spores of several nonendangered chlorophyllous and nonchlorophyllous species were tested for their ability to withstand LN exposure, and storage in LN was compared with other low temperature storage methods. Results from tests with these common species should provide direction for

future work with rare or endangered germplasm, which is often in limited supply.

Materials and Methods

Spores were obtained from a variety of sources, including from the American Fern Society Spore Exchange and from fronds collected in northwest Trinidad, at the Krohn Conservatory (Cincinnati) and at the Cincinnati Zoo and Botanical Garden.

When fronds were collected, they were air dried for several days under ambient conditions in the laboratory in paper envelopes, with spores collected from the envelopes and by scraping the fronds. Samples of the spores were then transferred to 2 ml polypropylene cryovials and immersed directly into LN. After 1 hr, the vials were removed and placed on the benchtop to warm at ambient temperature for 20 min.

LN exposed and control nonchlorophyllous spores of 33 species were tested for germination using one of the two following methods.

1) Spores of some species were sown on moist sterile soil (Metro Mix 250) in Magenta boxes or baby food jars with Magenta caps, and incubated at 26°C under CoolWhite fluorescent lights in a 16/8 hr light/dark cycle.

2) Spores of other species were germinated in vitro. Spores were wrapped in a small package made from Whatman No. 1 filter paper, and surface sterilized in a 1:20 dilution of commercial sodium hypochlorite for 5 minutes, followed by two rinses in sterile, ultra-pure water. The spores were then blotted onto medium consisting of half-strength Linsmaier and Skoog (1965) (LS) medium with 1.5% sucrose and 0.22% Phytagel (Sigma Chemical Co.), in 60 × 15 mm disposable petri dishes, approximately 15 ml/dish.

For longer storage, nonchlorophyllous spores of three species were placed in multiple cryovials and stored at three temperatures: LN (−196°C), −20°C, and 4°C. The cryovials stored at −20°C and 4°C were placed inside 20 ml borosilicate scintillation vials with plastic screw caps, containing 2–2.3 g of silica gel. Spores from the three storage temperatures were sampled at 7, 13, 34, and 75 months by germinating on soil, as described above. Spores from four species collected in Trinidad were also cryostored in LN.

Chlorophyllous spores of five species were also tested. These were obtained either from the Spore Exchange or from plants growing at the Cincinnati Zoo and Botanical Garden. In most cases these were air dried, as for the nonchlorophyllous spores.

However, *Osmunda regalis* spores which were collected from plants grown at the Cincinnati Zoo and Botanical Garden were tested using two other procedures. One portion of the spores was dried in a desiccator with silica gel overnight, placed in a cryovial, and frozen and thawed as described above. The spores were then placed in a 100% humidity chamber for 3 hours, and surface sterilized and germinated in vitro as described above. A second portion of the spores was first surface sterilized and then encapsulated in alginate according to the procedure of Fabre and Dereuddre (1990). The spores were

blotted into a 3% solution of low viscosity alginic acid (Sigma Chemical Co.) and the spore suspension was added dropwise to a 100 mM solution of calcium chloride, where the drops gelled and formed alginate beads containing the spores. After 20 min the beads were transferred to a solution of LS medium containing .75M sucrose and incubated on a gyratoray shaker, 125 rpm, for 18 hours. They were then dried on filter paper under the air flow of a laminar flow hood for 4 hours, transferred to 2 ml polypropylene cryovials, immersed directly into LN, and maintained there for 1 hour. The beads with spores were warmed at room temperature for 20 minutes and then transferred to half-strength LS medium for rehydration and growth. Some dried beads were also put into long-term LN storage, and a sample was removed after 18 months for growth and evaluation.

Only a qualitative evaluation of spore germination was made, both on soil and in vitro, although an attempt was made to maintain approximately equal amounts of control and LN exposed spores. Positive spore germination was recorded if any germination was observed. With the nonchlorophyllous spores, no great differences in the rate of germination were observed between control and frozen spores of any species, as determined by gross observation. With chlorophyllous spores, a distinction was made between the germination of many spores and the germination of one or only a few spores, as indicated in Table 3.

RESULTS

The germination of air-dried nonchlorophylous spores exposed to LN was equivalent to that of nonexposed spores for the species tested (Table 1). Germination rates varied between species, as determined by visual examination of the germination of similar amounts of spores, but there were no obvious differences between LN exposed and control spores of the same species. In the case of three species, there was no germination from either control or LN exposed spores.

There was also no apparent difference between the germination of spores of three species stored at 4°C, −20°C, or in LN, after more than six years of storage (Table 2). Four species from Trinidad (*Adiantum tenerum, Cyclopeltis semicordata, Macrothelyptris torresiana,* and *Tectaria incisa)* were also placed into long-term LN storage and germinated well after 52 months of storage.

Chlorophyllous spores of five species were also tested (Table 3). There was no germination observed for *Blechnum nudum, Matteuccia struthiopteris,* or *Osmunda cinnamomea* spores, that were obtained after storage at the Spore Exchange for several months. With *Onoclea sensibilis* spores obtained from the Spore Exchange, only one spore germinated in the nonexposed samples and none in the LN exposed sample, suggesting a low viability of the spores overall. However, when fresh spores of *O. sensibilis* were collected locally and tested, there was germination of the dried spores, both with and without LN exposure. Similarly, when spores of *Osmunda regalis* were obtained from a nonlocal source after about a year of dry storage, there was very little germi-

TABLE 1. Survival of 33 species with nonchlorophyllous spores through LN exposure after air drying; germination = +; no germination observed = −; contaminated, germination could not be observed = C. Spore sources: K = Krohn Conservatory; SE = Spore Exchange; T = Trinidad collection; Z = collection at the Cincinnati Zoo and Botanical Garden.

Species	Source	Germination	
		Control	LN expose
Germinated in vitro			
Adiantum caudatum L.	K	C	+
Adiantum tenerum Sw.	K	+	+
Adiantum trapeziforme L.	K	+	+
Cibotium glaucum (Sm.) Hook & Arn.	K	+	+
Cyrtomium falcatum (L.f.) C. Presl	K	+	+
Davallia fejeensis Hook.	K	+	+
Drynaria quercifolia (L.) J. Sm.	K	+	+
Phlebodium aureum (L.) J. Sm.	K	+	+
Germinated on soil			
Adiantum tenerum Sw.	T	+	+
Adiantum tetraphyllum Humb. & Bonpl. ex Willd.	T	+	+
Asplenium ruta-muraria L.	SE	+	+
Asplenium platyneuron (L.) Britton, Sterns, & Poggenb.	Z	+	+
Athyrium filix-femina (L.) Roth	Z	+	+
Athyrium thelypteroides (Michx.) Desv.	Z	+	+
Bolbitis sp.	K	−	−
Cibotium schiedei Schltdl. & Cham.	K	+	+
Cyathea arborea (L.) Sm.	SE	+	+
Cyclopeltis semicordata (Sw.) J. Sm.	T	+	+
Cyrtomium falcatum (L.f.) C. Presl.	Z, K	+	+
Cyrtomium fortunei John Sm.	Z	+	+
Dryopteris carthusiana (Vill.) H. P. Fuchs	Z	+	+
Dryopteris celsa (W. Palmer) Small	SE	+	+
Dryopteris clintoniana (D.C. Eaton) Dowell	SE	+	+
Dryopteris goldiana (Hook.) A. Gray	Z	+	+
Dryopteris marginalis (L.) A. Gray	Z	+	+
Macrothelyptris torresiana (Gaud.) Ching	K, T	+	+
Nephrolepis sp.	Z	+	+
Phegopteris connectilis (Michaux) Watt	SE	−	−
Polystichum acrostichoides (Michx.) Schott	Z	+	+
Polystichum aculeatum (L.) Roth	Z	+	+
Polystichum braunii (Spenn.) Fée	Z	+	+
Polystichum tsus-simense (Hook.) J. Sm.	K	+	+
Pteris sp.	K	+	+
Rumohra adiantiformis (G. Forst.) Ching	K	−	−
Tectaria incisa Cav.	T	+	+

nation with or without LN exposure. However, when locally collected spores were processed immediately after collection, there was good survival through drying and LN exposure, using either drying over silica gel or the encapsulation dehydration procedure. In addition, fresh spores of *O. regalis* that were cryostored using the encapsulation dehydration procedure retained good viability after 18 months of LN storage (Table 3).

TABLE 2. Survival of nonchlorophyllous spores of three species for up to 75 months at three storage temperatures.

Species	Storage time (months)	Storage temperatures		
		4°C	−20°C	−196°C
Pteris sp.	7	+	+	+
	13	+	+	+
	34	+	+	+
	75	+	+	+
Cyrtomium falcatum	7	+	+	+
	13	+	+	+
	34	+	+	+
	75	+	+	+
Polystichium tsus-sinense	7	+	+	+
	13	+	+	+
	34	+	+	C
	75	+	+	+

DISCUSSION

These results demonstrate that dried spores from a number of species of ferns can survive exposure to liquid nitrogen. In addition, dry, nonchlorophyllous spores of four species survived LN storage for 52 months, and three other species survived well for over 75 months when kept at either 4°C, −20°C, or −196°C (in LN). Chlorophyllous spores of *Osmunda regalis* maintained viability in LN for at least 18 months.

It is known that nonchlorophyllous spores of many species can be maintained in the dry state at room temperature for a number of years, or even decades (e.g., Windham *et al.,* 1986). Viability does decrease over time, and this loss has been correlated with a loss of protein, sugar, and amino acids in *Pteris vittata* (Beri and Bir, 1993). Hydrated storage has also been explored and has been shown to maintain viability longer than dry storage with spores of several species (Lindsay *et al.,* 1992). Spores of *Cryptogramma crispa* showed a decreased rate of germination when subjected to freezing at −18°C, although the degree of moisture in the spores was not determined (Pangua *et al.,* 1999).

Nonchlorophyllous spores are similar to orthodox seeds, which can be dried and stored for a number of years. The longevity of orthodox seeds is extended proportionately with a decrease in the storage temperature, and such seeds have traditionally been stored at 4°C or −20°C. Dried orthodox seeds are also generally tolerant of LN storage, which may extend longevity significantly (Stanwood, 1985; Pence, 1991). Nonchlorophyllous spores appear to have a similar tolerance for cryostorage, and, in the case of three species, there was no apparent difference in the germination of spores stored at 4°C, −20°C, or in LN for up to six years. More detailed studies are needed to determine if there are differences in the rate or the quality of germination after longer storage times, but all three temperatures should extend the storage life of nonchlorophyllous spores well beyond the mean of 3 years at room temperature, determined by Lloyd and Klekowski (1970).

TABLE 3. Survival of spores of five chlorophyllous species through LN exposure, prepared either with air drying, drying under the air flow of the laminar flow hood or with the encapsulation/pretreatment/dehydration procedure (Fabre and Dereuddre, 1990). Spore sources: SE = Spore Exchange; Z = collection at the Cincinnati Zoo and Botanical Garden. Germination of many spores = +; germination of one or only a few spores = (+); no germination observed = −.

Species	Source	Treatment	Time in LN	Survival
Blechnum nudum (Labill.) Matt ex Lerss.	SE	Air drying	0 hr	
			1	−
Matteuccia struthiopteris (L.) Tod.	SE	Air drying	0	−
			1	−
Onoclea sensibilis L.	SE	Air drying	0	(+)
			1	−
	Z	Air drying	0	+
			1	+
Osmunda cinnamomea L.	SE	Air drying	0	−
			1	−
Osmunda regalis L.	SE	Air drying	0	−
			1	(+)
	SE	Air drying	0	(+)
			1	(+)
	SE	Air drying	0	−
			1	−
	Z	Drying over silica gel	0	+
			1	+
	Z	Encapsulation/dehydration	0	+
			1	+
			0	+
			1	+
			18 mos	+

In contrast with nonchlorophyllous spores, chlorophyllous spores germinate rapidly, within an average of two days, and have a mean storage life of only 48 days (Lloyd and Klekowski, 1970), although there is evidence that in the moist soil spore bank they can survive at least 10 months (Dyer and Lindsay, 1992). No more than 5% viability was reported for *Osmunda regalis* and *O. claytoniana* spores maintained for 3.5 years at 4°C (Stokey, 1951). The limited viability of dry spores of *Equisetum hyemale* has been shown to result from damage to photosynthetic function that occurs within two weeks of storage (Lebkuecher, 1997), although their longevity has been increased by storage at 4°C and at −70°C (Lloyd and Klekowski, 1970; Whittier, 1996). Of the chlorophyllous species studied here, spores that had been stored for a year had lost most or all viability by the time of testing, whereas fresh spores were viable and maintained viability through desiccation and LN storage. In the case of *Osmunda regalis* spores, viability was maintained for at least 18 months in LN storage, and it is likely that this may extend for even longer periods of time. Freeze storage of dried chlorophyllous spores might be used by spore banks to maintain viability in these species over longer periods of time, al-

though further research is needed to determine whether conventional freezing temperatures are equally as effective as storage in LN.

Chlorophyllous spores that can tolerate desiccation but have limited viability under natural conditions resemble seeds classified as sub-orthodox (Bonner, 1986). Seeds of species of *Populus* and *Salix,* for example, can tolerate drying, but are generally short-lived either as dried or hydrated seeds. However, when they are dry, they can also tolerate exposure to freezing temperatures (−20°C and LN), and when stored under these conditions their longevity is increased compared with storage at 4°C (Pence, 1996, and unpublished). LN storage should offer a similar advantage for the long-term storage of the generally short-lived chlorophyllous spores.

The results of these studies suggest that low temperature storage, including cryopreservation, is an option for the storage of both nonchlorophyllous and chlorophyllous fern spores. Although each species is unique, these results with nonendangered species provide an indication that there is a high probability of success with spores from a variety of species, including rare or endangered species, for which spores may be in limited supply. Low temperature spore banking could be used to supplement traditional spore storage, as well as sporophyte collections, soil spore banks (Dyer, 1994), and LN storage of fern gametophytes (Pence, 2000) for the maintenance of rare or endangered species. For the long-term ex situ preservation of rare or endangered germplasm, low temperature storage, including cryopreservation, can be used as one tool in an integrated approach to fern species preservation.

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Morphology of Gametophytes and Young Sporophytes of *Sphaeropteris lepifera*

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Abstract.—*Sphaeropteris lepifera* is one of the largest tree ferns in Taiwan. On average, it produces 50.7 sporangia per sorus, and 64 spores per sporangium. Spore germination, after 2 years of storage at 4°C was over 95%. The pattern of spore germination was "Cyathea-type", and the gametophytes exhibited mainly Drynaria-type development with occasional Adiantum-type development. Typical gametophytes were heart-shaped but had the potential to elongate and become elliptical. Multicellular hairs on the dorsal and ventral surfaces of the midrib cushion increased in size and changed shape with age. They were usually uniseriate when young, and became multiseriate with age. Gametophytes initiated antheridia about 1 month after spores were sown, and did not become hermaphrodites until 7 weeks later. During ontogeny, the gametangial sequence was from the male to hermaphroditic. Antheridia formed on the wings of the ventral and dorsal surfaces of gametophytes. The wall of each antheridium was composed of 5 cells. Archegonia appeared on the cushion of the ventral surface of gametophytes. Some gametophytes initiated clone-formation through vegetative regeneration. When sufficient water was provided, young sporophytes began to appear 12 weeks after spores were sown. The first fronds were midribless. The uniseriate, multicellular hairs on young sporophytes were similar to those on gametophytes.

Gametophyte morphology, including mature forms, spore germination, and types of early development, trichomes, and gametangia, have been used to characterize fern taxa (Stokey, 1918, 1930; Atkinson and Stokey, 1964; Nayar and Kaur, 1971; Atkinson, 1973). These characteristics provide information relevant to fern phylogeny (e.g., Nayar and Kaur, 1971) and the ecology and reproductive biology of different species (Chiou and Farrar, 1997a, b; Chiou *et al.*, 1998; Masuyama, 1975a, b, 1979).

In the Cyatheaceae, spore germination is Cyathea-type and gametophyte development is Adiantum-type or nearly Drynaria-type (Nayar and Kaur, 1971). Gametophytes are long-lived (2–6 years) and tend to elongate slightly with age. The multicellular, bristle-like hairs that appear on gametophytes in this family indicate the Cyathaceae is phylogenetically close to the Loxsomaceae (Atkinson and Stokey, 1964). Antheridia and archegonia typically occur on the same gametophytes, with some vigorous gametophytes being strictly archegonial (Stokey, 1930). Apogamy was reported in some species in the Cyatheaceae

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TABLE 1. Spore sources, usage, culture medium and storage period.

Spores	Location	Observation	Medium	Stored (years)
Huang 25	Wulai, 370 m	sporangium and spore number	—	0
Huang 26	Wulai, 370 m	sporangium and spore number	—	0
Huang 27	Yamingshan, 250 m	sporangium and spore number	—	0
Huang 25	Wulai, 370 m	germination rate	agar	0
Chiou 14495	Wulai, 150 m	germination rate	agar	2
Chiou 14787	Hsinihsien, 200 m	germination rate	agar	1
Huang 25	Wulai, 370 m	gametophyte morphology	peat	0
Huang 26	Wulai, 370 m	gametophyte morphology	peat	0
Huang 27	Yamingshan, 250 m	gametophyte morphology	peat	0

(Stokey, 1918), but Walker (1966) reinvestigated two of these species and found them to be sexual diploids.

Sphaeropteris lepifera (Hook.) Tryon (Cyatheaceae) is the largest, and one of the most abundant, species in this family in Taiwan. Wang *et al.* (1977) described its sporophyte morphology, scales, stomata, epidermal cells, and the anatomy of the stipes and trunks. However, except for the gametangial sequence (Masuyama, 1975b), characteristics of the gametophyte of *S. lepifera* have not been described.

The number of spores per sporangium has also been used in the systematic classification of ferns (Sen, 1964; Gastony, 1974). Spore viability, gametangial sequence, and gametophyte growth habits are important components of fern reproductive biology (Chiou and Farrar, 1997a; Chiou *et al.*, 1998). Complex interactions among gametophytes in multispore cultures may affect the sequence of gametangial development in individual plants. Detailed analysis of sexual status, in relation to the age and size of gametophytes, is essential for determining the most probable breeding system employed by *S. lepifera* (Chiou and Farrar, 1997b; Chiou *et al.*, 1998). The present study explores spore production, spore viability after cold storage, gametangial sequence, morphology and growth habits of the gametophytes and the morphology of young sporophytes of *S. lepifera*.

MATERIALS AND METHODS

Spores were collected from sporophytes growing on mountain slopes in northern Taiwan (Table 1). Voucher specimens were deposited in the Herbarium of Taiwan Forestry Research Institute (TAIF). Spores were stored in the refrigerator at 4°C.

The number of sporangia per sorus (from 50 randomly sampled sori per plant) and the number of spores per sporangium (from 50 randomly sporangia per plant) were estimated from three sporophytes (Table 1).

Spores were sown on peat or agar medium. The viability of fresh spores and spores stored at 4°C for one or two years was assessed (Table 1). Spores were sterilized with 2.5% Clorox for 5 min, rinsed with sterilized water for 10 min,

TABLE 2. Mean germination (%) of fresh and stored (4°C) *S. lepifera* spores sown on agar medium.

Storage (years)	Days after sowing spores			
	5	7	9	11
0	0.0 (0)	81.2 (7.2)	96.7 (4.0)	99.0 (1.2)
1	35.8 (26.0)	97.9 (2.0)	98.7 (1.5)	98.9 (1.5)
2	1.3 (1.9)	29.7 (16.8)	84.0 (7.4)	95.4 (4.0)

[1] Standard error in parentheses.

and then sown on 1.1% agar-solidified medium containing Gantt and Arnotts' (1965) mineral nutrients and one drop of 1% $FeCl_3$ per 400 ml of medium. The pH of the media ranged from 5.0–5.2. Germination rates were estimated from six agar-medium petri dish cultures with 50 spores per dish. Rupture of the spore wall was used as an indicator of germination. Gametophyte morphology was observed for 8 months. The sexual expression, age and size of 50 gametophytes from each of 3 multispore peat cultures were recorded every 2 weeks. Gametophyte width was measured at the widest point.

Because the peat medium provided conditions more similar to those in the field than the agar medium, gametophyte morphology and sexual expression were assessed only for gametophytes in peat-grown cultures. However, gametophyte cultures on agar medium were used to estimate germination because spores and their germination status could be seen more easily on agar medium. All cultures were maintained at 20–25°C under white fluorescent illumination of 1000–1500 lux for 12 hrs per day.

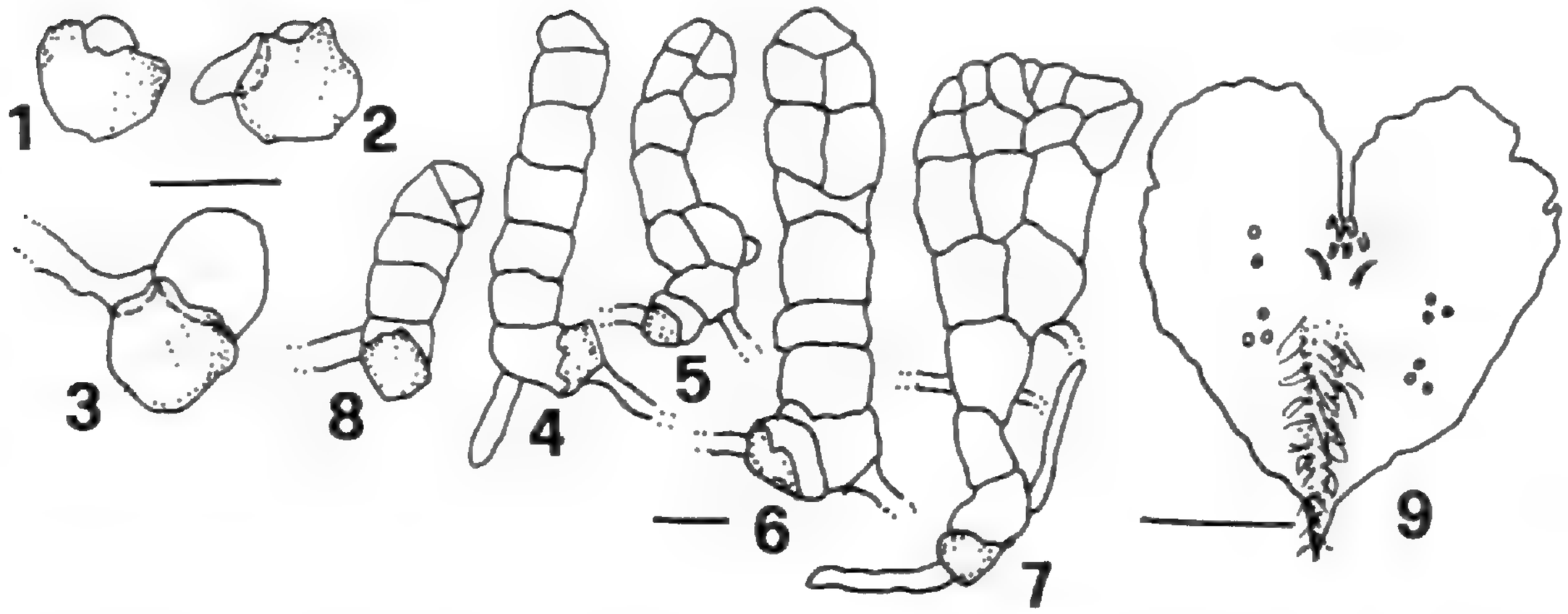
Microscopes (Lecica, Wild M8; Leitz, Dialux 20) were used to make morphological observations of gametophytes and young sporophytes. Pictures were made with the aid of a drawing tube or photomicrography. Gametangia were also observed with a scanning electron microscope. Gametophytes were dry mounted on double-stick mending tape, coated with gold, and observed with a Hitachi S2400 Scanning Electron Microscope.

RESULTS

The number of sporangia per sorus averaged 50.7 ± 13.1 s.d. and ranged from 19 to 78. Each sporangium contained 64 spores.

On agar medium, germination of spores stored at 4°C for one or two years began about 5 days after sowing, whereas germination of fresh spores did not begin until seven days after sowing. The viability of spores maintained for one or two years in cold storage was not significantly less than that of fresh spores. Two weeks after sowing, over 95% of fresh and stored spores had germinated (Table 2).

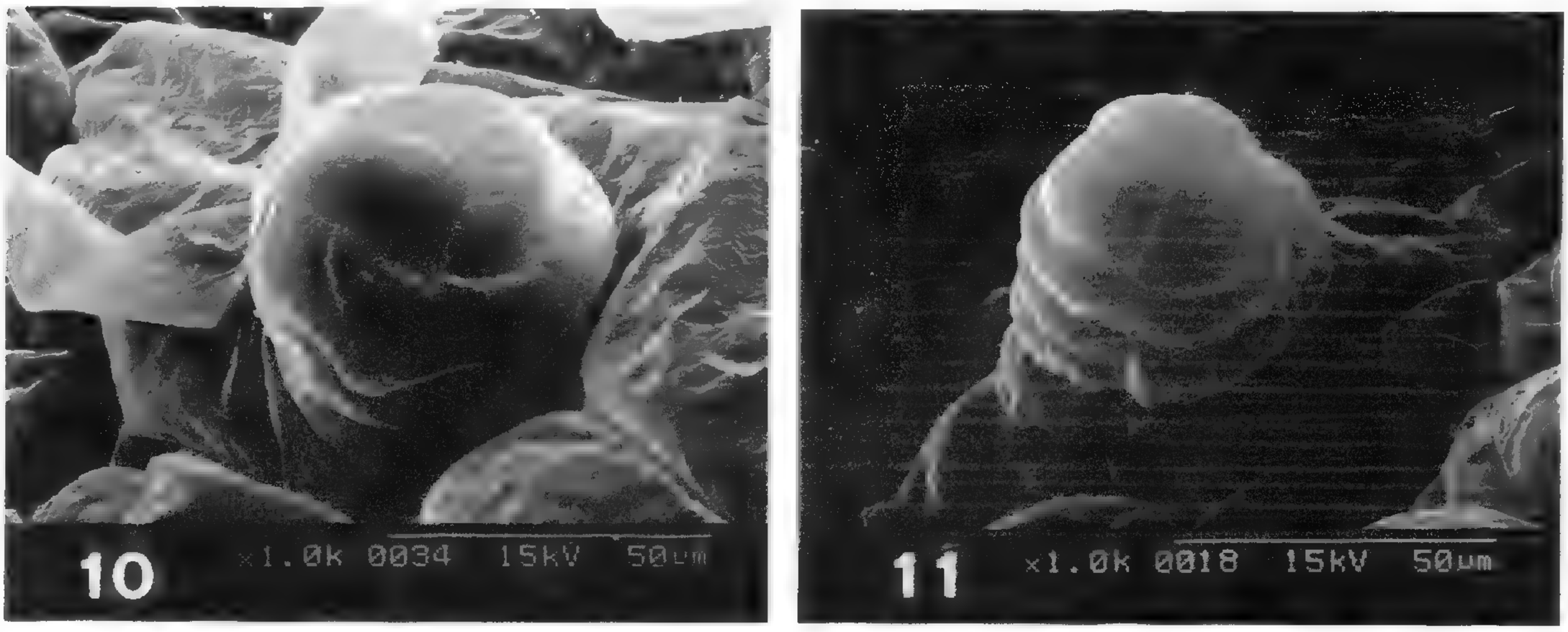
Spores of *S. lepifera* are tetrahedral. When spores germinate, the spore wall ruptures at the triradiate ridge (Fig. 1). The first cell division was parallel to the spore axis and formed the first rhizoid (Fig. 2), which elongated rapidly and did not contain chloroplasts. The second division was perpendicular to



FIGS. 1–9. Morphology of different stages of *S. lepifera* gametophytes. Figs. 1–3. Spore germination. Figs. 4–6. Filamentous stages. Fig. 7. A spatulate gametophyte. Fig. 8. A filament with a wedge-shaped apical cell. Fig. 9. A heart-shaped gametophyte. Bar = 50μm for Figs. 1–8; 1 mm for Fig. 9.

the first division (Fig. 3) and, by a series of transverse divisions, initiated a uniseriate filament that was usually 4–8 cells long (Figs. 4–8). About 4 days after spore germination, the apical, and sometimes subapical, cells divided parallel to the long axis of the filament (Fig. 5). However, in a few gametophytes the apical cell of the filament became sluggish and did not divide (Fig. 6). This apical cell was pushed to the subapical margin by intercalary divisions. Next, a spatulate plate formed. When gametophytes were about 5 cells wide, a wedge-shaped meristematic cell formed in the apical region (Fig. 7). At the filament stage, some gametophytes produced an apical meristematic cell (Fig. 8) that underwent repeated oblique divisions until it was replaced by a pluricellular meristem, the cells of which divided actively, producing a notched apex. About 5 weeks after spores were sown, a midrib formed behind the meristem in the median part, and a symmetrical, heart-shaped gametophyte formed (Fig. 9). The wings were one cell thick and were usually flat, but some curved upward at the margin. Rhizoids were restricted mostly to the ventral surface of the midrib. They were transparent but became light brown to brown with age.

Gametophytes initiated antheridium production in the spatulate stages (<0.5 mm wide). In heart shaped gametophytes, antheridia usually appeared on the ventral surface of the wings (Fig. 9), with a few forming on the dorsal surface. If the apical notch had not formed, antheridia sometimes formed near the apical region (Fig. 17), or on the margin, especially on filamentous proliferations (Fig. 20). Mature antheridia were about 50 μm wide and 40 μm high (Fig. 10). The antheridium wall was composed of five cells: a basal cell, a lower ring cell, an upper ring cell, a crescent-shaped cell, and an elliptical opercular cell. Upon dehiscence, the opercular cell was thrown off, and sperm were released. Gametophytes did not produce archegonia until 7 weeks after spore sowing (Table 3). Archegonia were restricted to the midrib behind the notch on the ventral surface, and no female only gametophytes were observed (Table 3).



FIGS. 10–11. SEM of *S. lepifera* gametangia. Fig. 10. An antheridium. Fig. 11. An archegonium.

Mature archegonia were about 40 µm wide and 50 µm high (Fig. 11), and the neck was 4 to 5 cells long. Antheridia continued to form after archegonia were initiated. Male gametophytes were usually less than 3 mm wide, whereas hermaphroditic gametophytes were usually greater than 3 mm wide. By 11 weeks after spore sowing, all gametophytes bore antheridia, that is they were male or hermaphroditic (Table 3).

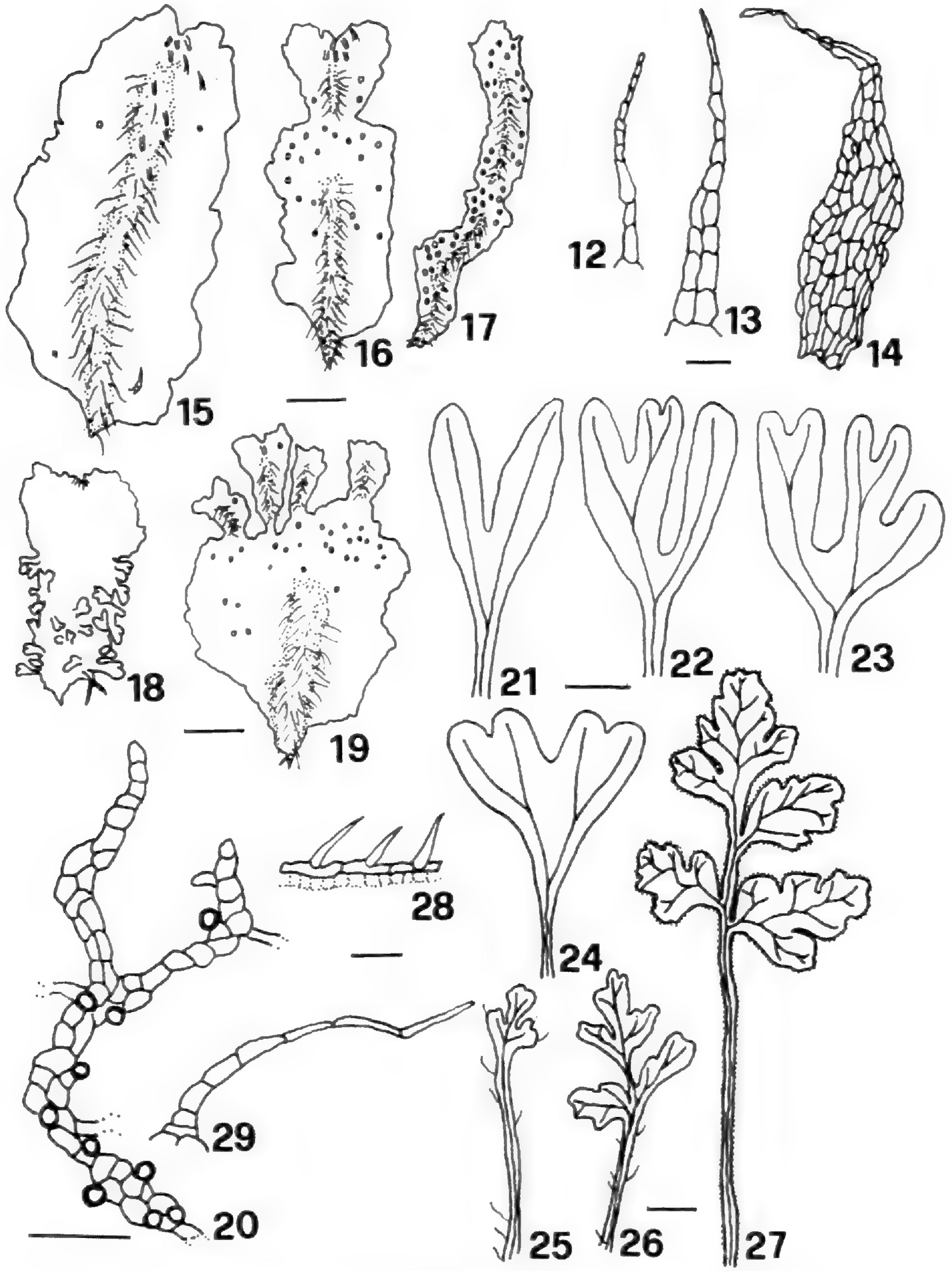
Trichomes occurred on both the dorsal and ventral surfaces of the gametophyte midrib. Usually, they were mixed with the archegonia on the cushion, especially near the apical notch of the gametophyte. Trichomes did not appear until the cushion and archegonia formed. The size of the pluricellular hairs varied greatly. Those on young gametophytes were uniseriate or, occasionally, bi- or triseriate (Figs. 12, 13), and usually were composed of less than 20 cells. Hairs began as uniseriate structures that grew by intercalary division into larger, multiseriate structures with age. On older gametophytes (e.g., 8 months old), the hairs could grow to 8 cells wide and be comprised of more than 50 cells in total, which were with a uniseriate, needle-like tip (Fig. 14).

Some gametophytes of *S. lepifera* elongated and became elliptical as they aged (e.g., 15 weeks old) (Figs. 15–17). Older gametophytes often became pale, or even necrotic, at their posterior end but kept growing at their anterior end. Some were slender, with many antheridia, but without archegonia or an apparent apical meristem. Other elongate gametophytes formed discrete cushions (Figs. 16, 17).

Some gametophytes began to produce vegetative proliferations when they were 13 weeks old. Daughter gametophytes formed from one or a few cells on the dorsal or ventral surfaces or on the margin (Fig. 18). Each of the daughter gametophytes developed a pluricellular meristem and gametangia and eventually produced large clones. Some gametophytes formed many filamentous proliferations, one to several cells wide (Fig. 20), that produced only antheridia. Occasionally, the anterior wings of gametophytes elongated and proliferated (Fig. 19). As proliferations grew, their posterior died, separating them from the parent gametophyte.

TABLE 3. Sexual status (%) and size (mm) of *S. lepifera* gametophytes of different ages grown in multispore cultures on peat media. A = asexual, M = male, F = female, H = hermaphroditic.

	5 wk				7 wk				9 wk				11 wk				13 wk				15 wk			
Width	A	M	F	H	A	M	F	H	A	M	F	H	A	M	F	H	A	M	F	H	A	M	F	H
0.5	32	38	—	—	5	30	—	—	—	23	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	1	24	—	—	1	36	—	—	1	8	—	—	—	21	—	—	—	11	—	—	—	5	—	—
2	—	5	—	—	—	25	—	—	—	40	—	—	—	44	—	5	—	38	—	3	—	25	—	1
3	—	—	—	—	—	1	—	2	—	19	—	9	—	14	—	16	—	13	—	25	—	9	—	19
4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	9	—	1	—	24
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
Total	33	67	—	—	6	92	—	2	1	90	—	9	—	79	—	21	—	63	—	37	—	40	—	60



FIGS. 12–29. Morphology of gametophytes and juvenile sporophytes of *S. lepifera*. Figs. 12–14. Multicellular hairs of gametophytes. Figs. 15–17. Elongate gametophytes. Figs. 18, 19. Gametophytes with proliferations. Fig. 20. Filamentous proliferation with antheridia. Figs. 21–24. Midribless first few fronds. Figs. 25–27. Midribbed fronds. Fig. 28. Unicellular hairs of a young sporophyte. Fig. 29. Multicellular hair of a young sporophyte. Bar = 100μm for Figs. 12–14, 28, 29; 0.2 mm for Fig. 20; 1 mm for Figs. 15–17, 21–27; 2 mm for Figs. 18, 19.

If water sufficient for fertilization was supplied, young sporophytes appeared in peat cultures about 12 weeks after spore sowing. The gametophyte usually senesced and disappeared after the sporophyte formed. However, some gametophytes with a young sporophyte formed proliferations that produced additional sporophytes. Laminae of the first few fronds of young sporophytes branched dichotomously (Figs. 21–24). Subsequent juvenile fronds were pinnately divided (Figs. 25–27). Except for the first few fronds, unicellular hairs and scales were evenly distributed on the frond margin (Figs. 25–29).

Discussion

Sen (1964) noted that the diagnostic characters for the sporangia of most genera in the Cyathaceae are very similar. In the Cyathaceae, the number of spores in each sporangium is 64 or 16. The sporangia of *Lophosoria, Sphaeropteris, Trichipteris, Cyathea, Cnemidaria* and, probably, *Metaxya* contain 64 spores, whereas those of most species of *Alsophila* and all species of *Nephelea* have 16 spores (Gastony, 1974). In this study, 64 spores were found in *S. lepifera* sporangia, the same number found in the sporangia of the majority of more highly evolved leptosporangiate ferns (Holttum and Sen, 1961; Gastony, 1974).

Page (1979) reported that the spores of some Cyatheaceae lost their viability after a few weeks, but did not describe the conditions under which they were stored. Germination of *Cyathea delgadi* spores was less than 20% after 2 years of dry storage at −12°C (Simabukuro *et al.,* 1998). However, the high germination rates of *S. lepifera* spores, even after 2 years of cold storage, suggest that the viability of Cyatheaceae spores varies from species to species and may be dependent on the storage conditions.

After *S. lepifera* spores germinated, the filament grew along the polar axis, while the first rhizoid elongated along the equatorial plane. This pattern of spore germination has been classified as "Cyathea-type" and is considered typical of ferns in the Cyatheaceae (Nayar and Kaur, 1971).

Using the definitions of Nayar and Kaur (1971), *S. lepifera* gametophyte development was mostly of the Drynaria-type, but with some Adiantum-type features. Our results are somewhat different from those of Nayar and Kaur (1971), who described *S. lepifera* gametophyte development as Adiantum-type or nearly Drynaria-type.

Although most *S. lepifera* gametophytes were typical heart-shaped, some became more elongated. Elongate gametophytes were paler and more slender than heart-shaped gametophytes, and some remained in the antheridial stage for 8 months. Stokey (1930) reported that elongate gametophytes of *Cyathea arborea* were very much pronounced and produced only antheridia when they grew in weak light. If low light intensity causes *S. lepifera* gametophytes to elongate, the slender, elongate gametophytes observed in this study may have developed after being overgrown by other gametophytes. This simulates partial burial, which may occur in nature. Although elongate gametophytes are in-

capable of supporting sporophytes, they may play a significant role in providing sperm and thus promoting cross-fertilization.

In the Cyatheaceae, the antheridial wall is usually composed of 5 cells, but some variation occurs (Stokey, 1930; Atkinson and Stokey, 1964). The wall of each antheridum of *S. lepifera* in this study was always comprised of 5 cells, including a basal cell, 2 ring cells, a crescent-shaped cell and an elliptical opercular cell that was usually shed at antheridial dehiscence. The archegonium neck is straight, more or less, as has been reported for other Cyatheaceae and some less advanced ferns (Stokey, 1930; Atkinson and Stokey, 1964).

The peculiar, multicellular hairs on the archegonial cushion of the gametophyte are characteristic of Cyatheaoid gametophytes (Stokey, 1930). Outside the Cyatheaceae, similar multicellular hairs are found only on gametophytes in the Loxsomaceae (Stokey, 1960; Atkinson and Stokey, 1964). Homology of the multicellular hairs on gametophytes in the Cyatheaceae and Loxsomaceae is supported by molecule-based cladistic analysis (Pryer *et al.*, personal communication).

Gametophytes of *S. lepifera* can produce large clones and enhance their longevity by vegetative growth, as has been described for some other species in the Cyatheaceae and many other fern taxa (Stokey, 1930; Atkinson and Stokey, 1964; Chiou and Farrar, 1997a). The type of vegetative regeneration present in *S. lepifera,* in which new gametophytes formed on the surface or margins of established gametophytes, also was observed in *Alsophila excelsa* by Atkinson and Stokey (1964). They suggested that this type of vegetative regeneration usually occurred on old or injured gametophytes. However, many clones of *S. lepifera* formed from gametophytes that were green and very healthy. Although each new gametophyte could produce male and female gametangia, few produced sporophytes while still attached to the parent gametophyte if the parent plant had produced a sporophyte. Perhaps, the developing embryo of the parent gametophyte inhibited the fertilization of eggs of the daughter gametophytes, or inhibited zygote development in fertilized eggs. The mechanism for this inhibition needs further investigation.

The sexual expression of *S. lepifera* is related to gametophyte size and development stages. Most asexual gametophytes were less than 0.5 mm wide, while male only gametophytes were 0.5–3.0 mm wide, and hermaphroditic gametophytes were usually more than 3.0 mm in width. Initial antheridium formation was followed by the attainment of a prolonged hermaphroditic phase during which antheridia continued to form. However, in a previous study of the gametangial sequence of *S. lepifera* (= *Cyathea lepifera*), antheridium formation ceased once plants became hermaphroditic (Masuyama, 1975b). Gametophyte sexual expression may be affected by the culture medium (Masuyama, 1975a). Masuyama's (1975b) observations were on gametophytes grown on agar medium, whereas gametophytes in this study were grown on peat medium.

A gametangial sequence from male to hermaphroditic may favor intragametophytic selfing (Klekowski, 1969; Masuyama, 1975a). However, this type of mating system is not consistent with another study of *S. lepifera* in Taiwan.

Based on isozyme data, Chen (1995) suggested that the mating system of *S. lepifera* tended toward intergametophytic crossing. Similarly, three other tree ferns, *Alsophila firma* (Cyatheaceae), *Cyathea stipularis* (Cyatheaceae) and *Lophosoria quadripinnata* (Lophosoriaceae) are outcrossing (Soltis *et al.*, 1991). It has been suggested that inbreeding of bisexual gametophytes may be limited by high genetic load (Klekowski, 1969, 1973; Masuyama, 1979; Peck *et al.*, 1990; Hooper and Haufler, 1997). Thus, if bisexual gametophytes are common in natural populations of *S. lepifera,* genetic load may be the most important factor favoring outcrossing in *S. lepifera.*

Young sporophytes were produced when sufficient water was supplied to sexually mature plants. Gametophytes would not bear sporophytes when water was not added to cultures, although they had mature gametangia. In addition, young sporophytes were always born on the archegonia, suggesting that *S. lepifera* sporophytes likely resulted from fertilization. The 64-spore sporangium also indicates that it is not an apogamous species.

In *S. lepifera,* the blades of juvenile leaves are dichotomously branched, whereas those of succeeding juvenile fronds are pinnately dissected. This is the most common type of frond ontogeny in leptosporangiate ferns (Wagner, 1952).

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The authors thank two anonymous reviews for their useful comments, Dr. Alan Warneke for editing the manuscript, and Miss Chien-Rong Huang for SEM assistance. This research was supported by the National Science Council of Taiwan (87-2313-B-054-004) and by the Taiwan Forestry Research Institute (contribution No. 137).

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Notes on *Lellingeria oreophila* (Grammitidaceae), a Poorly Known Species from Colombia

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Abstract.—*Lellingeria oreophila* (Maxon) A.R.Sm. & R.C.Moran, a poorly known species from Colombia, is described, illustrated, and discussed on the basis of a new find of this collection from Northern Andes in Colombia.

During a study of Neotropical Grammitidaceae, I found a herbarium specimen of *Lellingeria oreophila* (Maxon) A.R.Sm. & R.C.Moran, a species previously known only from the type collection in Colombia. Since the original description of this species by Maxon (1947), it has been mentioned in the literature only once, by Smith and Moran (1991), when they made the combination for it in *Lellingeria*. To aid future monographers, I discuss and illustrate this poorly known species and elaborate the original description of it by Maxon.

Lellingeria oreophila (Maxon) A. R. Sm. & R. C. Moran, Amer. Fern J. 81: 85. 1991. *Polypodium oreophilum* Maxon, Contr. Gray Herb. 165: 72. 1947. TYPE: COLOMBIA. Santander: Cerro Armas, 6° 21′ N, 73° 50′ W, 1200–1500 m, 26 July 1936, *Haught 1959* (HOLOTYPE US; ISOTYPE K). Fig. 1. A–F

Stems short-creeping, c. 0.5 cm thick, scaly, the scales 0.2–0.3 cm long, castaneous, linear-lanceolate, with hyaline, ciliate margins. Fronds 10–23 × 2–3 cm, erect; stipes 2–4 × 0.04 cm, dark brown, densely covered with simple, castaneous hairs ca. 0.1 mm long; laminae pinnatisect, lanceolate to slightly elliptic, chartaceous, gradually tapering to the base and to the apex; rachises sclerenchymatous, black; segments 1–2 × 0.15–0.2 cm, linear-deltate, slightly asymmetrical at the base, oblique to the costa, the margins flat, entire, the apex obtuse to slightly acute; indument of small, appressed, castaneous, unbranched hairs (up to 1 mm) on the rachises and abaxial laminar tissue; sinuses narrower than the width of the segments; veins free, 1-furcate, ending submarginally, inconspicuous. Sori round, arising at the vein apex, superficial or slightly sunken, glabrous. Sporangia non setose.

ADDITIONAL SPECIMEN EXAMINED.—**Colombia.** Antioquia: Guatapé, Vereda Santa Rita, finca Montepinar, 06° 15′N, 75° 10′W, 1850 m, 06 Feb 1990, *Contreras & Echeverri 159* (NY).

Lellingeria oreophila is apparently restricted to the northern region of the Andes, between 1200 and 1850 meters in elevation, where it occurs as an

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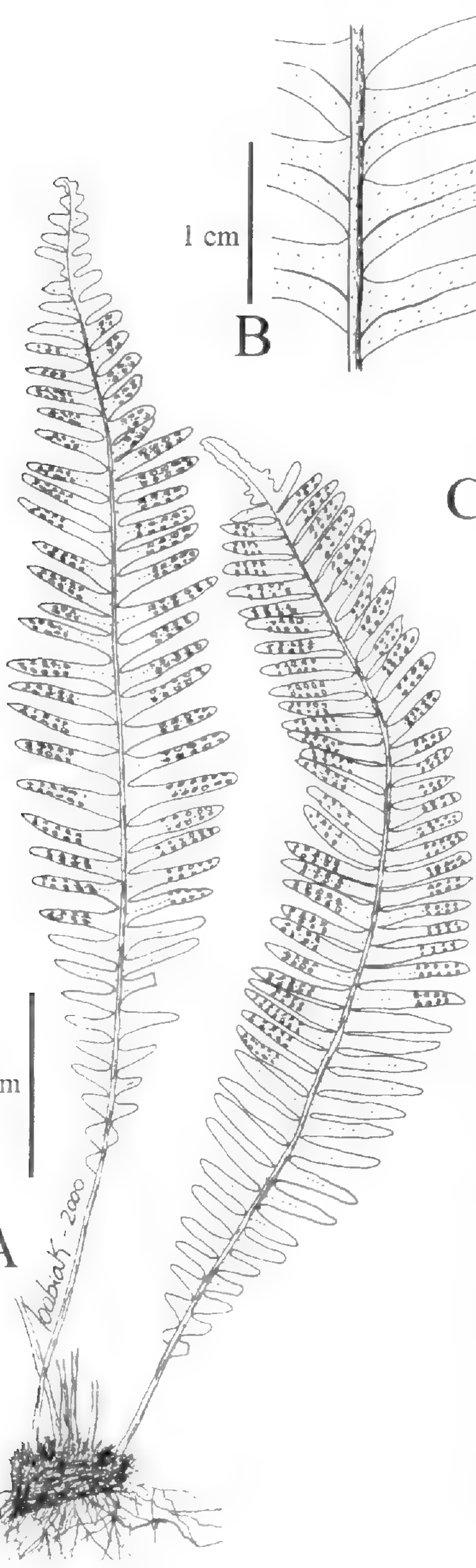

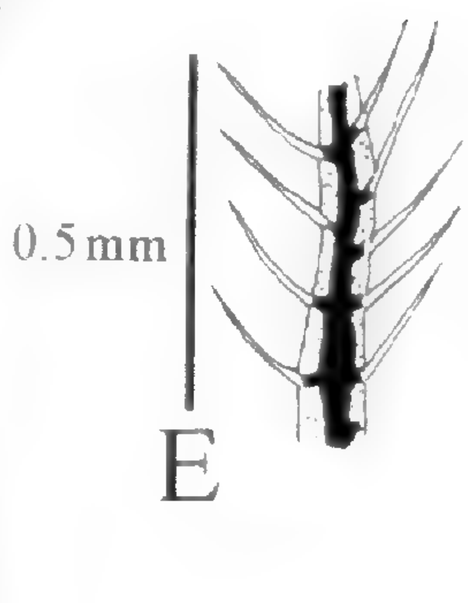

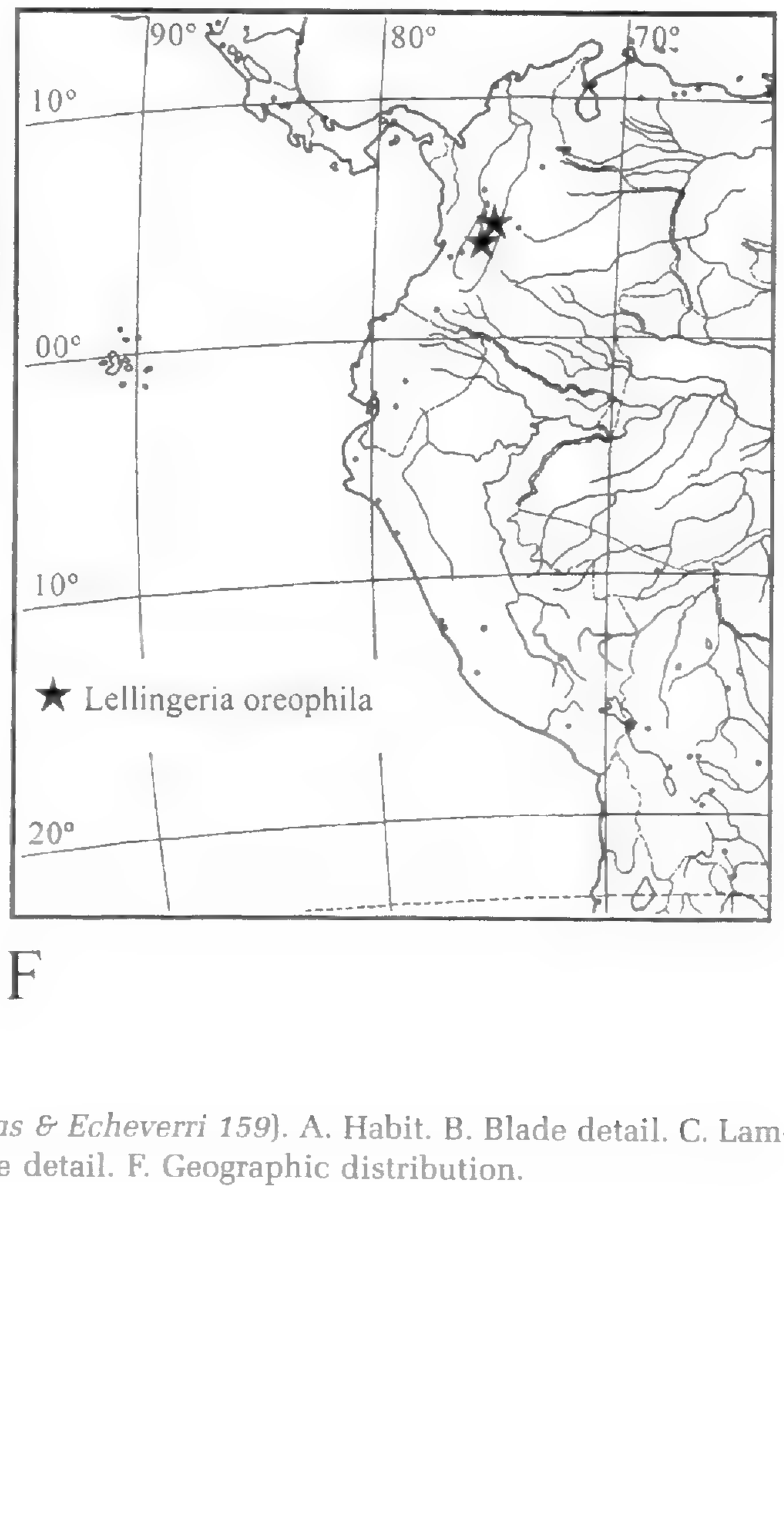

FIG. 1. A–F. *Lellingeria oreophila* (*Contreras & Echeverri 159*). A. Habit. B. Blade detail. C. Laminar hair detail. D. Stem scale. E. Stem scale detail. F. Geographic distribution.

epiphyte in cloud forests. This is the only specimen I found of this species after a search for it at BM, BR, GH, K, NY and US. This suggests the species is rare, but field work is needed to substantiate this.

Lellingeria oreophila is characterized by having glabrescent rachises or having only scattered hairs abaxially, stem scales that are linear-lanceolate, castaneous centrally with hyaline, ciliate margins, and by having appressed, unbranched, castaneous hairs on the abaxial surface of the laminae and rachises.

It is related to *Lellingeria apiculata* (Kunze ex Klotzsch) A. R. Sm. & R. C. Moran, which can be distinguished by its rachises densely covered by hairs on both sides, and its abaxial laminar surfaces glabrous or with scattered, hyaline, branched hairs, these acicular and spreading. *Lellingeria oreophila* although similar to *L. hirsuta* A. R. Sm. & R. C. Moran, can be recognized by having castaneous, unbranched hairs on the abaxial surface of the laminae; while *L. hirsuta* is glabrous.

Lellingeria stuebelii (Hieron.) A. R. Sm. & R. C. Moran is also similar but, according to Maxon (1947), can be distinguished by having conspicuous hairs on the rachises, margins, and abaxial laminar tissue, stems only 1 mm thick, and by the small and few ciliate scales on the stems.

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I thank the Andrew W. Mellon Foundation for support of a six-month studies at the New York Botanical Garden (including the GH and US herbaria in the United States), and the Margaret Mee Foundation, which provided financial support for a visit to the BM, BR and K herbaria in Europe. I also thank Dr. Robbin C. Moran and Dr. Jefferson Prado by their helpful comments on the manuscript. The drawings were prepared by the author.

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Shorter Notes

Kaempferol and quercetin 3-*O*-(X″,X″-di-protocatechuoyl)-glucuronides from *Pteris vittata*.— Previous work on the flavonoids of *Pteris vittata* L. has shown the presence of an anthocyanin (luteolinidin 5-*O*-glucoside) which was isolated by Harborne (Phytochemistry 5:589–600, 1966); in addition acid hydrolysis of extracts of this fern has led to the identification of kaempferol, quercetin, leucocyanidin and leucodelphinidin by Voirin (Ph.D. thesis, University of Lyon, p. 151, 1970). Recently 3-*C*-(6‴-acetyl-cellobiosyl)-apigenin (Am. Fern J. 89: 217–220, 1999) and 6-*C*-cellobiosylisoscutellarein 8-methyl ether together with quercetin 3-*O*-glucuronide and rutin (Am. Fern J. 90:42–47, 2000) have been reported from *P. vittata* by Imperato and Telesca.

In the present paper four flavonoids (I–IV) have been isolated from *Pteris vittata* L. This fern was collected in the Botanic Garden of the University of Naples and has been identified by Dr. R. Nazzaro (University of Naples); a voucher specimen (149.001.001.01) has been deposited in the Herbarium Neapolitanum (NAP) of the University of Naples.

Flavonoids (I–IV) have been isolated by preparative paper chromatography in BAW (*n*-butanol-acetic acid-water, 4:1:5, upper phase), 15% HOAc (acetic acid) and BEW (*n*-butanol-ethanol-water, 4:1:2.2) from an ethanolic extract of aerial parts of *Pteris vittata*. Further purification was carried out by Sephadex LH-20 column chromatography eluting whith methanol.

Ultraviolet spectral analysis with the customary shift reagents λ_{max} (nm) (MeOH) 257, 296 (sh), 348; +$AlCl_3$ 273, 303, 347, 398; +$AlCl_3$/HCl 273, 303, 346, 393; +NaOAc 271, 302, 367; +NaOMe 272, 324, 392 (increase in intensity); +NaOAc/H_3BO_3 258,351 suggested that flavonoid (I) may be a flavonol glycoside with free hydroxyl groups at positions 5, 7 and 4'.

Chromatographic behaviour on Whatman No 1 paper (R_f values: 0.65 in BAW; 0.40 in 15% HOAc; 0.82 in H_2O) suggested that flavonoid (I) may be a flavonoid *O*-glucuronide since it has a high mobility in H_2O according to an observation of Harborne (pp. 376–441 *in* J.B. Harborne, T.J. Mabry and H. Mabry, eds., *The Falvonoids,* Chapman and Hall, London, 1975).

Both total acid hydrolysis (2N HCl/MeOH 1:1; 1 hr at 100°C) and controlled acid hydrolysis (10% HOAc; 3.5 hr under reflux) gave kaempferol, glucuronic acid, glucuronolactone and 3,4-dihydroxybenzoic acid (protocatechuic acid). Alkaline hydrolysis (2N NaOH; 2 hr at room temperature in a sealed tube) gave kaempferol 3-*O*-glucuronide and protocatechuic acid. Since electrospray mass spectrum (ESMS) showed a pseudomolecular ion at m/z 779 $[(M+H)+2Na]^+$, two 3,4-dihydroxybenzoyl groups are present as acyl substituents. The above results show that flavonoid (I) is kaempferol 3-*O*-(X″, X″-di-protocatechuoyl)-glucuronide (Fig. 1), a new natural product.

Protocatechuic acid is reported for the first time as acyl substituent in flavonoid glycosides.

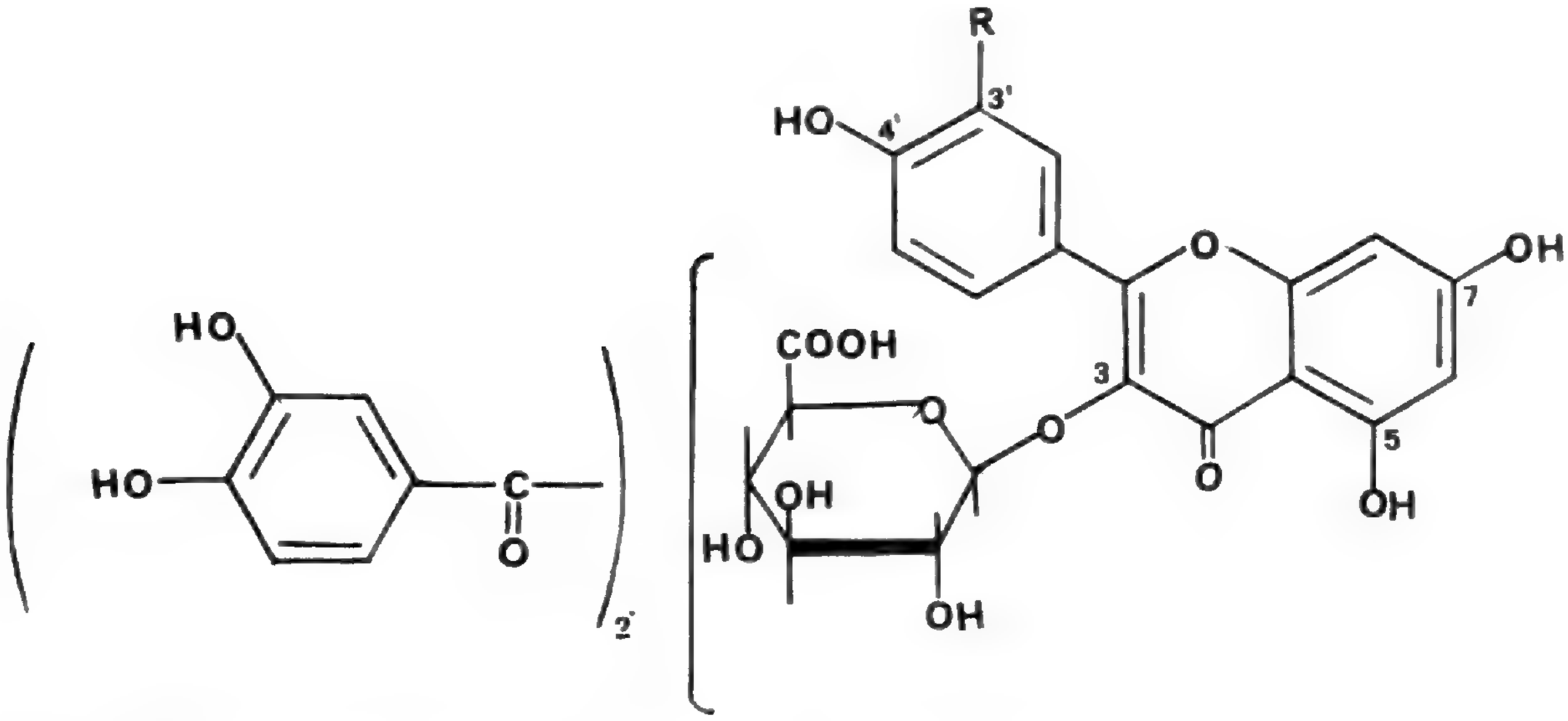

FIG. 1. The structure of flavonoid I (R=H) and flavonoid II (R=OH), kaempferol and quercetin 3-*O*-(X″,X″-di-protocatechuoyl)-glucuronides.

Ultraviolet spectral analysis with the usual shift reagents (λ_{max} (nm) (MeOH) 256, 265 (sh), 306 (sh), 355; +$AlCl_3$ 271, 301 (sh), 370 (sh), 427; +$AlCl_3$/HCl 269, 300 (sh), 399; +NaOAc 270, 327 (sh), 367; +NaOMe 271, 330 (sh), 400 (increase in intensity); +NaOAc/H_3BO_3 260, 374) suggested that flavonoid (II) may be a flavonol glycoside with free hydroxyl groups at positions 5, 7, 3′ and 4′. Chromatographic behaviour (R_f values on Whatman No 1 paper: 0.48 in BAW; 0.42 in 15% HOAc; 0.81 in H_2O) suggested that flavonoid (II) may be a flavonoid *O*-glucuronide since it has high R_f value in H_2O according to Harborne and Williams (pp. 376–441 *in* J.B. Harborne, T.J. Mabry and H. Mabry, eds., *The Flavonoids,* Chapman and Hall, London, 1975).

Both total acid hydrolysis (2N HCl/MeOH 1:1; 1 hr at 100°C) and controlled acid hydrolysis (10% HOAc; 3.5 hr under reflux) gave quercetin, glucuronic acid, glucuronolactone and protocatechuic acid. Alkaline hydrolysis (2N NaOH; 2 hr at room temperature in a sealed tube) gave quercetin 3-*O*-glucuronide and protocatechuic acid. Electrospray mass spectrum showed a pseudomolecular ion at m/z 795 $[(M+H)+2Na]^+$ and an ion at m/z 1523 $[(M\times2)+H+Na]^+$ which corresponds to a dimer; hence two 3,4-dihydroxybenzoyl groups are present in flavonoid (II) which is identified as quercetin 3-*O*-(X″,X″-di-protocatechuoyl)-glucuronide (Fig. 1), a new natural product.

Flavonoid (III) was identified as kaempferol 3-*O*-glucuronide by ultraviolet spectral analysis with the customary shift reagents, acid hydrolysis, ^{1}H NMR spectrum, ^{13}C NMR spectrum, DEPT experiments, ESMS and co-chromatography with an authentic sample. The electrospray mass spectrum showed a pseudomolecular ion at m/z 507 $[(M+H)+2\ Na]^+$ and ions m/z 992 $[(M\times2)+H+3\ Na]^+$, m/z 1475 $[(M\times3)+H+4\ Na]^+$ and 1959 $[(M\times4)+H+5\ Na]^+$ which showed that kaempferol 3-*O*-glucuronide forms a dimer, a trimer and a tetramer. Kaempferol 3-*O*-glucuronide has previously been found in several species of *Adiantum* as shown in a review of Markham (pp. 427–468 *in* J.B.

Harborne, ed., *The Flavonoids, Advances in Research since 1980,* Chapman and Hall, London and New York, 1988).

Flavonoid (IV) was identified as kaempferol 3-*O*-glucoside (astragalin) by ultraviolet spectral analysis with the usual shift reagents, acid hydrolysis, cochromatography with an authentic sample and ESMS. The electrospray mass spectrum showed a quasimolecular ion at m/z 471 $[(M+H)+Na]^+$ and an ion at m/z 919 $[(M\times2)+H+Na]^+$ which showed that kaempferol 3-*O*-glucoside forms a dimer. Astragalin has been found previously in a large number of fern species as shown in a review of Markham (above reference) but is here reported for the first time in the genus *Pteris*.

Until 1985, flavonoid glucuronides have been reported in only one fern genus (*Adiantum*) as shown in the review by Markham. More recently, quercetin 3-*O*-glucuronide has been found in *Pteris vittata* (above reference), luteolin 7-*O*-glucuronide has been isolated from *Pyrrosia serpens* by Markham and Andersen (Phytochemistry 29: 3919–3920, 1990), and eriodyctiol 7-*O*-glucuronide has been found in *Davallia mariesii* by Cui et al. (Chem. Pharm. Bull. 38: 3218–3220, 1990).

The presence of flavonoid glucuronides (I-III) in *Pteris vittata* as well as isolation of quercetin 3-*O*-glucuronide from this fern by Imperato and Telesca (above reference) show advancement from the phylogenetic point of view since glycosylation with sugars other than glucose and rhamnose may be considered phylogeneticly advanced. Acylated flavonoids (I, II) from *P. vittata* and the previous report of two acetylated flavonol glycosides from *P. grandifolia* by Tanaka et al. (Chem. Pharm. Bull. 26: 3580–3582, 1978) also indicate phylogenetic advancement. In addition, recent isolation of flavone *O*-glycosides from *P. cretica* by Imperato (Phytochemistry 37: 589–590, 1994; Experientia 50: 1115–1116, 1994) and by Imperato and Nazzaro (Phytochemistry 41: 337–338, 1996) and isolation of *O*-glucosides of *C*-glycosylflavones from *P. vittata* by Imperato and Telesca (above references) confirm the phylogenetic advancement of the genus *Pteris* since the presence of flavone *O*-glycosides and *O*-glycosides of *C*-glycosylflavones may be considered phylogeneticly derived according to the review by Markham.

The electrospray mass spectra showed that flavonoids (II and IV) form dimers whereas flavonoid (III) forms dimers, trimers and tetramers. These data are of interest since dimers and polimers based on hydrogen bonding have been observed in crystal structures of flavonoid aglycones as reported by Cantrell (pp. 391–394 *in* V. Cody, E. Middleton and J.B. Harborne, eds., *Plant Flavonoids in Biology and Medicine,* Alan R. Liss, New York, 1986) whereas data relating to crystal structures of flavonoid glycosides are scanty.

The author thanks University of Basilicata (Potenza, Italy) for finacial support (ex 60%). Mass spectral data were provided by SESMA (Naples).—FILIPPO IMPERATO, Dipartimento di Chimica, Università della Basilicata, I-85100 Potenza, Italy.

***Dryopteris filix-mas* New in Pennsylvania.**— The male fern *Dryopteris filix-mas* (L.) Schott has not been recorded previously in Pennsylvania. The species is generally rare in the eastern part of North America, but does occur in calcareous areas and moist woods around the Great Lakes. Closest to Pennsylvania, but still 300+ km distant, are sites in Michigan and Ontario (e.g., the Bruce Peninsula).

Recently, the author discovered a natural population of male fern near Pittsburgh, in the Dead Man's Hollow Preserve of the Allegheny Land Trust. In the northeastern section of the preserve there is a thriving colony of 19 plants on a 36.6m span of limestone talus. Seventeen of the plants are scattered along 7.6 m of the 4.5 m high, east-facing slope, and two more are disjunct about 29 m south of the main cluster. Most of the plants are fully fertile with frond lengths averaging 66 cm. The colony appears to be between 15–20 years old, possibly older, estimated from growth rates for cultivated specimens in Pittsburgh. The origin of *Dryopteris filix-mas* here is unknown. Fern spores can travel on prevailing winds over considerable distances, and it is possible that a "lake effect" windstorm carried this species south. It may be a relict population from a colder era or it may have started from spores locally produced from cultivated specimens in our general area.

Dead Man's Hollow is located along the Youghiogheny River about 1.6 km north of Boston Bridge (Route 48) in Lincoln and Liberty Boroughs of Allegheny County. The preserve was acquired in sections starting in 1996 and consists of about 160 hectares (400 acres) that have been largely untouched since the mid-20th century. Prior to that, the hollow was quarried, logged, farmed, and most recently housed a clay sewer pipe factory, the remains and shards from which are still quite evident. However, no roads or utility lines penetrated the hollow, and today it is a quiet place of small streams in bottom land that is surrounded by relatively steep slopes. The secondary forest has mixed eastern deciduous trees, introduced white pine and eastern hemlock, and native as well as exotic undergrowth species. Residential neighborhoods and two community parks border the preserve on three sides. An abandoned rail line (a rails to trails site) and the Youghiogheny River form the remaining border. The area where the male fern grows is near a footpath, but appears little disturbed. The author found the colony during an exploratory hike in the preserve on November 12, 2000, at which time a mature frond was collected as a voucher and deposited with the herbarium of the Carnegie Museum in Pittsburgh, Pennsylvania (Sheet No. 498068, CM). Growing along with *D. filix-mas* are dozens of robust plants of *Asplenium platyneuron,* but the rest of the fern flora in the hollow consists of common western Pennsylvania species, e.g., *D. intermedia, D. carthusiana, D. marginalis,* and *Polystichum acrostichoides.*—JOAN EIGER GOTTLIEB, 2310 Marbury Road, Pittsburgh, PA 15221.

American Fern Journal 90(4):145–146 (2000)

Review

Helechos de Mbaracayú, by María Peña-Chocarro, Griselda Marín, Belén Jiménez, and Sandra Knapp. 1999. The Natural History Museum, London. 142 pages. Softcover (ISBN 0 565 09137 9) £3.00. Available from The Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom, and from Fundación Moisés Bertoni, Casilla 714, Asunción, Paraguay.

This book, written in Spanish, is a popular identification guide to the 115 species of pteridophytes growing in Mbaracayú, a 650 km^2 reserve in eastern Paraguay. Nearly 80% of the reserve consists of relatively undisturbed wet forest, and the rest is mostly savanna, or *cerrado,* that occurs on well-drained soils. Both habitats harbor pteridophytes.

What distinguishes this guide is its highly visual approach. There are *a lot* of illustrations. Each species treatment is allotted a full page, and that page is filled with illustrations, including silhouettes of leaves and drawings of venation and sorus shape. The rhizomes are usually shown in the silhouettes if the plant is of small size. Toward the bottom of the page is a short description with the main characters in boldface, followed by a statement of geographic distribution and notes. Below this, at the bottom of the page, are five boxes containing standardized pictures that give the plant's habit and size (first box), sorus shape, habitat, growth habit, and (last box) any characteristic helpful in identifying the plant.

The main key at the beginning of the book repeats these five boxes so that, again, identification is primarily visual. The key is divided into ten groups based primarily on leaf cutting, from entire to more highly divided. The species treatments are grouped in the text by this characteristic, and in the middle of the fore-edge of each page a shaded side-box encloses a leaf symbol showing the degree of cutting. This allows you to flip through the guide and quickly find the main leaf-division sections.

At the end of the book is a list of the pteridophytes ordered by family (22 families and 46 genera are recognized). This is followed by a bibliography and index to scientific names. There is no glossary because technical terms have been kept to a minimum. Unfamiliar terms can be sought in the introduction that explains the parts of the fern plant.

My only complaint is that several of the drawings are inaccurate or of poor quality. For instance, the drawing of *Salvinia minima* does not accurately show the arrangement of the sori on the submerged leaf, nor does it depict the numerous and conspicuous hairs on the submerged segments. Also, one of the sori appears to be fused with another sorus—something that does not occur. In the illustration of *Psilotum nudum,* the synangia are poorly drawn, the bifid enation beneath it is missing, and a long stalk is shown attaching to the side, not the base, of one synangium (were the synangia really that long-stalked?). The indusia on *Adiantum raddianum* are shown separated from (i.e., within)

the leaf margin, not formed from an enrolling of it. I find it hard to believe that *Cyathea atrovirens,* or any extant tree fern, has thick prop roots as is illustrated for that species.

Despite problems with some of the illustrations, this guide will be useful to botanists working in Paraguay and nearby regions in adjacent countries. At a price of £3.00, it is easily affordable. The authors should be congratulated on the care they have taken in making this guide easy-to-use and for their highly visual approach.—ROBBIN C. MORAN, The New York Botanical Garden, Bronx, NY 10458–5126, U.S.A.

American Fern Journal 90(4):147 (2000)

Referees for 2000

All papers submitted to the journal are peer reviewed. Members of the editorial board and the Society, as well as additional scientists in cognate areas, do these reviews on a voluntary basis. It is their work that contributes to the high quality of articles in the American Fern Journal and to its continued success. The American Fern Society and I extend our thanks to the following reviewers for their assistance, diligence, and patience in the year 2000—R. James Hickey.

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