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Adiantum mariposatum (Pteridaceae), a New Species from Ecuador

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Abstract.—Approximately 40 species of *Adiantum* are represented in Ecuador. A new species is described here from Pastaza province, Ecuador. It has been confused with *A. anceps*, but differs in having only once-pinnate blades and pubescent rachises, segment stalks, and indusia.

Key Words.—*Adiantum*, Ecuador, new species

The genus *Adiantum* comprises about 200 species distributed worldwide (Mickel and Smith, 2004) and is particularly abundant in the New World tropics. Several new *Adiantum* species have been described from South America (Zimmer, 2007; Prado, 2003; Prado and Smith, 2002; Prado, 2000) including one from Ecuador (Smith and Prado, 2004). In this paper yet another new species of *Adiantum*, from Pastaza Province, is described.

Adiantum mariposatum M. McCarthy & Hickey, ***sp. nov.*** TYPE.—Ecuador. Pastaza, c. 5 km E of Mera, on road to Shell-Mera, 78°5′W 1°28′S, rocky escarpment, road bank and riverside vegetation, 1050 m, 30 July 1980, *B. Øllgaard, S. Roth, & C. Sperling 35582* (holotype: AAU!; isotypes: GH!, UC). **Figs 1, 2.**

Folia pinnata, 16–23 cm longa, 6–12 cm lata; stipites atropurpurei usque ebenei, longitudinaliter 1/2–2/3 folia aequantes, glabri; rhachides atropurpureae usque ebeneae, supra hirsutae; pinnae 1–8, dimidiatae usque trapeziformes, 52–74 mm longae, 34–47 mm latae; petioluli brevi, 0.5–2 mm longi, hirsuti; indusia discreta, 0–5 per pinnam, ovata usque late ovata, hirsuta.

Plants terrestrial. *Rhizomes* short-creeping, 4–5 mm in diameter, densely scaly; *scales* triangular to narrowly triangular, lustrous, rigid, bullate, darkened centrally, castaneous along margins and apices, bases auriculate, margins with spreading to recurved teeth, apices attenuate. *Leaves* monomorphic, 16–32 × 6–12 cm; *stipes* 50–60% the length of the fronds, atropurpureous to ebeneous, mainly glabrous, with scattered scales near the base, shining to weakly glaucous; *stipe scales* narrowly triangular, stramineous proximally, castaneous distally, margins with spreading to recurved teeth; *blades* oblong to broadly obovate, pinnate (entire when young), glabrous, pinna bases overlapping the rachis; *rachises* atropurpureous to ebeneus, abaxially glabrous, adaxially with minute scattered hairs 0.1–0.2 mm long, hairs castaneous proximally, stramineous distally, extending onto segment stalks; *segments* dimidiate to trapeziform, papyraceous, not articulate, 50–75 × 34–47 mm, stalks 0.5–2.1 mm long, darkened color passing into segment bases,

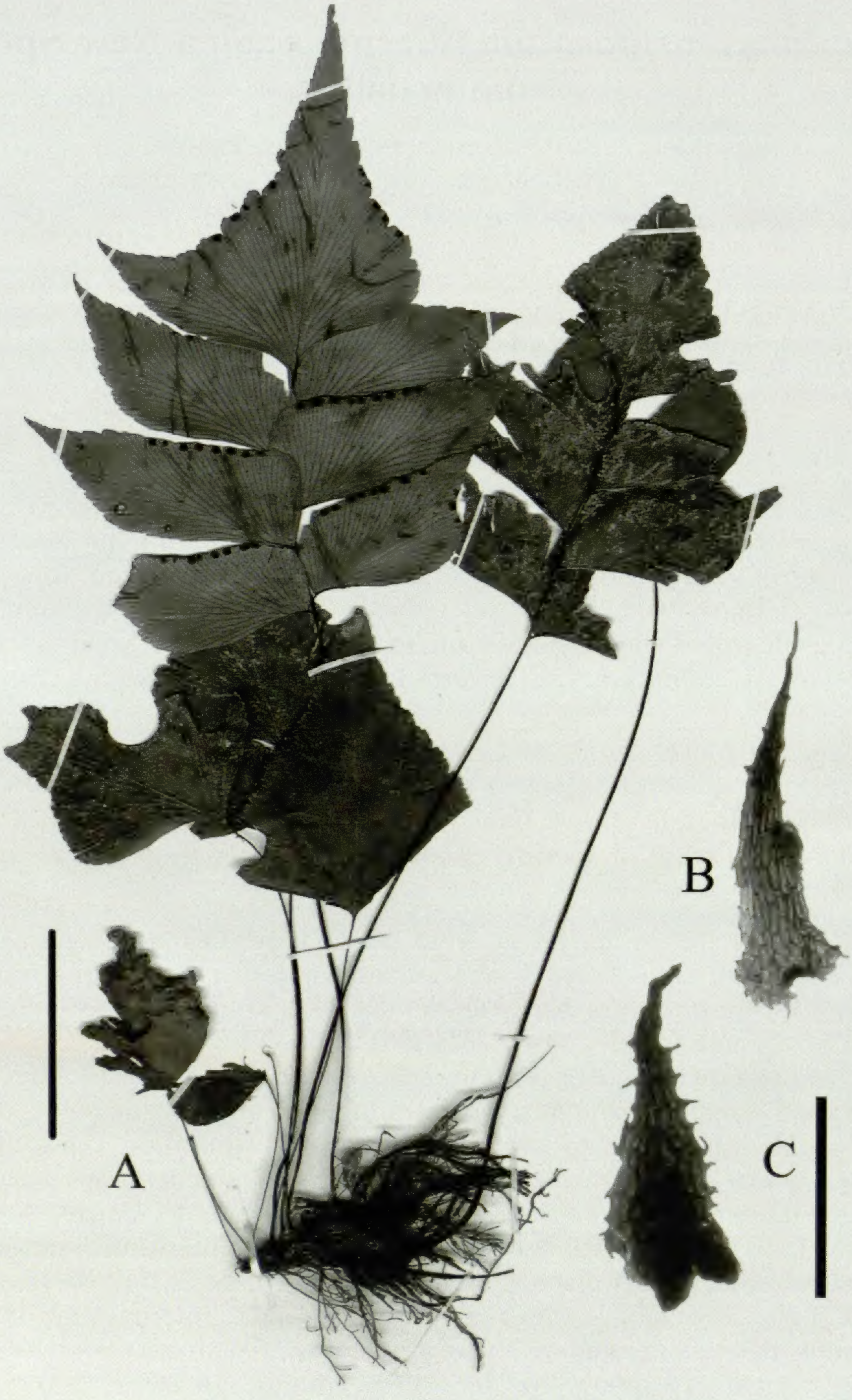

FIG. 1. Holotype of *Adiantum mariposatum* (*B. Øllgaard, S. Roth, & C. Sperling 35582*, AAU). A. Habit showing entire and pinnate fronds (scale bar equals 5 cm). B. and C. Stipe and rhizome scales, respectively, showing margins with spreading and recurved spines (scale bar equals 1 mm).

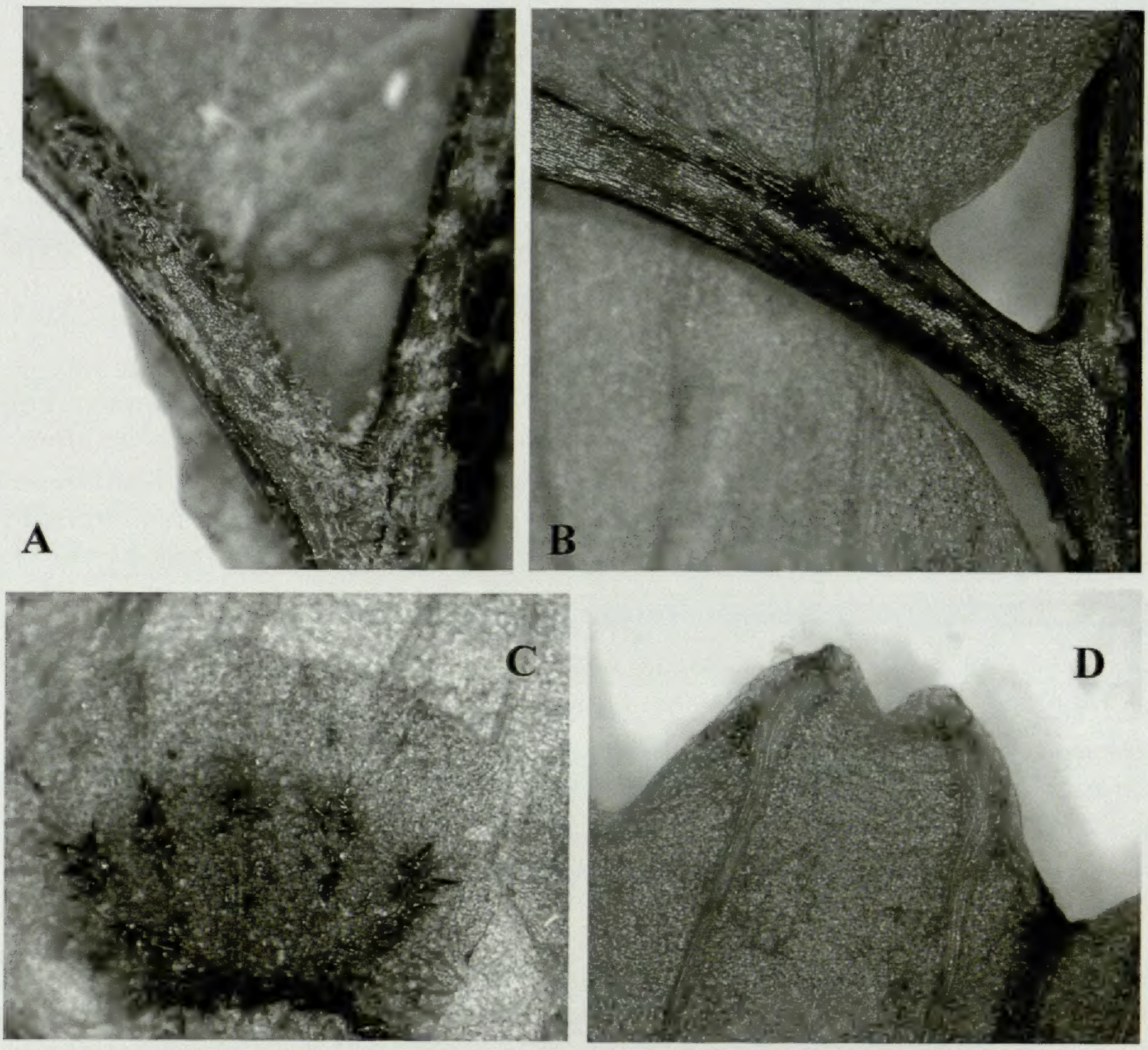

FIG. 2. Holotype of *Adiantum mariposatum* (*B. Øllgaard, S. Roth, & C. Sperling 35582*, AAU). A. Adaxial view of rachis and segment with minute scattered hairs. B. Abaxial view of glabrous rachis and segment stalks. C. Sorus with minute red-brown hairs following receptacle lines. D. Segment margin with distally dilated veins.

bases basiscopically excavate, acroscopically truncate, margins shallowly dentate, apex acuminate; *veins* free, dilated distally, ending in marginal teeth or arcuate toward the nearest distal tooth, prominulous, markedly so adaxially, lacking venuloid idioblasts between veins; *terminal blade segment* broadly trullate, 80–100 × 65–80 mm; *sori* discontinuous, 2–16 per segment, widely depressed ovate, 2–3 × 2–3 mm, false indusia stramineous, transparent when young, becoming brittle and black with age, with small reddish-brown hairs along receptacle lines, margins erose; *spores* trilete, stramineous-gold, 23–44 μm.

This species is known only from Pastaza Province in the eastern foothills of the Ecuadorian Andes. It grows in wet forests and on rocky riverbanks, in shade.

The epithet for this new species makes reference to the large butterfly-shaped segments.

PARATYPE.—ECUADOR. **Pastaza**. Mera-Shell Mera, ca. 2 km E of Mera, at bridge over Río Alpayacu, 78°06′W 01°28′S, 1100 m, 21 Jan 1992, *B. Øllgaard et al. 99574* (AAU).

Adiantum mariposatum can be distinguished by having compact, pinnate fronds that reach about 30 cm, large glabrous segments that overlay the rachis, adaxial pubescence along the rachises and segment stalks, and by the broadly ovate, sparsely pubescent indusia. It can be confused with *A. anceps*, which reaches 2 m, has 1–3 pinnate fronds, and is completely glabrous along the rachises, segment stalks, and indusia.

As currently circumscribed, *Adiantum mariposatum* falls within the *A. tetraphyllum* group as delineated by Tryon and Tryon (1982). This group has 1- or 2-pinnate blades, axes with scales or adaxial pubescence, sessile to short-stalked segments, and few to many indusia. *Adiantum humile* Kunze., *A. latifolium* Lam., *A. obliquum* Kaulf., *A petiolatum* Desv., *A. tomentosum* Klotzsch, *A. pulverulentum* L., and *A. tetraphyllum* Humb. & Bonpl. ex Willd. are other members in this group. All of these species however, have conspicuous venuloid idioblasts (silica bodies) on laminar surfaces between veins (Sundue, 2009), a character that is lacking in *A. mariposatum*. This character was not mentioned in Tryon's 1982 circumscription of the Adiantoid groups, but is now thought to be relevant in determining systematic relationships (Sundue, 2009).

Adiantum mariposatum may be more closely allied to the more widespread *A. urophyllum* Hook., which lacks visible venuloid idioblasts, has 2-pinnate leaves (juvenile leaves may be 1-pinnate), more numerous and smaller segments with long tapering apices, densely pubescent stipes, rachises, and segment stalks, and sparsely pubescent to often glabrous indusia. The small red-brown hairs on the indusia of *A. mariposatum* tend to follow the receptacle lines, whereas the hairs of *A. urophyllum* appear randomly scattered across the indusia. No other *Adiantum* species observed during this study displayed a similar linear arrangement of pubescence along the indusia. This character appears to be unique to *A. mariposatum*. Additional *Adiantum* species with pubescent indusia include, *A. terminatum* Kze. *ex* Miq., *A. trichochlaenum* Mickel & Beitel, *A. tricholepis* Fée, and *A. curvatum* Kaulf. *Adiantum terminatum* and *A. trichochlaenum* are 2- pinnate, have indument abaxially along the rachises, and venuloid idioblasts between the veins on both surfaces of the pinnules. *Adiantum tricholepis* and *A. curvatum* are 3- or 4-pinnate, and lack venuloid idioblasts.

LITERATURE CITED

MICKEL, J. T. and A. R. SMITH. 2004. Pteridophytes of Mexico. Mem. New York Bot. Gard. 88:21–45.

PRADO, J. 2000. A new species of *Adiantum* (Pteridaceae) from Bahia, Brazil. Brittonia. 52:210–212.

PRADO, J. 2003. New species in *Adiantum* from Brazil. Amer. Fern J. 93:76–80.

PRADO, J. and A. R. SMITH. 2002. Novelties in Pteridaceae from South America. Amer. Fern J. 92:105–111.

SMITH, A. R. and J. PRADO. 2004. New species in *Adiantum* and *Pteris* (Pteridaceae) from the Andes. Brittonia 55:310–316.

SUNDUE, M. A. 2009. Silica bodies and their systematic implications in Pteridaceae (Pterdiophyta). Bot. J. Linn. Soc. 161:422–435.

TRYON, R. M. and A. F. TRYON. 1982. *Ferns and allied plants with special reference to Tropical America.* Springer-Verlag, New York.

ZIMMER, B. 2007. *Adiantum krameri* (Pteridaceae), a new species from French Guiana. Willdenowia. 37:557–562.

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The Gametophyte of *Ophioglossum pendulum* in Culture

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ABSTRACT.—The spores of *Ophioglossum pendulum* ssp. *falcatum* germinated after six weeks in the dark on a nutrient medium containing inorganic nutrients and glucose. The gametophytes grew on the same nutrient medium to give globular, teardrop-shaped, and finally cylindrical gametophytes. The mature gametophytes were cylindrical and highly branched. Other aspects of these gametophytes were normal for *Ophioglossum* gametophytes with sunken antheridia and short-necked archegonia. The gametangia were functional because fertilization took place in older cultures. Mature gametophytes of *O. pendulum* ssp. *falcatum* from culture had the same structure as those of *O. pendulum* ssp. *pendulum* from nature. The differing conditions under which the gametophytes of both subspecies grew did not alter their stellate form.

KEY WORDS.—*Ophioglossum*, gametophytes, fern development

In a study by Whittier and Moyroud (1993), spores of *Ophioglossum palmatum* L. were used to determine if gametophytes of an epiphytic *Ophioglossum* species could grow in culture. The spores germinated but only when the pH was low, which made it difficult to keep an agar-based culture medium solid. Hundreds of small multicellular gametophytes formed, but few advanced beyond the 12-celled stage. About 30 macroscopic gametophytes developed and continued to grow when transferred to new cultures. Only two of these macroscopic gametophytes became mature after two and a half years in culture. Why the nutrient medium on which hundreds of spores germinated did not support the growth of a significant number of older gametophytes is not understood. It was expected with modifications to the nutrient medium that greater numbers of mature gametophytes would develop, however this did not happen.

Because the study on *O. palmatum* gave inconclusive results, this study using spores from the other epiphytic species of the genus, *Ophioglossum pendulum* L., was initiated. The aim of this experiment, as before, was to grow mature gametophytes of an epiphytic species on the solid agar surface of a nutrient medium. Also, it was of interest to determine whether the growth pattern and structure of these gametophytes was the same as those of gametophytes growing under natural conditions.

MATERIALS AND METHODS

Spores of *Ophioglossum pendulum* L. ssp. *falcatum* (Presl) Clausen were obtained from plants on Mt. Tantalus in Oahu, Hawaii. A voucher is on deposit

at TENN. The spores obtained had the typical shape and internal organization for *Ophioglossum* spores (Whittier, 1981; 2003).

The spores were surface sterilized with 20% Clorox (1.1% sodium hypochlorite) for 2 min by the method of Whittier (1964). Under sterile conditions, the spores were rinsed with water, collected on filter paper, suspended in water and sown on 12 ml of nutrient medium in culture tubes (20 mm × 125 mm) with screw caps that were tightened to reduce moisture loss.

The nutrient medium contained 100 mg $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2$, 100 mg K_2HPO_4, and 25 mg arginine per liter. The medium was completed with 2.5 g of glucose, 0.5 ml of a minor element solution (Whittier and Steeves, 1960) and 4 ml of a FeEDTA solution (Sheat *et al.*, 1959). The medium was solidified with 1.1% agar and was at pH 5.7 before autoclaving. The sown spores were maintained in darkness or under a 12 hr photoperiod (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) from cool white fluorescent lamps at 22 ± 1°C. To promote the development of mature gametophytes, young gametophytes were transferred to fresh nutrient medium containing 0.5% glucose instead of 0.25% glucose. Later, mature gametophytes were transferred from the culture tubes to petri plates with 50 ml of nutrient medium containing 0.5% glucose for additional growth.

The mature gametophytes were fixed with Randolph's modified Navashin fluid (CRAF; Johansen, 1940). After fixation, the gametophytes were embedded in paraffin and sectioned by conventional techniques (Johansen, 1940). The sections were stained with Heidenhain's hematoxylin, safranin O, and fast green.

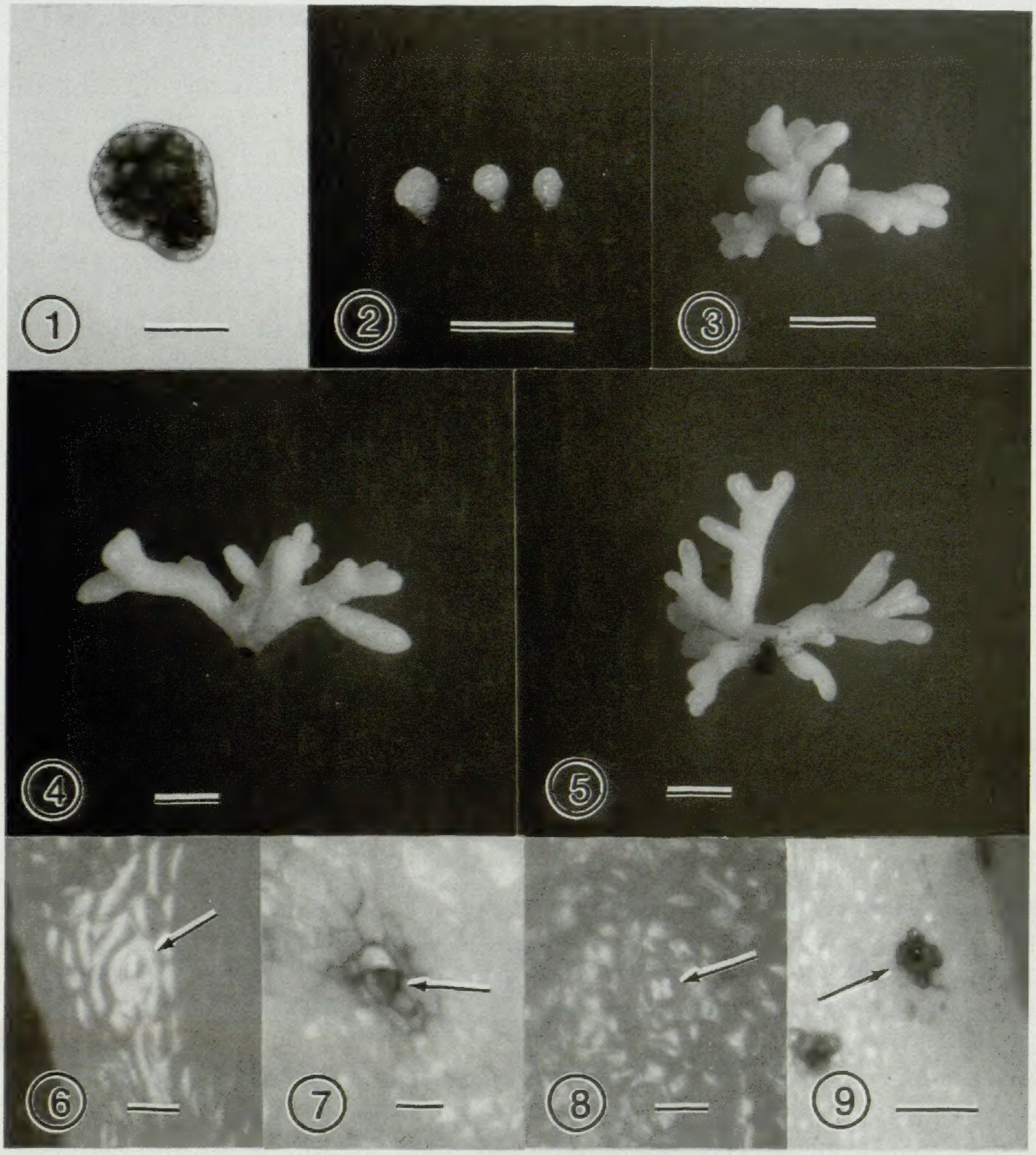
Results

After six weeks (42 days) in the dark, 6.3% of the spores had germinated. Germination must have initiated during the sixth week because germination had not occurred by the end of week five (35 days). No germination occurred in illuminated cultures after one year.

About a month after the first germinating spores were found, small globular gametophytes (Fig. 1) had developed. Six months later, larger teardrop-shaped gametophytes (Fig. 2) were observed on the surface of the nutrient medium.

The teardrop-shaped gametophytes enlarged to initiate short cylindrical gametophytes. At this time the gametophytes began to branch forming cylindrical branches in several directions. These branches had antheridia and archegonia interspersed along their length and thus were sexually mature. Besides increasing in length through the action of meristems each with a single apical cell (not illustrated), the original branches underwent more branching (Figs. 3, 4, 5). Relatively quickly a gametophyte with numerous radiating branches was formed. The branches were brittle and tended to detach if the gametophytes were handled.

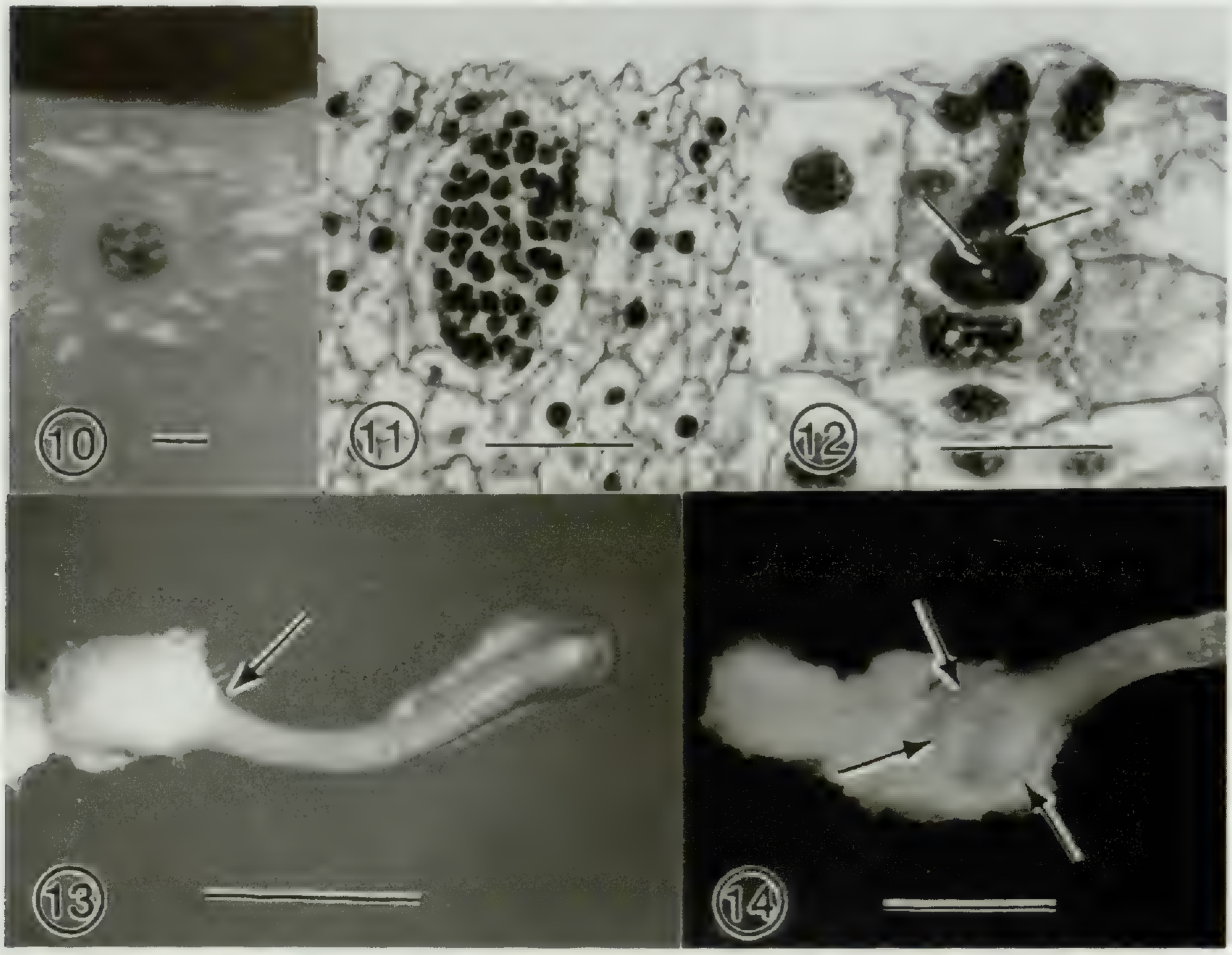
The antheridia were difficult to see on the living gametophytes without the use of a dissecting microscope at high magnification (Fig. 6). The exposed portion of the antheridium was in a shallow depression on the surface of the gametophyte (Fig. 6). It was composed of 4–5 cells with a more or less centrally



FIGS. 1–9. Gametophytes of *Ophioglossum pendulum*. 1. Globular gametophyte, bar = 100 µm. 2. Teardrop-shaped gametophyte, bar = 2 mm. 3–5. Branched gametophytes, bars = 2 mm. 6. Surface view of an antheridium (arrow), bar = 100 µm. 7. Surface view of an old antheridium with opercular cell (arrow), bar = 100 µm. 8. Surface view of neck of young archegonium (arrow), bar = 200 µm. 9. Open archegonium with reflexed neck cells (arrow), bar = 200 µm.

located single opercular cell. The triangular surface wall of the opercular cell was best seen with a postmature antheridium having dark walls (Fig. 7).

The archegonia (Fig. 8) were also difficult to find on the surface of these gametophytes. They were best identified with light reflecting off the gametophyte surface. The neck of the archegonium sits in a shallow depression at the gametophyte surface. It had a short neck that extends about 30 µm above



FIGS. 10–14. Gametophytes and embryos of *Ophioglossum pendulum*. 10. Open archegonium without reflexed neck cells, bar = 100 μm. 11. Longitudinal section of an antheridium, bar = 100 μm. 12. Longitudinal section of an archegonium with two nuclei in neck canal cell (arrows), bar = 50 μm. 13. Gametophyte with protruding primary root of embryo with arrow showing exit point of root, bar = 2 mm. 14. Handsection of embryo with main mass of embryonic tissue embedded in gametophyte (arrows), bar = 1 mm.

the gametophyte surface. It was composed of a fully exposed terminal tier of four neck cells attached to a partially exposed subterminal tier of neck cells. When an archegonium opened, the neck cells of the terminal tier spread apart to expose the neck canal to the external environment. This was easiest to observe with an old open archegonium that had dark walls (Fig. 9). The reflexed neck cells were easily displaced in handling the gametophyte to show the four neck cells to which the terminal neck cells were attached (Fig. 10).

The antheridia were sunken in the parenchymatous tissue that makes up the body of the cylindrical gametophyte. Close to maturity each antheridium contained an ellipsoidal or spherical mass of spermatocyctes (Fig. 11) in longitudinal section. Sections of archegonia clearly demonstrated a short neck, a binucleate neck canal cell and an egg for each archegonium (Fig. 12). A ventral canal cell was not observed. It may have been missed because the correct stage of archegonial development was not sectioned.

Young embryos were formed on gametophytes in old cultures. Enough water existed on the surface of the nutrient medium for the spermatozoa to reach the archegonia and bring about fertilization. The embryos were recognized by the primary root growing out of the gametophyte (Fig. 13). A large portion of the young embryo at this stage remained embedded in the gametophyte (Fig. 14). There was no stem or primary leaf as part of the embryo at this stage. The later development of these young sporophytes was not followed.

Discussion

The spores of *O. pendulum* ssp. *falcatum* from Hawaii started germinating in the dark at about the average time for previously studied spores of *Ophioglossum* (Whittier, 2003). Also, their time for germination was close to that reported by Campbell (1907) for spores of ssp. *pendulum* from Sri Lanka and Java. He reported germinated spores 36 days after sowing. In the present study the earliest spore germination needed more than 35 days but less than 42 days in the dark. Because 6% germination and some gametophyte growth had occurred at 42 days, the earliest germination would have been initiated prior to day 42.

The earliest stages of gametophyte development were not examined in this study. However, the young globular gametophytes of *O. pendulum* ssp. *falcatum* (Fig. 1) had the same structure as those of other *Ophioglossum* species (Whittier, 1981; 2003). It appeared from the occurrence of typical globular gametophytes that there was nothing unusual about early gametophyte development in this subspecies.

Highly branched gametophytes of *O. pendulum* ssp. *pendulum* were found by Lang (1902) and Campbell (1907) in Sri Lanka and Java. Both reported that the branches of these gametophytes extended in all directions. Gametophytes of *O. pendulum* ssp. *falcatum* with the same basic structure as those of ssp. *pendulum* were grown in culture. In nature the gametophytes grow embedded in tangles of roots between persistent leaf bases of some ferns or in humus on tree branches, whereas in culture the gametophytes grow on the solid surface of the nutrient medium. Whether the gametophytes grew in nature or in culture did not alter the structure of these highly branched gametophytes.

In addition to the normal growth pattern, the anatomy of these gametophytes was the same as that of gametophytes from nature. The apices had a single apical cell type of meristem and the bulk of the gametophyte was composed of parenchyma tissue. Sunken antheridia with ellipsoidal or spherical masses of spermatocytes were illustrated by Lang (1902) and Campbell (1907). The archegonium with its short neck, binucleate neck canal cell and egg was known from earlier studies. One difference was the apparent absence of the ventral canal cell in the archegonia on the cultured gametophytes of ssp. *falcatum*. Campbell (1907) reported its presence in ssp. *pendulum* but acknowledged that it was difficult to find because it was extremely inconspicuous. Because it forms late in development just before archegonial maturity, it may be evanescent. This along with the small size can explain why

the ventral canal cell was missed in this study and also by Lang (1902). Other than the difficulty in finding the ventral canal cell, the archegonia on the cultured gametophytes of ssp. *falcatum* are the same as those of ssp. *pendulum* from nature.

In support of the normalcy of the cultured gametophytes was the occurrence of sexual reproduction. Several young embryos with the primary root piercing the gametophyte to contact the surface of the nutrient medium occurred on these gametophytes. The embryos consisted of a mass of tissue embedded in the gametophyte and the elongating primary root. No stem or primary leaf was observed at this stage. This structure was described for young embryos of ssp. *pendulum* (Campbell, 1907). Embryos with precocious roots are not unusual for the genus, but where the stem and primary leaf arise on these young embryos is open to question (see Mesler *et al.*, 1975).

This study has demonstrated that the gametophytes of an epiphytic *Ophioglossum* species, *O. pendulum*, can be grown in culture. This study was successful in part because the nutrient medium on which the spores germinated also supported gametophyte development to maturity. The branched gametophytes of ssp. *falcatum* had the same branched structure as those of ssp. *pendulum* from nature. The substrates on which gametophytes of the two subspecies grew had little affect on their basic structure. Stellate gametophytes formed under the growing conditions in nature and those in culture.

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LITERATURE CITED

CAMPBELL, D. H. 1907. Studies on the Ophioglossaceae. Ann. Jard. Bot. Buitenzorg 6:138–194.

JOHANSEN, D. A. 1940. *Plant Microtechnique.* McGraw-Hill, New York.

LANG, W. H. 1902. On the prothalli of *Ophioglossum pendulum* and *Helminthostachys zeylandica.* Ann. Bot. 16:23–56.

MESLER, M. R., R. D. THOMAS and J. G. BRUCE. Mature gametophytes and young sporophytes of *Ophioglossum nudicaule.* Phytomorphology, 25:156–165.

SHEAT, D. E. G., B. H. FLETCHER and H. E. STREET. 1959. Studies on the growth of excised roots. VIII. The growth of excised tomato roots supplied with various inorganic sources of nitrogen. New Phytologist 58:128–154.

WHITTIER, D. P. 1964. The effect of sucrose on apogamy in *Cyrtomium falcatum.* Amer. Fern J. 54:20–25.

WHITTIER, D. P. 1981. Spore germination and young gametophyte development of *Botrychium* and *Ophioglossum* in axenic culture. Amer Fern J. 71:13–19.

WHITTIER, D. P. 2003. Rapid gametophyte maturation in *Ophioglossum crotalophoroides.* Amer. Fern J. 93:137–145.

WHITTIER, D. P. and R. MOYROUD. 1993. The promotion of spore germination and gametophyte development in *Ophioglossum palmatum* by low pH. Amer. Fern J. 83:41–46.

WHITTIER, D. P. and T. A. STEEVES. 1960. The induction of apogamy in the bracken fern. Canad. J. Bot. 38:925–930.

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Studies on the Gametophytes of Eight Chinese Species of *Dryopteris* (Dryopteridaceae)

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Abstract.—The gametophyte morphology and development of eight Chinese species of *Dryopteris* (Dryopteridaceae) were studied and described. Spores of all species were monolete and reniform. The germination pattern was the *Vittaria*-type. Germinal filaments were uniseriate, sometimes biseriate and the prothallial development was the *Aspidium*-type. Adult gametophytes in culture were cordiform, elongate-cordiform to cordiform-reniform, having wings with marginal and superficial trichomes. Gametangia belong to leptosporangiate fern type. Spore size, germination time, numbers of trichomes, morphology of rhizoids, formation time of the gametangia and gametophyte margin shape were different among the studied species.

Key Words.—*Dryopteris*, gametophytes, fern development

Dryopteris consists of about 230 species, which are distributed from temperate to tropical regions, with the highest species abundance and diversity in eastern Asia, especially in China (Li and Lu, 2006). Species in this genus are terrestrial with creeping rhizomes; petioles with numerous round, vascular bundles arranged in a ring; stems short–creeping to erect; leaves monomorphic, 1–3–pinnate-pinnatifid, gradually reduced distally to pinnatifid apex, herbaceous to somewhat leathery; pinnae not articulate to rachis; segment margins entire, crenate or serrate; sori round with a peltate indusium; spores brownish, coarsely rugose or with folded wings; and the chromosome number of x = 41 is common in the Dryopteridaceae.

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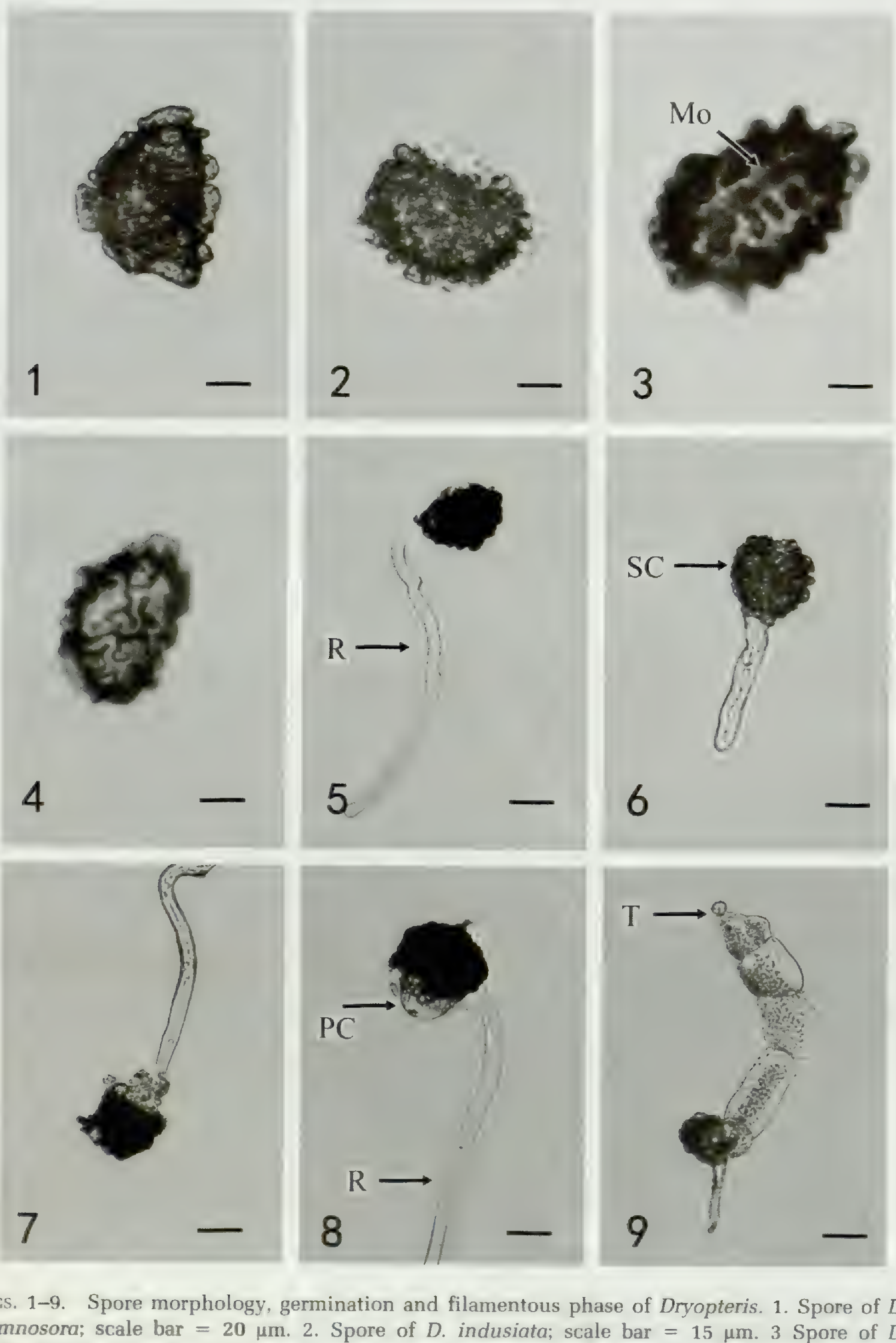
TABLE 1. Collection data.

Dryopteris species	Collector name	Collection no. and date	Site location	Deposit herbarium	Spore numbers
D. expansa	X. C. Zhang	3079, 9/2003	Yunnan	Herbarium of Chinese National Herbarium, IBCAS (PE)	60
D. championii	X. C. Zhang	4142, 10/2006	Guangxi, Huaping	IBCAS (PE)	61
D. gymnosora	X. C. Zhang	4119, 10/2006	Guangxi, Huaping	IBCAS (PE)	60
D. indusiata	X. C. Zhang	4145, 10/2006	Guangxi, Huaping	IBCAS (PE)	60
D. subtriangularis	X. C. Zhang	3083, 09/2003	Yunnan, Pingbian	IBCAS (PE)	59
D. fructuosa	X. C. Zhang	2749, 09/2002	Sichuan, Panzhihua	IBCAS (PE)	60
D. atrata	X. Cheng	2011, 01/2000	Yunnan, Kunming	Herbarium of Kunming Institute of Botany, CAS (KUN)	60
D. integriloba	B. D. Liu	567, 12/2004	Hainan, Wuzhishan	Herbarium of Harbin Normal Univeristy	60



FIGS. 1–9. Spore morphology, germination and filamentous phase of *Dryopteris*. 1. Spore of *D. gymnosora*; scale bar = 20 µm. 2. Spore of *D. indusiata*; scale bar = 15 µm. 3 Spore of *D. subtriangularis* with monolete (Mo) scar (arrow); scale bar = 15 µm. 4. Spore of *D. integriloba*; scale bar = 15 µm. 5. Germination of *D. fructuosa* with rhizoid (R); scale bar = 22 µm. 6. Germination of *D. indusiata* with spore coat (SC); scale bar = 15 µm. 7. Germination of *D. championii*; scale bar =

Although much taxonomical and systematic research on the genus *Dryopteris* has been performed (Tryon and Tryon, 1982; Kramer and Green, 1990; Geiger and Ranker, 2005) and the Chinese taxa have been described (Ching, 1965, 1978; Li and Lu, 2006; Liu *et al.*, 2007), the relationships among *Dryopteris* species remain poorly understood. Since gametophyte morphology of ferns is considered to be significant and characteristic of fern taxa (Chen *et al.*, 2008), studies on the gametophytes of *Dryopteris* species should be performed.

Gametophyte morphology and development of several *Dryopteris* species have been studied and summarized (Cousens, 1975; Cousens and Horner, 1970; Duncan, 1943; Kanamori, 1967; Kaur, 1977; Loyal, 1959; Momose, 1937, 1939; Pérez-García *et al.*, 1999, 2001). However, there have been no detailed reports on several species of *Dryopteris*. Hence, this paper describes the morphogenetic study of *D. atrata* (Wall) Ching, *D. championii* (Benth.) C. Chr., *D. expansa* (C. Presl) Fraser-Jenk. & Jermy, *D. gymnosora* (Makino) C. Chr., *D. fructuosa* (Christ) C. Chr., *D. indusiata* Makino & Yamam, *D. integriloba* C. Chr., *D. subtriangularis* (C. Hope) C. Chr.

Materials and Methods

Materials for research were obtained from several China localities (Table 1). Spores were collected from fertile pinnae of eight different living plants (*Dryopteris atrata*, *D. championii*, *D. expansa*, *D. gymnosora*, *D. fructuosa*, *D. indusiata*, *D. integriloba*, and *D. subtriangularis*) and leaves with spores were kept in clean paper bags under dry conditions. The remains of sporangia and indusia were eliminated by a mesh with pores 0.054 mm in diameter one week later. Spores were spread evenly in plastic basins (measuring 25 cm × 20 cm × 5 cm) with a sieved mixture of black soil and sand (Zhang *et al.*, 2008) at an average density of 100–150 spores per cm^2. Basins were covered with transparent plastic film to avoid contamination and desiccation, under a diurnal cycle of 12/12 hr, with fluorescent light (10,000 $\mu mol \cdot m^{-2} \cdot sec^{-1}$), at 25 °C.

Spore sizes were measured from material in water with a compound microscope (No. XTS 20130, Beijing Tech Instrument Co., LTD) equipped with an ocular micrometer. An average of sixty measurements of the spore length and width per species were made. Spore morphology was observed under the compound microscope from material in water.

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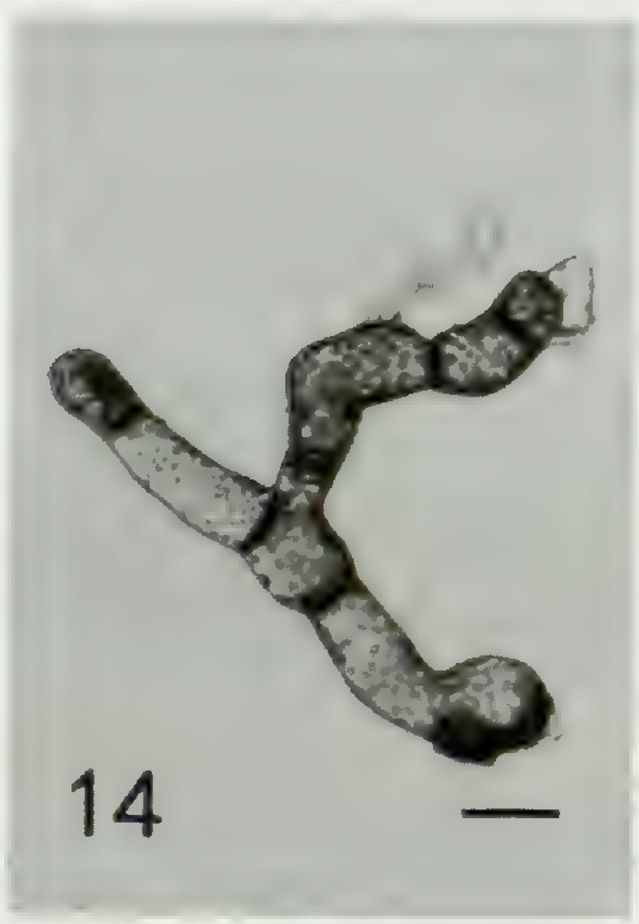
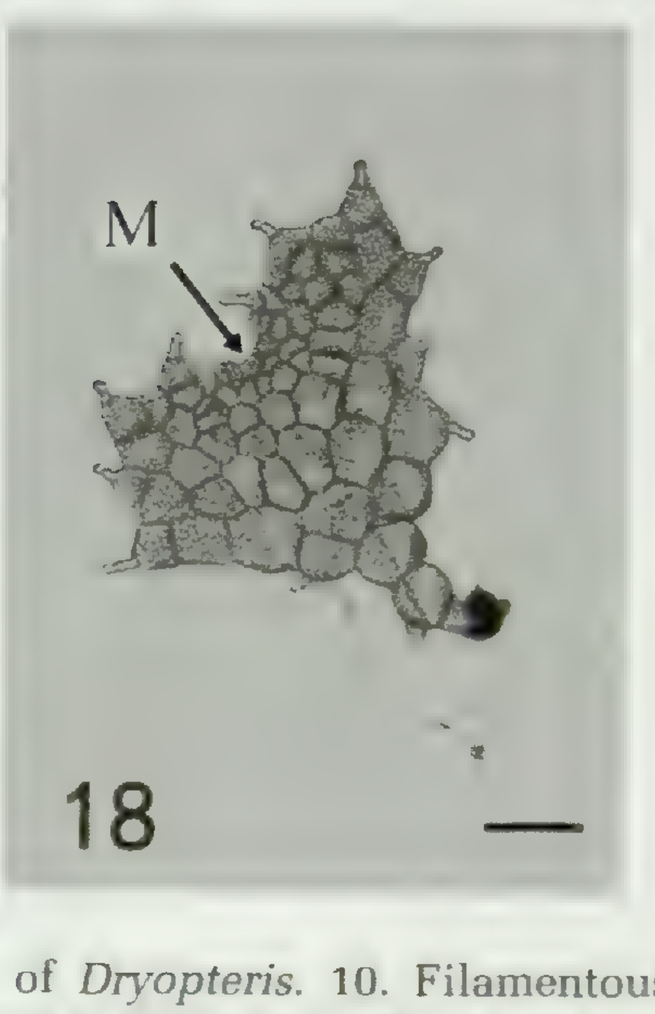
30 μm. 8. Germination of *D. fructuosa* with prothallial cell (PC); scale bar = 25 μm. 9. Filamentous phase of *D. gymnosora* with a trichome (T); scale bar = 40 μm.





FIGS. 10–18. Filamentous phase and young plate morphology of *Dryopteris*. 10. Filamentous phase of *D. championii*; scale bar = 30 μm. 11. Filamentous phase of *D. indusiata* with rhizoid (R); scale bar = 30 μm. 12. Filamentous phase of *D. championii* with trichome (T); scale bar = 30 μm. 13. Biseriate Filament of *D. subtriangularis*; scale bar = 60 μm. 14. Filamentous phase of *D. atrata*; scale bar = 30 μm. 15. Filamentous phase of *D. subtriangularis* with trichome (T); scale bar =

Results

Spores.—Spores of all species were homosporous, reniform and monolete. Size was variable among species. Spore measures were (21) 24 (30) × (31) 36 (40) μm in *D. expansa*, (25) 29 (33) × (50) 55 (60) μm in *D. championii*, (36) 40 (45) × (61) 72 (83) μm in *D. gymnosora*, (27) 32 (36) × (51) 60 (62) μm in *D. indusiata*, (60) 65 (70) × (100) 112.5 (120) μm in *D. subtriangularis*, (29) 32 (34) × (32) 40 (45) μm in *D. fructuosa*, (28) 31 (36) × (42) 46 (49) μm in *D. atrata* and (29) 33 (36) × (33) 38 (43) μm in *D. integriloba* (Figs. 1–4). Perine was winged (Tryon and Lugardon, 1991).

Germination.—The germination rate in spores was 90%. The germination process began after about 20 days in *D. expansa*, 7–12 days in *D. championii*, about two weeks for *D. gymnosora*, 8–11 days for *D. indusiata*, about one week for *D. subtriangularis*, about 25 days for *D. fructuosa*, about 20 days for *D. atrata* and about 24 days for *D. integriloba*. All the species share *Vittaria*-type (Nayar and Kaur, 1971) germination after sowing (Figs. 5–7). The short and hyaline rhizoid appeared first and initially had a wall perpendicular to the polar axis, and was followed by the first prothallial cell (Fig. 8). The initial prothallial cell was characterized by a large number of chloroplasts.

Filamentous phase.—In most species, with the division of the first prothallial cell by a transverse wall, an apical cell was produced, and finally a uniseriate germ–filament formed, which was 2–20 cells long (Figs. 9–12). Cells were barrel–shaped and had abundant chloroplasts. However, in *D. subtriangularis*, the germ-filament was uniserial or biseriate (Fig. 13). In *D. atrata*, there were two types of germ-filament: uniseriate and branched (Fig. 14). The germ–filament developed an apical unicellular trichome in the early phases of gametophyte development, which was observed in *D. gymnosora*, *D. indusiata* and *D. subtriangularis* (Figs. 9, 11, 12, 15). In *D. championii*, *D. expansa*, *D. atrata* and *D. fructuosa*, trichomes formed in the laminar phase. Compared with the other species, trichomes were produced earliest in *D. subtriangularis* when the germ-filament was only 2 cells long (Fig. 15). The spore coat remained attached.

Laminar phase.—The differentiation of the laminar phase was asynchronous and development occurred between days 17 (*D. championii*) and 41 (*D. gymnosora*). In *D. subtriangularis*, although the terminal cell of the germ-filament had produced a trichome, it still took part in the laminar formation. It divided longitudinally into a larger and a smaller cell. The larger one remained inactive and the smaller one divided actively, contributing to the development of the prothallial plate (Fig. 16). As a result, the plate was slightly lopsided at the anterior end (Fig. 17). A meristematic cell was differentiated in one of the

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35 μm. 16. Filament develops towards prothallial plate stage of *D. subtriangulari* with trichome (T); scale bar = 35 μm. 17. Plate phase of *D. subtriangularis*; scale bar = 35 μm. 18. Young gametophyte of *D. championii* with meristem (M); scale bar = 60 μm.


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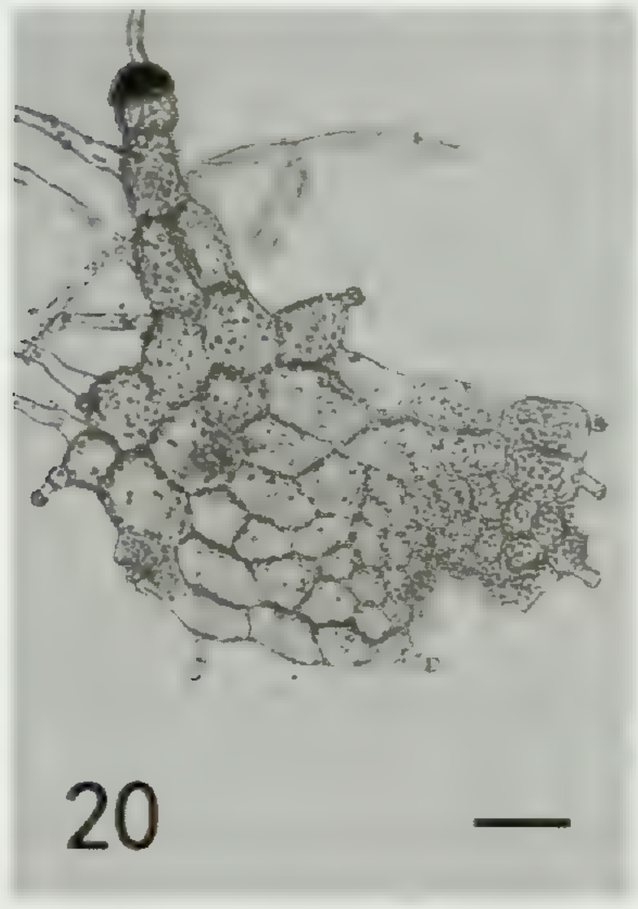
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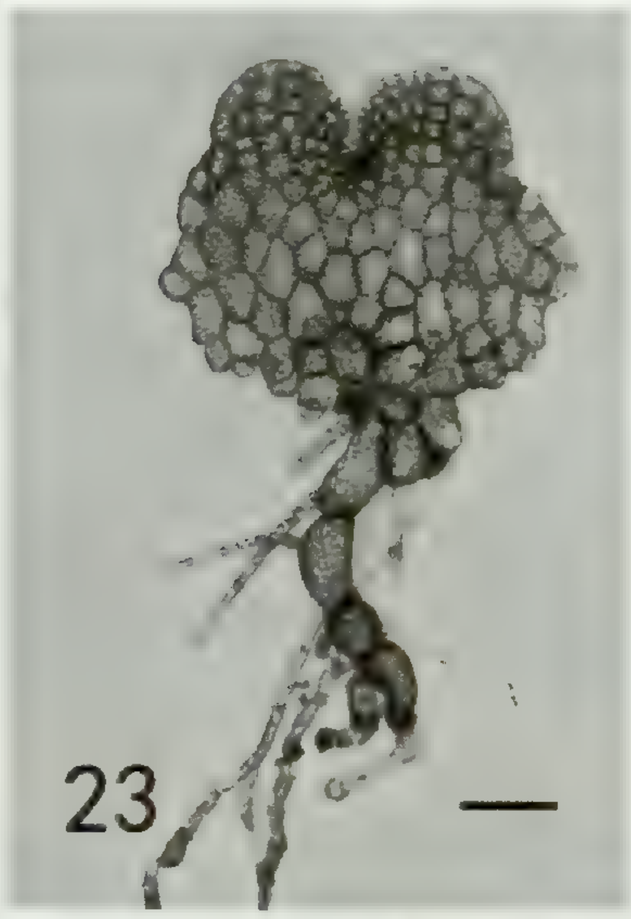
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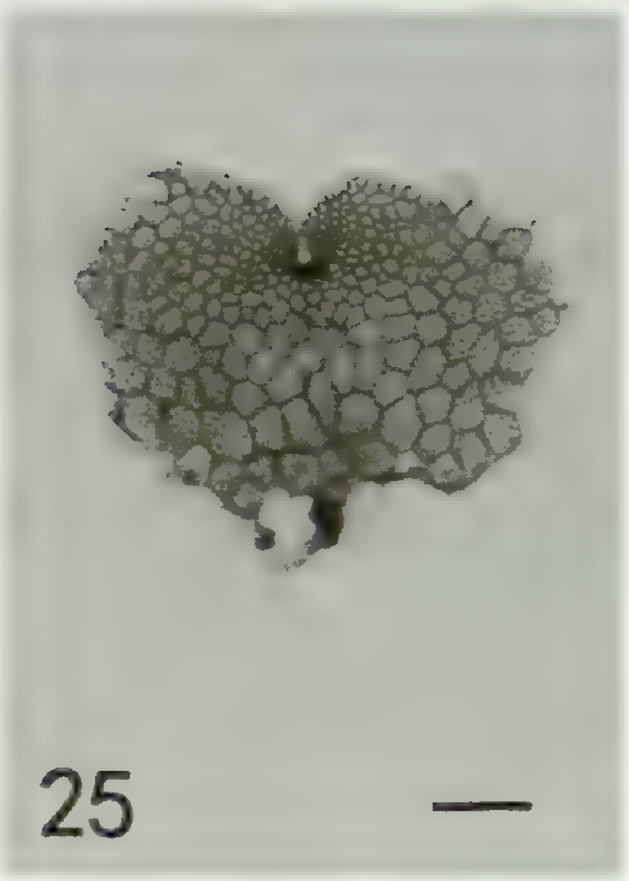
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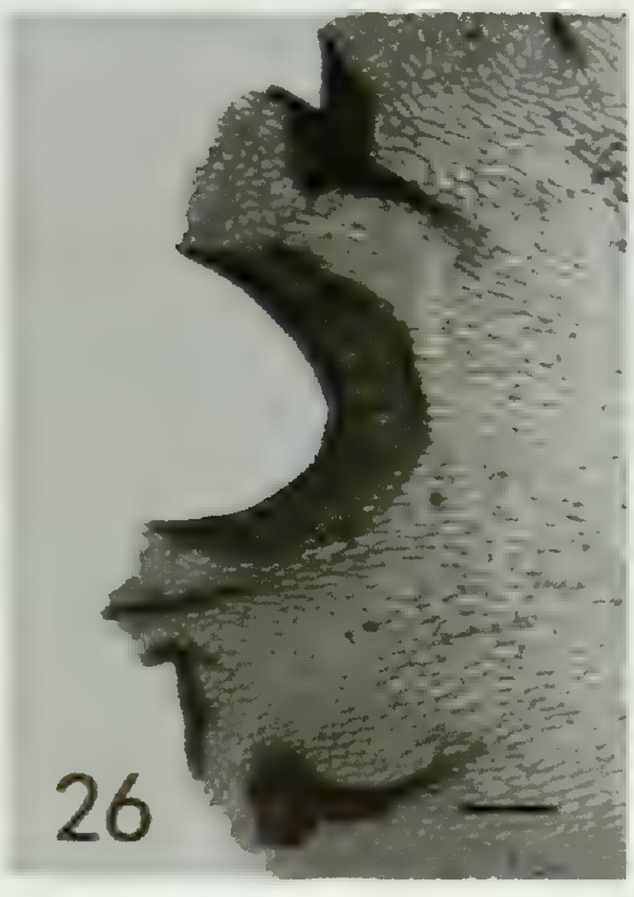
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FIGS. 19–27. Plate phase of *Dryopteris*. 19. Young plate of *D. gymnosora*; scale bar = 80 μm. 20. Gametophyte of *D. indusiata*; scale bar = 60 μm. 21. Gametophyte of *D. subtriangularis*; scale bar = 200 μm. 22. Mature gametophyte of *D. atrata*; scale bar = 70 μm. 23. Young gametophyte of *D. expansa*; scale bar = 120 μm. 24. Cordiform gametophyte of *D. expansa*; scale bar = 150 μm. 25. Mature gametophyte of *D. expansa*; scale bar = 150 μm. 26. Gametophyte with folded margins of *D. expansa*; scale bar = 350 μm. 27. Diverse plate phase of *D. fructuosa*; scale bar = 150 μm.

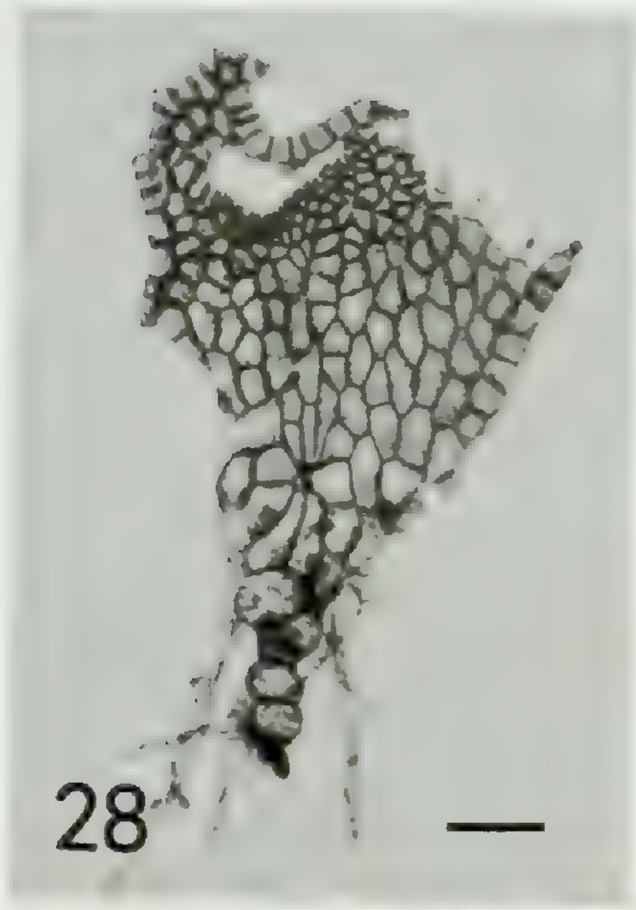
marginal cells formed from the active smaller cell. Prothallial development in all species was *Aspidium*-type (Nayar and Kaur, 1969).

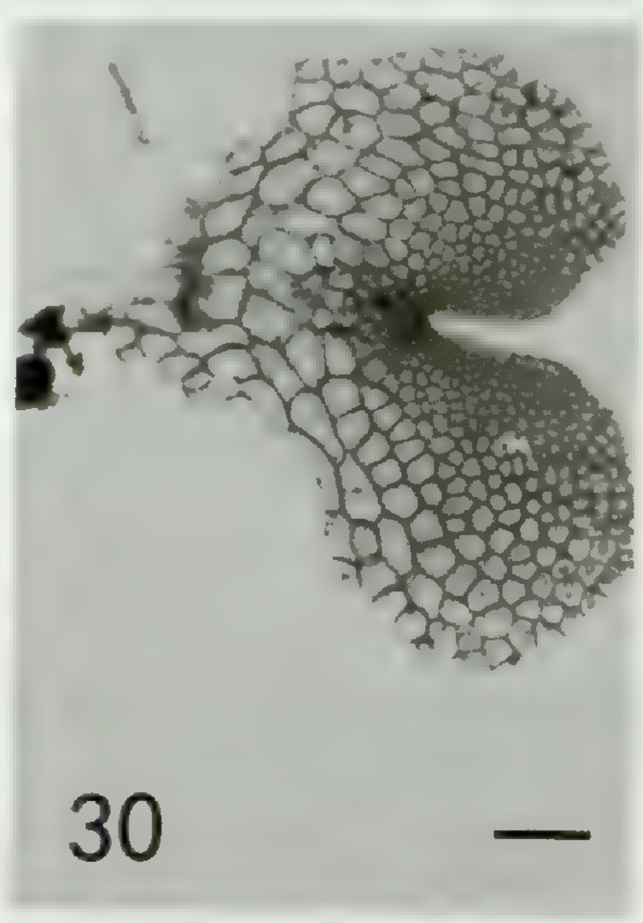
Young gametophytes of *D. championii* were cordiform-spatulate, becoming cordiform with age, with underdeveloped wings and irregular margins (Fig. 18). The young plate phase of *D. gymnosora* was cordiform-reniform (Fig. 19), but at maturity was mostly cordiform, with strongly developed wings and smooth to slightly irregular margins. Gametophytes in *D. indusiata* and *D. subtriangularis* were spatulate when young and cordiform when mature (Figs. 20–21), with wide wings and smooth to slightly irregular margins. Young gametophytes of *D. atrata* were spatulate with very irregular margins and filamentous extensions 2–6 cells long. When mature they became cordiform-reniform with well–developed wings (Fig. 22). Gametophytes in *D. expansa* were spatulate when young, becoming cordiform to cordiform-reniform when mature, with smooth to irregular margins (Figs. 23–25). Folded margins were observed in some gametophytes (Fig. 26). Gametophytes in *D. fructuosa* were the most variable: they were irregular spatulate with small extensions when young (Fig. 27), and when mature they were elongate-cordiform, with irregular margins with filamentous extentions 3–20 cells long, or plate extensions 10–30 cells long (Fig. 28). Sometimes cordiform-reniform gametophytes with irregular margins developed (Fig. 29). Gametophytes in *D. integriloba* were spatulate when young and cordiform to cordiform-reniform when mature, with wide wings and smooth to slightly irregular margins (Fig. 30).

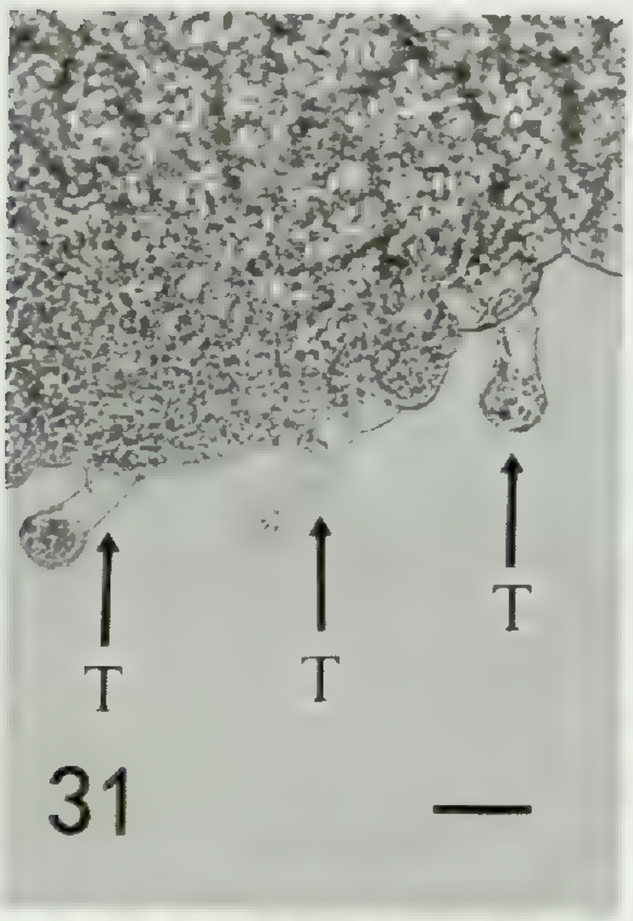
Adult gametophyte.—The time for the first adult gametophytes of all species to differentiate varied from days 21 (*D. subtriangularis)* to 52 (*D. fructuosa*). Under our cultural conditions, the largest adult gametophyte belonged to *D. subtriangularis* (7 × 4 mm).

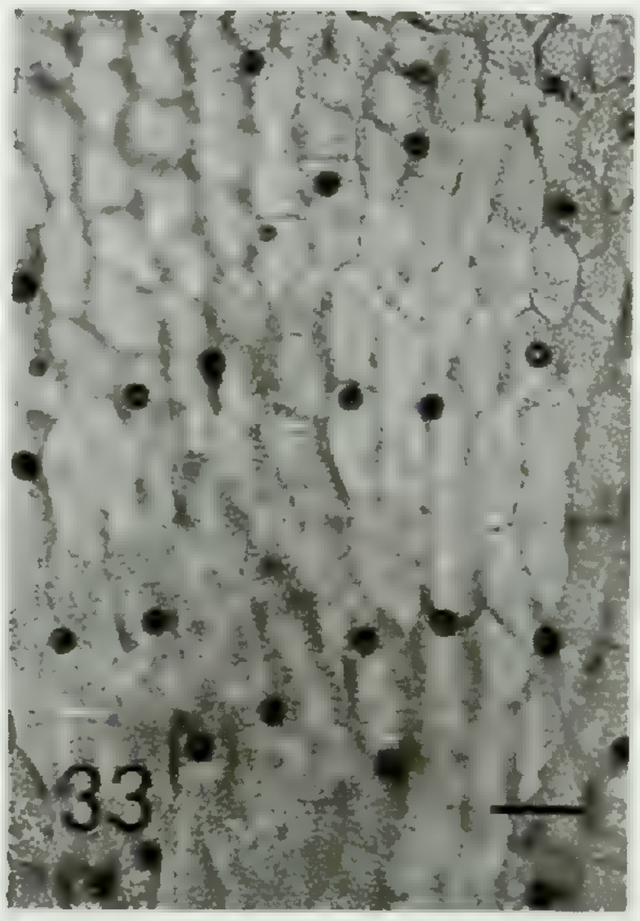
For most species, the first trichome originated from the terminal cell of the filament. With the development of the gametophyte, more and more trichomes were produced by division of the other prothallial cells. Trichomes were found on the margin and surfaces of the gametophytes, and were papillate to slender claviform, with or without glands (Figs. 31–32). They were most abundant in gametophytes of *D. subtriangularis* at an average of 100 (Fig. 33), compared with those of *D. indusiata* (11), *D. gymnosora* (23), *D. championii* (12), *D. expansa* (26), *D. atrata* (20), *D. fructuosa* (17), and *D. integriloba* (10). Chloroplasts were mainly distributed at the apex of the trichome, which are generally smaller in size compared to those of other prothallial cells. Chloroplasts in the marginal cells were mostly disk-shape and in the central cells were oval, disk-shape and dumbbell-shape (Fig. 34). Some marginal cells connecting to trichomes were prominent.

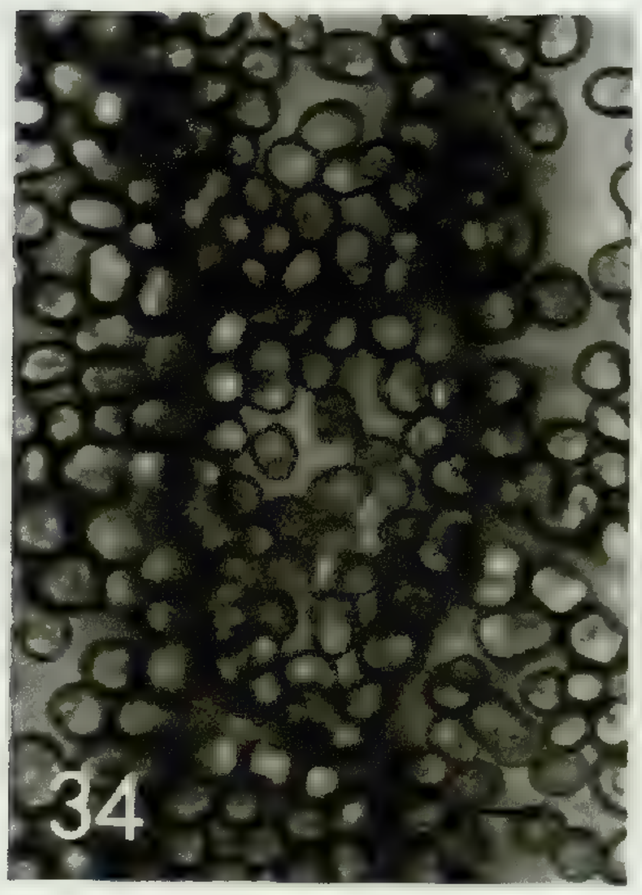
Rhizoids were formed by the cell divisions of the prothallial cells. In all species, the first rhizoids were hyaline. With the development of the prothallus, the rhizoids of all species became brown and curved. Rhizoids developed mainly on the ventral surface of the young prothallus but they also could be observed on the mature prothallial margins.

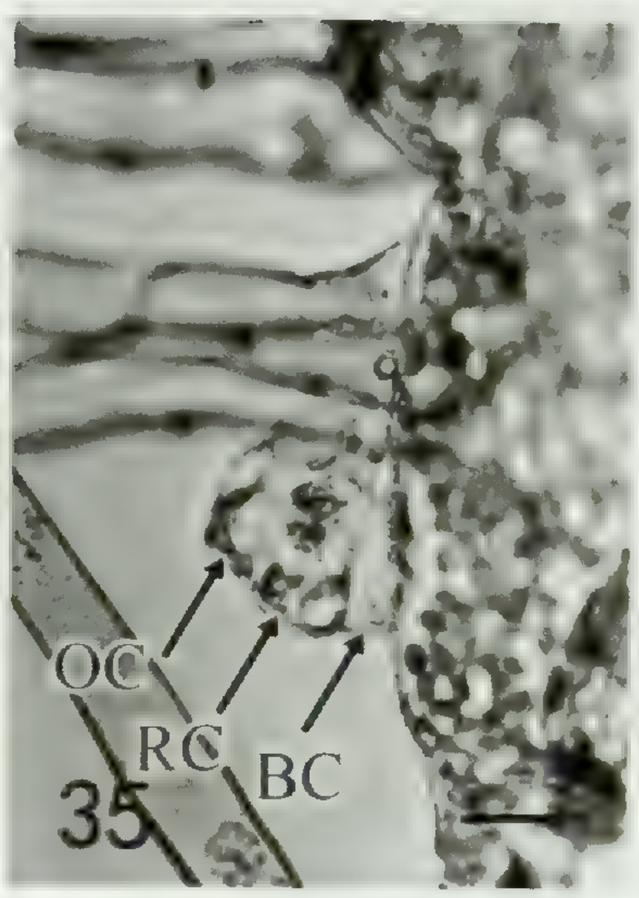


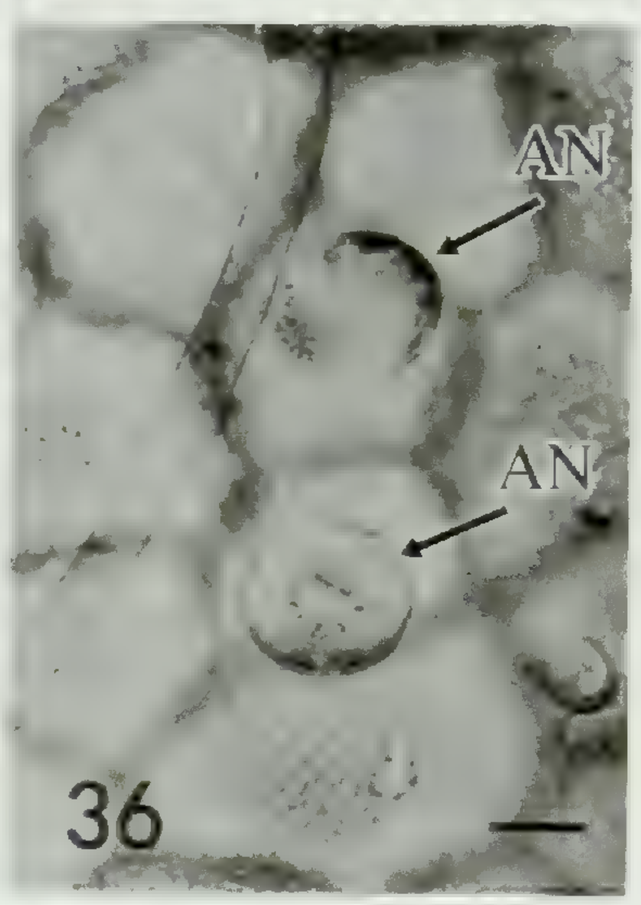












FIGS. 28–36. Gametophyte morphology, trichome, and antheridia of *Dryopteris*. 28. Diverse prothallium phase of *D. fructuosa*; scale bar = 150 μm. 29. Cordiform-reniform gametophyte of *D. fructuosa*; scale bar = 150 μm. 30. Gametophyte of *D. integriloba*; scale bar = 150 μm. 31. Trichomes (arrows) of *D. gymnosora*; scale bar = 100 μm. 32. Trichome of *D. fructuosa*; scale bar = 30 μm. 33. Distribution of the trichomes on the mature prothallus of *D. subtriangularis* (The black

Gametangia.—The gametophytes are sexually mature once the gametangia form. Time for gametangia formation varied from 25 days in *D. indusiata* to 80 days in *D. gymnosora*. The antheridia were restricted to the basal part of the gametophyte (between the rhizoids). The number of the antheridia varied from 10–40 per gametophyte. Morphologically, the antheridia were globose, and consisted of a basal cell, an annular or ring cell and an opercular cell (Fig. 35). Antherozoids were liberated by detachment of the operculum (Fig. 36).

The archegonia developed on the cushion near the meristematic zone of the gametophyte. Their necks were elongated, composed of 3–5 tiers of cells with each tier having four cells. The archegonia became brown when they were post-mature (Figs. 37).

Sporophytes.—The first sporophytes were observed by about 8–12 weeks after sowing. In all species, the first leaves were spatulate, bilobed to trilobed (Fig. 38). Fertilization occurred on almost all gametophytes to produce sporophytes (Fig. 39).

Discussion

The spores of all species shared features such as monolete spores and ornamented perine. However, the spore sizes of the studied species were different.

Germination times differed in the different species, corresponding with previous studies for other species of Dryopteridaceae (Chandra and Nayar, 1970; Mendoza *et al.*, 1999a, 1999b, 2002; Pérez-García *et al.*, 1999, 2001; Mendoza and Pérez-García, 2003; Mendoza, 2001). The germination pattern in all species was of the *Vittaria*-type, as observed by Nayar and Kaur (1971), Chandra and Nayar (1970), Mendoza *et al.* (1999a, 1999b, 2002, 2003), Pérez-García *et al.* (1999, 2001), Mendoza (2001) in Dryopteridaceae. It is the most common type in ferns. In this type, the rhizoid develops first after the formation of a wall perpendicular to the polar axis of the spores (Nayar and Kaur, 1971).

Germinative uniseriate filaments were 2–10 cells long in all species. Biseriate filaments in *D. subtriangularis* were 4–12 cells long in our cultural conditions. Uniseriate and branched germ–filaments appeared in *D. atrata.* The prothallial development was *Aspidium*–type, which is also observed by Pérez–García *et al.* (1999, 2001) for *Dryopteris* species in Mexico. In this type, the apical cell of the germ–filament produced a unicellular papillate trichome crowning it. Commonly, the apical cell becomes sluggish. However, in some cases, this cell remains active and divides, taking part in prothallial plate formation (Nayar and Kaur, 1971). The prothallial development of the studied

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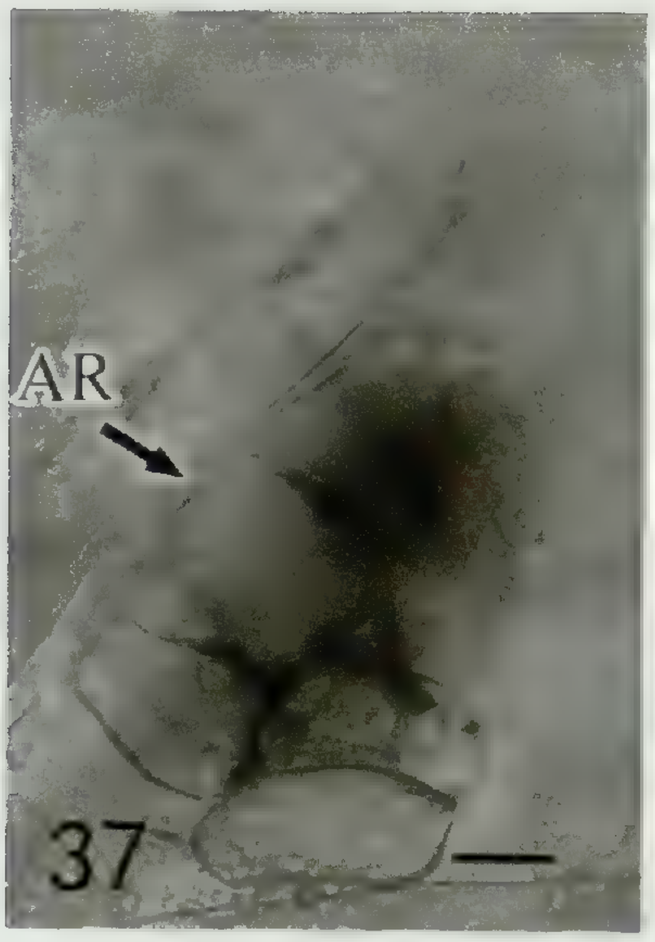
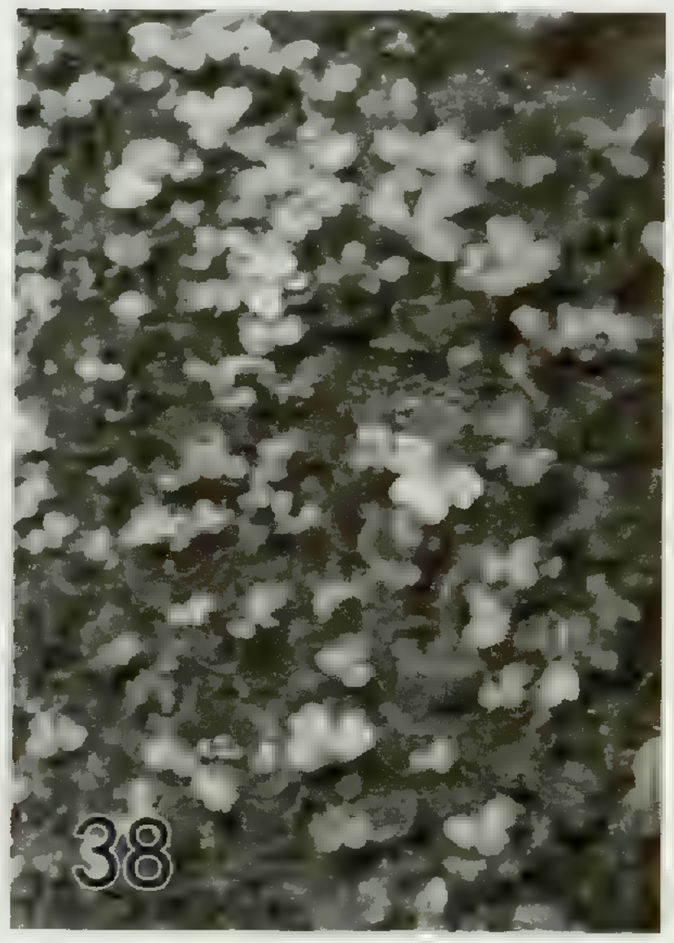
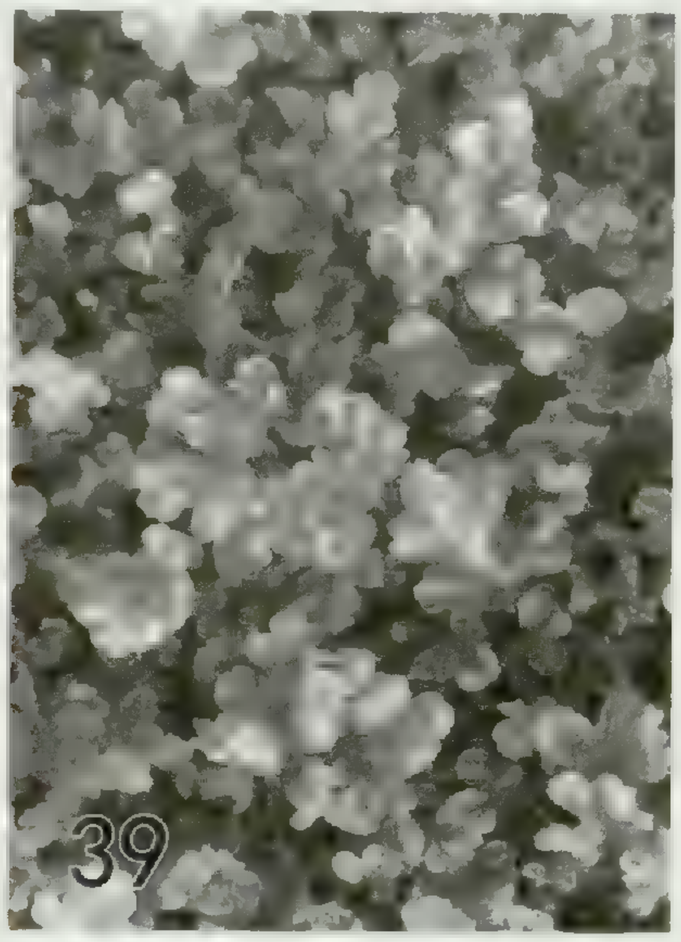
spots show the trichomes); scale bar = 500 μm. 34. Prothallial cells showing the chloroplasts of *D. fructuosa*; scale bar = 15 μm. 35. Antheridia of *D. integriloba* with basal cell (BC), ring cell (RC) and opercular cell (OC); scale bar = 30 μm. 36. Antheridia (arrows) of *D. atrata*; scale bar = 30 μm.







FIGS. 37–39. Archegonia and sporophyte of *Dryopteris*. 37. The lateral view of the mature archegonium (arrow) of *D. subtriangularis*; scale bar = 10 μm. 38. Young sporophytes of *D. gymnosora*. 39. Young sporophytes of *D. subtriangularis*.

species occurred according to the latter route. The adult gametophyte developed faster in *D. subtriangularis* than in the other species.

Trichomes of all studied taxa were papillate to slender claviform, with or without glands, contrasting with the observations of Pérez–García *et al.* (1999, 2001), who noted that trichomes of *Dryopteris* are unicellular, capitate rounded apex, with a layer of extracellular secretion. Trichomes were found on the margin and surfaces of the gametophytes, agreeing with the observations by Pérez–García *et al.* (1999, 2001) for other species of *Dryopteris*.

The observed morphology supports the description of Pérez–García *et al.* (1999, 2001), who found that gametophytes of the genus *Dryopteris* are spatulate, cordiform-spatulate, reniform, or cordate.

Sex organs of the studied taxa are of the typical leptosporangiate type, similar to the description of Dryopteridaceae given by Nayar and Kaur (1971).

The rhizoids in all species developed on the ventral surface and margin of the prothallus; they were initially hyaline and subsequently became brown in mature and older prothallia. Nevertheless, differences in the morphology of the rhizoids in some species were observed. Furthermore, number and length of the rhizoids in all species were different.

The *Vittaria*-type germination, the *Aspidium*–type prothallial development, the presence of unicellular trichomes, and the morphology of the adult gametophytes, are diagnostic characteristic features for the genus *Dryopteris*. Distinguishing features among the studied species are size of the spores, germination time, time of formation of the gametangia, and thallus margin shape.

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Literature Cited

Chandra, P. and B. K. Nayar. 1970. Morphology of some polystichoid ferns. I. The gametophytes of *Arachniodes*, *Cyrtomium* and *Polystichum*. Bot. J. Linn. Soc. 63:265–276.

Chen, G. J., X. Cheng, B. D. Liu and Y. Jiao. 2008. Comparative studies on gametophyte morphology and development of seven species of Cyatheaceae. Amer. Fern J. 98:83–95.

Ching, R. C. 1965. Dryopteridaceae–a new fern family. Acta Phytotax. Sin. 10:1–5.

Ching, R. C. 1978. The Chinese fern families and genera: systematic arrangement and historical origin. Acta Phytotax. Sin. 16:1–59.

Cousens, M. I. 1975. Gametophyte sex expression in some species of *Dryopteris*. Amer. Fern J. 60:13–27.

Cousens, M. I. and H. T. Horner. 1970. Gametophyte ontogeny and sex expression in *Dryopteris ludoviciana*. Amer. Fern J. 60:13–27.

Duncan, R. E. 1943. Origin and development of embryos in certain apogamous forms of *Dryopteris*. Bot. Gaz. 105:202–211.

Geiger, J. M. O. and T. A. Ranker. 2005. Molecular phylogenetics and historical biogeography of Hawaiian *Dryopteris* (Dryopteridaceae). Mol. Phylogenet. Evol. 3:392–407.

Kanamori, K. 1967. Origin and early development of apogamous embryos in the prothallia of *Dryopteris chinesis*. J. Jap. Bot. 42:111–118.

Kaur, S. 1977. Morphology of the prothallus and juvenile sporophytes of some species of *Dryopteris*. Proc. Indian Acad. Sci. 85:163–171.

Kramer, K. U. and P. S. Green. 1990. Pteridophytes and gymnosperms. Pp. 74–80. *In:* The families and genera of vascular plants, K. Kubitzki, (ed.). Springer–Verlag, Berlin.

Li, C. X. and S. G. Lu. 2006. Phylogenetics of Chinese *Dryopteris* (Dryopteridaceae) based on the chloroplast *rps*4-*trn*S sequence data. J. Plant Res. 119:589–598.

Liu, H. M., X. C. Zhang, W. Wang, Y. L. Qiu and Z. D. Chen. 2007. Molecular phylogeny of the fern family Dryopteridaceae inferred from chloroplast *rbc*L and *atp*B genes. Int. J. Plant Sci. 689:1311–1323.

Loyal, D. S. 1959. Some observations on the cytology and apogamy of Himalayan *Dryopteris paleacea* (Don.) Hand. Mazz. J. Indian Bot. Soc. 39:608–613.

Mendoza-Ruiz, A. 2001. *Morfogénesis de la fase sexual de pteridofitas mexicanas, familia Dryopteridaceae*. M. Sc. thesis, Facultad de Ciencias, Universidad Nacional Autónoma de México, Mexico, D.F.

Mendoza-Ruiz, A. and B. Pérez-García. 2003. Comparative analysis of the sexual phase of *Phanerophlebia* (Dryopteridaceae) in Mexico. Can. J. Bot. 81:501–516.

Mendoza-Ruiz, A., B. Pérez-García and R. Riba. 1999a. Morfología yanatomía del gametofito de *Didymochlaena truncatula* (Dryopteridaceae). Rev. Biol. Trop. 47:87–93.

Mendoza-Ruiz, A., B. Pérez-García and R. riba. 1999b. Morfogénesis de la fase sexual del helecho *Arachniodes denticulata* (Dryopteridaceae). Rev. Biol. Trop. 47:791–797.

Mendoza-Ruiz, A., B. Pérez-García and R. Riba. 2002. Comparative research of gametophytes of *Olfersia alata* and *Olfersia cervina* (Dryopteridaceae). Amer. Fern J. 92:45–54.

Momose, S. 1937. Studies on the gametophytes of ferns. I. On the prothallial development and prothallium of *Dryopteris varia* O. Kuntze. J. Jap. Bot. 12:113–120.

Momose, S. 1939. Studies on the gametophytes of ferns. IX. Further observations on the prothallium of eudryopteroid ferns. J. Jap. Bot. 15:280–292.

Nayar, B. K. and S. Kaur. 1969. Type of prothallial development in homosporous ferns. Phytomorphology 19:179–188.

Nayar, B. K. and S. Kaur. 1971. Gametophytes of homosporous ferns. Bot. Rev. 37:295–396.

PÉREZ-GARCÍA, B., A. MENDOZA, J. I. REYES and R. RIBA. 1999. Morfogénesis de la fase sexual de seis especies mexicanas de helechos del género *Dryopteris* (Dryopteridaceae). Rev. Biol. Trop. 47:69–81.

PÉREZ-GARCÍA, B., A. MENDOZA, J. I. REYES and R. RIBA. 2001. Morfogénesis de la fase sexual de seis especies mexicanas del género *Dryopteris* (Dryopteridaceae), Parte II. Rev. Biol. Trop. 49:265–278.

TRYON, A. F. and B. LUGARDON. 1991. *Spores of the pteridophyta: surface, wall structure, and diversity based on electron microscope studies*. Springer Verlag, New York, USA.

TRYON, R. M. and A. F. TRYON. 1982. *Ferns and allied plants, with special reference to tropical America*. Springer Verlag, New York, USA.

ZHANG, K. M., L. SHI, X. C. ZHANG, C. D. JIANG and W. L. TIM-CHUN. 2008. Gametophyte morphology and development of six Chinese species of *Pteris* (Pteridaceae). Amer. Fern J. 98:33–41.

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In Vitro Regeneration of Leatherleaf Fern (*Rumohra adiantiformis* (G.Forst.) Ching)

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Abstract.—*Rumohra adiantiformis,* also known as "leatherleaf fern", is an ornamental species that, because of its long display life, is widely used in floral arrangements. In this study, a new protocol for *in vitro* regeneration of the leatherleaf fern was established. For spore germination, two culture media (MS and Knop) were assessed with presence or absence of 1g L^{-1} activated charcoal (AC) under different light and dark conditions. Frond, frond microcuttings, and prothallus explants were evaluated on Knop regeneration medium supplemented with 1g L^{-1} AC, 0.5% agar, pH 5.0 in combination with 2,4 dichlorophenoxyacetic acid (2,4-D: 0.0, 0.1, 0.5 and 1.0 mg L^{-1}) and 6-benzylamino purine (BA: 0.0, 0.1, 0.5 and 1.0 mg L^{-1}). For rooting, four levels of α-naphthaleneacetic acid (NAA: 0.0, 0.01, 0.1 and 0.2 mg L^{-1}) were tested. After 18 days of culture, spore germination rate was 100% on Knop medium with AC and 8 h light/16 h dark. After 120 days of culture, sporophytes 1.7 ± 0.4 cm in length developed on Knop medium, while those spore-cultured on MS medium never produced sporophytes. From those germinated sporophytes, prothallus explants cultivated on Knop medium with AC and 0.5 mg L^{-1} BA showed the highest regeneration rate with 235.7 gametophytes. The best sporophyte rooting response was obtained with 0.01 mg L^{-1} NAA. Complete, regenerated sporophytes were obtained 183 days after culture initiation. By this procedure it would be possible to obtain up to 2 million sporophytes from one fertile frond. To determine the origin of the regenerated gametophytes, a histological analysis was performed with scanning electron microscopy (SEM). The analysis revealed that the gametophytes were regenerated from explant epidermal tissues on either the adaxial or abaxial surface.

Key Words.—Leatherleaf fern, *Rhumora,* Sporophyte regeneration

Ferns are conventionally propagated both sexually and asexually. In asexual, or vegetative, propagation, new plants are produced from rhizomes, stolons, tubers, stipules, roots, buds, cuttings, and attached aerial stems (layering). Asexual fern propagation also includes apospory and apogamy

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(Kottackal *et al.*, 2006), deviations from the "normal" life cycle in ferns. Apospory is the development of a gametophyte from an epidermal cell or cells of a sporophyte (Ambrožic-Dolinšek *et al.*, 2002), while apogamy is the development of a sporophyte directly from a gametophyte without sexual fusion (Kottackal *et al.*, 2006). In either direction, since only mitotic divisions are involved, the number of chromosomes remains the same (Foster and Gifford, 1974).

Rumohra adiantiformis (G.Forst.) Ching, also known as the "7-weeks-fern", "leatherleaf" or "samambaia-preta", is highly valued on the international florist greenery market because of its long post-harvest display life (D'Souza *et al.*, 2006). *Rumohra adiantiformis* extractivism began in the 1970s as a major survival strategy for small-scale African and Brazilian farmers. It is a low-growing species indigenous to many parts of the Southern Hemisphere (Australia, South and Central America, Southern Africa and some Islands in the Indian Ocean). It is now cultivated commercially in American nurseries for the cut flower industry (Poole and Conover, 1978; Milton and Moll, 1988; Schumann and Mills, 1996). Most of the world's production of *R. adiantiformis* occurs in Florida where it is grown in controlled environments (Stamps *et al.*, 1994; Stamps, 2004).

Commercial propagation of *R. adiantiformis* is done by rhizome division, but frequent replanting is necessary, making it difficult to satisfy the great demand for its leaves (Chen and Read, 1983; Strandberg, 2003). Improved regeneration procedures of *in vitro* culture are therefore desirable (Fernández and Revilla, 2003). Little research on the *in vitro* culture of *R. adiantiformis* through rhizomes (Chen and Read, 1983; Amaki and Higuchi 1991) or spores (Brum and Randi, 2002; Brum and Randi, 2006) has been reported. To our knowledge, no attempt to increase the *in vitro* regeneration rate or to reduce the regeneration period has been reported. Therefore, the objectives of this study were to increase the regeneration rate and to reduce the time of regeneration of *R. adiantiformis* by *in vitro* culture.

Materials and Methods

In vitro *sorus germination and initial explants.*—Mature *Rumohra adiantiformis* fronds bearing sori were used as the source of explants. For disinfection, fronds were washed under running tap water for 5 min, then the surface was sterilized sequentially, first in 70% (v/v) ethanol (30 sec), 0.1% sodium hypochloride solution with two drops of Tween 20® (10 min), and rinsed three times with sterile distilled water (3 min each). Five sori were dissected and placed in glass flasks containing 30 mL of Knop (Knop, 1865) or MS (Murashige and Skoog, 1962) medium, supplemented with 0 or 1 g L^{-1} activated charcoal (AC) Hycel®. Each culture medium was adjusted to pH 5.0 with 1 N NaOH or HCl. Both media were gelled using 0.5% agar (Bioxon®) before autoclaving at 121°C and 1.2 kg cm^{-2} for 20 min. The cultures were incubated at 25 ± 1°C for one month and maintained under a cycle of 16 h light and 8 h dark, with a light intensity of 39 µmol m^{-2} s^{-1}. After spore germination

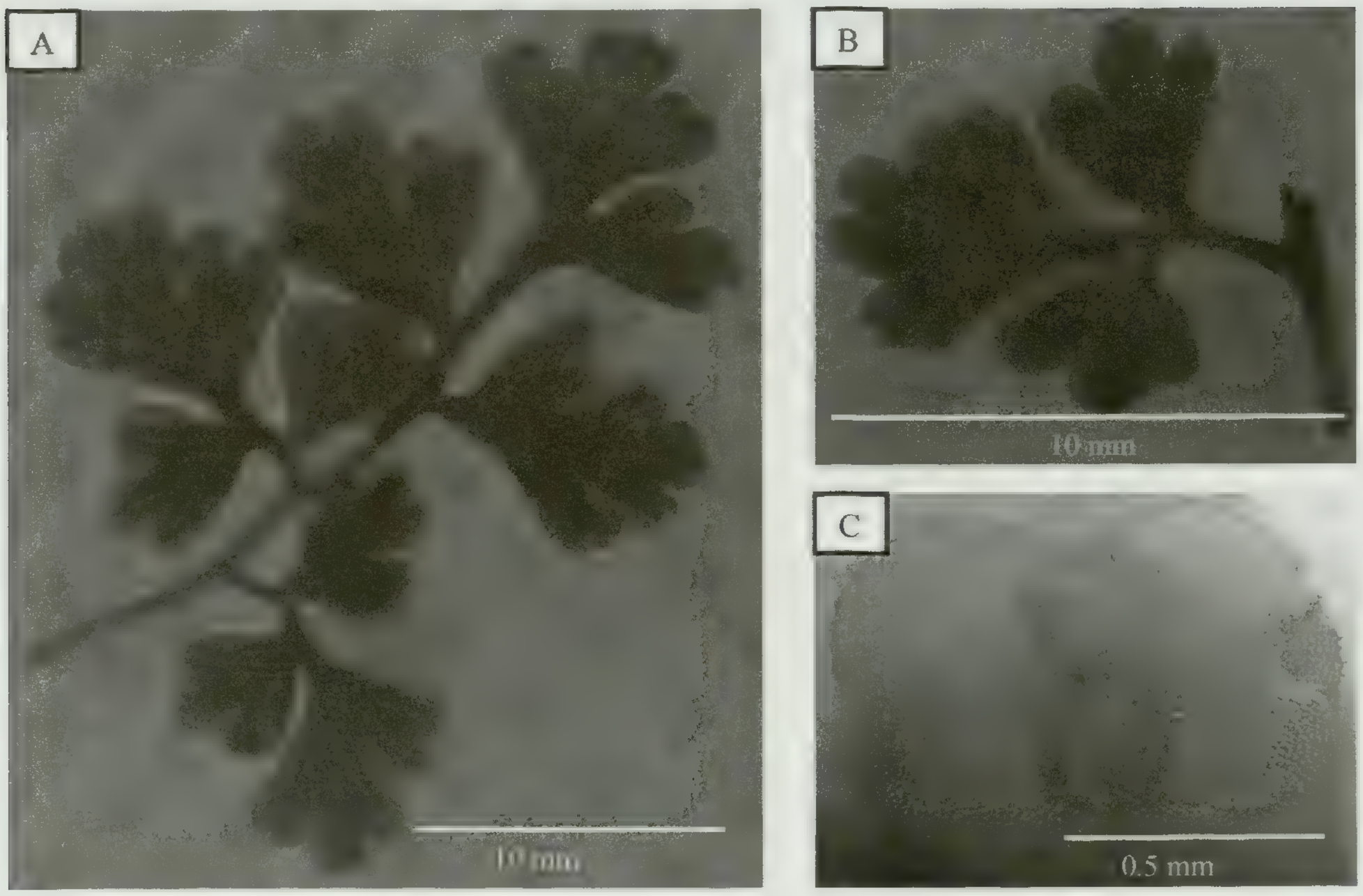

FIG. 1. Type of explants of *Rumohra adiantiformis* used for *in vitro* regeneration. A) frond, B) frond microcutting and C) prothallus. All were obtained one month after culture initiation on Knop medium under light.

and sporophyte regeneration, fronds (Fr) 20–30 mm long (Figure 1A) and frond microcuttings (MicFr) 10 mm long (Figure 1B) were used as initial explants for *in vitro* gametophyte regeneration. For a third explant, fronds bearing sori were cut into 2 mm pieces and placed in glass flasks containing sterilized distilled water. Subsequently, sori were centrifuged (Menéndez *et al.*, 2006) in Eppendorf tubes containing 70% ethanol for 1 min; 0.01% sodium hypochloride was then added, and the mixture was centrifuged for 3 min. Finally, the sori were washed three times with sterilized distilled water, and the obtained spores were placed on Whatman No. 42 filter paper (International Ltd Maidstone England) discs 1 cm in diameter and cultivated for one month on Knop medium supplemented with 1g L^{-1} AC under the same light conditions as described above. The resulting prothalli (Pr) (Figure 1 C) were used as the third type of explants for gametophyte regeneration.

Sporophyte regeneration rate and time reduction.—In order to increase the rate and reduce time of sporophyte regeneration, the effect of 2,4-dichlorophenoxyacetic acid (2,4-D Sigma®) (0.0, 0.1, 0.5 and 1.0 mg L^{-1}) in combination with 6-benzylamino purine (BA Sigma®) (0.0, 0.1, 0.5 and 1.0 mg L^{-1}) on Fr, MicFr and Pr was evaluated. Each group of explants was placed on Knop medium supplemented with 1 g L^{-1} AC, 0.5% agar, at pH 5.0. All cultures were maintained under conditions of 16/8 h light/dark for three months at 25 ± 1°C. A total of 32 treatments were tested.

Rooting test.—For rooting, regenerated sporophytes were transferred to MS medium (half-strength) supplemented with α-naphthaleneacetic acid (NAA

Sigma®) (0.0, 0.01, 0.1 and 0.2 mg L^{-1}), 3% sucrose and 1g L^{-1} AC at pH 5.0. All media were gelled using 0.8% agar (Bioxon®). The cultures were incubated for one month at 25 ± 1°C under the same conditions described for the second stage.

Transfer to soil conditions and acclimatization.—Each well-rooted sporophyte was transferred to a pot containing cosmopeat® as substrate. The pots with transparent covers were maintained at a temperature of 25–28°C under a 16 h photoperiod. The covers were removed after 8 days.

Histological study.—In order to determine the origin of regenerated gametophytes from Fr, MicFr and Pr explant epidermal tissues on either the adaxial or abaxial surface, scanning electron microscopy (SEM) was used. For SEM observations, the selected explants were fixed in 2.5% glutaraldehyde for 12 h at 4°C then washed three times (30 min at 4°C) with Sorensen's 0.1 M phosphate buffer, pH 7.2. The explants were then dehydrated in a series of different grades of ethanol (30, 40, 50, 60, 70, 80, 90%) for 40 min and transferred to 100% ethanol (3 times, 3 min). After the samples were dehydrated in a critical-point dryer (Sandri-708A), the samples were sputter-coated with gold for 4 min in the ionizer (Ion Sputter JFC-1100, Jeol Fine Coat). All observations were documented on digital images using SEM (Jeol JSM 6390) at 15 Kv.

Statistical analysis.—Analysis of variance (ANOVA) and a test of least significant differences (LSD) were performed to assess germination rate, germination period, prothallus mass diameter, number of sporophytes, sporophyte height, number of regenerated sporophytes per explant (Fr, MicFr and Pr), length and number of roots, length and number of fronds, percentage of adaptation, and adaptation period, in a randomized-block design. Each flask contained five explants and was regarded as one block. Each test was replicated four times. All data were processed with the Statistical Analysis System V8.0 (SAS Institute, 1999).

Results and Discusion

In vitro *sorus germination and initial explants.*—In the present study, using the same MS medium, 70% spore germination was obtained both with and without AC, but 28 and 30 d after culture initiation, respectively, were necessary for sporophyte regeneration (Table 1). In contrast, a rate of 100% spore germination was obtained on Knop medium. When this medium was supplemented with AC, 18 d were required for germination, while 23 d were required without AC (Table 1). It was also noted that sporophytes formed only on gametophytes from spores cultured on Knop medium. Thus, it appears that medium salts and AC have an important effect on the rate and period of germination, as well as on sporophyte formation. The major difference between the two culture media is probably the amount of nitrogen. MS medium contains eight times the ammonium concentration contained in Knop medium.

TABLE 1. Effect of two culture media on spore germination rate and sporophyte regeneration of *Rumohra adiantiformis*. Values were obtained 30 d (for germination) and 120 d (for sporophyte) after culture initiation.

Treatment	Germination (%)	Germination (days)	Diameter of prothallus mass (cm)	Number of sporophytes	Height of sporophyte (cm)
Knop + 1 gL^{-1} AC	100	18 ± 0.0	1.6 ± 0.2	5.9 ± 1.9	1.7 ± 0.4
Knop + 0 gL^{-1} AC	100	23 ± 0.0	1.0 ± 0.3	3.1 ± 0.9	0.7 ± 0.2
MS + 1 gL^{-1} AC	70	28 ± 0.0	1.4 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
MS + 0 gL^{-1} AC	70	30 ± 0.0	1.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.0

± SD = Standard deviation
MS = Murashige and Skoog medium
Knop = Knop medium
AC = Activated charcoal

It was reported in *Nephrolepis exaltata* (L.) Schott fern that 32% of spore germination took place within 28–30 d after culture initiation on MS media (González *et al.*, 2006). In *Drynaria fortunei* (Kunze) J.Sm. a spore germination rate of 15.3% was obtained after 7 d on MS medium (Chang *et al.*, 2007). The influence of absolute and relative amounts of nitrate and ammonium on induction and differentiation of plant cell cultures has been reported for a number of *in vitro* systems (Ramage and William, 2002). Ammonium used as the sole source of nitrogen appears to have a negative effect on growth and morphogenesis (Walch-Liu *et al.*, 2000). In addition, activated charcoal absorbs vitamins, cytokinins, auxins and inhibitory substances, thus altering the ratios of medium components and subsequently affecting plant regeneration (Fridborg *et al.*, 1978; Ebert and Taylor, 1990; Druart and Wulf, 1993; Arzate *et al.*, 2007).

All spore cultures germinated under light, but the spores cultured in the dark, on either Knop or MS medium and with or without AC, did not germinate even after 200 d of culture. However, when the cultures were kept under a dark to light cycle, all spores germinated after 15 d on Knop medium and after 18 d on MS medium. Weinberg and Bruce (2007) mentioned that the spore culture of *Anemia phyllitidis* (L.) Sw. needed light to induce its germination. Likewise, Chang *et al.* (2007) observed that spores of *D. fortunei* germinated only under light, indicating that light is one of the most important factors that affect events in the life cycle of a fern, functioning as a signal to awaken the dormant fern spore.

In the present study, after spore germination, a prothallus mass 1.4 cm in diameter was observed (Table 1, Figure 2A), but sporophytes were never induced on MS cultures, either with or without AC. In contrast, prothallus masses with developed sporophytes 1.6 cm tall (Figure 2B) were observed at 16 weeks on the Knop medium with AC (1.7 cm in height) or without AC (Table 1). Nevertheless, Brum and Randi (2006) reported that *R. adiantiformis* sporophyte induction took place after 19 weeks. This is probably consistent, as

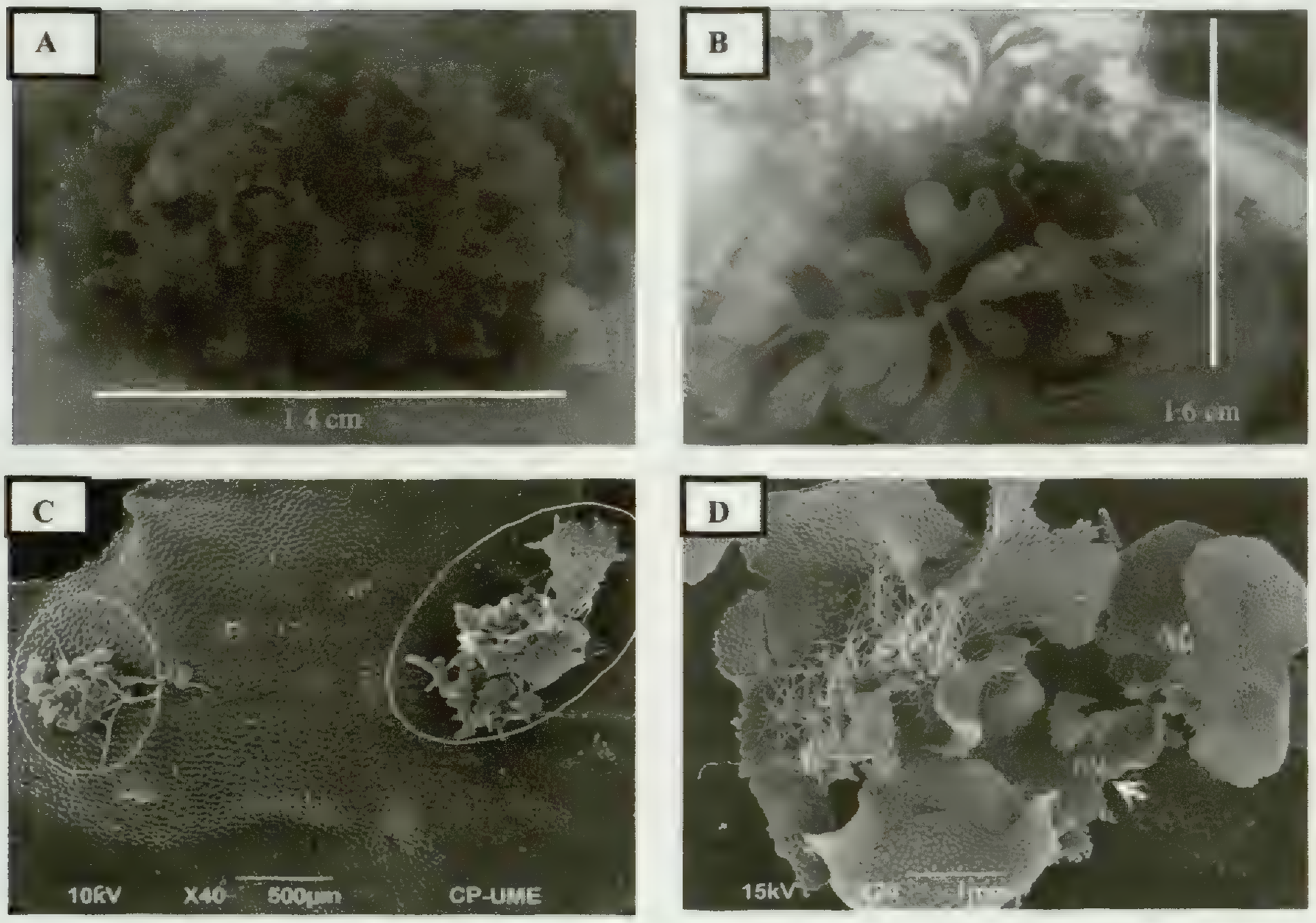

FIG. 2. *Rumohra adiantiformis* during *in vitro* regeneration. A) Gametophyte obtained in MS medium without activated charcoal. B) Obtained sporophytes on Knop medium with 1 g L^{-1} activated charcoal. Scanning electron micrographs of gametophyte regeneration of *R. adiantiformis* formed on different explants. C) Masses of gametophytes (circles) developing on the adaxial surface of the Frond (Fr) obtained under light on Knop medium supplemented with 0.1 mg L^{-1} 2,4-D and

it was mentioned by Teng (1997) that the addition of AC to the culture media greatly improved efficiency of sporophyte regeneration.

Sporophyte regeneration rate and time reduction.—When Fr explants were cultured on Knop medium, masses of gametophytes were induced with 1 g L^{-1} AC, 0.1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BA (Table 2, Figure 2C). In addition, the masses of gametophytes were observed on both sides (adaxial and abaxial) of Fr explants. Using MicFr explants, the best gametophyte regeneration response (13.5 gametophytes) was observed with 1g L^{-1} AC, 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BA (Table 2, Figure 2D). On these explants, aposporous gametophytes developed. This physiological response is probably due to an effect caused by the exogenous growth regulators used. In contrast, when Pr explants were cultured with 1g L^{-1} AC and 0.5 mg L^{-1} BA, 235.7, apogamous sporophytes developed (Table 2, Figure 2E). Pr explants produced 17 times more gametophytes than MicFr and 22 times more than Fr. Extrapolating this result, it would be possible to obtain up to 2 million regenerated sporophytes from one fertile *R. adiantiformis* frond. Moreover, Pr explants did not require the presence of 2,4-D, probably due to its high content of endogenous auxins and the regeneration capacity of the vegetative cells which were observed in those explants (Figure 2F). The differences in response to 2,4-D and BA of assayed explants obtained in the present study might depend on the type, age and the initial size of the explants.

The aposporously produced gametophytes were similar in appearance to gametophytes produced from spores. According to Bhojwani and Razdan (1983), the exogenous requirements for growth regulators depend on endogenous hormone levels in the plant system. Thus, the formation of adventitious buds on leaves without growth regulators may be a result of appropriate endogenous hormone levels (Camloha *et al.,* 1994). These observations are in agreement with those of Ong and Ng (1998) on the fern *Pyrrosia pilosellodes* (L.) M. Price, in which regeneration of drought-stressed gametophytes was detected through the formation of unicellular protonemata on the surfaces of living cells; the unicellular protonemata continued normal development as if they were sporelings (gametophytes that develop following

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←

0.1 mg L^{-1} BA 108 days after culture initiation. D) A prothallus mass (PrM) (arrow) developing on the abaxial surface of the Frond Microcutting (MicFr) cultured on Knop medium supplemented with 1.0 mgL^{-1} 2,4-D and 0.1 mg L^{-1} BA under light 108 d after culture initiation. E) Prothallus (Pr) explant on Knop medium supplemented with 0.5 mg L^{-1} BA 108 d after culture initiation, regenerated gametophytes (arrows) developing on the Pr surface. F) Successive stages of gametophyte development, observed *in vitro* on Knop medium supplemented with 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BA. A new cordate type of gametophyte originating from vegetative cells (Vc) is observed on the prothallus. Early formation of trichomes (Tri) which begin as an initial cell and end as the terminal cell of the prothallial filament (ProFil). G) Sporophyte rooting response to 0.01 mg L^{-1} α-naphthaleneacetic acid. H) Regenerated sporophyte 183 days after culture initiation.

TABLE 2. Number of regenerated gametophytes from three kinds of explants in response to plant growth regulators and Knop medium with 1 g L^{-1} active charcoal. Values were obtained 108 d after culture initiation and represent the means ± standard deviations (n ≥ 50 cultured explants per treatment).

PGR: 2, 4-D: BA (mgL-1)	Frond (Fr)	Frond Microcutting (MicFr)	Prothallus (Pr)
0.0: 0.0	1.5 ± 1.0 f g h	4.5 ± 1.3 c d e	68.5 ± 6.4 h i
0.0: 0.1	4.0 ± 1.8 c d	6.5 ± 1.2 c	82.5 ± 9.8 g h
0.0: 0.5	2.0 ± 0.8 f g	0.0 ± 0.0 h	235.7 ± 15.3 a
0.0: 1.0	3.5 ± 0.6 d e	6.2 ± 1.2 c d	122.0 ± 14.7 c d e
0.1: 0.0	1.2 ± 0.5 f g h i	4.2 ± 2.8 d e	146.7 ± 15.6 b
0.1: 0.1	10.5 ± 1.2 a	2.0 ± 1.4 f g h	135.5 ± 21.0 b c
0.1: 0.5	1.0 ± 0.0 g h i	2.0 ± 1.6 f g h	91.7 ± 24.0 f g
0.1: 1.0	0.2 ± 0.5 h i	2.7 ± 1.2 e f g	61.5 ± 5.0 h i j
0.5: 0.0	5.0 ± 0.8 c	4.0 ± 1.8 e f	120.5 ± 10.8 c d e
0.5: 0.1	2.2 ± 0.9 f g	3.2 ± 1.7 e f g	45.2 ± 3.1 j
0.5: 0.5	1.0 ± 0.8 g h i	10.0 ± 1.8 b	105.2 ± 7.3 e f
0.5: 1.0	7.2 ± 1.7 b	1.5 ± 1.0 g h	137.5 ± 19.8 b c
1.0: 0.0	2.5 ± 1.2 e f	0.2 ± 0.5 h	131.0 ± 25.3 b c d
1.0: 0.1	0.5 ± 0.5 h i	13.5 ± 2.5 a	111.7 ± 23.3 d e f
1.0: 0.5	2.5 ± 1.2 e f	4.5 ± 1.2 c d e	59.0 ± 7.4 i j
1.0: 1.0	0.0 ± 0.0 i	3.5 ± 1.2 e f g	58.7 ± 9.0 i j
2,4-D (A)	17.71 ***	8.22 ***	16.22 ***
BA (B)	19.47 ***	12.45 ***	15.07 ***
A × B	38.63 ***	25.78 ***	52.93 ***
Variation Coefficient	35.92	36.75	14.36

Means with same letters are not statistically different (LSD, 0.05).
*** = ≤ 0.001

spore germination). These results show the beneficial effect of the combination auxin/citokinin in enhancing cell division (Camloha *et al.*, 1994).

Chen and Read (1983) worked with rhizome tips of *Rumohra adiantiformis* on modified Prague's medium, using 2iP, kinetin and zeatin; they reported that the potential annual propagation rate from a single rhizome tip can be as high as 16,000,000 plantlets ready for potting and transfer to the greenhouse. Amaki and Higuchi (1992) reported that *R. adiantiformis*, in order to regenerate plants from segments of rhizome in MS medium, it was necessary to add 0.5 mg L^{-1} NAA and to eliminate the use of BA.

Rooting test.—Regenerated sporophytes with an average of 7.3 fronds and 6.5 roots were obtained after 4 weeks. However, a better response was observed when 0.01 mg L^{-1} NAA was added, resulting in 9.6 fronds and 8.2 roots in the same period. It was also observed that higher levels of NAA decreased number and length of both roots and fronds (Table 3, Figure 2G). These results are similar to those reported by Kottackal *et al.* (2006), who also increased the number of rhizoids in gametophytes of *Pityrogramma calomelanos* (L.) Link by adding NAA. On the other hand, Thakur *et al.* (1998) obtained 10.5 roots per regenerated *Matteuccia struthiopteris* plantlet in gelled half-strength MS medium supplemented with 1.0% AC after 8 weeks.

TABLE 3. Effect of NAA on *Rumohra adiantiformis* root number, root length, frond number and frond length. Values were obtained at transplant and are means ± standard deviations (n ≥ 50).

Treatment NAA (mg L^{-1})	Root number	Root length (cm)	Frond number	Frond length (cm)
0	6.5 ± 1.9 c	0.5 ± 0.2 c	7.3 ± 2.0 c	2.0 ± 0.4 a
0.01	8.2 ± 0.8 a	0.7 ± 0.2 a	9.6 ± 2.8 b	2.1 ± 0.6 a
0.1	8.1 ± 2.4 a	0.5 ± 0.2 c	10.1 ± 4.4 a	1.9 ± 0.5 b
0.2	7.6 ± 1.5 b	0.6 ± 0.1 b	9.5 ± 3.8 b	1.8 ± 0.4 b

Transfer to soil conditions and acclimatization.—Acclimatization of 98% of the regenerated sporophytes with 10 cm long fronds was achieved after 15 days. Chang *et al.* (2007) reported that 5-month-old *D. fortunei* gametophytes were transferred to the greenhouse and juvenile sporophytes developed after 14 weeks. Chen and Read (1983) observed that preconditioning of intact rhizome tips of *R. adiantiformis* (4 weeks) to the air in the greenhouse is very important.

Histological study.—Induced aposporous gametophytes were observed on the adaxial surface of Fr (Figure 2C) and on the abaxial surface of MicFr (Figure 2D), while sporophyte regeneration by "apogamy" was observed on Pr explants (Figure 2E). Likewise, different stages of gametophyte regeneration were also observed in the vegetative cells (Figure 2F). Kottackal *et al.* (2006) in *Pityrogramma calomelanos* observed that, using crosier explants, gametophytes were induced from epidermal hair.

Conclusions.—The Knop medium supplemented with 1 g L^{-1} AC under light was the best condition for spore germination and formation of sporophytes in *Rumohra adiantiformis*. The highest number of gametophytes (235.7) was obtained by culturing prothallus explants on Knop medium with 1 g L^{-1} AC and 0.5 mgL^{-1} BA, 183 d after culture initiation. The addition of 0.01 mg L^{-1} NAA improved the rooting of regenerated sporophytes. The histological study revealed that gametophytes originated from vegetative cells on either the adaxial or abaxial surfaces of the assayed explants. Aposporous gametophytes were observed on Fr and MicFr explants and apogamous sporophytes on Pr explants. The high percentage (98%) of acclimatization and the relatively short time (183 d) of regeneration make this procedure applicable for obtaining up to 2 million completely regenerated sporophytes.

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Literature Cited

Amaki, W. and H. Higuchi. 1992. A possible propagation system of *Nephrolepis, Asplenium, Pteris, Adiantum* and *Rumohra* (Arachniodes) through tissue culture. Acta Horticulturae. 300:237–243.

Ambrožic-Dolinšek, J., M. Camloh, B. Borhancec and J. Žel. 2002. Apospory in leaf culture of staghorn fern *Platycerium bifurcatum*. Plant Cell Report. 20:791–796.

Arzate-F, A-M., M. Miwa, T. Shimada, T. Yonekura and K. Ogawa. 2007. *In vitro* propagation of Miyamasukashi-Yuri (*Lilium maculatum* Thunb. Var. Bukosanense), an endangered plant species. Revista Fitotecnia Mexicana. 30:373–379.

Bhojwani, S. S. and M. K. Razdan. 1983. Plant Tissue Culture: Theory and Practice. Developments in Crop Science. Elsevier Science Publishers, Amsterdam, p. 71–90.

Brum, F. M. R. and A. M. Randi. 2002. High irradiance and temperature inhibit the germination of spores of the fern *Rumohra adiantiformis* (Forst.) Ching (Dryopteridaceae). Revista Brasil. Bot. 25:391–396.

Brum, F. M. R. and A. M. Randi. 2006. Germination of spores and growth of gametophytes and sporophytes of *Rumohra adiantiformis* (Forst.) Ching (Dryopteridaceae) after spore cryogenic storage. Revista Brasil. Bot. 29:489–495.

Camloha, M., N. Gogola and J. Rode. 1994. Plant regeneration from leaf explants of the fern *Platycerium bifurcatum in vitro*. Scientia Horticulturae. 56:257–266.

Chang, H. C., D. C. Agrawal, C. L. Kuo, J. L. Wen, C. C. Chen and H. S. Tsay. 2007. *In vitro* culture of *Drynaria fortunei*, a fern species source of Chinese medicine "Gu-Sui-Bu". In vitro Cell. Dev. Biol. Plant. 43:133–139.

Chen, S. Y. and P. E. Read. 1983. Micropropagation of leatherleaf fern (*Rumohra adiantiformis*). Proceeding of the Florida State Horticultural Society. 96:266–269.

Druart, P. and O. Wulf. 1993. Activated charcoal catalyses sucrose hydrolysis during autoclaving. Plant Cell Tissue Organ Culture. 32:97–99.

D'souza, G. C., R. Kubo, L. Guimaraes and E. Elisabetsky. 2006. An ethnobiological assessment of *Rumohra adiantiformis* (samambaia-preta) extractivism in Southern Brazil. Biodiversity and Conservation. 15:2737–2746.

Ebert, A. and H. F. Taylor. 1990. Assessment of the changes of 2, 4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. Plant Cell Tissue Organ Culture. 20:165–172.

Fernández, H. and M. A. Revilla. 2003. *In vitro* culture of ornamental ferns. Plant Cell Tissue and Organ Culture. 73:1–13.

Fridborg, G., M. Pedersen, L. E. Landstrom and T. Eriksson. 1978. The effect of activated charcoal on tissue culture: absorption of metabolites inhibiting morphogenesis. Physiologia Plantarum. 43:104–106.

Foster, A. S. and E. M. Gifford. 1974. Comparative Morphology of Vascular Plants. An invaluable reference on the morphologies of past and present plant types. San Francisco Wm, Freeman & Co. (2nd Edition). 555 p.

González, R. H., J. A. Herrera and A. C. Ramos. 2006. Multiplicación *in vitro* de *Nephrolepis exaltata* (L.) Schott, a partir de esporas. Revista Chapingo. Serie Horticultura. 12:141–146.

Knop, W. 1865. Quantitative Untersuchungen Über die Ernahrungsprozesse der Pflanzen. Landwirtsch. Versuchssat. Stn. 7:93–107.

Kottackal, P. M., S. Sini, C. L. Zhang, A. Slater and P. V. Madhusoodanan. 2006. Efficient induction of apospory and apogamy *in vitro* in silver fern (*Pityrogramma calomelanos* L.). Plant Cell Report. 25:1300–1307.

Menéndez, V., N. F. Villacorta, M. A. Revilla, V. Gotor, P. Bernanrd and H. Fernández. 2006. Exogenous and endogenous growth regulators on apogamy in *Dryopteris affinis* (Lowe) Fraser-Jenkins sp. *affinis*. Plant Cell Report. 25:85–91.

Milton, S. J. and E. J. Moll. 1988. Effects of harvesting on frond production of *Rumohra adiantiformis* (Pteridophyta: Aspidiaceae) in South Africa. Journal of Applied Ecology. 25:725–743.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassys with tobacco tissue culture. Physiologia Plantarum. 15:473–497.

Ong, B. L. and M. L. Ng. 1998. Regeneration of drought-stressed gametophytes of the epiphytic fern, *Pyrrosia pilosellodes* (L.) Price. Plant Cell Report. 18:225–228.

Poole, R. T. and C. A. Conover. 1978. Feasibility of harvesting leatherleaf fern by clear cutting. Proceedings of the Florida State Horticultural Society. 91:230–231.

Ramage, C. M. and R. R. Williams. 2002. Mineral nutrition and plant morphogenesis. In Vitro Cell. Dev. Biol.-Plant. 38:116–124.

SAS. 1999. SAS User's guide: Statistics version 8.0. Cary, NC: SAS Institute Inc.

Schumann, A. W. and H. A. Mills. 1996. Injury of leatherleaf fern and tomato from volatilized ammonia after fertilizer application. Journal of Plant Nutrition. 19:573–593.

Sheffield, E. 1984. Apospory in the fern *Pteridium aquilinum* (L.) Kuhn. Cytobios. 39:171–176.

Soare, L. C., E. Visoiu and M. Andrei. 2007. Researches concerning the *in vitro* differentiation of the fern *Phegopteris connectilis* (Michx.) Watt. Not. Bot. Hort. Agrobot. Cluj. 35:7–14.

Stamps, R. H. 2004. Effects of postharvest dip treatments on leatherleaf fern (*Rumohra adiantiformis*) frond vase life. Proc. VII Int. Symp. On Postharvest. Physiology Ornamentals. Acta Horticulturae. 543:299–303.

Stamps, R. H., T. A. Nell and J. E. Barret. 1994. Production temperatures influence growth and physiology of leatherleaf fern. HortScience 29:67–70.

Strandberg, J. O. 2003. Seasonal variations in production and development of leatherleaf fern leaves. Ann appl. Biol. 143:235–243.

Teng, W. L. 1997. Activated charcoal affects morphogenesis and enhances sporophyte regeneration during leaf cell suspension culture of *Platycerium bifurcatum*. Plant Cell Report. 17:77–83.

Thakur, R. C., Y. Hosoi and K. Ishii. 1998. Rapid *in vitro* propagation of *Matteuccia struthiopteris* (L.) Todaro – an edible fern. Plant Cell Report. 18:203–208.

Weinberg, E. S. and B. R. Voeller. 2007. Induction of fern spore germination. Proc. Nat. Acad. Sci. USA. 64:835–842.

Walch-Liu, P., G. Neumann, F. Bangerth and G. Engels. 2000. Rapid effects of nitrogen form on leaf morphogenesis. J. Exp. Bot. 51:227–237.

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Pteridophytes of Mo'orea, French Polynesia: Additional New Records

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ABSTRACT.—The ferns and lycophytes of Mo'orea, French Polynesia were surveyed as part of the Mo'orea Biocode Project during September 12–13, 2006, September 6–27, 2008, and August 11–28, 2010, resulting in collection of 42 species that had not been previously reported to occur on Mo'orea. Specimen citations, previously known distributions, and taxonomic notes (when appropriate) are given for each new record. New records include 16 species collected for the first time during the Mo'orea Biocode Project, and 26 species collected prior to the project but not included in published species lists. This brings the total number of known Mo'orean pteridophytes to 132 species.

KEY WORDS.—Mo'orea, Mo'orea Biocode Project, Ferns, Lycophytes, Pteridophytes

The purpose of the Mo'orea Biocode Project is to "create the first comprehensive inventory of all non-microbial life in a complex tropical ecosystem" (http://mooreabiocode.org). The inventory will include molecular, morphological, and ecological data, and serve as a valuable reference library for species identification and further ecological and systematic studies. Studies of ferns and lycophytes (i.e., "pteridophytes"), in particular, will benefit from this inventory because it will enable rapid identification of gametophytes based on DNA sequences, thus enabling detailed field studies of this ecologically important yet poorly known life stage. Initial field work has focused on collecting and identifying sporophytes based on morphology; DNA data and further collections of sporophytes and gametophytes will be added as the project progresses.

Murdock and Smith (2003) compiled a list of 80 Mo'orean pteridophytes based on previous literature reports (8 species) and examination of herbarium specimens (72 species); further records were added by Ranker *et al.* (2005; 8 species), Nitta (2008; 3 species), and Rouhan *et al.* (2008; 1 species). However, previous lists of Mo'orean pteridophytes lacked sufficient sampling of the tallest

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peak on the island, Mt. Tohiea (1207 m). Additional field surveys were conducted during September 12–13, 2006, September 6–27, 2008, and August 11–28, 2010 as part of the Mo'orea Biocode Project that included explorations of Mt. Tohiea and yielded 32 new species records from this site; an additional 10 new species records were found on other parts of the island, bringing the total number of Mo'orean pteridophytes to 132 species (including at least 122 native species; the above species counts do not add up exactly due to taxonomic changes). Furthermore, examination of herbarium specimens at PAP revealed many earlier collections made on Mt. Tohiea and Mt. Mou'aputa by J. Florence and others which had not been included in the lists compiled by Murdock and Smith (2003) or Ranker *et al.* (2005), and are listed here (26 species; including several species listed on the PAP website [Florence *et al.*, 2007] for which specimens were unavailable at the time we visited the herbarium). Collection data, previously known distributions based on published records and specimens available at UC, and taxonomic notes (when appropriate) are given for each new record. Species identifications are based on morphology and may be subject to revision as molecular data become available, especially for uncertain taxa. Family treatment follows Smith *et al.* (2006), except for the acceptance of Athyriaceae as distinct from Woodsiaceae. All data of specimens collected as part of the Mo'orea Biocode Project, including images, are freely available online at http://biocode.berkeley.edu.

Athyriacae

Deparia confluens (Kunze) M. Kato

Mo'orea, Mt. Mou'aputa, 17.53483°S, 149.79965°W, ca. 331 m, 15 Aug 2010, *J. Nitta 592* (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 17.53259°S, 149.80127°W, ca. 397 m, 19 Sep 2008, *J. Nitta 290*, with S. Vinette (P, PAP, TNS, UC). Epipetric, rock face next to stream. Previously known from the Philippines, Borneo, Java, Bali, Sumbawa, Ternate, Sulawesi, Fiji, Samoa, and Tahiti (Kato, 1984).

Deparia petersenii (Kunze) M. Kato subsp. ***congrua*** (Brack.) M. Kato

Mo'orea, Mt. Tohiea, 17.55486°S, 149.81502°W, ca. 636 m, 24 Aug 2010, *J. Nitta 650*, with V. Liao, J.-Y. Meyer, and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.54984°S, 149.82214 W, ca. 1195 m, 25 Aug 2010, *J. Nitta 685*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55393°S, 149.8168°W, ca. 803 m, 25 Aug 2010, *J. Nitta 699*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 28 Aug 2005, *F. Jacq FJ442* (PAP); Mo'orea, Mt. Tohiea, vallon entre les deux sommets, 17.55°S, 149.82°W, ca. 1160 m, 9 Jul 1987, *J. Florence 8352* (P, PAP); Mo'orea, flanc S de l'épaulement SE du Mt. Tohiea, 17.55°S, 149.82°W, ca. 660 m, 21 Oct 1986, *J. Florence 7917* (P, PAP). Epipetric on damp rock walls in cloud forest. Previously known from eastern Australia, the Solomon Islands, Norfolk Island, New Caledonia, Vanuatu, Fiji, Samoa, Rarotonga, and Tahiti; adventive in New Zealand (Kato, 1984).

Diplazium proliferum (Lam.) Thouars

Mo'orea, Ancestor's Trail, 17.53246°S, 149.82974°W, ca. 115 m, 9 Oct 2009, *M. Fourdrigniez 590* (UC). Epipetric and terrestrial along trails. Previously known from tropical Africa, Madagascar, Mauritius, Malesia, Australia (Queensland), Fiji, Samoa, and Tahiti. Introduced.

Blechnaceae

Blechnum pacificum Lorence & A. R. Sm., ined.

Mo'orea, pass between Mt. Tiura and Mt. Tatiri, 17.52728°S, 149.87703°W, ca. 761 m, 17 Aug 2010, *J. Nitta 616* (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55336°S, 149.81862°W, ca. 994 m, 25 Sep 2008, *J. Nitta 320*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, Mt. Rotui, 17.5106°S, 149.8383°W, ca. 916 m, 10 Sep 2008, *J. Nitta 212*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, flanc Sud de Mt. Mou'aputa, ca. 610 m, 19 Aug 1983, *Florence 4994* (PAP; not examined by us). Terrestrial in cloud forest. Previously known only from the Marquesas and Society Islands (Tahiti).

Blechnum capense Burm. f. (synonym *Blechnum sylvaticum* Schelpe), from South Africa, has been historically applied to this species, we believe erroneously.

Blechnum patersonii (R. Br.) Mett.

Mo'orea, Mt. Tohiea, 17.55077°S, 149.82263°W, ca. 1190 m, 17 Sep 2008, *J. Nitta 265*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, UC); Mo'orea, Mt. Mou'aputa, 17.52644°S, 149.80339°W, ca. 823 m, 19 Sep 2008, *J. Nitta 288*, with S. Vinette (P, UC); Mo'orea, Mt. Tohiea, vallon entre les deux sommets, 17.55°S, 149.82°W, ca. 1160 m, 9 Jul 1987, *J. Florence 8351* (P, PAP). Epipetric or epiphytic in cloud forest. Previously known from eastern Australia (McCarthy and Orchard, 1998), Indonesia, Malaysia, New Caledonia, Vanuatu, Fiji, and Tahiti.

Blechnum vulcanicum (Blume) Kuhn

Mo'orea, Mt. Rotui, 17.5106°S, 149.8383°W, ca. 760 m, 10 Sep 2008, *J. Nitta 217*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55103°S, 149.82198°W, ca. 1188 m, 17 Sep 2008, *J. Nitta 271*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, UC); Mo'orea, crête SE du Mt. Tohiea, 17.55°S, 149.82°W, ca. 1130 m, 10 Jul 1987, *J. Florence 8372* (P, PAP); Mo'orea, flanc Sud de Mt. Mou'aputa, 17.53°S, 149.82°W, ca. 610 m, 19 Aug 1983, *J. Florence 4995* (P, PAP). Epiphytic in cloud forest. Previously known from southeastern Asia, southeastern Australia, Tasmania, New Zealand, and in the Pacific as far east as the Cook Islands, as far north as the Marquesas Islands (Chambers and Farrant, 2001).

Cyatheaceae

Cyathea epaleata (Holttum) Holttum

Mo'orea, Mt. Tohiea, 17.55379°S, 149.81633°W, ca. 778 m, 25 Aug 2010, *J. Nitta 703*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt.

Mou'aputa, 17.52644°S, 149.80339°W, ca. 823 m, 19 Sep 2008, *J. Nitta 287* (P, UC), with S. Vinette. Terrestrial in cloud forest. Endemic to the Society Islands, previously known from Tahiti (Holttum, 1964) and Raiatea (J.-Y. Meyer, unpublished data).

Davalliaceae

Humata anderssonii (Mett. ex Kuhn) C. Chr.

Mo'orea, Mt. Mou'aputa, 17.52728°S, 149.8033°W, ca. 778 m, 15 Aug 2010, *J. Nitta 606* (P, PAP, TNS, UC); Mo'orea, Mt. Rotui, 17.5064°S, 149.8408°W, ca. 760 m, 10 Sep 2008, *J. Nitta 210*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea summit area, 17.54944°S, 149.8225°W, ca. 1176 m, 13 Sep 2006, *J.-Y. Meyer 3127*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, crête SE du Mt. Tohiea, ca. 1010 m, 22 Oct 1986, *J. Florence 7957* (PAP; not examined by us); Mo'orea, flanc Sud de Mt. Mou'aputa, arête terminale, ca. 780 m, 19 Aug 1983, *J. Florence 4996* (PAP; not examined by us). Epiphytic in cloud forest. Endemic to the Society Islands, previously known from Tahaa (J.-Y. Meyer, unpublished data) and Tahiti.

Humata repens (L. f.) Diels (synonym *Davallia repens* (L. f.) Kuhn), has been applied in a broad sense to this species (Nooteboom, 1994), but *H. anderssonii* seems specifically distinct. If *H. repens* is circumscribed to include *H. anderssonii*, it has nearly 50 synonyms, based on types that range from Africa, southeastern Asia, Malesia, and Polynesia (Nooteboom, 1994).

Dennstaedtiaceae

Histiopteris incisa (Thunb.) J. Sm.

Mo'orea, Mt. Tohiea, 17.55066°S, 149.82231°W, ca. 1213 m, 16 Sep 2008, *J. Nitta 679*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55331°S, 149.8188°W, ca. 1004 m, 16 Sep 2008, *J. Nitta 258*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC). Terrestrial. Pantropical in the broad sense, as treated here, occurring throughout the Neotropics (southern Mexico and Antilles to Bolivia), Juan Fernández Islands, southern Africa, eastern and southeastern Asia, Malesia, New Zealand, and Tasmania (Mickel and Smith, 2004); in the Society Islands, previously known from Tahiti (Copeland, 1932).

Hypolepis dicksonioides (Endl.) Hook.

Mo'orea, Mt. Tohiea, 17.55068°S, 149.82233°W, ca. 1212 m, 16 Sep 2008, *J. Nitta 260*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, crête SE du Mt. Tohiea, 17.55°S, 149.82°W, ca. 1100 m, 23 Oct 1986, *J. Florence 7968* (P, PAP). Terrestrial. Previously known from New Zealand, Norfolk Island, Samoa, the Marquesas Islands, and Tahiti (Brownsey and Smith-Dodsworth, 1989).

Paesia divaricatissima (Dryand.) Copel.

Paesia tahitensis Copel.

Mo'orea, Mt. Mou'aputa, 17.5275°S, 149.80313°W, ca. 760 m, 15 Aug 2010, *J. Nitta 594* (P, PAP, TNS, UC); Mo'orea, Mt. Rotui, 17.5106°S, 149.8383°W, ca. 760 m, 10 Sep 2008, *J. Nitta 219*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, crête SE du Mt. Tohiea, ca. 1100 m, 22 Oct 1986, *J. Florence 7967* (PAP; not examined by us). Terrestrial; common on ridges in cloud forest above ca. 750 m. Heretofore, known only from Tahiti (Copeland, 1932).

DRYOPTERIDACEAE

Dryopteris dicksonioides (Mett. ex Kuhn) Copel.

Mo'orea, Mt. Tohiea, 17.55126°S, 149.82184°W, ca. 1170 m, 17 Sep 2008, *J. Nitta 274*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, crête sommitale SE du Mt. Tohiea, ca. 1170 m, 23 Oct 1986, *J. Florence 7973* (PAP; not examined by us). Terrestrial. Endemic to the Society Islands (previously known from Raiatea and Tahiti; Copeland, 1932).

Dryopteris macrolepidota Copel.

Mo'orea, Mt. Tohiea, 17.55343°S, 149.81813°W, ca. 939 m, 24 Aug 2010, *J. Nitta 655*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 17.52637°S, 149.80334°W, ca. 823 m, 15 Aug 2010, *J. Nitta 597* (P, PAP, TNS, UC). Terrestrial. Heretofore, known only from Tahiti (Copeland, 1932).

Leucostegia pallida (Mett.) Copel.

Mo'orea, Mt. Tohiea, 17.55449°S, 149.8152°W, ca. 680 m, 24 Aug 2010, *J. Nitta 651*, with V. Liao, J.-Y. Meyer, and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, face of Mt. Tohiea above town of Maatea, 17.5567°S, 149.8225°W, ca. 390 m, 14 Sep 2008, *J. Nitta 240*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, flanc S de l'épaulement SE du Mt. Tohiea, ca. 660 m, 21 Oct 1986, *J. Florence 7916* (PAP; not examined by us). Epiphytic. Widespread, from continental Asia (India, Sikkim, Bhutan, Burma, Thailand, China, Vietnam), throughout Malesia, and Polynesia (Samoa, Tahiti; Nooteboom, 1998).

Formerly included in Davalliaceae, but molecular data support placement in Dryopteridaceae (Smith *et al.*, 2006; Tsutsumi and Kato, 2006).

GLEICHENIACEAE

Diplopterygium longissimum (Blume) Nakai

Mo'orea, Mt. Tohiea, 17.55342°S, 149.81895°W, ca. 1035 m, 24 Aug 2010, *J. Nitta 667*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55339°S, 149.81891°W, ca. 1017 m, 24 Sep 2008, *J. Nitta 313*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, crête SE du Mt. Tohiea, 17.55°S, 149.82°W, ca. 1150 m, 10 Jul 1987, *J. Florence 8369* (PAP). Terrestrial at high elevation on ridges. Previously known from Malaysia to Polynesia and Australia (Holttum, 1968); in the Society Islands, from Tahiti.

Sticherus tahitensis (Copel.) H. St. John

Mo'orea, Mt. Tohiea summit area, 17°33′00°S, 149°49′21°W, ca. 1180 m, 13 Sep 2006, *J.-Y. Meyer 3134*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, Mt. Tohiea, crête sommitale du sommet N, ca. 1190 m, 9 July 1987, *J. Florence 8360* (PAP; not examined by us). Terrestrial at high elevations on ridges. Endemic to the Society Islands (Tahiti) pending further study.

Sometimes confused with *Sticherus brackenridgei* (E. Fourn.) H. St. John from Melanesia.

HYMENOPHYLLACEAE

Hymenophyllum flabellatum Labill.

Mo'orea, Mt. Tohiea, 17.55332°S, 149.81876°W, ca. 1007 m, 24 Aug 2010, *J. Nitta 665*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 17.52644°S, 149.80339°W, ca. 823 m, 19 Sep 2008, *J. Nitta 283*, with S. Vinette (P, PAP, TNS, UC). Pendant epiphyte in cloud forest. Previously known from Australia, New Zealand, Vanuatu, Fiji, Samoa, and Tahiti (Brownsey and Smith-Dodsworth, 1989; McCarthy and Orchard, 1998).

Hymenophyllum cf. ***javanicum*** Spreng.

Mo'orea, Mt. Tohiea, 17.55158°S, 149.82132°W, ca. 1148 m, 24 Aug 2010, *J. Nitta 675*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55126°S, 149.82184°W, ca. 1170 m, 17 Sep 2008, *J. Nitta 273*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea summit area, 12 Sep 2006, *J.-Y. Meyer 3126*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, haut vallon de la face SE du Mt. Tohiea, ca. 1120 m, 23 Oct 1986, *J. Florence 7990* (PAP; not examined by us). Epiphytic in cloud forest. Widespread; previously known from Vietnam, Borneo, Indonesia, New Guinea, New Caledonia, and Fiji.

Hymenophyllum javanicum and *Hymenophyllum samoense* Baker form a continuous series of morphotypes, including both specimens with flat and undulating laminae (Ebihara, 2008). Mo'orean specimens have undulating laminae, but further study is needed to circumscribe species within this group.

Hymenophyllum aff. ***multifidum*** (G. Forst.) Sw.

Mo'orea, Mt. Tohiea, 17.55021°S, 149.82254°W, ca. 1178 m, 25 Aug 2010, *J. Nitta 680*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55188°S, 149.82111°W, ca. 1143 m, 25 Aug 2010, *J. Nitta 696*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55103°S, 149.82198°W, ca. 1188 m, 17 Sep 2008, *J. Nitta 269*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, vallon entre les deux sommets, ca. 1150 m, 9 Jul 1987, *J. Florence 8342* (PAP; not examined by us). Epiphytic in cloud forest. Involucres at right angles to lamina.

Originally considered endemic to New Zealand, but Ebihara (2008) reported that it is also known from Vanuatu and Fiji. Mo'orean material differs from New Zealand material in its smaller size, winged stipes, and broader involucres.

Polyphlebium endlicherianum (C. Presl) Ebihara & K. Iwats.

Trichomanes endlicherianum C. Presl; *Crepidomanes endlicherianum* (C. Presl) P. S. Green

Mo'orea, Mt. Tohiea, 17.55376°S, 149.81693°W, ca. 804 m, 24 Aug 2010, *J. Nitta 653*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, face of Mt. Tohiea above town of Maatea, 17.5554°S, 149.82254°W, ca. 474 m, 22 Aug 2010, *J. Nitta 638* (P, PAP, TNS, UC); Mo'orea, face of Mt. Tohiea above town of Maatea, 17.55725°S, 149.82242°W, ca. 319 m, 22 Aug 2010, *J. Nitta 629* (P, PAP, TNS, UC). Epipetric at lower elevations in cloud forest. Previously known from New Zealand, Fiji (Brownlie, 1977), the Austral Islands, and the Marquesas Islands.

Polyphlebium endlicherianum is occasionally confused with *Polyphlebium borbonicum* (Bosch) Ebihara & Dubuisson, but differs by an obvious row of elongate marginal cells (Nitta, 2008; Ebihara *et al.*, 2009).

Vandenboschia maxima (Blume) Copel.

Trichomanes maximum Blume; *Crepidomanes maximum* (Blume) K. Iwats.

Mo'orea, Mt. Tohiea, 17.54984°S, 149.82214°W, ca. 1195 m, 25 Aug 2010, *J. Nitta 686*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 17.52716°S, 149.80351°W, ca. 776 m, 15 Aug 2010, *J. Nitta 604* (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55362°S, 149.81644°W, ca. 794 m, 25 Sep 2008, *J. Nitta 326*, with S. Vinette (P, UC); Mo'orea, Mt. Tohiea, 17.55366°S, 149.81741°W, ca. 867 m, 16 Sep 2008, *J. Nitta 252*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, UC); Mo'orea, Mt. Tohiea summit area, 12 Sep 2006, *J.-Y. Meyer 3125*, with R. Taputuarai and E. Poroi (PAP). Terrestrial and epipetric. Widespread, from southeastern Asia (Thailand), throughout Malesia, to the Society Islands (Tahiti, Huahine; Copeland, 1932).

Lindsaeaceae

Lindsaea rigida J. Sm.

Mo'orea, Mt. Rotui, 17.5106°S, 149.8383°W, ca. 916 m, 10 Sep 2008, *J. Nitta 216*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, face of Mt. Tohiea above town of Maatea, 17.5567°S, 149.8225°W, ca. 390 m, 14 Sep 2008, *J. Nitta 237*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 17.52705°S, 149.80333°W, ca. 777 m, 19 Sep 2008, *J. Nitta 284*, with S. Vinette (P, UC); Mo'orea, Mt. Tohiea summit area, 17.55139°S, 149.82139°W, ca. 1150 m, 13 Sep 2006, *J.-Y. Meyer 3135*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, Mt. Tohiea, crête sommitale du sommet N, 17.55°S, 149.82°W, ca. 1190 m, 9 Jul 1987, *J. Florence 8359* (P, PAP). Once to twice pinnate. Previously known from Micronesia (Pohnpei), the Solomon Islands, Vanuatu, and the Society Islands (Tahiti; Kramer, 1970).

Lindsaea propinqua Hook. and *Lindsaea pacifica* K. U. Kramer are morphologically similar and included in the same clade as *L. rigida* in a recent molecular study (Lehtonen *et al.*, 2010); Kramer (1970) described *L.*

propinqua and *L. pacifica* as terrestrial and *L. rigida* as epiphytic, but Mo'orean plants were observed to be both epiphytic and terrestrial.

Lycopodiaceae

Huperzia ribourtii (Herter) Holub

Lycopodium ribourtii Herter

Mo'orea, Mt. Tohiea, 17.55376°S, 149.81693°W, ca. 804 m, 24 Aug 2010, *J. Nitta 652*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55345°S, 149.81804°W, ca. 932 m, 25 Sep 2008, *J. Nitta 324*, with S. Vinette (P, UC); Mo'orea, Mt. Tohiea, 19 Jul 2004, *F. Jacq FJ296*, with J.-F. Butaud and F. Laure (PAP; not examined by us); Mo'orea, Mt. Tohiea, 19 Jul 2004, *F. Jacq FJ317*, with J.-F. Butaud and F. Laure (PAP; not examined by us). Epiphytic in cloud forest. Previously known from the Cook Islands and Society Islands (Tahiti; Copeland, 1932).

Oleandraceae

Oleandra sibbaldii Grev.

Mo'orea, Mt. Tohiea, 17.5534°S, 149.8192°W, ca. 1045 m, 24 Aug 2010, *J. Nitta 670*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55335°S, 149.81927°W, ca. 1045 m, 16 Sep 2008, *J. Nitta 259*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 19 Jul 2004, *F. Jacq FJ274* (PAP); Mo'orea, Mt. Tohiea, 19 Jul 2004, *F. Jacq FJ320* (PAP); Mo'orea, crête SE du Mt. Tohiea, 17.55°S, 149.82°W, ca. 1020 m, 22 Oct 1986, *J. Florence 7961* (P, PAP). Epiphytic; common on ridges above ca. 1000 m. Previously known from the Philippines (Tryon, 2000), Indonesia, Fiji (Brownlie, 1977), Samoa (Christensen, 1943), the Marquesas Islands, and the Society Islands (Tahiti; Copeland, 1932).

Polypodiaceae

Calymmodon cf. ***orientalis*** Copel.

Mo'orea, Mt. Tohiea, 17.55021°S, 149.82254°W, ca. 1178 m, 25 Aug 2010, *J. Nitta 682*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55158°S, 149.82132°W, ca. 1148 m, 24 Aug 2010, *J. Nitta 673*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea summit area, 17.54944°S, 149.8225°W, ca. 1176 m, 12 Sep 2006, *J.-Y. Meyer 3124*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, Mt. Tohiea, vallon entre les deux sommets, ca. 1150 m, 9 Jul 1987, *J. Florence 8343* (PAP; determined as *Calymmodon grantii* Copel., not seen by us). Epiphytic in cloud forest. Endemic to the Society Islands, previously known only from Tahiti (Copeland, 1932).

Copeland (1932) described two species of *Calymmodon* from Tahiti which he split on the basis of growth habit (compact in *C. grantii* vs. lax in *C. orientalis*) and paleae length (under 2 mm in *C. grantii* vs. over 2 mm in *C. orientalis*); however, several misdetermined specimens used by Copeland are present at UC, and distinctions between the two are doubtful.

Grammitis marginelloides (J. W. Moore) Copel.

Mo'orea, Mt. Tohiea, 17.55342°S, 149.81895°W, ca. 1035 m, 24 Aug 2010, *J. Nitta 666*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55148°S, 149.82146°W, ca. 1159 m, 25 Sep 2008, *J. Nitta 318*, with S. Vinette (P, PAP, UC); Mo'orea, Mt. Tohiea, 17.5506°S, 149.82262°W, ca. 1199 m, 17 Sep 2008, *J. Nitta 261*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea summit area, 17.54944°S, 149.8225°W, ca. 1176 m, 12 Sep 2006, *J.-Y. Meyer 3121*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, Mt. Tohiea, vallon entre les deux sommets, 17.55°S, 149.82°W, ca. 1150 m, 9 Jul 1987, *J. Florence 8345* (P, PAP). Epiphytic in cloud forest. Endemic to the Society Islands, previously known only from Tahiti.

Microsorum powellii (Baker) Copel.

Phymatosorus powellii (Baker) Pic. Serm.

Mo'orea, Mt. Tohiea, 17.55351°S, 149.81743°W, ca. 877 m, 24 Aug 2010, *J. Nitta 654*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55341°S, 149.81837°W, ca. 964 m, 24 Sep 2008, *J. Nitta 311*, with S. Vinette (P, UC). Epiphytic. Previously known from the Austral and Society Islands (Tahiti).

Oreogrammitis raiateensis (J. W. Moore) Parris

Grammitis raiateensis (J. W. Moore) Copel.

Mo'orea, Mt. Tohiea, 17.55021°S, 149.82254°W, ca. 1178 m, Aug 2010, *J. Nitta 681*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55185°S, 149.82114°W, ca. 1138 m, 24 Aug 2010, *J. Nitta 672*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea summit area, 12 Sep 2006, *J.-Y. Meyer 3123*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, Mt. Tohiea summit area, 12 Sep 2006, *J.-Y. Meyer 3140*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, Mt. Tohiea summit area, 12 Sep 2006, *J.-Y. Meyer 3141*, with R. Taputuarai and E. Poroi (PAP). Epiphytic in cloud forest. Endemic to the Society Islands, previously known only from Raiatea (Copeland, 1932).

Selliguea plantaginea Brack.

Mo'orea, Mt. Tohiea, 17.55335°S, 149.81854°W, ca. 989 m, 24 Aug 2010, *J. Nitta 657*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55341°S, 149.81821°W, ca. 949 m, 16 Sep 2008, *J. Nitta 255*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea summit area, 13 Sep 2006, *J.-Y. Meyer 3128*, with R. Taputuarai and E. Poroi (PAP); Mo'orea, crête SE du Mt. Tohiea, 17.55°S, 149.82°W, ca. 1020 m, 22 Oct 1986, *J. Florence 7960* (P, PAP). Epiphytic. Previously known from Sulawesi, New Guinea, Fiji, and various Pacific island groups; in the Society Islands from Tahiti and Huahine (Copeland, 1932; Hovenkamp, 1998).

There has been some confusion between *Selliguea feeoides* Copel. and *S. plantaginea*; according to Hovenkamp (1998), although the name *S. feeoides* has been applied commonly for specimens from the Pacific area, the two differ

by hydathodes of the adaxial surface frequent in *S. feeoides* vs. infrequent or absent in *S. plantaginea*.

Psilotaceae

Psilotum complanatum Sw.

Mo'orea, face of Mt. Tohiea above town of Maatea, 17.55588°S, 149.82289°W, ca. 445 m, 22 Aug 2010, *J. Nitta 642* (P, PAP, TNS, UC); Mo'orea, south side of Mt. Muaroa, 17.54653°S, 149.84776°W, ca. 513 m, 12 Aug 2010, *J. Nitta 575* (P, PAP, TNS, UC); Mo'orea, trail from Belvedere to Three Coconuts, 17.5403°S, 149.8267°W, ca. 251 m, 12 Sep 2008, *J. Nitta 221*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, Haapiti, 4 Jul 1973, *J. Raynal 18039* (PAP; not examined by us); Mo'orea, *J. Vesco s.n.* (PAP; not examined by us); Mo'orea, *G. K. Barclay 3323A* (PAP; not examined by us). Epiphytic. Widespread throughout the Neotropics, Malaysia, and Oceania (e.g., Hawaii); in the Society Islands from Tahiti, Borabora (Copeland, 1932).

Pteridaceae

Cheilanthes cf. ***nudiuscula*** (R. Br.) T. Moore

Mo'orea, rocky outcropping above town of Papetoai, 17.49824°S, 149.86832°W, ca. 211 m, 19 Aug 2010, *Nitta 619*, with M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, Temae Rocher à l'extrémité de la chaîne de Paaru Iti, ca. 80 m, 12 Dec 2004, *Butaud 825* with É. Lagouy (PAP; determined as *Cheilanthes tenuifolia* (Burm.) Sw., not examined by us); Mo'orea, Vallée Ouest Papeahi, crête rocheuse menant au trou de Tearai, ca. 488 m, 13 Mar 2005, *Butaud 961* (PAP; determined as *C. tenuifolia*, not examined by us). Previously known from Hong Kong, Australia, the Philippines, Timor, New Caledonia, and Fiji (Chambers and Farrant, 1991); one specimen from Tahiti (*Setchell 75*) at UC.

Material from the Society Islands has historically been called *C. tenuifolia*; *C. nudiuscula* is closely related and may differ in density of laminar hairs (Chambers and Farrant, 1991). Available Mo'orean material was immature and depauperate; additional research is needed to determine which name should be applied.

Pteris ensiformis Burm. f. cv. 'Victoriae'

Mo'orea, road from Vaiare to Paopao, 17.51716°S, 149.81104°W, ca. 69 m, 22 Sep 2008, *J. Nitta 299*, with S. Vinette (P, PAP, TNS, UC). Terrestrial on disturbed road cuts. Widespread, including southern China, the Himalayas, India, Ceylon, Burma, Malaysia, southern Japan, the Ryukyus, Taiwan, the Philippines, Australia, Micronesia, and Polynesia (Shieh, 1994). Introduced.

Pteris mertensioides Willd.

Mo'orea, Mt. Tohiea, 17.55362°S, 149.81644°W, ca. 794 m, 25 Sep 2008, *J. Nitta 325*, with S. Vinette (P, PAP, TNS, UC). Terrestrial. Previously known

from Peninsular Malaysia, the Philippines, New Guinea, Fiji, Samoa, and Tahiti.

Selaginellaceae

Selaginella laxa Spring

Mo'orea, face of Mt. Tohiea above town of Maatea, 17.5567°S, 149.8225°W, ca. 390 m, 14 Sep 2008, *J. Nitta 244*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, Moyenne vallée d'Afareaitu, 1ère cascade, ca. 50 m, 30 Dec 1981, *J. Florence 2228* (PAP; not examined by us). Epipetric on rocks in streams. Historically thought to be widespread in Polynesia (Samoa to the Marquesas).

The type specimen of *S. laxa* is from the Society Islands; however, the conspecificity of material from Hawaii is in doubt.

Tectariaceae

Arthropteris palisotii (Desv.) Alston

Mo'orea, Maraarii Valley, 17.52479°S, 149.89342°W, ca. 127 m, 19 Aug 2010, *J. Nitta 618*, with M. Fourdrigniez and P. S. S. Way (P, PAP, TNS, UC); Mo'orea, trail to pass between Mt. Tiura and Mt. Tatiri, 17.53046°S, 149.86578°W, ca. 124 m, 17 Aug 2010, *J. Nitta 607*, with M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, trail from Vaiare/Paopao pass to Three Pines, 17.52669°S, 149.80777°W, ca. 242 m, 22 Sep 2008, *J. Nitta 303*, with S. Vinette (P, PAP, TNS, UC). Epipetric. Widespread, from tropical Africa to southeastern Asia, Australasia, Malesia, and Fiji (McCarthy and Orchard, 1998).

Previously thought to be closely related to either *Oleandra* (Kramer, 1990) or *Nephrolepis* (Pichi Sermolli, 1977); however, molecular data support inclusion in Tectariaceae (Hasebe *et al.*, 1995; Smith *et al.*, 2006; Tsutsumi and Kato, 2006; Schuettpelz and Pryer, 2007).

Tectaria decurrens (C. Presl) Copel.

Mo'orea, Mt. Mou'aputa, 17.52689°S, 149.80348°W, ca. 807 m, 15 Aug 2010, *J. Nitta 600* (P, PAP, TNS, UC); Mo'orea, face of Mt. Tohiea above town of Maatea, 17.5622°S, 149.8217°W, ca. 197 m, 14 Sep 2008, *J. Nitta 248*, with S. Vinette (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 28 Aug 2005, *F. Jacq FJ443* (PAP; not examined by us); Mo'orea, moyenne vallée de Maatea, flanc gauche, ca. 220 m, 13 Aug 1983, *J. Florence 4877* (PAP; not examined by us); Mo'orea, moyenne vallée de Maatea, flanc gauche, ca. 130 m, 29 Dec 1981, *J. Florence 2220* (PAP; not examined by us). Terrestrial. Widespread in tropical mainland Asia, the Philippines, New Guinea, in the Pacific eastward to Tahiti (Holttum, 1991).

Tectaria dissecta (G. Forst.) Lellinger

Mo'orea, Mt. Mou'aputa, 17.52689°S, 149.80348°W, ca. 807 m, 15 Aug 2010, *J. Nitta 603* (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 17.52687°S, 149.80345°W, ca. 789 m, 19 Sep 2008, *J. Nitta 289*, with S. Vinette (P, PAP,

TNS, UC); Mo'orea, Mt. Tohiea, 17.55148°S, 149.82146°W, ca. 1159 m, 24 Sep 2008, *J. Nitta 316*, with S. Vinette (P, PAP, TNS, UC). Terrestrial. Previously known from Taiwan, Indonesia, the Philippines, New Guinea, and in the Pacific east to Tahiti (Holttum, 1991).

THELYPTERIDACEAE

Amphineuron opulentum (Kaulf.) Holttum

Cyclosorus opulentus (Kaulf.) Nakaike

Mo'orea, road from Vaiare to Paopao, 17.51716°S, 149.81104°W, ca. 69 m, 22 Sep 2008, *J. Nitta 300*, with S. Vinette (P, PAP, UC); Mo'orea, Maharepa, 23 May 2004, *F. Jacq FJ294* (PAP; not examined by us). Terrestrial. Widely distributed; previously known from East Africa, Seychelles, Sri Lanka and southern India, southeastern Asia, throughout Malesia, northeastern Australia, and in the Pacific from the Bonin Islands to New Caledonia and eastward to the Marquesas and the Austral Islands (Holttum, 1977). Widely naturalized in the Neotropics. In the Society Islands, previously known from Tahiti and Raiatea.

Chingia longissima (Brack.) Holttum

Cyclosorus longissimus (Brack.) Ching

Mo'orea, valley going up to Mt. Uui from Hauru, 17.50928°S, 149.9046°W, ca. 87 m, 21 Aug 2010, *J. Nitta 620* (P, PAP, TNS, UC); Mo'orea, south side of Mt. Muaroa, 17.54653°S, 149.84776°W, ca. 513 m, 12 Aug 2010, *J. Nitta 576* (P, PAP, TNS, UC); Mo'orea, face of Mt. Tohiea above town of Maatea, 17.5567°S, 149.8225°W, ca. 390 m, 14 Sep 2008, *J. Nitta 246*, with S. Vinette (UC). Terrestrial. Previously known from the Caroline, Solomon, and Marquesas Islands, as well as from Tahiti (Holttum, 1977).

Coryphopteris sp.

Mo'orea, Mt. Tohiea, 17.55332°S, 149.81876°W, ca. 1007 m, 24 Aug 2010, *J. Nitta 663*, with V. Liao and R. Taputuarai (P, PAP, TNS, UC); Mo'orea, Mt. Mou'aputa, 17.5275°S, 149.80313°W, ca. 760 m, 15 Aug 2010, *J. Nitta 593* (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea, 17.55068°S, 149.82233°W, ca. 1045 m, 16 Sep 2008, *J. Nitta 279*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC); Mo'orea, Mt. Tohiea summit area, 13 Sep 2006, *J.-Y. Meyer 3136*, with R. Taputuarai and E. Poroi (PAP). Terrestrial; common on ridges above ca. 1000 m. Endemic to Mo'orea pending further study.

The combination of prominent yellow glands on the underside of the lamina and costae with scales but relatively sparse hairs differentiates this species from other *Coryphopteris* from the Society Islands such as *C. pubirachis* var. *tahitensis* Holttum and *C. raiateana* Holttum.

Plesioneuron sp.

Mo'orea, Mt. Mou'aputa, 17.52822°S, 149.80258°W, ca. 678 m, 19 Sep 2008, *J. Nitta 281*, with S. Vinette (P, PAP, TNS, UC). Terrestrial. Endemic to Mo'orea pending further study.

The sole Mo'orean collection of this species differs from *Plesioneuron tahitense* Holttum, endemic to Tahiti, in lacking sporangial setae and the basal pinna lobes only slightly, if at all, reduced. The only other *Plesioneuron* occurring in the Society Islands (Tahiti, Mo'orea) is *P. attenuatum* (Brack.) Holttum, also known from the Bismarck Archipelago and Samoa (Holttum, 1977). This differs by its larger fronds, broader and longer pinnae (to 25 × 4 cm; to 8 × 2 cm in *Nitta 281*) with more oblique and falcate segments, basal segments of at least the distalmost pinnae strongly reduced, persistent stipe base scales, and relatively large, tan indusia.

Pneumatopteris mesocarpa (Copel.) Holttum

Cyclosorus mesocarpus (Copel.) Ching

Mo'orea, face of Mt. Tohiea above town of Maatea, 17.5567°S, 149.8225°W, ca. 390 m, 14 Sep 2008, *J. Nitta 245*, with S. Vinette (P, UC). Mo'orea, Mt. Tohiea, 17.55511°S, 149.81454°W, ca. 589 m, 16 Sep 2008, *J. Nitta 249*, with J.-Y. Meyer, R. Taputuarai, and M. Fourdrigniez (P, PAP, TNS, UC). Terrestrial. Previously known only from the Society Islands, including Huahine (Holttum, 1977) and Tahiti.

Closely related *Pneumatopteris glandulifera* (Brack.) Holttum, known from Rarotonga, Samoa, the Solomon Islands, and New Hebrides (Holttum, 1977), may occasionally be confused with *P. mesocarpa*, but differs in absence of indusia.

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Literature Cited

Brownlie, G. 1977. The Pteridophyte Flora of Fiji. Beih. Nova Hedwigia 55:1–397.

Brownsey, P. J. and J. C. Smith-Dodsworth. 1989. New Zealand Ferns and Allied Plants. David Bateman, Auckland.

Chambers, T. C. and P. A. Farrant. 1991. A re-examination of the genus *Cheilanthes* (Adiantaceae) in Australia. Telopea 4:509–557.

Chambers, T. C. and P. A. Farrant. 2001. Revision of *Blechnum* (Blechnaceae) in Malesia. Blumea 46:283–350.

Christensen, C. 1943. A Revision of the Pteridophyta of Samoa. Bull. Bernice P. Bishop Mus. 177:1–138, + plates 1–4.

Copeland, E. B. 1932. Pteridophytes of the Society Islands. Bull. Bernice P. Bishop Mus. 93:1–87, + plates 1–16.

Ebihara, A. 2008. Hymenophyllaceae. Pp. 39–58. *In*: National Museum of Nature and Science, eds. Illustrated Flora of Ferns & Fern-Allies of South Pacific Islands. Tokai University Press.

EBIHARA, A., J. H. NITTA, D. LORENCE and J.-Y. DUBUISSON. 2009. New records of *Polyphlebium borbonicum*, an African filmy fern, in the New World and Polynesia. Amer. Fern J. 99:200–206.

FLORENCE, J., H. CHEVILLOTTE, C. OLLIER and J.-Y. MEYER. 2007. Base de données botaniques Nadeaud de l'Herbier de la Polynésie française (PAP). http://www.herbier-tahiti.pf

HASEBE, M., P. G. WOLF, K. M. PRYER, K. UEDA, M. ITO, R. SANO, G. J. GASTONY, J. YOKOYAMA, J. R. MANHART, N. MURAKAMI, E. H. CRANE, C. H. HAUFLER and W. D. HAUK. 1995. Fern phylogeny based on *rbcL* nucleotide sequences. Amer. Fern J. 85:134–181.

HOLTTUM, R. E. 1964. The tree-ferns of the genus *Cyathea* in Australasia and the Pacific. Blumea 12:241–274.

HOLTTUM, R. E. 1968. A Revised Flora of Malaya. Vol. II. Ferns of Malaya, 2nd ed. Government Printing Office, Singapore.

HOLTTUM, R. E. 1977. The family Thelypteridaceae in the Pacific and Australasia. Allertonia 1:169–234.

HOLTTUM, R. E. 1991. *Tectaria* Group. Pp. 1–132. *In*: Flora Malesiana, Ser. II, Vol. 2 (1). Rijksherbarium & Hortus Botanicus, Leiden.

HOVENKAMP, P. 1998. An account of the Malay-Pacific species of *Selliguea* (Polypodiaceae). Blumea 43:1–108.

KATO, M. 1984. A taxonomic study of the athyrioid fern genus *Deparia* with main reference to the Pacific species. J. Fac. Sci. Univ. Tokyo, Sect. 3, Bot. 13:375–429.

KRAMER, K. U. 1970. The lindsaeoid ferns of the Old World V. The smaller Pacific islands. Blumea 18:157–194.

KRAMER, K. U. 1990. Oleandraceae. *In*: K. Kubitzki, ed. The families and genera of vascular plants. Vol. I. Pteridophytes and Gymnosperms. K. U. Kramer and P. S. Green, vol. eds. Springer Verlag, Berlin.

LEHTONEN, S., H. TUOMISTO, G. ROUHAN and M. J. M. CHRISTENHUSZ. 2010. Phylogenetics and classification of the pantropical fern family Lindsaeaceae. Bot. J. Linn. Soc. 163:305–359.

MCCARTHY, P. M. and A. E. ORCHARD, eds. 1998. Flora of Australia. Vol. 48. Ferns, Gymnosperms and Allied Groups. ABRS/CSIRO, Melbourne.

MICKEL, J. T. and A. R. SMITH. 2004. The pteridophytes of Mexico. Mem. New York Bot. Gard. 88:1–1054.

MURDOCK, A. G. and A. R. SMITH. 2003. Pteridophytes of Moorea, French Polynesia, with a new species, *Tmesipteris gracilis* (Psilotaceae). Pacific Sci. 57:253–265.

NITTA, J. H. 2008. Exploring the utility of three plastid loci for biocoding the filmy ferns (Hymenophyllaceae) of Moorea. Taxon 57:725–736.

NOOTEBOOM, H. P. 1994. Notes on Davalliaceae II. A revision of the genus *Davallia*. Blumea 39:151–214.

NOOTEBOOM, H. P. 1998. Davalliaceae. Pp. 235–276. *In*: Flora Malesiana, Ser. II, Vol. 3. Rijksherbarium & Hortus Botanicus, Leiden.

PICHI SERMOLLI, R. E. G. 1977. Tentamen Pteridophytorum genera in taxonomicum ordinem redigendi. Webbia 31:313–512.

RANKER, T. A., P. G. TRAPP, A. R. SMITH, R. C. MORAN and B. S. PARRIS. 2005. New records of lycophytes and ferns from Moorea, French Polynesia. Amer. Fern J. 95:126–127.

ROUHAN, G., D. H. LORENCE, T. J. MOTLEY, J. G. HANKS and R. C. MORAN. 2008. Systematic revision of *Elaphoglossum* (Dryopteridaceae) in French Polynesia, with the description of three new species. Bot. J. Linn. Soc. 158:309–331.

SCHUETTPELZ, E. and K. M. PRYER. 2007. Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. Taxon 56:1037–1050.

SHIEH, W. C. 1994. Pteridaceae. Pp. 206–233. *In*: T. C. Huang, ed. Flora of Taiwan (2nd ed.), Vol. I. Editorial Committee of the Flora of Taiwan, Taipei.

SMITH, A. R., K. M. PRYER, E. SCHUETTPELZ, P. KORALL, H. SCHNEIDER and P. G. WOLF. 2006. A classification for extant ferns. Taxon 55:705–731.

TRYON, R. 2000. Systematic notes on the Old World fern genus *Oleandra*. Rhodora 102:428–438.

TSUTSUMI, C. and M. KATO. 2006. Evolution of epiphytes in Davalliaceae and related ferns. Bot. J. Linn. Soc. 151:495–510.

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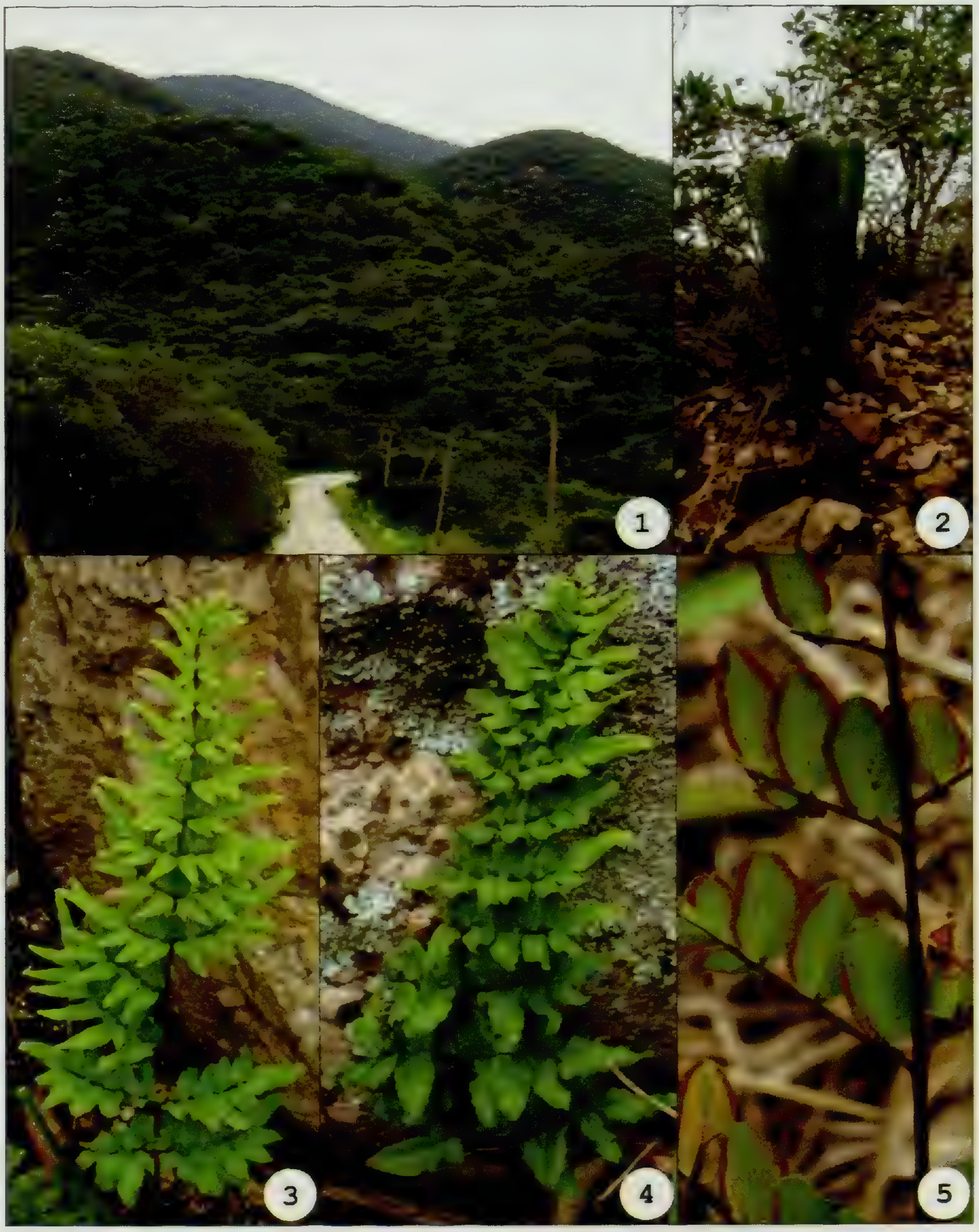
Shorter Notes

***Pellaea flavescens* Fée in Rio de Janeiro, its Lectotypification, and its New Record for São Paulo State, Brazil.**—The type specimens of *Pellaea flavescens* Fée (*Habitat in Brasilia fluminensi, Glaziou 2473*, K-000633009 p.p., K-000633010, P-00252273, P-00252274) came from the Serra dos Órgãos, located in the state of Rio de Janeiro, southeastern Brazil. These mountains have an elevational range of 80–2280 m, and the Atlantic rain forest is the main vegetation. Beyond the specimens cited in the original publication of this species by Fée (Crypt. Vasc. Brés. 1:44, t. 22, f. 2. 1869), only a few collections of this taxon were known from Rio de Janeiro, all from the vicinity of the type-locality: Brazil: **Rio de Janeiro**, Tijuca, Pedra José Cineiro, 21 Jan 1871, *Glaziou 3546* (P); *Ex sylvis montanis Brasiliae prope Petropolis*, 2000–3000 feet, 10 Jul 1882, *Ball s.n.* (K-000633008). The *Glaziou 3546* collection was mentioned by Fée (Crypt. Vasc. Brés. 2: 28. 1873).

Later, the species was collected in the following localities: **Rio de Janeiro**, Santa Maria Magdalena, Alto do Desengano, 2000 m, 3 Mar 1934, *Santos Lima and Brade 13149* (RB); id, Frade de Macaé, 19 Jun 1937, *Brade 15803* (RB); id., Base do Pico da Tijuca, 15 Jun 1948, *Duarte and Pereira 1142* (RB); id., Nova Friburgo, Duas Pedras, 1951, *Capell s.n.* (RB). More recently, on 9 Apr 2006, *P. flavescens* was re-collected (*Moraes 78*, RB), in Petrópolis, Distr. Araras, Área de Proteção Ambiental of Petrópolis, Serra da Maria Comprida. This last locality is also in the Serra dos Órgãos complex. These eight collections represent all records for this taxon to Rio de Janeiro and in spite of its scattered records, *Pellaea flavescens* was not included in a recent list of the Brazilian endangered plants (MMA, Instrução Normativa n. 06, 23 Sep 2008).

In October 2009, the present authors found a small population of *Pellaea flavescens* [*Prado et al. 2036* (DUKE, MO, SP)] in another range of mountains (Serra do Japi, municipality of Jundiaí), in São Paulo State. This area also has the Atlantic rain forest as the predominant vegetation. The plants were growing at ca. 790 m elevation, among grasses and rocks, in an open area, near some plants of *Cereus peruvianus* (L.) Mill. (Cactaceae) (Figs. 1, 2). This is the first record for this species outside of Rio de Janeiro. It is probable that the distribution of *P. flavescens* will be more extensive than is presently known, but it seems to be very rare in nature, as evidenced by the few specimens that have been collected since the species was first discovered in the 19th century.

Pellaea flavescens can be easily distinguished from other Brazilian cheilanthoid ferns by the combination of its short-creeping rhizomes; erect fronds to ca. 50 cm tall; dark brown to blackish petioles and rachises, the rachises not flexuous, bearing hairs and filiform scales, adaxially slightly sulcate and with two narrow wings; the laminae light green, 2-3-pinnate at the base, sometimes the proximal pinnules only lobate, subcoriaceous and glabrous on the laminar tissue; pinnules varying from ovate to lanceolate (Figs. 3–5).



FIGS. 1–5. **1**. General view of Serra do Japi, SP. **2**. *Cereus peruvianus* (L.) Mill. (Cactaceae). **3**, **4**. Habit of *Pellaea flavescens* Fée. **5**. Abaxial pinnule surface (Photos by J. Prado, Oct 2009).

To fix the application of this epithet, a lectotype for this taxon is chosen here, as follows below:

Pellaea flavescens Fée, Crypt. Vasc. Brés. 1: 44, t. 22, f. 2. 1869. LECTOTYPE (here designated).—BRAZIL: **Rio de Janeiro**, 7 Aug 1869, *A. Glaziou 2473*

(P-00252274; duplicates K-000633009 p.p., K-000633010, MO-1803633 not seen, P-00252273).

Pellaea flavescens Fée var. *macahensis* Brade, Arch. Jard. Bot. Rio de Janeiro 11: 28, t. 8. 1951. TYPE.—BRAZIL, **Rio de Janeiro**, Frade de Macaé, 700 m, 19 Jun 1937, *A. C. Brade 15803* (RB).

The variety *macahensis* is merely a bigger plant of *Pellaea flavescens* with the pinnules more widely spaced. In all other features the type of *macahensis* matches with the type of *P. flavescens*.

According to Tryon and Tryon (*Ferns and allied plants, with special reference to tropical America*, Springer-Verlag, New York, pg. 288. 1982), *Pellaea flavescens* belongs to the Section *Ormopteris*. However, in the modern sense of the genus (Gastony and Rollo, Amer. Fern J. 85(4):341–360. 1995[1996]) this taxon is not a *Pellaea*. This taxon also differs from the other members of Section *Ormopteris* by the slightly sulcate axis adaxially. Molecular studies are currently underway to assess its correct position into Brazilian cheilanthoid ferns.

This species is endemic to southeastern Brazil and now it is know from Rio de Janeiro and São Paulo States, from disjunct populations along the Atlantic rain forest.

We are grateful to Dr. João Vasconcellos Neto (UNICAMP) for invitation to participate of the project to collect ferns in the Serra do Japi, São Paulo.—Jefferson Prado and Regina Yoshie Hirai, Instituto de Botânica, Herbário SP, C. P. 3005, 01031-970 São Paulo, SP, Brazil.

***In Situ* Gametophyte Morphology of the Tropical Epiphyte *Oleandra articulata*.**—The genus *Oleandra* is a poorly understood lineage of ferns that is comprised of epiphytic, hemiepiphytic, and terrestrial species (Moran and Riba, Eds, Psilotaceae a Salvinaceae. Flora Mesoamericana. Universidad Nacional Autonoma de Mexico, Mexico City. 1995; Tsutsumi and Kato, Bot. J. Lin. Soc. 151:495–510. 2006). In spite of the fact that the genus has been variably treated as a member of the Polypodiaceae, Dryopteridaceae, and Nephrolepidaceae several early authors recognized the unique nature of the group. Hooker (Genera Filicum. H.G. Bohn, London.1840) remarked that *Oleandra* was a "highly beautiful and very natural genus." Others from Greville (Trans. Bot. Soc. Edin. 3:49–50.1848) to Pichi-Sermolli (Webbia 20:765–769. 1965) and Tryon (*Rhodora* 99:335–343. 1997; *Rhodora* 102:428–438. 2000) have all commented on the unique morphological nature of this lineage. Indeed, recent molecular analyses have confirmed these observations and *Oleandra* is now placed in the monogeneric Oleandraceae as sister to the clades containing the old world Davalliaceae and cosmopolitan Polypodiaceae (Tsutsumi and Kato, 2006; Schuettpelz and Pryer, Fern Phylogeny. In T. A.

Ranker and C. H. Haufler [eds.], The Biology and Evolution of Ferns and Lycophytes, 395–416. Cambridge Univ. Press, Cambridge. 2008). Whereas there has been no detailed monographic revision of this group, it is estimated that 40 (Smith *et al.*, *Taxon* 55:705–731. 2006) to 80 species have been described from the neo- and paleotropics and Polynesia (Moran and Riba, 1995). In Mesoamerica there are four (Tryon, 1997) to five (Moran and Riba, 1995) species recognized. *Oleandra articulata* (Sw.) C. Presl (Figure 1a) is one of the most abundant epiphytes at the La Selva Biological Station in Costa Rica (Watkins Jr. and Cardelús, Am. Fern J. 99:162–175. 2010).

The morphology and habit of *Oleandra articulata* are unlike that of most epiphytic ferns: its leaves are paper-thin (Watkins, Rundel, and Cardelus, Oecologia 153:225–232. 2007) and produce long and un-branched parallel veins; the species also produces a long-traipsing rhizome that grows over surrounding epiphytes in the canopy habitat. Indeed, it can be difficult to find the origin of many plants as rhizomes can grow 3–5m (J.E. Watkins, Jr. Pers. Obs.). The species is often found in great densities in some host trees and represents a significant component of the canopy flora (Watkins Jr. and Cardelús, 2010). Given the unusual nature of this species it is surprising that little is known of its reproductive ecology.

Recently, we had the great fortune to discover a large number of gametophytes and young sporophtyes of this species growing in the canopy of a *Hyeronima alchorneoides* (Euphorbiaceae) at La Selva Biological Station in Costa Rica (Fig. 1A–D). To our knowledge, the gametophyte of this species has yet to be described. In addition, none of the descriptions that exist for the genus have used material collected *in situ* (e.g., Atkinson and Stokey, *Phytomorphology* 14:51–70. 1964; Nayar and Kaur, *Bot. Rev.* 37:295–396. 1971), that is the goal of this note.

Approximately 50 gametophytes were collected and observed under compound and stereoscopic microscopes. In general, the gametophytes are cordiform-thalloid with broad wings (Fig. 2I). Most strikingly, the thallus surface and margins were covered with copious unicellular papillate secretory hairs (Fig. 2A–F, J). The occurrence of such hairs on the gametophyte has been reported for the genus in the past; however, here we describe a novel secretory pattern previously undescribed. From many hairs, the secretions form unusual finger-like projections that extend out in several dimensions (Fig. 2A–F). Even more notable is that these secretory projections often connect several hairs together in a manner resembling electrical lines (Fig. 2A & B). Some of these strands can obtain lengths of nearly 1 cm. The exact chemical nature of the secretion is unknown as we did not have adequate tools for such analyses in the field setting. However, one concern was that these strands could be fungal in origin. We were unable to locate septa in any of the strands and the physical appearance is similar to that produced on the hairs without such outgrowths. Several studies have shown such secretions to be lipidic in nature (Nayar and Kaur, 2007, and references therein). Future work on these secretions would benefit from the simple Sudan test for lipid presence. Speculation as to the function of such hairs is premature; however, they are remarkably reminiscent

FIG. 1. **A.** Sporophytes of *Oleandra articulata* growing on a branch *Hyeronima alchorneoides* (Euphorbiaceae) at La Selva Biological Station in Costa Rica. **B.** Developmental series from gametophytes (upper left of image) to young sporophyte with elongated rhizome. C–D. Collection of *in situ* gametophytes and young sporophytes growing in the canopy of *Hyeronima alchorneoides* (Euphorbiaceae), G = gametophytes, S = sporophytes.

of the branched hair network on the surface of *Salvinia* sporophytes that render those leaves "unwettable." Nevertheless, these hairs serve as an important species specific character allowing for identification of the gametophytes.

Unlike many epiphytic gametophytes, the thallus exhibited no tendency to become elongate or three dimensional (Farrar *et al.*, Gametophye Ecology. *In* T. A. Ranker and C. Haufler [eds.], Biology and Evolution of Ferns and Lycophtyes, 222–251. Cambridge University Press, Cambridge. 2008). This discovery is quite exciting given the phylogenetic placement of the genus. As discussed in detail, Farrar *et al.* (2008) have pointed out that many epiphytes rely on gametophytes that produce elongate/strap-shaped growth forms that can persist in the complex and highly competitive matrix that makes up moss mats in epiphytic habitats. It has been suggested that as ferns radiated into the canopy this morphology could have been useful in spreading within moss mats and potentially in increasing thallus water holding capacity (Watkins *et*

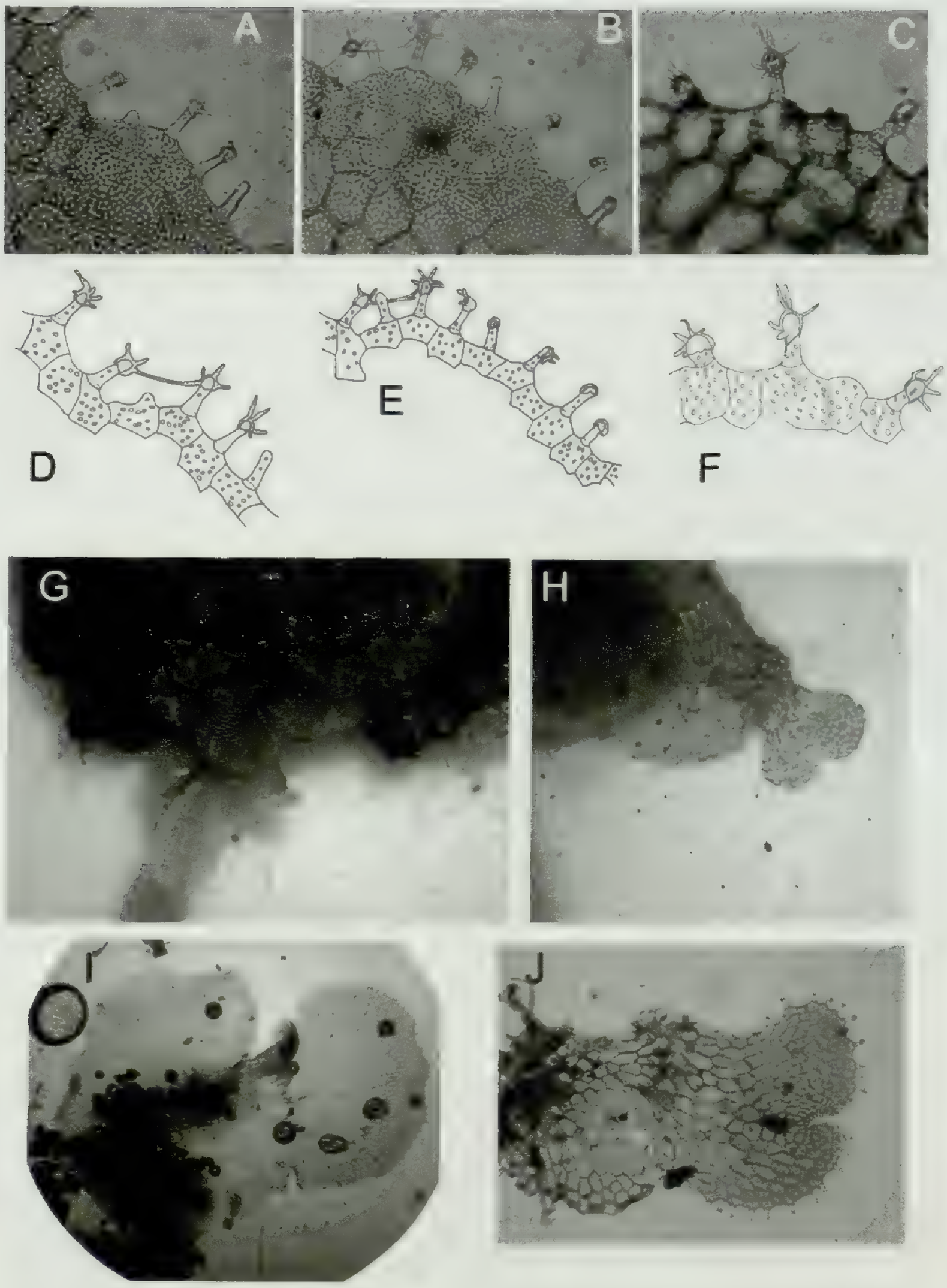


FIG. 2. Details of the gametophytes of *Oleandra articulata* gametophytes collected on a branch of *Hyeronima alchorneoides* (Euphorbiaceae) at La Selva Biological Station in Costa Rica. **A–F.** Hair morphology in this species is unusual via production of secretions that yield finger-like projections. In some cases, these secretions link several hairs together. (A, B, D, E, 10×; C,F 20×). **G–H.** The species produces what appears to be asexual buds from the thallus margins. Buds resemble small versions of mature gametophytes. **I.** Example of mature gametophyte with emerging sporophyte (sp) and surface hairs (sh). **J.** Detail of young gametophyte demonstrating production of marginal hairs (4×).

al., New Phytologist 176:708–717. 2007). *Oleandra* occurs at the phylogenetic base of a major radiation event into the canopy (Tsutsumi and Kato, 2006; Schuettpelz and Pryer, 2008). Thus, the gametophytes of this species may resemble the archetypical form found in early pre-epiphyte progenitors.

Another aspect of gametophyte morphology that is common in epiphytic taxa is asexual reproduction (Chiou and Farrar, *Am. Fern J.* 87:77–88. 1997).

We did observe this phenomenon in *O. articulata* though it appeared to be less frequent than in other taxa such as the Vittariaceae and Hymenophyllaceae at this site (J.E. Watkins, Jr. Pers. Obs.). While difficult to quantify, we recorded asexual proliferations on approximately 8% of the thalli observed. The formation of these proliferations was not unusual, and resembled cordiform outgrowths of the parent thallus (Fig. 2G & H). Such proliferations are unlikely to act in dispersal, but may result in a long lived perennial gametophyte as has been described in some members of the Polypodiaceae (Chiou and Farrar, 1997).

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Narrow Substrate Niche of *Cheilanthes lanosa*, the Hairy Lip Fern, is Determined by Carbohydrate and Lipid Contents in Gametophytes

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ABSTRACT.—*Cheilanthes lanosa* is a xerophytic fern that inhabits rock cliffs and crevices. Morphological features such as cuticle and trichomes assist sporophyte survival. However, the gametophyte stage appears to lack any water-saving features. Previous studies suggest that the gametophyte may balance its water through carbohydrate production and a strong internal osmotic gradient. To investigate the basis for gametophyte survival, lipids and carbohydrates were quantified using the vanillin and anthrone assays. Results suggest that lipids and carbohydrates increase in percent of total biomass (w/w) throughout development. In addition, lipid and carbohydrate content can change with varying culture conditions. Young gametophytes, with high carbohydrate and low lipid content, are relegated to substrates with a potential for a small but continuous water source.

KEY WORDS.—*Cheilanthes lanosa*, gametophyte physiology, water balance

Cheilanthes, the Rock Ferns, is a genus of xerophytic ferns that is generally restricted to rocky habitats (Mickel, 1979; Yatskievych, 1999). *Cheilanthes* sporophytes have evolved morphological characteristics and reproductive mechanisms that aid survival in dry climates and could potentially survive in numerous xeric habitats. For example, *Cheilanthes* sporophytes produce numerous trichomes that help to prevent water loss (Quirk and Chambers, 1981). They produce a thick cuticle, and several species appear bluish-green as a result of this production (Cobb *et al.*, 2005). In addition, *Cheilanthes* species exhibit reduced surface area to volume of leaf cells (Gratani *et al.*, 1998; Hevly, 1963; Pickett, 1931). Thus, there is a relatively small amount of surface area for water loss. Another characteristic that makes these ferns very hardy is their ability to desiccate and still maintain viability. Like many desiccation-tolerant ferns, *Cheilanthes* sporophytes can dry and rehydrate (Quirk and Chambers, 1981). These ferns also engage in mycorrhizal relationships (Palmieri and Swatzell, 2004). Mycorrhizae typically aid in water and nutrient uptake in exchange for photosynthates (Al-Karaki, 1998; Harley and Smith, 1983).

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Survival for ferns, however, also includes reproduction, and sexually reproducing ferns generally require at least a film of water for male gametes to reach the female gametophyte structures, the archegonia (Raven *et al.*, 2005). Some *Cheilanthes* species circumvent this need for water through apogamy (Hevly, 1963; Steil, 1933; Whittier, 1965). Thus, *Cheilanthes* sporophytes possess a multitude of characteristics that enable these ferns to persist through the diploid portion of their life cycles.

Gametophytes, however, lack the majority of these features. They do possess a lipid-based substance that appeared to be exuded by wax glands (Lingle *et al.*, 2004). In addition, the gametophytes can desiccate fully, revive, and produce new gametophytes. Under a slow drying regime, gametophytes may abandon peripheral, vulnerable cells and dry to a bright green, almost crystalline group of cells. These cells can revive when rehydrated and produce new filaments and prothalli (Diamond and Swatzell, 2003). However, these gametophytes are haploid, and only one cell thick (Steil, 1939). Therefore, it is still unclear how the gametophytes, which appear as highly vacuolate, unprotected cells in a single layer, can survive without desiccation or even through it.

Clearly, there must be some physiological mechanism, or combination of mechanisms, that aids in gametophyte survival, but the nature of this mechanism has not been elucidated. The obvious candidate would be a physiological mechanism that can control water uptake and prevent water loss. Previous research has shown that the protonema of at least some *Cheilanthes* species have specific water requirements to germinate (Nondorf *et al.*, 2003) and remain viable as they mature. For example, slender lip ferns germinate and survive optimally on limestone substrate with a specific retention of 20–30 $\mu l \cdot cm^{-3}$ (Dooley and Swatzell, 2002; Nondorf *et al.*, 2003). Studies also show that hairy lip fern gametophytes manage water in their cells through control of aquaporin-like proteins, or water-specific plasma membrane channels (Diamond, 2007). Still, control of aquaporins alone cannot maintain water balance in a desiccating environment. Osmosis, water diffusion across a membrane, although slow, does occur and would place vulnerable gametophyte cells at risk. In hairy lip fern gametophytes, aquaporin-like proteins appear to increase in quantity throughout development (Ricks and Swatzell, 2006), as do lipids and carbohydrates (Diamond *et al.*, 2003, Lingle *et al.*, 2004). Previous studies have shown that gametophytes that are grown in conditions closely resembling the fern's natural environment have higher levels of mono- and disaccharides than gametophytes or protonemal callus grown on agar medium (Abney, 2004). Diamond *et al.* (2003) showed that the total osmotic potential in gametophytes grown under natural conditions is five times that of agar-grown gametophytes. Therefore, it is possible that increased lipid concentrations that block osmosis, increased carbohydrate concentrations that promote water uptake and inhibit water loss, and increased aquaporin-like proteins and/or control of water flow may constitute the survival mechanism for hairy lip fern gametophytes.

To determine if lipids and carbohydrates play a pivotal role in the water balance mechanism of *Cheilanthes* gametophytes, the developmental stages of *Cheilanthes lanosa* (Michx.) D.C. Eat. gametophytes were examined for changes in total lipid and carbohydrate contents. We predicted that as gametophytes mature, lipids and carbohydrates would increase in concentration during their development. We also predicted that the environment would be an influence on lipid and carbohydrate production, with the mature gametophytes grown on dry sand substrate exhibiting a higher level of both lipids and carbohydrates.

Methods

Plant Collection

Hairy lip fern sporophylls were collected in the fall after the first frost from a sandstone bluff 0.25 mi north of Makanda, Illinois, placed in glass 9 cm-diameter Petri dishes, and stored in the dark at 4°C. After several months, sporophylls were crushed using a mortar and pestle. *Cheilanthes lanosa* spores average 40 μm in diameter (Devi *et al.*, 1970) and spores were separated from the plant material using a 65 μm brass mesh sieve. Spores were stored at 4°C in the dark.

Plant Growth and Materials

Wet grown (WG) gametophytes and protonemal callus.—Spores were surface sterilized in a 7% (v/v) commercial bleach solution with 0.1% (v/v) Triton X-100 for 10 min. Spores were then rinsed in sterile ddH_20 and sown on a modified (Smith, 1992) tissue culture medium (TCM) in 9 cm-diameter disposable, sterile Petri dishes. Spores were incubated at 25°C in 0.175 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of continuous far red light (650–705 nm) for 10 days.

Following germination and protonemal development, the plates were separated. Some plates were left in the far red light to induce and maintain protonemal callus growth. The remaining plates were then exposed to continuous white light (405–710 nm) and the protonemata began planar growth into gametophytes.

Dry grown (DG) gametophytes.—Spores were sown on fine grain white sand (Décor Sand, Activa Products Inc., Marshall, TX) in 9 cm glass Petri dishes, wetted with 20 ml of TCM, and incubated first in far red light, then white light as the WG cultures described above. However, unlike the sterile cultures of WG and callus, the DG cultures were not sealed. After DG gametophytes reached the prothallus stage, they were wetted erratically upon drying with ddH_2O. DG gametophytes were grown to maturity, that is, until the first sporophytes appeared and wax glands were abundant.

Sampling Procedures

Each sample consisted of clumps of gametophytes, approximately the same size, withdrawn haphazardly from approximately 12 plates of each treatment.

To avoid potential desiccation, samples were placed immediately into preweighed 0.5 ml microcentrifuge tubes and frozen at −80°C. After all samples were gathered, they were removed from storage, but not opened until the wet weight of each had been determined. Thus, any potential water loss within the tube during storage was also taken into account. Following determination of the sample weights, vanillin or anthrone assays were then performed.

Anthrone Assay

Gametophytes (n = 98 for each stage) were collected from agar or sand using needle-tipped tweezers. Gametophytes were placed in 1 ml microcentrifuge tubes, weighed to obtain total sample weight, and stored at −80°C. Gametophytes were then placed in 1.5 ml microcentrifuge tubes and homogenized with a micropestle in 62.5 µl of 0.5 M Na_2SO_4. Following homogenization, 1.25 ml anthrone reagent (Judd, 2006; van Handel, 1985a) was added to the sample and vortexed. Tubes were then heated at 100°C for 12 min, vortexed again, and cooled to room temperature. The absorbance of each sample was measured at 625 nm using a Beckman DU 640B spectrophotometer. Sucrose standards (0–100 µg/ml) were prepared from a 0.1 g/L stock solution and absorbance was measured as above.

Vanillin Assay

Gametophytes (n = 100 for each stage) were collected and weighed as above. Samples were stored in 1.0 ml microcentrifuge tubes at −80°C. Samples were then transferred to 1.5 ml microcentrifuge tubes and homogenized with a micropestle. Following homogenization, 100 µl of a 1:1 chloroform/methanol was added to each sample. The samples were centrifuged at approximately 10,000 rpm for 10 min. The supernatant was retained and allowed to dry overnight. Samples were vortexed and heated at 100°C for 10 min in 200 µl of concentrated H_2SO_4. Samples were cooled to 25°C and vortexed. Samples were then transferred to glass shell vials, mixed with 3 ml of vanillin reagent (Barnes and Blackstock, 1973; van Handel, 1985b) and incubated for 30 min. Absorbance was determined at 525 nm using a Beckman DU 640B spectrophotometer. The lipid standard, almond oil (NowFoods, Bloomingdale, IL), was prepared and measured as above. Stock concentration was 1:1,000 in a 1:1 chloroform/methanol mixture. Average weight (n = 100) of standards was 4.83 µg/ml. Standard dilutions were 1X, 1/2X, and 1/4X. Absorbance was used to calculate lipid weights in samples.

Data Analysis

Statistical differences in percent lipid and carbohydrate content per total weight were determined by analysis of variance (ANOVA) followed by a

Tukey's studentized test using the SAS General Linear Model Procedure (SAS 1999–2000).

Results

Plant Culture

It was important to produce protonema *en masse* without altering the uniseriate, filamentous growth. Following spore germination on TCM, protonema developed into callus under extended red light treatment (Fig. 1A). Blue light wavelengths in white light exposure induced planar growth (Fig. 1B). However, under red light treatment in tissue culture, cells were continuously produced in numerous filaments.

Gametophytes in agar culture with blue light developed normally (Fig. 1C; WG). The protonemal remnant persisted in many of the gametophytes. Gametophytes produced antheridia, but no archegonia, similar to previous reports of *Cheilanthes feei* T. Moore gametophytes in the wild (Steil, 1933). The WG gametophytes resembled dry sand cultured gametophytes in size and morphology (Fig. 1D; DG). However, DG gametophytes on sand generally completed the haploid portion of their life cycle including wax gland and sporophyte production (WG gametophytes did produce wax glands when left long enough in culture so that the agar substrate began to dry from age; this was rare and WG gametophytes never produced sporophytes; Fig. 2).

Anthrone Assay

Calculation of the means (Table 1) from the anthrone assay revealed that the mean percentage of carbohydrates per total weight was 23.53% ±15.21% for the WG treatment, 12.21% ±30.29% for the DG treatment, and 17.31% ± 9.73% for the protonemal callus treatment (Fig. 3). Analysis of variance (ANOVA) followed by a Tukey's studentized test revealed that there was a significant difference in percent carbohydrates between the WG and DG treatments, but there was no significant difference between protonemal callus and the other treatments.

Vanillin Assay

Lipid concentration in callus, WG, and DG gametophytes was assessed using a vanillin assay. Assay results show a mean percent lipid concentration of 0.72% ± 0.46% for the WG treatment, 0.22% ± 0.20% for the DG treatment, and 0.10% ± 0.14% for the protonemal callus treatment (Fig. 4; Table 1). An ANOVA followed by a Tukey's studentized test revealed that there was a significant difference between each treatment.

Overall, the percent carbohydrates of total weight were much higher in all three treatments than the percent lipids of total weight. Means of lipid concentrations per total weight ranged below 1.0%. However, carbohydrate concentrations were as much as 23.0% of total weights.

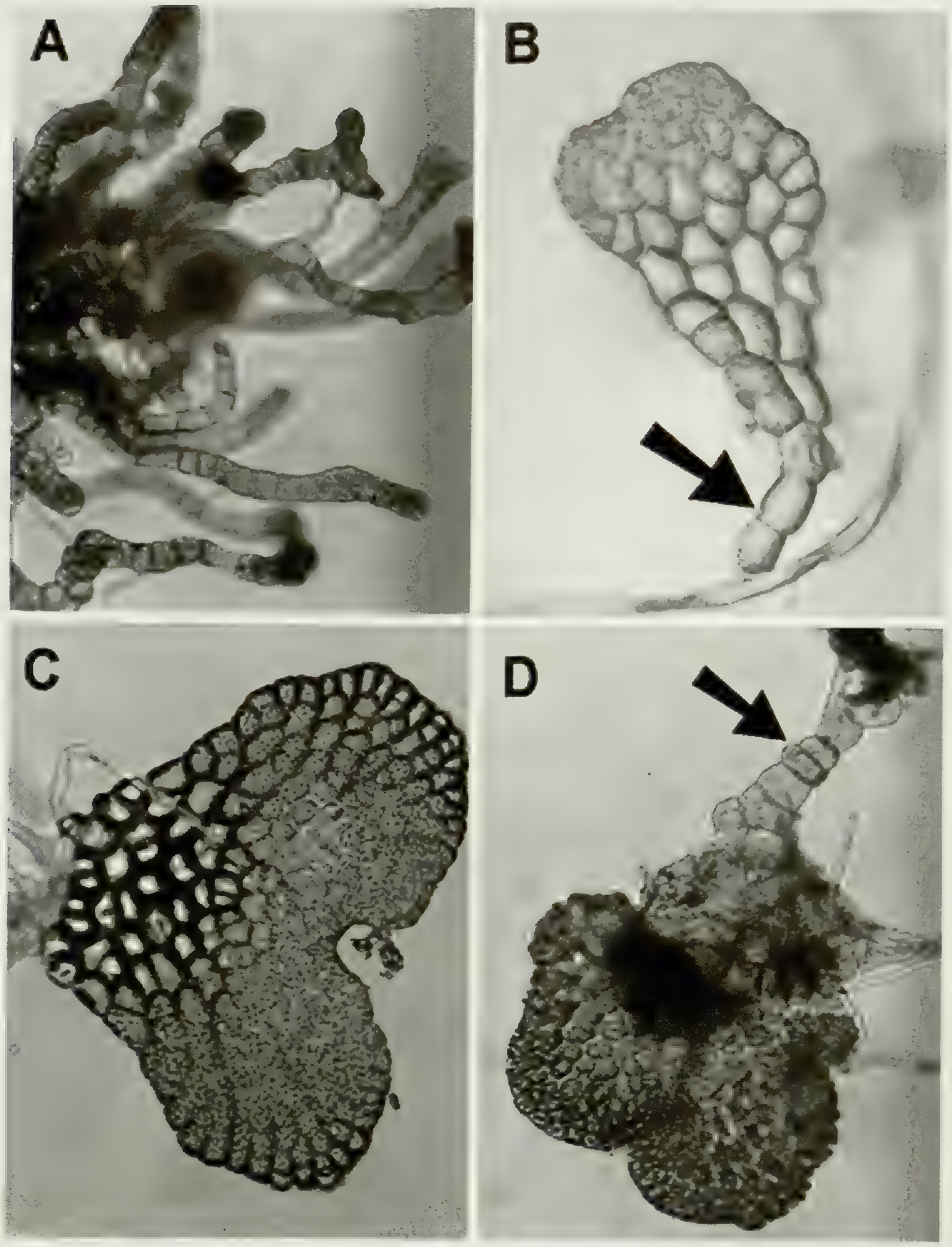

FIG. 1. Cultured Gametophytes of *Cheilanthes lanosa*. Gametophytes in agar culture underwent normal development from germination, through the protonemal stage **(A)**, to prothallus development **(B)**. Mature gametophytes on agar **(C)** and in dry culture **(D)** still bear the remnant of the protonemal stage **(arrow)**. Mature gametophytes produced antheridia.

DISCUSSION

Effects of Environment and Development on Lipid and Carbohydrate Production

Previous research on hairy lip fern gametophytes revealed an increased ability to manage water balance as gametophytes mature (Diamond *et al.*, 2003; Diamond, 2007). Therefore, we hypothesized that carbohydrate and lipid concentrations would also increase through the gametophyte generation with maturity. Lipids in cuticle materials or intracellular lipids that line cell

FIG. 2. Mature gametophytes of *Cheilanthes lanosa* grown under dry conditions (DG). Wax glands are commonly produced on cell surfaces. Prior to forming a sporophyte, which will draw resources from the gametophyte, a thickened sporophyte pad will develop in the center of the gametophyte.

peripheries could potentially block osmosis, and carbohydrates, utilized as an internal osmotic gradient, could increase water uptake and inhibit water loss (Lingle *et al.*, 2004; Schneider *et al.*, 2003). We predicted that normal gametophyte development would include an increase in lipids and carbohydrates that could protect the gametophyte from desiccation and prepare it to manage water in an unpredictable environment. This prediction was only partially accurate. Gametophytes grown in a consistent environment, such as the wet agar-grown gametophytes (the protonematal callus and the WG gametophytes) steadily increased in lipids and carbohydrates. However, we did not predict that gametophytes grown in substantially dryer, unpredictable conditions (DG) would be significantly lower in percent lipid and carbohydrate content than their mature wet grown counterparts or that the samples would vary so widely. Instead, the results of this study suggest that carbohydrate and lipid concentrations in different gametophyte stages could be the result of both environmental and developmental factors.

The wet agar-grown cultures showed a pattern of increase in both carbohydrate and lipid concentrations from protonemal callus to mature

TABLE 1. Mean % carbohydrate and lipid concentration in gametophyte culture. Analysis of variance (ANOVA) followed by a Tukey's studentized test ($P<0.05$) showed a significant difference between percent carbohydrates in wet-grown (WG) and dry-grown (DG) treatments. There was no significant difference between percent carbohydrates in callus and other treatments. ANOVA followed by a Tukey's studentized test revealed a significant difference between each treatment. Overall, gametophytes contained far more carbohydrates than lipids.

Gametophyte Culture	% Carbohydrate (n=98)	% Lipids (n=100)
Callus	**17.3±9.7**	**0.1±0.1**
WG	**23.5±15.2**	**0.7±0.5**
DG	**12.2±30.3**	**0.2±0.2**

gametophytes (WG). Protonemal callus contained 17.31% carbohydrates per total weight and this level rose to 23.53% upon maturity. In an arid environment, a strong internal carbohydrate concentration would allow gametophytes to maintain a strong internal osmotic gradient. The gametophytes can control whether or not water can flow through their plasma membrane aquaporins (Diamond, 2007), so that a gametophyte could draw water hygroscopically in a humid atmosphere. However, this steady increase occurred only as the gametophytes matured in a protected environment and this osmotic gradient was able to develop without depletion or risk of

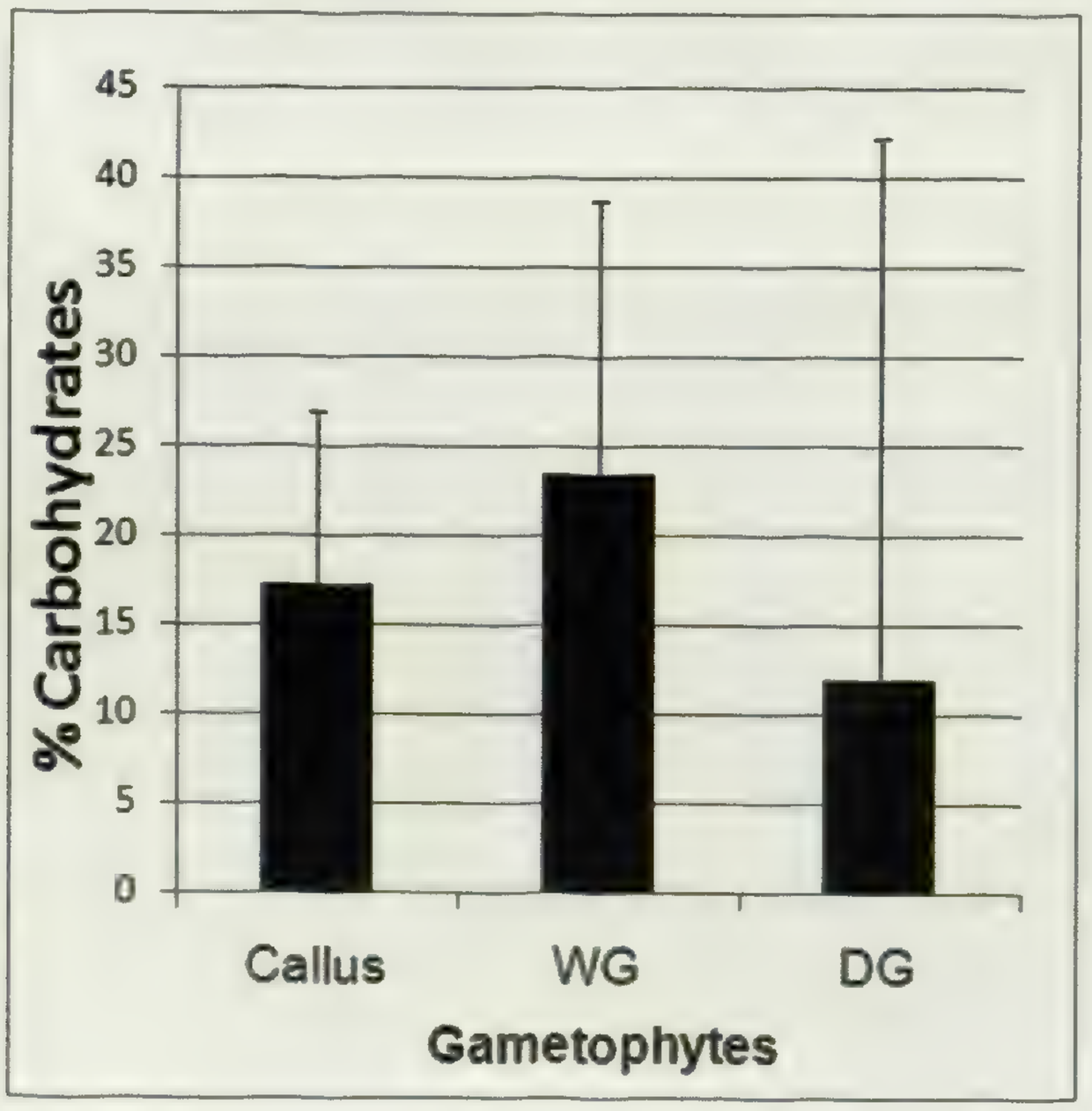

FIG. 3. Mean (n = 98) carbohydrates content per total weight in gametophytes of *Cheilanthes lanosa*. There was no statistical difference ($P < 0.05$) between callus and WG samples. There was also no significant difference between callus and DG samples. There was a statistical difference between the DG and WG samples. Solid = growth on wet agar-based nutrient medium. Stripe = growth on fine sand with erratic watering. WG = wet-grown gametophytes. DG = dry-grown gametophytes.

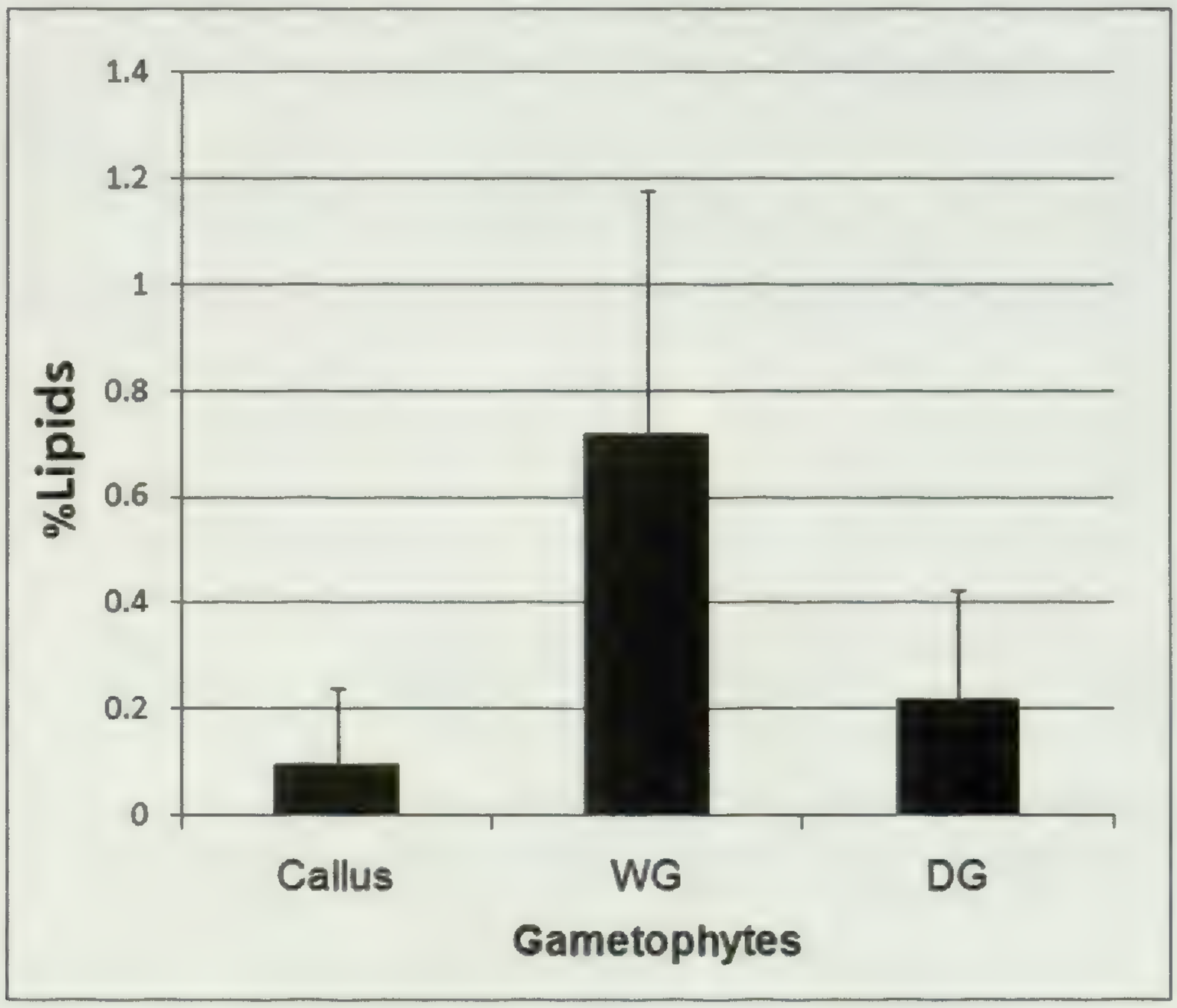

FIG. 4. Mean percent (n = 100) lipids per total volume weight of callus, wet grown (WG), and dry grown (DG) gametophytes. There was a statistical difference ($P < 0.05$) among all sample means. Solid = growth on wet agar-based nutrient medium. Stripe = growth on fine sand with erratic watering.

desiccation. The large standard deviation in these samples is likely due to the variations in maturity of gametophytes sampled as batches and not as individuals (which would have been very difficult). Thus, sample collection, in which each sample was a clutch of individuals grasped from one of a dozen plates with tweezers, and collected at a precise time and date, would naturally introduce variation. Still, the standard deviation was consistent between the WG and callus stages. This suggests a constant range of response within the wet agar environments.

DG gametophytes exhibited a much lower concentration of carbohydrates and lipids than expected. These gametophytes produce lipid-based exudates from trichome-like structures, and these structures appear to be similar in morphology to wax glands of related species which are also xerophytic (Diamond *et al.* 2003; Lingle *et al.* 2004). Though the trichome-like structures in hairy lip ferns have not been fully characterized microscopically, it is likely that they are also wax glands. Trichomes produced a halo of lipid-based exudates and this exudate appears to cover the entire gametophyte surface (Lingle *et al.*, 2004). Thus, a high concentration of lipids in the DG gametophytes was expected. In addition, other xerophytes can mobilize lipids to the cell periphery of vessel elements and thereby block transpiration (Schneider *et al.*, 2003). However, *Cheilanthes lanosa* gametophytes do not possess vascular tissue, but are single celled in thickness. Lipid localization

was beyond the scope of this study, but regardless of localization, all lipids would still be included in the total percentage of tissue mass. With respect to carbohydrate concentrations and because DG gametophytes are impervious to high solute perturbation (up to 500 mM NaCl for 1 hr; Diamond *et al.*, 2003), we predicted a high concentration of carbohydrates as well.

The lower concentration of lipids and carbohydrates found in the DG gametophytes in this experiment could be due to several factors. WG gametophytes and protonemal callus were grown in an environment with consistent water and nutrient availability, and their resources may have been committed to growth and development. Alternatively, DG gametophytes may have utilized their resources differently, perhaps for stress responses and secondary metabolic pathways. The high variation in the DG treatments is characteristic of a plant population exhibiting a high level of signaling activity and response in a stressful environment (Fitter and Hay, 1987; Taiz and Zeiger, 2006). Although WG gametophytes rarely complete their life cycle to sporophyte formation, but dry down with the agar and slowly produce wax glands, DG gametophytes consistently produce sporophytes in culture (Diamond and Swatzell, 2003). At the time the DG gametophytes were collected, they were poised to complete the haploid stage and begin sporophyte production through apogamy. Because sporophytes are nutritionally dependent on gametophytes, it is possible that the gametophytes invested their resources for cell division machinery and proteins (or for other cell components that were not measured in this study) instead of starch storage or cytoplasmic sugars. For example, Abney (2004) showed using HPLC that dry-grown gametophytes contained a higher soluble concentration of mono- and disaccharides than wet-grown gametophytes or protonemal callus. Mono- and disaccharides were immeasurable in protonemal callus (Abney, 2004). In addition, Diamond *et al.* (2003) showed that total osmotic potential in DG gametophytes is five times that of WG gametophytes. Taken together with the results of this study, this suggests that a portion of the osmotic gradient in DG gametophytes has still not been described.

Another explanation of the differences between the two types of mature gametophytes could be the differences between lipid and carbohydrate extraction methods and the nature of the cell wall and cuticle. In the anthrone assay (Judd, 2006; van Handel, 1985a), all cell components were liberated with H_2SO_4. However, in the vanillin assay (Judd, 2006; van Handel, 1985b), sample preparation involved a lipid extraction from the cell wall surfaces. Following extraction and solubilization, cell wall materials were pelleted and discarded. Lipids still bound to and within the cuticular layer of the cell wall, if a cuticle was actually present, would have been discarded. Thus, lipids in DG gametophytes that were committed to cuticle production would have been discarded and not measured in the assay. Still, there is no evidence yet whether the DG gametophytes actually produce cuticle, or whether they simply produce lipid substances via wax glands. Regardless of the basis for the differences between dry grown and wet agar-grown stages, it appears that the

environment is as much of a factor in lipid and carbohydrate production as is development.

Changes in Development Leave Protonema Vulnerable

Development may also be responsible for the establishment of a strong, internal osmotic gradient within gametophytes. Mature DG and WG gametophytes have higher concentrations of carbohydrates and lipids than the respective protonema. Gametophytes appear to be developmentally programmed to increase the concentration of these molecules as they mature. The high concentration of carbohydrates and lipids allows mature gametophytes to remain impervious to desiccation. The limiting stage for hairy lip fern gametophytes appears to be the protonematal stage. When a hairy lip fern spore germinates, the first few cells emerge as uniseriate, filamentous protonema (Raghavan, 1980, 1989). There appears to be no cuticle on these cells, which must grow and divide rapidly as the prothallus develops. Throughout the gametophyte stage, water uptake is controlled through aquaporin-like proteins; plants can potentially control aquaporin function based on a blue-light responsive phosphorylation switch (Johansson *et al.*, 1996; Johnson and Chrispeels, 1991; Kaldenhoff *et al.*, 1995). Thus, protonemata are able to close their aquaporins when stressed, but this does not make them impervious to desiccation (Diamond, 2007). Eventually, because the protonema has very few lipids and no lipid-producing trichomes, osmosis across the plasma membrane and cell wall will lead to desiccation. Conversely, if too much water is present, the water eventually moves by osmosis, in spite of aquaporin control, across membranes into protonemal cells in high amounts, compromising the protonema (Nondorf *et al.*, 2003; Diamond, 2007). Overall, data suggest that the high internal osmotic gradient that drives rapid water uptake through aquaporin-like proteins is also a factor limiting the protonemal stage. Consequently, they must live in an environment that contains the right amount of water (Dooley and Swatzell, 2002).

Hairy lip ferns are primarily found on sedimentary rocks, in which there is a small amount of water continuously present. Because the protonemata is limited by the lack of cuticle, a low lipid content, and a high carbohydrate osmotic gradient, it is also limited to environments that offer a constant, low level of moisture. The high internal osmotic gradient that drives rapid water uptake through aquaporin-like proteins for prothallus production is also the factor that limits the protonemal stage. These ferns are typically relegated to sedimentary substrate by the narrow conditions under which the protonema can live.

Conclusion

Overall, data from this study suggest that hairy lip ferns increase carbohydrates and lipids as a normal process of development. However, there is also a difference in lipid and carbohydrate content between mature

gametophytes on agar and on dry sand, and environmental factors also play a role. DG gametophytes, which consistently develop into sporophytes, contain fewer carbohydrates and lipids than those grown on wet agar. Interestingly, although the mature gametophytes are fairly impervious to desiccation because of their high internal osmotic gradients and lipid content, protonemata are vulnerable and limited. Because they contain a high internal osmotic gradient but lack external lipid protection, protonema can readily become hypotonic when exposed to the water required for quick prothallus development. Therefore, although they enjoy a large range throughout the eastern United States, hairy lip fern gametophytes are typically limited to a narrow niche of sedimentary rock.

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Literature Cited

Abney, T. S. 2004. Analytical Service Report. Complex Carbohydrate Research Center, University of Georgia, GA, USA.

Al-Karaki, G. N. 1998. Benefit, cost and water-use efficiency of arbuscular mycorrhizal durum wheat grown under drought stress. Mycorrhiza 8:41–45.

Barnes, H. and J. Blackstock. 1973. Estimation of lipids in marine animals and tissues: Detailed investigation of the sulphophosphovanillin method for 'total' lipids. Journal of Experimental Marine Biology and Ecology 12:103–118.

Cobb, B., E. Farnsworth and C. Lowe. 2005. *Ferns of Northeastern and Central North America, 2nd ed.* New England Wild Flower Society, New York. 417 pp.

Devi, S., B. K. Nayar and I. W. Knobloch. 1971. Spore morphology of some American species of *Cheilanthes* and *Notholaena*. Grana 11:27–35.

Diamond, H. L. and Swatzell, L. J.. 2003. Cultivation of *Cheilanthes* species with focus on *Cheilanthes feei* and *Cheilanthes lanosa*. Available online at http://amerfernsoc.org/pages/swatzell/index.html. Accessed October 30, 2007.

Diamond, H. L., H. R. Jones, J. Jones and L. J. Swatzell. 2003. Water balance in the xerophytic fern *Cheilanthes lanosa*. Midwest Section for American Society of Plant Biologists, Ames, IA. March.

Diamond, H. L. 2007. *Water balance in* Cheilanthes lanosa *gametophytes*. Masters Thesis. Southeast Missouri State University, Cape Girardeau, MO. 65 pp.

Dooley, M. A. and L. J. Swatzell. 2002. Porosity and specific retention of Eminence-Potosi Dolomite Limestone from *Cheilanthes feei* habitat in southeast Missouri. Botanical Society of America, Madison, WI. August.

Fitter, A. H. and R. K. M. Hay. 1987. Environmental Physiology of Plants. *2nd Edition*. Academic Press Limited, London. 423 pp.

Gratani, L., M. F. Crescente and G. Rossi. 1998. Photosynthetic performance and water use efficiency of the fern *Cheilanthes persica*. Photosynthetica 35(4):507–516.

Judd, T. M. 2006. Relationship between food stores and foraging behavior of *Pheidole ceres* (Hymenoptera: Formicidae). Annals of the Entomological Society of America 99(2):398–406.

HARLEY, J. L. and S. E. SMITH. 1983. *Mycorrhizal Symbiosis*. Academic Press, New York. 483 pp.

HEVLY, R. H. 1963. Adaptations of cheilanthoid ferns to desert environments. Journal of the Arizona Academy of Science 2:164–175.

JOHNSON, K. and M. CHRISPEELS. 1991. Tonoplast-bound protein kinase phosphorylates tonoplast intrinsic protein. Plant Physiology 100:1787–1795.

JOHANSSON, I., C. LARSSON, B. EK and P. KJELLBOM. 1996. The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. The Plant Cell 8(7):1181–1191.

KALDENHOFF, R., A. KŠLLING, J. MEYERS, U. KARMANN, G. RUPPEL and G. RICHTER. 1995. The blue light-responsive AthH2 gene of *Arabidopsis thaliana* is primarily expressed in expanding as well as in differentiating cells and encodes a putative channel protein of the plasmalemma. Plant Journal 7:87–95.

LINGLE, M. A., H. L. DIAMOND and L. J. SWATZELL. 2004. Trichomes of *Cheilanthes lanosa* gametophytes. Student Research Conference, Southeast Missouri State University, Cape Girardeau, MO. April.

MICKEL, J. T. 1979. *How to Know the Ferns and Fern Allies*. WCB McGraw-Hill, St. Louis, MO. 229 pp.

NONDORF, S. L., M. A. DOOLEY, M. PALMIERI and L. J. SWATZELL. 2003. The effects of pH, temperature, light intensity, light quality, and moisture levels on spore germination in *Cheilanthes feei* of Southeast Missouri. American Fern Journal 93(2):55–68.

PALMIERI, M. and L. J. SWATZELL. 2004. Mycorrhizal fungi associated with the fern *Cheilanthes lanosa* in southeast Missouri and southern Illinois. Northeastern Naturalist 11(1):57–66.

PICKETT, F. L. 1931. Notes on xerophytic ferns. American Fern Journal 21(2):49–57.

QUIRK, H. and T. C. CHAMBERS. 1981. Drought tolerance in *Cheilanthes* with special reference to the gametophyte. Fern Gazette 12(3):121–129.

RAGHAVAN, V. 1980. *Cytology, Physiology, and Biochemistry of Germination of Fern Spores. International Review of Cytology, Vol 62*. Academic Press, Inc. pp 69–118.

RAGHAVAN, V. 1989. *Developmental Biology of Fern Gametophytes*. Cambridge University Press, Cambridge, MA. 361 pp.

RAVEN, P. H., R. F. EVERT and S. E. EICHHORN. 2005. *The Biology of Plants, Ed 6*. WH Freeman and Company, New York, NY. 686 pp.

RICKS, D. C. and L. J. SWATZELL. 2006. Changes in *Cheilanthes lanosa* PIP1 aquaporin expression between spore germination and mature gametophyte development. American Society of Plant Biologists, Boston. August.

SCHNEIDER, H., B. MANZ, M. WESTHOFF, S. MIMIETZ, M. SZIMTENINGS, T. NEUBERGER, C. FABER, G. KROHNE, A. HAASE, F. VOLKE and U. ZIMMERMANN. 2003. The impact of lipid distribution, composition and mobility on xylem water refilling of the resurrection plant *Myrothamnus flabellifolia*. New Phytologist 159:487–505.

SMITH, R. H. 1992. Plant Tissue Culture: Techniques and Experiments. Academic Press, Inc, San Diego, CA. 231 pp.

STEIL, W. N. 1939. Apogamy, apospory and parthenogenesis in the pteridophytes. The Botanical Review 8:433–453.

STEIL, W. N. 1933. New cases of apogamy in certain homosporous leptosporangiate ferns. Botanical Gazette 95:164–167.

TAIZ, L. and E. ZEIGER. 2006. Plant Physiology. *4th Edition*. Sinauer Associates, Inc., Sunderland, Massachusetts. 650 pp.

VAN HANDEL, E. 1985a. Rapid determination of glycogen and sugars in mosquitoes. Journal of the American Mosquito Control Association 1(3):299–300.

VAN HANDEL, E. 1985b. Rapid determination of total lipids in mosquitoes. Journal of the American Mosquito Control Association 1(3):302–304.

WHITTIER, D. 1965. Obligate apogamy in *Cheilanthes tomentosa* and *C. alabamensis*. Botanical Gazette 126(4):275–281.

YATSKIEVYCH, G. 1999. *Steyermark's Flora of Missouri*. Missouri Department of Conservation with Missouri Botanical Gardens, Jefferson City, MO. 991 pp.

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Intraspecific Variation in Four Distinct Populations of *Anemia villosa* Humb. & Bonpl. ex Willd. (Anemiaceae) Occurring in Rio de Janeiro, Brazil

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Abstract.—Leaf samples of *Anemia villosa* (Anemiaceae) were collected in four distinct places in Rio de Janeiro State, Brazil. Two coastal land populations are located in Itacoatiara and Imbuhy rocky outcrops, municipality of Niterói, and two populations in the inland mountain region of the state, Pedra Dubois, municipality of Santa Maria Madalena, and in Lumiar, municipality of Nova Friburgo. Five leaves of each one of five randomly chosen individuals were collected in each site. The four populations analyzed were very similar in anatomic qualitative characteristics. ANOVA results for the quantitative variables showed significant differences between all populations. The PCA separated the populations analyzed in two groups: one formed by the coastal land and other formed by the inland mountain.

Key Words.—Intraspecific variation, *Anemia*, fern, leaf anatomy

The diversity of plant communities that comprise the Brazilian Atlantic rain forest complex has prompted a number of studies on functional variation of species that are widespread in various habitats within this complex. All of these studies have indicated a large degree of intraspecific variation between distinct populations at different habitats, regarding both anatomical and physiological traits (Scarano *et al.*, 2002; Scarano *et al.*, 2005; Mantuano *et al.*, 2006).

Anatomical and morphological characters may serve as reliable indicators in the study and understanding of ecological adaptations of living organisms (Fahn, 1964). Environmental conditions, such as light intensity and nutrient availability, can influence the size and shape of leaves as well as anatomical aspects. For example, in high light habitats, leaves of many plants tend to be smaller and more deeply incised than under shadier conditions (MacLellan, 2000). In addition, there are other well known shifts in leaf traits such as

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cuticle thickness, epidermal cell size, stomatal frequency and length, and trichome frequency and length that are often related to environmental conditions (Olson and Carlquist, 2000; Rôças *et al.*, 2001; Hlwatika and Bhat, 2002).

Studies on fern morphology and anatomy are common (Kraus *et al.*, 1993, Graçano *et al.*, 2001; Chaerle and Viane, 2004; Pita *et al.*, 2006a, b), however studies on intraspecific variation in such traits in ferns are not usual (Liu *et al.*, 2006; Boeger *et al.* 2007). According to Mickel (1962), in the last hundred years, *Anemia* has been an explored subject for morphological studies that have been largely concerned to the taxonomy. Little work, other than that of Ribeiro *et al.* (2007), has been done on understanding how environmental conditions influence morphology in the genus *Anemia*.

Anemia villosa Humb. & Bonpl. ex Willd. is widely distributed in eastern Brazil, from Santa Catarina to Ceará, and also in northern South America from Peru to Surinam. In Rio de Janeiro State, large populations are commonly associated with vegetation islands on rocky outcrops (Santos and Sylvestre, 2006). According to Mickel (1962) this species has a considerable variation in size and form that can lead to confusion with other species. In Rio de Janeiro State, *A. villosa* populations exhibit two different specimen sizes. The aim of this study was to analyze anatomical variation and environmental parameters in *A. villosa* growing at four different sites in Rio de Janeiro State, Brazil to generate a better understanding of the degree of plasticity exhibited by this species across a variety of habitats.

Material and Methods

Leaf samples of *Anemia villosa* were collected in four distinct sites in Rio de Janeiro State, Southeastern Brazil: Pedra de Itacoatiara (Serra da Tiririca State Park) and Forte Imbuhy, on the coast of Niterói; Pedra Dubois at municipality of Santa Maria Madalena and Lumiar, municipality of Nova Friburgo. All populations grew in inselbergs, except for Lumiar, which was collected at the border of the Atlantic rain forest (Table 1). The two populations in municipality of Niterói are approximately 14 km apart from each other.

In each collection site, five plants were randomly chosen, except for the site of Pedra Dubois, where only three plants were selected. From each plant, five leaves were collected and immediately fixed in formalin-acetic acid-alcohol 70% (FAA_{70}) (Berlyn and Miksche, 1976). Fragments of each last pinna (0.5 cm^2) were gradually dehydrated in ethanol and embedded in glycol methacrylate resin (Feder and O'Brien, 1968). Five micrometer thick transverse sections were made with a rotary microtome, and were subsequently stained with 0.1% toluidine blue O (O'Brien and McCully, 1981). The tracheary elements were observed in material dissociated using the Franklin method (1945) and stained by hydroalcoholic safranin (Johansen, 1940). Identification of vascular bundle types followed Ogura (1972) and stomata Sen and De (1992).

TABLE 1. Climatic aspects of the four sites from where the samples of *Anemia villosa* Humb. & Bonpl. ex. Willd were collected (Rio de Janeiro-Brazil).

	Pedra Dubois	Lumiar	Forte Imbuhy	Pedra de Itacoatiara
Municipality	Santa Maria Madalena	Nova Friburgo	Niterói	Niterói
Sites	Inselberg	Atlantic Rain Forest (bordering)	Inselberg	Inselberg
Latitude e Longitude	21°45′S 41°41′W	22°21′S 42°27′W	22°56′S 43°07′W	22°58′S 43°01′W
Altitude	1090 m	1700m	50m	200m
Mean Temperature	20°C	18°C	23°C	23°C
Mean annual rainfall	1440mm	2205mm	1207mm	1207mm

Anatomical observations and measurements were made using an Olympus BX50 light microscope with the aid of Image-Pro Plus version 4 software. The images were acquired by a video camera Cool SNAP-Pro.

The following leaf anatomical parameters were measured for each sample: thickness of leaf blade, mesophyll, adaxial and abaxial epidermis; trichome length and density; stomata length and density; and lignified petiole area. Means and standard deviations were calculated from a sample size of 25 fields for each measurement (N=25). A nested ANOVA was used to study the association between the outcome variable and group. Principal Component Analysis (PCA) was used to find the higher variance components. To test the hypothesis that the analyzed sample was composed of morphologically different discrete groups, a Discriminant Function Analysis (DFA) was performed (Schlichting, 1986; Zar, 1996). Overall differences between the compared groups are presented by Mahalanobis distance. The statistical analyses were performed with Statistica v. 6.0 (Zar, 1996).

RESULTS

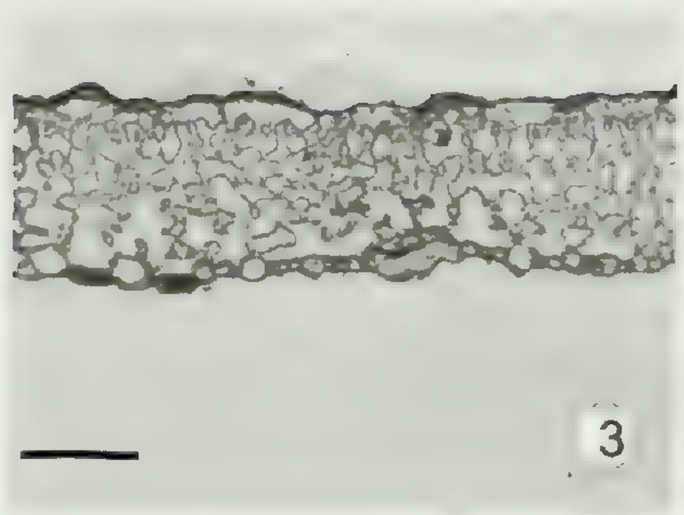
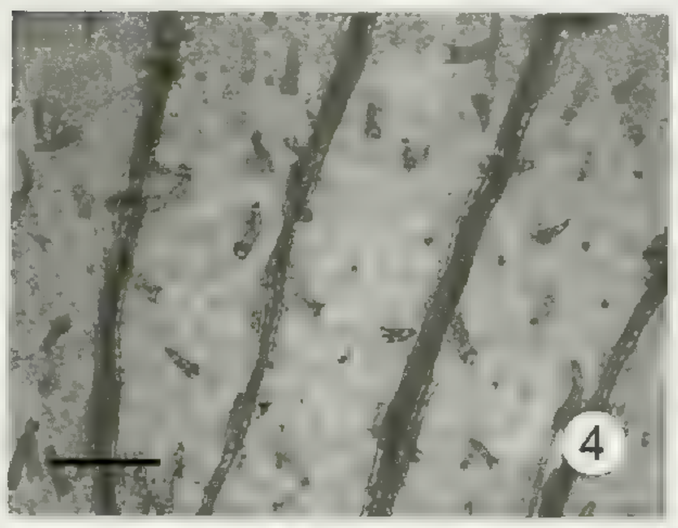
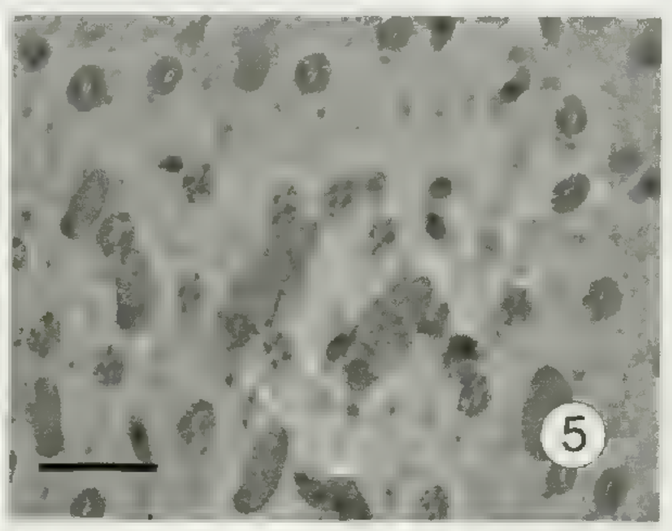
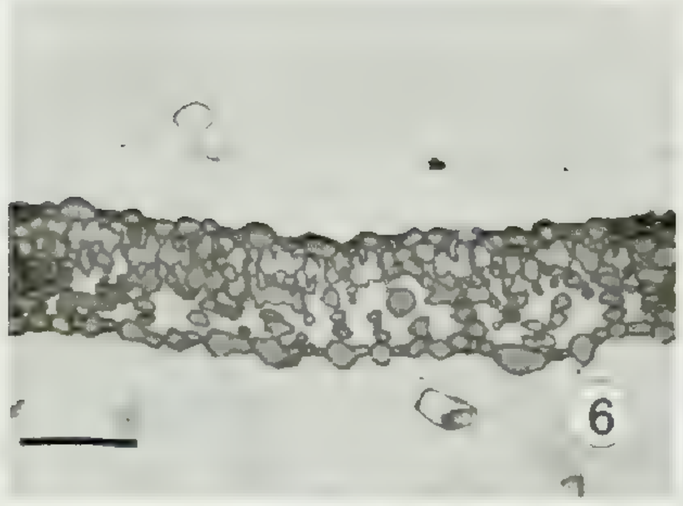
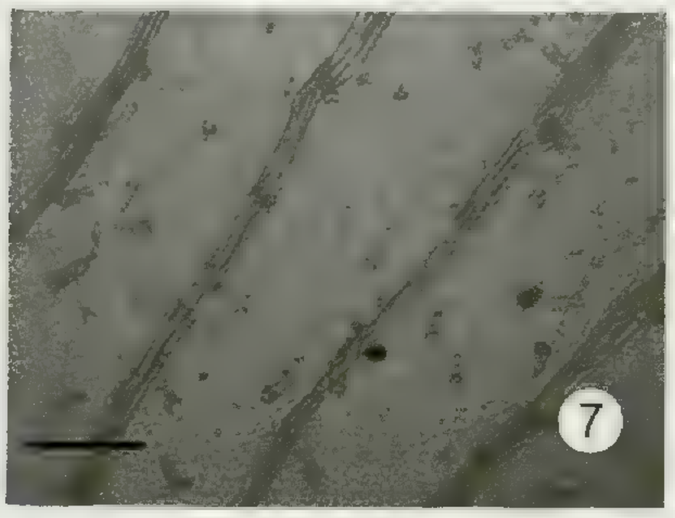
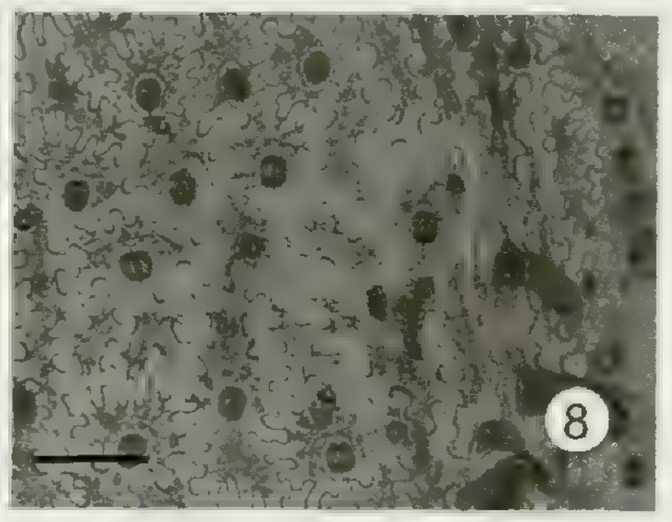
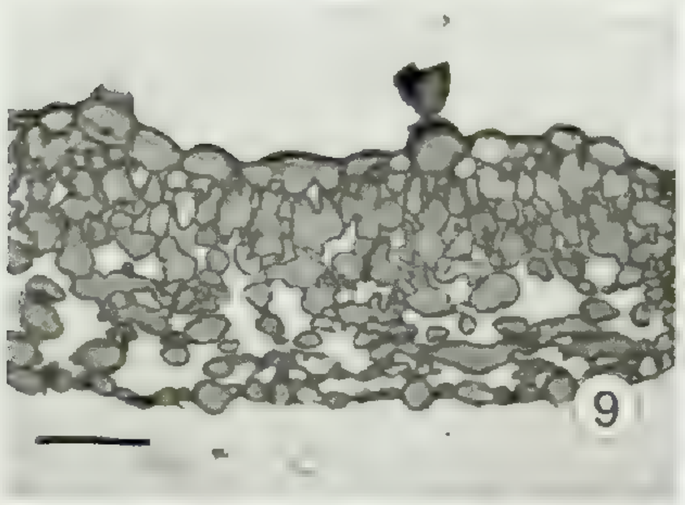
As described by Mikel (1962), different populations of *Anemia villosa* show differences in leaf morphology. The specimens from Lumiar and Pedra Dubois exhibit the typical oblong leaf blade with fertile pinnae close to the sterile pinnae, and the specimens from Niterói have triangular leaf blade with fertile pinnae that vary in their localization from remote to close to the sterile pinnae (Fig. 1).

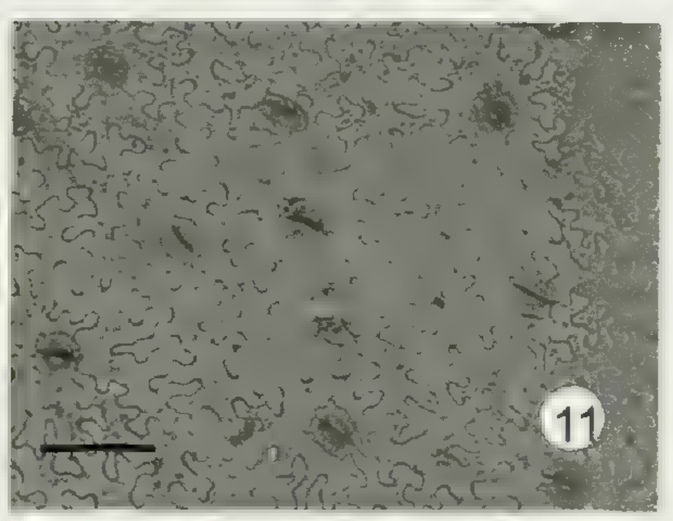
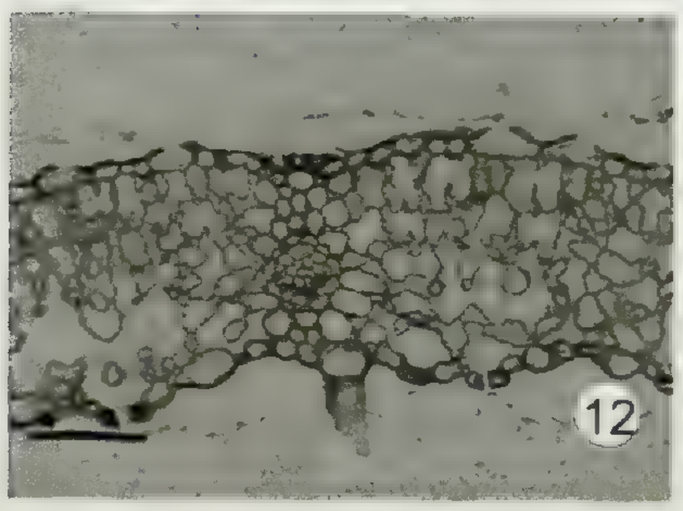
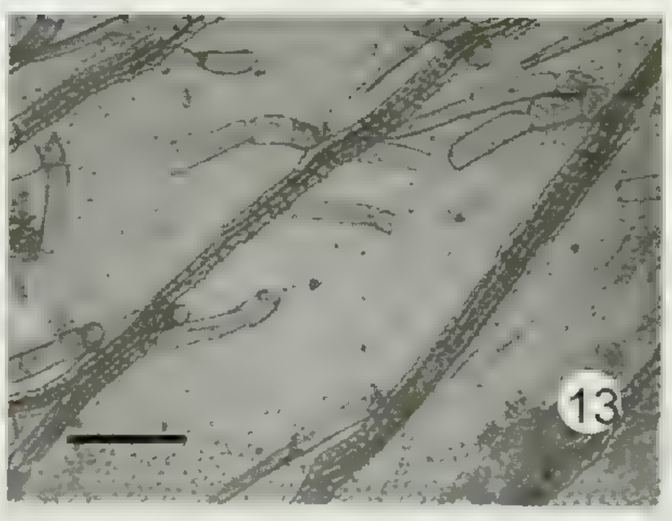
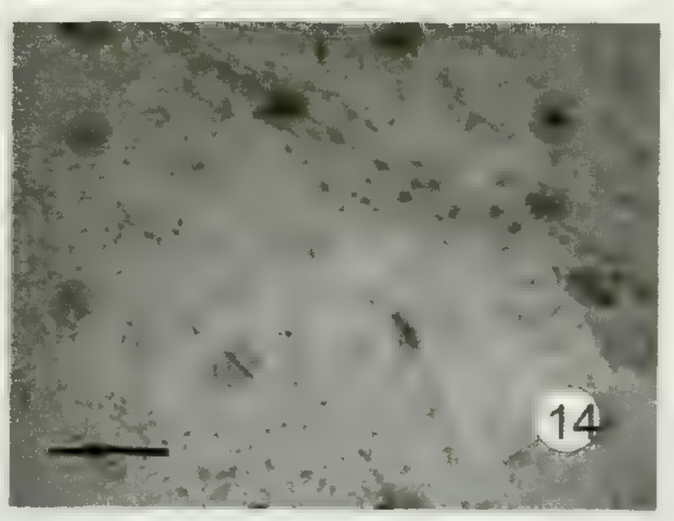
The four populations analyzed are similar in qualitative characters such as type of stomata, trichomes, and stele. The petiole has a uniseriated epidermis with lignified walls along with uniseriated trichomes. The stomata are parallel in the adaxial epidermis, making up two lines, and are supported above the common epidermal cells by cells with lignified walls. Below the epidermis there are four to five layers of sclerenchyma followed by parenchyma with some amyloplasts. The stele is V-shaped and the endodermis is clearly visible and shows evident Casparian strips; two layers of pericycle were observed.

FIG. 1. Specimens of *Anemia villosa* Humb. & Bonpl. ex Willd. from Pedra de Itacoatiara (a) and Pedra Dubois (b).

The xylem showed incurved margins and consists of scalariform tracheids and parenchyma cells. The sieve elements were observed almost completely surrounding the xylem; the phloem consists of sieve cells and parenchyma (Fig. 2).

FIG. 2. Basic petiole structure of four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil. Bar = 350 mm.

FIGS. 3–14. Sections of leaves of four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil. 3–5. Leaf of Itacoatira populations. 3) Cross section of leaf blades. 4) Adaxial epidermis surface. 5) Abaxial epidermis surface. 6–8. Leaf of Imbuhy populations. 6) Cross sections of leaf blades. 7) Adaxial epidermis surface. 8) Abaxial epidermis surface. 9–11. Leaf of Lumiar populations. 9) Cross sections of leaf blades. 10) Adaxial epidermis surface. 11) Abaxial epidermis surface. 12–14. Leaf of Pedra Dubois populations. 12) Cross sections of leaf blades. 13) Adaxial epidermis surface. 14) Abaxial epidermis surface. Bars = 10 μm in Figs. 3, 5, 6, 8, 9, 11, 12, 14; 200 μm in Figs. 4, 7, 10, 13.

All populations have hypostomatic leaves. The epidermis is comprised of one layer of cells with sinuous anticlinal walls and convex periclinal walls, in both abaxial and adaxial surfaces. The stomata are desmocytic and polocytic, and the guard cells of the stomata protrude a little from the surface of the epidermis. Pluricellular trichomes are present in both adaxial and abaxial surfaces. The mesophyll shows 3–4 layers of arm-cells, which are more compactly arranged near the adaxial epidermis (Figs. 3–14).

TABLE 2. Leaf anatomical differences of ecological populations of *Anemia villosa* Humb. & Bonpl. ex Willd (Mean ± standard deviation) and F values (NESTED-GLM).

	Pedra Dubois	Lumiar	Forte Imbuí	Pedra de Itacoatiara	F
Epidermis thickness of adaxial surface (µm)	21.28±6.3a	23.59±4.55a	21.34±4.83a	21.11±4.8a	8.19
Epidermis thickness of abaxial surface (µm)	23.95±5.85a	21.15±5.22ab	21.32±5.03ab	20.23±5.26b	14.07
Trichomes length of adaxial surface (µm)	512.12±84.98a	464.25±101.28a	191.50±53.52b	127.19±44.34b	446.77
Trichomes length of abaxial surface (µm)	507.28±92.80a	513.10±99.56a	149.09±40.73b	128.28±41.33b	25.81
Trichomes density of adaxial surface (mm)	6.57±1.73a	10.28±0.97b	18.63±2.4c	17.42±2.4d	520.51
Trichomes density of abaxial surface (mm)	13.57±3.63a	17.08±2.15a	56.96±5.18b	57.64±5.82c	121.95
Length of stomata (µm)	53.08±4.52a	52.99±6.01a	39.15±4.32b	41.48±3.95b	50.06
Density of stomata (mm)	47.83±3.56a	49.08±2.66a	72.65±7.22b	50.48±8.2c	296.10
Leaf thickness (µm)	207.88±16.61a	239.49±39.2b	159.38±21.71c	181.90±15.68d	187.84
Mesophyll thickness (µm)	155.53±11.61a	184.75±40.76b	113.15±17.65c	136.89±12.96d	170.72

The results of the nested ANOVA were significant among sites (F =115.67) and significant among individuals (F=5.66). Nested ANOVA results for the quantitative variables among groups are presented in Table 2. All populations were similar for one out of 10 variables (epidermis thickness of adaxial surface). All populations were different in three variables (trichome density on adaxial surface, leaf thickness and mesophyll thickness). The inland mountain (Pedra Dubois and Lumiar) and coastal land (Pedra de Itacoatiara and Forte Imbuhy) populations were statistically different in three variables (trichomes lengh of adaxial surface, trichomes length of abaxial surface and stomata length).

Factor loadings and eigenvalues for the first two components (PCs1 and 2) extracted in the PCA are shown in Table 3. These accounted for 57.71% of the total variance. Anatomical characters such as trichome density of abaxial surface, uniseriate trichome length of adaxial surface, uniseriate trichome length of abaxial surface, and trichome density of adaxial surface showed the highest (either positive or negative) correlations with PC1, and epidermis thickness of adaxial and abaxial surface showed the highest correlations with PC2. Figure 15 shows that PCA separated the populations analyzed in two groups: one formed by the coastal land populations of Imbuhy and Itacoatiara, and other formed by the inland mountain populations of Parque Estadual do Pedra Dubois and Lumiar.

Factor structure for the 10 variables and eigenvalues for the first two factors (DF1 and 2) extracted in the DFA are shown in Table 3. DF1 was positively

TABLE 3. Factor loadings in PCA and Factor structure coefficients in DFA for the variables of *Anemia villosa* Humb. & Bonpl. ex Willd.

Variables	PC1	PC2	DF1	DF2
Stomata length	−0.761	0.131	0.162	−0.070
Stomata density	0.597	−0.349	−0.184	0.855
Leaf thickness	−0.811	−0.301	0.191	0.053
Trichome density on adaxial surface	0.846	−0.197	−0.341	−0.074
Trichome density on abaxial surface	0.948	−0.827	−0.593	−0.293
Epidermis thickness on adaxial surface	−0.163	−0.696	0.014	0.0115
Epidermis thickness on abaxial surface	−0.169	0.548	0.021	−0.097
Unisseriate trichome length on adaxial surface	−0.881	0.019	0.272	0.367
Unisseriate trichome length on abaxial surface	−0.945	−0.067	0.354	0.278
Lignified area in petiole	−0.852	−0.094	0.190	0.094
Eigenvalues	6.34	1.12	2.84	1.53

correlated with trichome lengh of abaxial surface and negatively correlated with trichome density of abaxial surface, whereas DF2 was positively correlated with stomata density. DFA classification was 98.27% for individuals from groups I, II and III (Fig. 16).

DISCUSSION

In this study all populations of *A. villosa* showed similar qualitative characters but variation was demonstrated in the quantitative characters. This quantitative variation occurred between specimens at different latitudes, temperatures and altitudes in the Rio de Janeiro State showing two distinct groups: the coastal land populations and the inland mountain populations. These two groups were described by Mickel (1962) in Rio de Janeiro state as specimens with oblong leaf blades and fertile pinnae approximate to the sterile pinnae (inland mountain population) and those with broader leaf blades with fertile pinnae varying in their origin from remote to approximate to the sterile pinnae (coastal land populations).

Other studies have verified quantitative variation in populations of angiosperm species that occur in different habitats or micro-habitats (e.g., Rôças *et al.*, 1997, 2001; Hlwatika and Bhat, 2002; Mantuano *et al.*, 2006). The anatomical differences in *Anemia villosa* are evident in traits such as trichome density of adaxial and abaxial surfaces, leaf thickness and mesophyll thickness between populations and in trichome length of adaxial and abaxial surfaces and stomata length between the inland mountain and coastal land populations.

The inland mountain populations, submitted to lower temperatures and higher humidity, presented thicker leaves with longer stomata and trichomes, and low stomata frequency when compared to coastal land populations. The difference in thickness of the leaf and mesophyll was expressed in length but

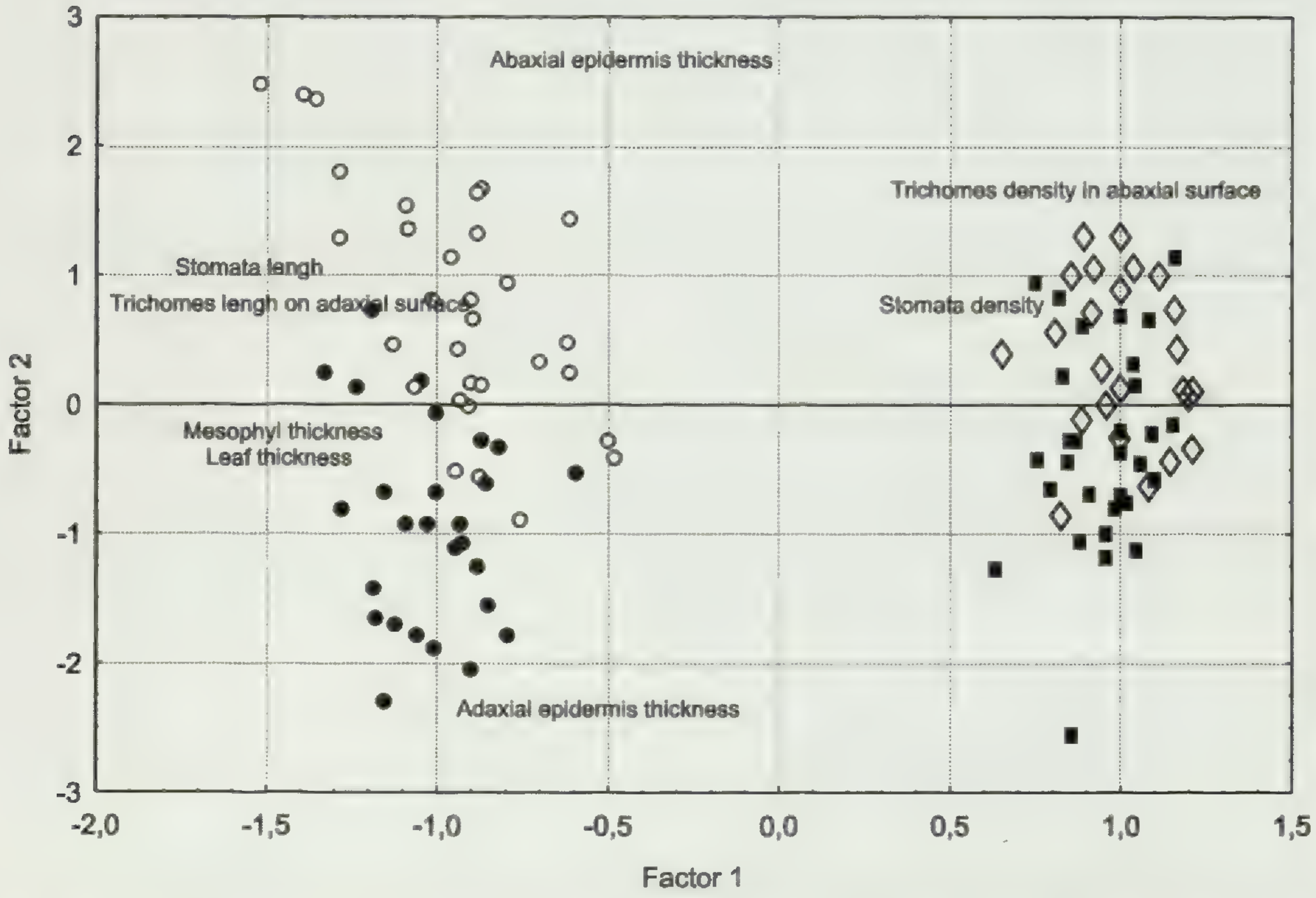


FIG. 15. Principal Components Analysis of four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil.

not in number of cell layers. According to some studies (Woodward, 1979; Willians and Black, 1993; Körner, 1999; Loeys *et al.*, 2002) biomass allocation is temperature sensitive, and exposure to low temperatures results in plants that exhibit reduced investment in the shoot and leaves that are thicker than their warm-growth counterparts. Differences in stomata frequency were also observed by Hlwatika and Bhat (2002) studying *Rapanea melanophloes* (L.) Mez and *Cunonia capensis* L. in distinct ecological sites. These authors suggest that the higher stomatal frequencies in sclerophyllous vegetation may be a reaction to the favorable photosynthetic conditions.

Rôças *et al.* (1997, 2001), studying intraspecific variation in *Alchornea triplinervia* (Spreng.) Müll. Arg., Mantuano *et al.* (2006) verifying intraspecific variation in *Erythroxylum ovalifolium* Peyr., and Pereira *et al.* (2009) studying variation of *Andira legalis* (Vell.) Toledo leaves, observed differences in epidermis thickness between the populations and related this to changes in light regimes. In *A. villosa* all populations analyzed are fully exposed to sunlight, which may explain why there is no difference in their epidermis thickness.

Although intraspecific variation in ferns has been poorly explored, it is crucial to understand the distribution of this group. According to Liu *et al.* (2006), the leaf anatomy of *Isoetes* shows less environmentally induced variation than the external leaf morphology. In this present study, however, it

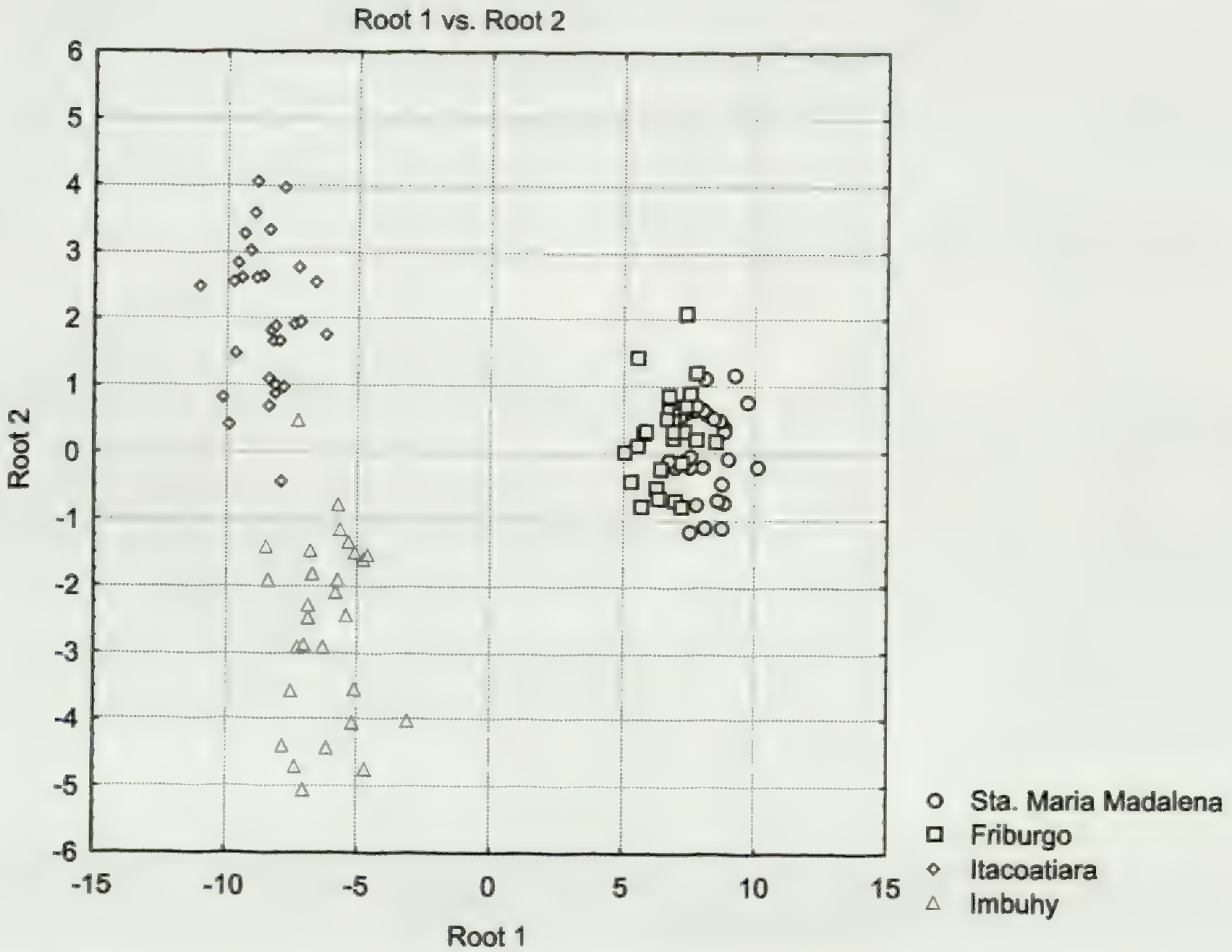


FIG. 16. Discriminant Function Analysis in four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil.

was verified that under different environmental conditions *Anemia villosa* populations showed both anatomical and morphological modifications.

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LITERATURE CITED

BERLYN, G. P. and J. P. MIKSCHE. 1976. *Botanical microtechique and cytochemistry*. Iowa State University Press, Ames.

BOEGER, M. R. T., L. E. CAVICHIOLO, M. W. PIL and P. H. LABIAK. 2007. Variabilidade fenotípica de *Rumohra adiantiformis* (G. Forst) Ching (Dryopteridaceae). Hoehnea 34(4):553–561.

CHAERLE, P. and R. L. L. VIANE. 2004. Leaf anatomy and the occurrence of false veins in *Asplenium* (Aspleniceae, Pteridophyta). Bot. J. Linn. Soc. 145:187–194.

FAHN, A. 1964. Some anatomical adaptations of desert plants. Phytomorphology 14:93–102.

FEDER, N. and T. P. O'BRIEN. 1968. Plant microtechnique: some principles and new methods. Amer. J Bot. 55(1):123–142.

FRANKLIN, G. L. 1945. Preparation of thin sections of synthetic resin and wood-resin composites, and a new macerating method for wood. Nature 155(3924):51.

GRAÇANO, D., A. A. AZEVEDO and J. PRADO. 2001. Anatomia foliar das espécies de Pteridaceae do Parque Estadual do Rio Doce (PERD) – MG. Revista Brasileira de Botânica 24:333–347.

HLWALTIKA, C. N. M. and R. B. BHAT. 2002. An ecological interpretation of the difference in leaf anatomy and its plasticity in contrasting tree species in Orange Kloof, Table Mountain, South Africa. Ann. Bot. 89(1):109–114.

JOHANSEN, D. 1940. *Plant microtechnique*. McGraw-Hill Book Company, Inc., New York-London.

KORNER, C. 1999. *Alpine plant life: functional plant ecology of high mountain ecosystems*. Springer-Verlog, Berlin.

KRAUS, J. E., G. MONTENEGRO and A. J. KIM. 1993. Morphological studies on entomogenous stem galls of *Microgramma squamulosa* (Kauf.) Sota (polypodiaceae). Amer. Fern J. 83(4):120–128.

LIU, H., Q. WANG and W. TAYLOR. 2006. Morphological and anatomical variation in sporophylls of *Isoetes sinensis* Palmer (Isoteaceae), an endangered quillwort in China. Amer. Fern J. 96(3):67–74.

LOEYS, B. R., I. SCHEWATER, I. PONS, A. FITTER and C. K. ATKIN. 2002. Growth temperature influences the underlying components of relative growth rate: an investigation using inherently fast- and slow- growing plant species. Plant, cell & environment 25:975–988.

MACLELLAN, T. 2000. Geographic variation and plasticity of leaf shape and size in *Begonia dregei* and *B. homonyma* (Begoniaceae). Bot. J. Linn. Soc. 132:79–95.

MANTUANO, D. G., C. F. BARROS and F. R. SCARANO. 2006. Leaf anatomy within and between three "restinga" populations of *Erythroxylum ovalifolium* Peyer. (Erythroxylaceae) in Southeast Brazil. Revista Brasil. Bot. 29:209–215.

MICKEL, J. T. 1962. A monographic study of the fern genus *Anemia*, subgenus *Coptophyllum*. Iowa State Coll. J. Sci. 36(4):349–482.

OGURA, Y. 1972. *Comparative anatomy of vegetative organs of the pteridophytes*. Gebruder Borntraeger, Berlin.

OLSON, M. E. and S. CARLQUIST. 2000. Stem and root anatomical correlations with life form diversity, ecology and systematics in *Moringa* (Moringaceae). Bot. J. Linn. Soc. 135:315–348.

O'BRIEN, T. P. and M. E. MCCULLY. 1981. *The study of plant structure: principles and select methods*. Ed. Termacarphi Pty. Ltda, Melbourne.

PEREIRA, D. C., C. F. BARROS and F. R. SCARANO. 2009. In situ variation in leaf anatomy and morphology of *Andira legalis* (Leguminosae) in two neighboring but contrasting light environments in a Brazilian sandy coastal plain. Acta bot. bras. 23:267–273.

PITA, P. B., N. L. MENEZES and J. PRADO. 2006a. External and internal morphology of the vegetative leaves, sporophylls and sporangia of *Huperzia* Bernh. species (Lycopodiaceae - Pteridophyta) from Brazil. Revista Brasil. Bot. 29(1):115–131.

PITA, P. B., N. L. MENEZES and J. PRADO. 2006b. Morfologia externa e interna de raiz e caule de espécies de *Huperzia* Bernh. (Lycopodiaceae - Pteridophyta) do Brasil. Hoehnea 33(4):495–510.

RIBEIRO, M. L. R. C., M. G. SANTOS and M. G. MORAES. 2007. Leaf anatomy of two *Anemia* Sw. species (Schizaeaceae-Pteridophyte) from a rocky outcrop in Niterói, Rio de Janeiro, Brazil. Revista Brasileira de Botânica 30(4):695–702.

ROÇAS, G., C. F. BARROS and F. R. SCARANO. 1997. Leaf anatomy plasticity of *Alchornea triplinervia* (Euphorbiaceae) under distinct light regimes in a Brazilian montane Atlantic rain forest. Trees 11(8):469–473.

ROÇAS, G., C. F. BARROS and F. R. SCARANO. 2001. Leaf anatomical variation in *Alchornea triplinervia* (Spreng) Müll. Arg. (Euphorbiaceae) under distinct light and soil water regimes. Bot. J. Linn. Soc. 136:231–238.

SANTOS, M. G. and L. SYLVESTRE. 2006. Aspectos florísticos e econômicos das pteridófitas de um afloramento rochoso do estado do Rio de Janeiro, Brasil. Acta Bot. Bras. 20:115–124.

SCARANO, F. R., H. M. DUARTE, G. RÔÇAS, S. M. B. BARRETO, E. F. AMADO, F. REINERT, T. WENDT, A. MANTOVANI, H. R. P. LIMA and C. F. BARROS. 2002. Acclimation or stress symptom? An integrated study of intraspecific variation in the clonal plant *Aechmea bromelifolia*, a widespread CAM tank-bromeliad. Bot. J. Linn. Soc. 140:391–401.

SCARANO, F. R., H. M. DUARTE, A. C. FRANCO, A. GESSLER, E. A. MATTOS, M. NAHN, H. RENNENBERG, H. L. T. ZALUAR and U. LUTTGE. 2005. Ecophysiology of selected tree species in different plant communities at the periphery of the Atlantic Forest of SE-Brazil.I. Behavior of three different species of *Clusia* in an array of plant communities. Trees 19:497–509.

SCHLICHTING, C. D. 1986. The evolution of phenotypic plasticity in plants. Annu. Rev. Ecol. Evol. Syst. 17:667–693.

SEN, U. and B. DE. 1992. Structure and ontogeny of stomata in ferns. Blumea 37:239–261.

WILLIANS, D. G. and R. A. BLACK. 1993. Phenotypic variation in contrasting temperature environments-growth and photosynthesis in *Pennisetum setaceum* from different altitudes on Hawaii. Funct. Eco. 7:623–633.

WOODWARD, F. I. 1979. The differential temperature responses of the growth of certain plant species from different altitudes. II Analyses of the control and morphology of leaf extensions and specific leaf area of *Phleum bertolonii* D.C. and *P. alpinum* L. New Phyt 82:397–405.

ZAR, J. H. 1996. Biostatistical analysis 3 ed. Prentice Hall, New Jersey.

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Distributional Patterns and Biogeographic Analysis of Ferns in the Sierra Madre Oriental, Mexico

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Abstract.—We analyzed the distributional patterns of 66 species of leptosporangiate ferns inhabiting in the Sierra Madre Oriental (SMO), Mexico, using grid-cells and endemism index values, parsimony analysis of endemicity (PAE) and track analysis. The strict consensus area cladogram obtained with PAE showed a polytomy and four main groups or clades, two of them including only two or three grid-cells. The largest group included grid-cells located along the SMO, mainly in the central-southern portions of the SMO; a second group was located at the southern portion of the SMO in the leeward zone of this mountain chain. The track analysis of the fern taxa allowed us to recognize five generalized tracks in the SMO and their convergence showed five nodes. With endemism index values, 11 grid-cells were identified as important areas for ferns, seven of them corresponding to the group 1 of PAE (from montane environments), and four grid-cells to the group 2 of PAE (from leeward areas of the Sierra Madre Oriental). The results obtained herein slightly agree with other published works using other kinds of organisms. The analyses used herein serve to generate useful information about the biogeographic history of this complex area, and led us to support, detect, test and propose areas, important from biogeographic viewpoint, for ferns.

Key Words.—biogeography, track analysis, parsimony analysis of endemicity, richness, Mexico, leptosporangiate ferns, Sierra Madre Oriental

Ferns are seedless vascular plants distributed worldwide with great species diversity, especially in tropical regions. Mexico represents one of the countries with a very diverse fern flora (Tryon, 1972; Tryon and Tryon, 1984; Mickel and Smith, 2004). According to Hassler and Swale (2001), there is a higher diversity of ferns in South America, with 3,281 species (2,271 endemic to the region), especially in Colombia, Ecuador, Brazil, Venezuela, and Peru. Central America is the second most diverse region, with 2,620 species (616 endemic to the region), mainly in Costa Rica, Panama, and Mexico. Mexico is one of the countries with a high proportion of endemic fern species (including 196 taxa). Mickel and Smith (2004) noted that 113 genera and 877 species of leptosporangiate ferns inhabit Mexico, of which nearly 2% of the genera and 17% of the species are endemic. In Mexico, these plants inhabit a broad range of habitats (Mickel and Smith, 2004) and are distributed mainly in tropical

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montane and temperate vegetation types, but also live in dry habitats (Siqueiros and González, 2006).

Ferns and lycopods are important throughout the forest strata, mainly in the humid mountain zone, because they play an important role in the hydric balance of the forest (Ambrose, 2004); they also play a crucial role in vegetation structure (Hill and Silander Jr., 2001; Paciencia and Prado, 2005). Additionally, ferns, especially in the gametophytic phase, are considered good indicators of environmental changes in the forest due to their sensitivity to the microclimatic and edaphic parameters (Page, 1979a, b).

From a biogeographical viewpoint, ferns have been considered interesting plants (Barrington, 1993; Kato, 1993; Wolf *et al.*, 2001) due to processes involved in their distributional patterns and because different explanations have been used to interpret their geographical distribution, mainly dispersal and vicariance. Early studies explained their distribution based on ecophysiological and reproductive traits; all attributed fern distribution mechanisms to dispersal (i.e., Lyell, 1870; Christ, 1910; Winkler, 1938; Tryon, 1970, 1972, 1985; Smith, 1972; Puentha, 1991; Barrington, 1993). Recently, Karst *et al.*, (2005) demonstrated the role of environmental variables in determining fern distribution at mesoscales, such as soil moisture and humid climatic conditions. According to Wolf *et al.* (2001), despite the fact that many ferns produce bisexual spores capable of travelling long distances, chances of establishing new populations are low; in this way, evidence of historical processes such as allopatric differentiation may sometimes be hidden by dispersal.

The Sierra Madre Oriental (SMO) physiographic and biogeographic province is a mountain chain located in the northeastern part of Mexico (Fig. 1), and from a biological viewpoint is one of the most important mountain systems in Mexico (González-Zamora *et al.*, 2007). The SMO province comprises parts of the following states: Coahuila, Nuevo León, Tamaulipas, Durango, Zacatecas, San Luis Potosí, Veracruz, Guanajuato, Querétaro, Hidalgo, and Puebla (Cervantes-Zamora *et al.*, 1990). This province extends to the east to the Gulf of Mexico, in the south to the Trans-Mexican Volcanic Belt, in the west it extends to the Mexican Plateau, and in the north to the northern part of the state of Coahuila, western part of the state of Nuevo León and to the southern part of the United States. The wetter slopes of this mountain chain are considered to be one of the places in the country where fern species diversity and abundance are concentrated (Mickel and Smith, 2004), and ferns constitute a frequent floristic component in temperate forests of the SMO. For many years, the SMO has been recognized as a biogeographic natural region by several authors based on different criteria, but its high biological richness has complicated its natural delimitation (Luna-Vega *et al.*, 2004). Many authors consider it as a natural unique area and other authors as an archipelago of areas (Luna-Vega *et al.*, 2004).

The Mesozoic rocks and the basement complex that constitute the SMO were uplifted, shortened and transported northeastward forming a fold and thrust belt during the Laramide orogeny (Eguiluz de Antuñano *et al.*, 2000). Also, this

FIG. 1. Study area. The gray area shows the location and limits of the Sierra Madre Oriental (SMO) in Mexico. Abbreviations are: Coah. = Coahuila, Dgo. = Durango, Gto. = Guanajuato, Hgo. = Hidalgo, N.L. = Nuevo León, Pue. = Puebla, Qro. = Querétaro, S.L.P. = San Luis Potosí, Tamps. = Tamaulipas, Ver. = Veracruz, Zac. = Zacatecas.

mountain chain may represent a generally non-extended backarc to the continental arc of the Sierra Madre Occidental (Ortega-Gutiérrez *et al.*, 1994). The climate in this province varies and most of the main climatic types of the country can be found in the region. Climatic diversity is largely due to its complex physiographic heterogeneity and meteorological phenomena, among others factors (Hernández-Cerda and Carrasco-Anaya, 2004).

Different vegetation types occur in the SMO province, but oak and cloud forests are characteristic, with suitable abiotic conditions for the development of ferns. Although the SMO is a montane area with a prevalence of temperate forests, dry habitats are represented in several places, mainly in the inland lowlands near the Mexican Plateau. In the Gulf of Mexico area and southeastern portions of the SMO, precipitation is greater than 1,200 mm, reaching 4,000 mm in the state of Puebla, with only two dry months in the year. Maximum annual temperature in the region ranges from 26° to 30°C, with the exception of the highest areas, and minimum annual temperature is less than 12 C (Hernández-Cerda and Carrasco-Anaya, 2004).

Several studies have been carried out in this region, including some biogeographic analyses with different taxa, including vascular plants (González-Zamora *et al.*, 2007; Luna-Vega *et al.*, 1999, 2000; Santa Anna del Conde *et al.*, 2009), macromycetes (Cifuentes *et al.*, 2004), birds (Navarro *et al.*, 2004), mammals (León-Paniagua *et al.*, 2004), herpetofauna (Canseco-Márquez *et al.*, 2004), and beetles (Márquez and Morrone, 2004). In these studies, the SMO province was divided into two or three subregions, and congruent distributional patterns among organisms with different dispersal capabilities were noted. If the SMO is divided into three sections, it is separated by two geographical features: the Pánuco basin, which is a natural barrier that divides the southern part from the central section, and a second feature, which is comprised of the Saltillo-Monterrey mountain system, consisting of faults and deformations located between the states of Nuevo León and Coahuila, which divide the central part from the northern section (González-Zamora *et al.*, 2007; Santa Anna del Conde *et al.*, 2009). If the SMO is divided into two sections, the Pánuco basin is a barrier that divides this province into two parts (Luna-Vega *et al.*, 1999; Márquez and Morrone, 2004).

In comparison with angiosperms, fern species have been used in few biogeographic works that analyze the distributional patterns of the Mexican biota; these studies took place in the Mexican states of México (Tejero-Díez, 1990) and Veracruz (Palacios-Ríos and Gómez-Pompa, 1997), but the whole Sierra Madre Oriental province was not included in either of these works. Despite showing wider distributional patterns than seed plants, some ferns also exhibit the restricted patterns of distribution shown by seed plants, and it seems that the same factors appear to have shaped these shared patterns. Considering that ferns generally have high dispersal capabilities and wide distribution ranges, in this study we compare the distributions of some species restricted or semi-restricted to the Sierra Madre Oriental to test if their patterns of distribution are congruent with other groups of organisms. This work represents the first biogeographical study in the SMO province based on fern distribution, applying three different historical biogeographic methods.

Our aim was to analyze the distributional patterns and to detect areas of richness and endemism in the Sierra Madre Oriental province based on semi-restricted species of leptosporangiate ferns, applying endemism indices to grid-cells, and biogeographic analyses applying a parsimony analysis of endemicity and the panbiogeographic method. These analyses should generate useful information on the distributional patterns of ferns in this region, and assist in detecting and proposing areas that are important from a biogeographic viewpoint for these vascular plants in northeastern Mexico.

Materials and Methods

Distributional Data.—We used 66 species of leptosporangiate ferns belonging to the Polypodiales (Smith *et al.*, 2006), which is one of the major groups of Monilophytes (sensu Pryer *et al.*, 2004). These 66 fern species belong to nine different families and are included in 24 genera. Species selected for this study

have distributional areas mainly within the SMO or with areas that extended slightly beyond those boundaries into adjacent areas, such as the Mexican Plateau, Trans-Mexican Volcanic Belt, southern Mexico, and some southern areas of the United States of America and northern Central America. In this study we used the delimitation proposal of the SMO produced by González-Zamora *et al.* (2007) as a framework. The selected species were taxonomically validated based on Mickel and Smith (2004) and do not have synonymy problems.

Distributional data were obtained from the review of more than 900 herbarium specimens deposited in the following collections: National Herbarium of the Instituto de Biología, UNAM (MEXU); Herbarium of the Escuela Nacional de Ciencias Biológicas, IPN (ENCB); Herbaria of the Instituto de Ecología A.C. in Pátzcuaro (IEB) and Xalapa (XAL); and Herbarium of the Facultad de Ciencias, UNAM (FCME). We also obtained some records from the Red Mundial de Información Biótica (REMIB) hosted on the web page of the Comisión Nacional para el Uso y Conocimiento de la Biodiversidad (CONABIO) (http//www.conabio.com.mx). With this information, we constructed a database that includes 1,244 georeferenciated records.

At least 400 fern species inhabit the SMO, most of them widely distributed in Mexico/or reaching adjacent countries in Central America. Fern species were selected based on the combination of the following criteria: (1) species endemic to the SMO, recorded in one or several grid-cells; (2) species endemic to Mexico, and well-represented in the SMO (e.g., *Astrolepis crassifolia*, *A. laevis*); those species well-distributed in continental Mexico, including the SMO, and also represented in one or both peninsulas (Baja California and Yucatán) were excluded, because they have a different biogeographic history from the rest of the country (Contreras-Medina *et al.*, 2007); (3) species represented in Mexico, well-distributed in the SMO, but also present in adjacent countries, considering those species distributed in only one or two states of the United States and one or two countries of Central America, based on the proposal of Megamexico (Rzedowski, 1991), because Mexico by itself is not a natural unit. Species with a wider distribution elsewhere but only with one record within the SMO were not considered (e.g. *Trichomanes bucinatum*), because they can indicate a false endemism to one grid-cell.

Biogeographic Analyses.—From distributional data, geographic distribution maps of each species were obtained using ArcView GIS (ESRI, 1999) and then these distribution maps were projected on a map of the Sierra Madre Oriental proposed by González-Zamora *et al.* (2007). We divided the Sierra Madre Oriental province in 34 grid-cells of 1° latitude × 1° longitude, which were used as area units in the different analyses. These 34 grid-cells contain almost one recorded species. We chose grid-cells of one geographical degree per side, partially to facilitate the data manipulation and to reduce the effect of sampling artefacts, such as mapping errors and unsampled grids in sparsely inhabited areas (Crisp *et al.*, 2001). This scale size was also chosen because it was tested in previous studies on areography and diversity of different groups of Mexican flora (Kohlmann and Sánchez, 1984; García-Mendoza, 1995;

Contreras-Medina and Luna-Vega, 2007; Santa Anna del Conde *et al.*, 2009) and fauna (Escalante *et al.*, 2004; Ochoa and Flores-Villela, 2006).

In the grid-cell analysis we counted the number of species recorded in each grid-cell (richness), and obtained the mean and median values; also we noted those species recorded in more grid-cells.

Parsimony analysis of endemicity (PAE) was developed by Rosen (1988) and Rosen and Smith (1988) to address the shortcomings of phenetic approaches used to assess area relationships of fossil or recent assemblages from different areas (Porzecanski and Cracraft, 2005). This method begins with presence/absence data for a set of sample localities and a particular taxon (Rosen and Smith, 1988). With this information, a data matrix of areas versus taxa is constructed and analysed (Luna-Vega *et al.*, 1999). Shared presences group areas according to the most parsimonious cladogram, which represents nested sets of areas (Morrone and Crisci, 1995). Compared to cladistic biogeography, PAE can be applied to taxa whose phylogenetic relationships are unknown.

In Mexico, PAE has been applied to different biological groups and using different geographic units, such as hydrological basins (Aguilar-Aguilar *et al.*, 2003), grid-cells (Morrone and Escalante, 2002; Rojas-Soto *et al.*, 2003; Méndez-Larios *et al.*, 2005; Contreras-Medina *et al.*, 2007; Santa Anna del Conde *et al.*, 2009), biogeographic provinces (Morrone *et al.*, 1999; Morrone and Escalante, 2002; Dávila-Aranda *et al.*, 2002; Contreras-Medina *et al.*, 2007), and transects (García-Trejo and Navarro, 2004; León-Paniagua *et al.*, 2004).

We used 59 taxa (columns) and 34 grid-cells (rows) to construct the data matrix of the PAE method. In this method, those species found in a single grid-cell are not useful for assessing relationships, and thus were not included in the matrix, because these taxa are equivalent to autapomorphies (Luna-Vega *et al.*, 1999). The matrix included one row coded with all zeros to root the area cladogram. The data matrix analysis was carried out using Nona (Goloboff, 1999) through Winclada (Nixon, 2002), applying multiple TBR, searching on 100,000 initial trees (mult*100), and holding 30 trees per replication (h/10). When more than one parsimonious cladogram resulted from the analysis, a strict consensus cladogram was constructed.

The panbiogeographic approach was originally proposed by Croizat (1958, 1964). The method consists of plotting the localities of organisms on maps and connecting their disjunct distribution areas or localities together with lines called individual tracks. Individual tracks of organisms are then superimposed and if they coincide, the resulting summary lines are considered generalized tracks. Generalized tracks indicate the pre-existence of ancestral biota, which subsequently become fragmented by tectonic and/or climatic changes (Morrone and Crisci, 1995). A baseline is a geological feature such as a river basin crossed by the track. Convergence or intersection of two or more generalized tracks produces a node, which represents a complex and composite geological area (Morrone and Crisci, 1995).

For track analysis (Craw *et al.*, 1999), we used the collection localities of each species, represented by points on the maps of each taxon, and then we drew individual tracks (not shown). For each taxon, the localities within a

track were joined by the line of minimum distance between each point (a minimum spanning tree). All the individual track maps were compared and superimposed to define several generalized tracks, using the software 'Trazos' (Rojas, 2004); this is an extension of ArcView GIS (ESRI, 1999) that works under the concept of minimum spanning tree, under Prim algorithm. To run the program, it is necessary to capture the longitude and latitude coordinates of all the collection localities, in order to draw the individual tracks.

Baselines were then identified, which are any geographic, climatic or geological features that spatially match a generalized track. In this work, these baselines were identified on a regional intra-continental scale from geographic, climatic and physiographic data of the SMO. On a regional scale, baselines are more difficult to identify (Contreras-Medina, 2006), because disjunct distributions of organisms are less evident than transoceanic disjunctions (González-Zamora *et al.*, 2007). In the areas of convergence or intersection among two or more generalized tracks, we recognized nodes, and these were compared with previous panbiogeographic studies that include the SMO (Escalante, 2003; Álvarez and Morrone, 2004; Márquez and Morrone, 2004; Morrone and Gutiérrez, 2005; González-Zamora *et al.*, 2007).

In order to evaluate endemism, we used the corrected weighted endemism index proposed and applied by Crisp *et al.* (2001) and Linder (2001) to Australian and African floras respectively. In Mexico, this index has been applied to the geographic distribution of seed plants such as Ternstroemiaceae (Luna-Vega *et al.*, 2004), gymnosperms (Contreras-Medina and Luna-Vega, 2007), and Cactaceae (Santa Anna del Conde *et al.*, 2009). We applied this index to grid-cells, in order to detect endemism centers of ferns. Species richness also known as 'unweighted species richness' was measured as the total count of species within each grid-cell (Linder, 2001). A first index termed 'weighted endemism' was calculated and comprised several steps (Crisp *et al.*, 2001). The first step consisted of dividing each grid-occurrence by the total number of grids in which one species occurs. Thus, a fern species restricted to a single grid scored '1' for that grid, and '0' for all other grids, whereas a fern species found in 10 grids was scored as '0.1' for each of the ten grids, and '0' for all remaining grids; then the sum of all score species values for each grid was obtained. A second index named 'corrected weighted endemism' (Crisp *et al.*, 2001), consisted in dividing the values of weighted endemism index (WE) by the total number of species in each grid cell. Those grid-cells with the highest scores are considered important biogeographic areas for the biological group under study. Grid-cells with only one species recorded were not considered for the analysis of corrected weighted endemism (CWE), because these cells do not include overlapping distributions. Those areas composed by sets of neighboring grid-cells or isolated grid-cells with high values in CWE index represent centers of endemism. Each species was scored as present in a grid-cell independently of whether it was recorded once or numerous times in that grid-cell (Linder, 2001). Because the WE index has been considered as a measure sensitive to diversity (Linder, 2001; Santa Anna del Conde *et al.*, 2009), we decided to work only with the CWE index, which is not significantly

correlated with grid diversity. Our study is interested in endemism values more than total richness, so values obtained on richness are based only in those species almost restricted to the SMO province.

Unfortunately Crisp *et al.* (2001) and Linder (2001) do not present a reference parameter to recognize those important areas in the application of CWE index; for this reason, some proposals have been developed such as the use of mean value (Contreras-Medina and Luna-Vega, 2007; Santa Anna del Conde *et al.*, 2009) or an Olmstead-Tukey test (Aguilar-Aguilar *et al.*, 2008). In this study, we evaluated the relationship between richness and endemism with an Olmstead-Tukey corner test of association (Steel and Torrie, 1980); this test was previously successful applied for this index in Mexico (Aguilar-Aguilar *et al.*, 2008). This test produces a graph in which each grid-cell is placed in one of four quadrants, where richness and CWE values are plotted. Extreme values are the best indicators of an association between variables and this test gives them special weight (Steel and Torrie, 1980). Grid-cells located in the upper-right quadrant were identified as the most important biogeographic areas for ferns in northeastern Mexico.

Results

The Sierra Madre Oriental province was divided in 34 grid-cells (Fig. 2), all of them including at least one record. For this study, we included 1244 occurrence records of 66 fern species, included in nine families and 24 genera (Table 1). The three fern species represented in most grid-cells were *Notholaena aschenborniana* in 19 grid-cells, and *Astrolepis crassifolia* and *Cheilanthes aemula* both recorded in 16 grid-cells. Other well-represented taxa were *Anemia mexicana*, *Argyrochosma microphylla* and *Llavea cordifolia*, recorded all of them in more than 10 grid-cells. Richness in the 34 grid-cells was in the range of 1 to 34 species per grid-cell (Table 2), with a mean value = 9.37 species.

Parsimony Analysis of Endemicity.—The analysis of the grid-cell matrix (available upon request) produced 38 most parsimonious cladograms of 179 steps, consistency index of 0.32 and retention index of 0.53. The strict consensus cladogram (Fig. 3) with 212 steps, consistency index of 0.27, and retention index of 0.40, showed a polytomy composed of 17 grid-cells, and four main groups or clades.

Panbiogeographic Analysis.—Distribution analysis of 66 fern species showed that seven species (*Asplenium dianae, A. semipinnatum, Cheilanthes apiacea, C. chipinquensis, Notholaena brachycaulis, N. leonina,* and *Pellaea ribae*) have a very restricted distribution within the Sierra Madre Oriental (endemic to one grid-cell), and thus were not included in the track analysis.

The congruence of the individual tracks of the fern taxa allow for the recognition of five generalized tracks in the SMO (Fig. 4). The individual tracks of *Cheilanthes mexicana, Elaphoglossum viridae, Pellaea notabilis, Polystichum ordinatum, Polypodium subpetiolatum* and *Woodsia mexicana* were not considered because they do not belong to any of the generalized

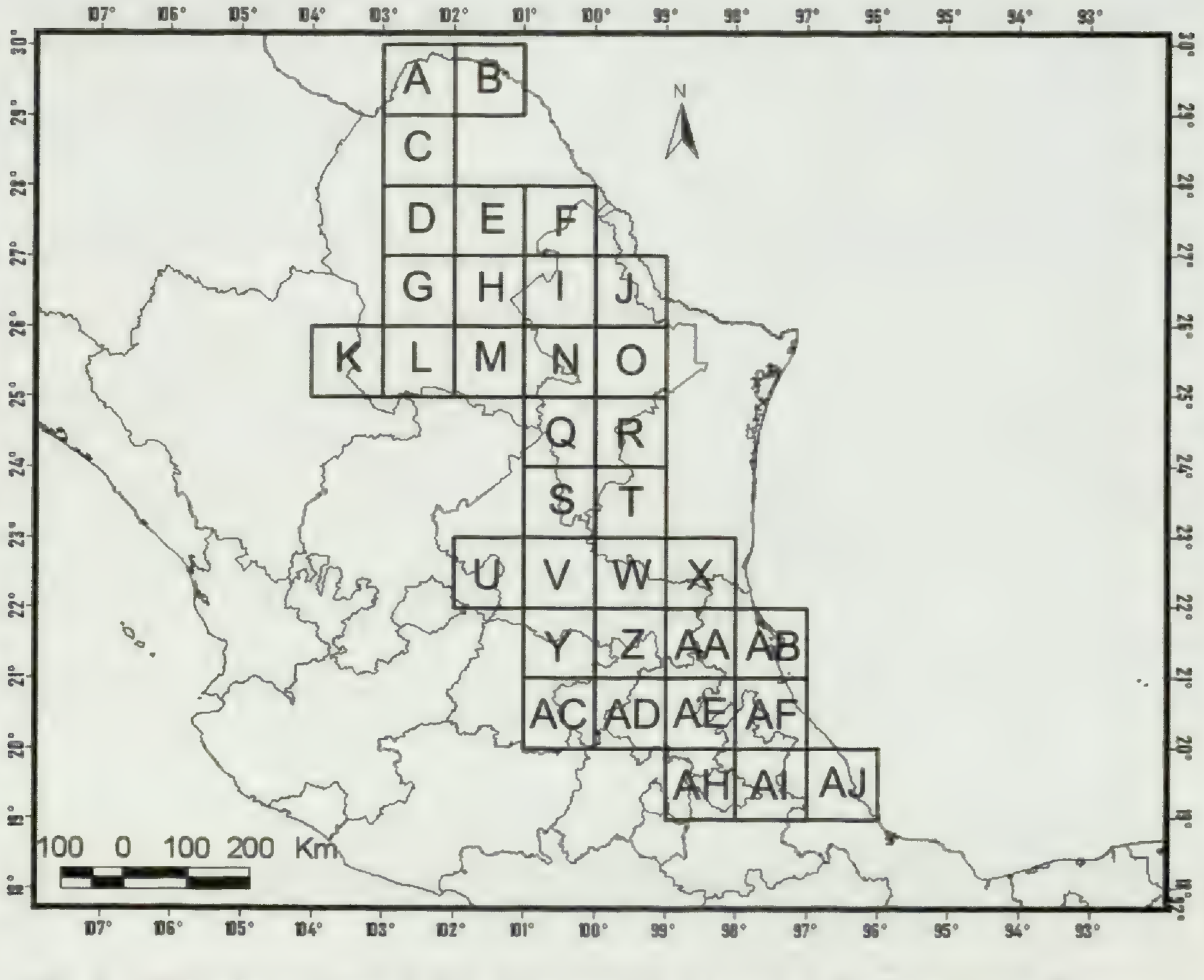

FIG. 2. Grid-cells employed in this study overlaid on a map of the Sierra Madre Oriental.

tracks found. Two generalized tracks are located in the northern part of the Sierra, including the states of Coahuila and Nuevo León, but one of them crosses the Pánuco river basin towards the Sierra Gorda area in the state of Querétaro. A third generalized track is located in the southern portion of the SMO and crosses the Pánuco river basin, and the last two are located in the southern part of this basin. All of these generalized tracks are connected among themselves.

The generalized tracks detected, the number of taxa that support them, and the areas involved are as follows:

(1) Southeastern generalized track (Fig. 4a). This track runs from the limit of the SMO along the Trans-Mexican Volcanic Belt through the central portion of the SMO and crosses the Pánuco river basin, and ends in the boundary between the states of Nuevo León and Tamaulipas. It includes the individual tracks of 20 species (Table 1). This is the largest track and is congruent with tracks obtained in the studies of Álvarez and Morrone (2004) with birds and González-Zamora *et al.* (2007) with Asteraceae.

(2) Northeastern generalized track (Fig. 4b). This track begins in the northern portion of the state of Coahuila and runs throughout the south and ends in the Sierra Gorda area in the state of Querétaro. It consists of the individual tracks of 15 species (Table 1). This track is supported by the tracks obtained by the work of Morrone and Gutiérrez (2005) with fleas.

(3) Southern generalized track (Fig. 4c). This track runs from the Pánuco river basin through the western limit of the SMO and connects with the Trans-Mexican Volcanic Belt. Includes the

TABLE 1. Selected fern species following the classification of Smith *et al.* (2006), species that support each generalized track, and species that belong to each group of the PAE.

Species	Generalized tracks	Groups
Aspleniaceae		
Asplenium dianae A.R. Sm.		
Asplenium semipinnatum (Hieron.) A.R.Sm.		
Asplenium soleirolioides A.R. Sm.	a	
Holodictyum ghiesbreghtii (E. Fourn.) Maxon	a	
Blechnaceae		
Woodwardia martinezii Maxon ex Weath.	a	2
Dryopteridaceae		
Ctenitis mexicana A.R. Sm.	e	
Dryopteris cinnamomea (Cav.) C.Chr.	a	
Dryopteris pseudofilix-mas (Fée) Rothm.	e	
Elaphoglossum obscurum (E. Fourn.) C. Chr.	a	2
Elaphoglossum vestitum (Schltdl. et Cham.) Schott ex T. Moore	a	2
Elaphoglossum viride (E. Fourn.) C. Chr.		
Elaphoglossum potosianum Christ	b	1
Phanerophlebia gastonyi Yatsk.	a	
Phanerophlebia nobilis (Schltdl. et Cham.) C. Presl	e	
Phanerophlebia remotispora E. Fourn.	b	2
Phanerophlebia umbonata Underw.	b	
Polystichum ordinatum (Kunze) Liebm.		2
Grammitidaceae		
Melpomene leptostoma (Fée) A.R. Sm. et R.C. Moran	a	
Polypodiaceae		
Polypodium arcanum Maxon	a	
Polypodium conterminans Liebm.	e	
Polypodium eatonii Baker	a	2
Polypodium liebmannii C. Chr.	c	
Polypodium longepinnulatum E. Fourn.	a	2
Polypodium madrense J. Sm.	c	
Polypodium martensii Mett.	a	
Polypodium rhodopleuron Kunze	e	2
Polypodium subpetiolatum Hook.		1
Polypodium villagranii Copel.	a	
Pteridaceae		
Argyrochosma delicatula (Maxon et Weath.) Windham	b	
Argyrochosma formosa (Liebm.) Windham	a	1
Argyrochosma microphylla (Mett. ex Kuhn) Windham	b	
Argyrochosma pallens (Weath. ex R.M. Tryon) Windham	c	
Aspidotis meifolia D.C. Eaton	b	1
Astrolepis crassifolia (T. Moore et Houlston) D.M. Benham et Windham	c	
Astrolepis laevis (M. Martens ET Galeotti) Mickel	b	
Bommeria ehrenbergiana (Klotzsch) Underw.	b	
Cheilanthes aemula Maxon	a	
Cheilanthes apiacea Mickel		
Cheilanthes chipinquensis Knobloch et Lellinger		
Cheilanthes cucullans Fée	a	

TABLE 1. Continued.

Species	Generalized tracks	Groups
Cheilanthes hintoniorum Mendenh. et Nesom	b	1
Cheilanthes horridula Maxon	d	
Cheilanthes leucopoda Link	b	
Cheilanthes mexicana Davenp.		
Cheilanthes purpusii T. Reeves	c	1
Cheiloplecton rigidum (Sw.) Fée	b	
Llavea cordifolia Lag.	a	
Mildella fallax (M. Martens et Galeotti) Nesom	a	
Notholaena affinis (Mett.) Hook. ex T. Moore	c	1
Notholaena aschenborniana Klotzsch	e	
Notholaena brachycaulis Mickel		
Notholaena bryopoda Maxon	d	
Notholaena copelandii C.C. Hall	b	
Notholaena galeottii Fée	e	
Notholaena jacalensis Pray	e	
Notholaena leonina Maxon		
Notholaena neglecta Maxon	b	
Notholaena rigida Davenp.	b	
Pecluma sursumcurrens (Copel.) M. G. Price	a	
Pellaea cordifolia (Sessé et Moç.) A.R. Sm.	c	
Pellaea notabilis Maxon		1
Pellaea ribae Mendoza et Windham		
Pellaea villosa (Windham) Windham et Yatsk.	e	
Schizaeaceae		
Anemia mexicana Klotzsch	b	
Thelypteridaceae		
Thelypteris schaffneri (Fée) C. F. Reed	a	
Woodsiaceae		
Woodsia mexicana Fée		

individual tracks of seven species (Table 1). This track is supported by the work of González-Zamora *et al.* (2007) with Asteraceae.

(4) Northern generalized track (Fig. 4d). This track is located at the northern part of the SMO and runs throughout the central part of Coahuila and Nuevo León. Only two species are included in this track (Table 1). This track is congruent with tracks obtained by González-Zamora *et al.* (2007) with Asteraceae.

(5) Sierra Gorda generalized track (Fig. 4e). Located in the southern portion of the SMO, this track is the shortest one and includes only the states of Hidalgo and Querétaro. There are nine species included in this track (Table 1). This track is also supported by the work of Morrone and Gutiérrez (2005) with fleas.

Intersection or convergence in generalized tracks allowed for the recognition of five nodes (Fig. 5), two of them located in northeastern portion of the SMO, two in the southern portion and the last one also located in the south, on the boundary with the Trans-Mexican Volcanic Belt. These nodes are: Nuevo León, Tamaulipas, Landa, Sierra Gorda and Orizaba.

Endemism Analysis.—The endemism analysis was performed with 32 grid-cells that contain at least two fern species (mean value of 10 and median value

TABLE 2. Species richness, endemism and number of records for ferns in each grid-cell. Values of corrected and weighted endemism of each grid-cell in the SMO.

Grid-cells	Number of species	Endemic species	Number of records	Corrected endemism	Weighted endemism
A	4	0	23	0.13	0.52
B	1	0	3	0.06	0.06
C	3	0	14	0.06	0.2
D	2	0	10	0.06	0.13
E	4	0	10	0.08	0.32
F	4	0	5	0.08	0.35
G	4	0	8	0.14	0.58
H	8	0	15	0.15	1.25
I	7	1	13	0.22	1.58
J	3	0	5	0.11	0.34
K	5	0	2	0.15	0.76
L	4	0	3	0.17	0.71
M	4	0	5	0.15	0.6
N	26	2	111	0.24	6.24
O	3	0	6	0.11	0.33
Q	9	0	36	0.12	1.06
R	19	0	72	0.17	3.39
S	4	0	7	0.09	0.39
T	21	2	71	0.24	5.05
U	1	0	3	0.05	0.05
V	14	1	19	0.25	3.49
W	5	0	7	0.12	0.64
X	2	0	1	0.19	0.39
Y	4	0	1	0.2	0.81
Z	34	1	374	0.23	8.06
AA	12	0	11	0.15	1.85
AB	3	0	6	0.15	0.46
AC	6	0	11	0.14	0.88
AD	24	0	81	0.21	5.17
AE	28	0	120	0.21	5.91
AF	11	0	14	0.22	2.43
AH	4	0	22	0.09	0.39
AI	26	0	105	0.21	5.65
AJ	18	0	50	0.26	4.75

of 5), because the concept of area of endemism implies the overlap in distribution areas of two or more endemic taxa. U and B grid-cells were not considered because in them are represented only one species. The resulting CWE values were in the range 0.06–0.26 (mean and median values of 0.15) (Table 2).

From Olmstead-Tukey test application (Steel and Torrie, 1980) with p-value of 0.001, and considering values above the medians (representing richness and CWE index), 11 grid-cells as important areas for ferns were identified, forming a continuum (Fig. 6), from the I grid-cell in the north to the AI-AJ grid-cells in the south.

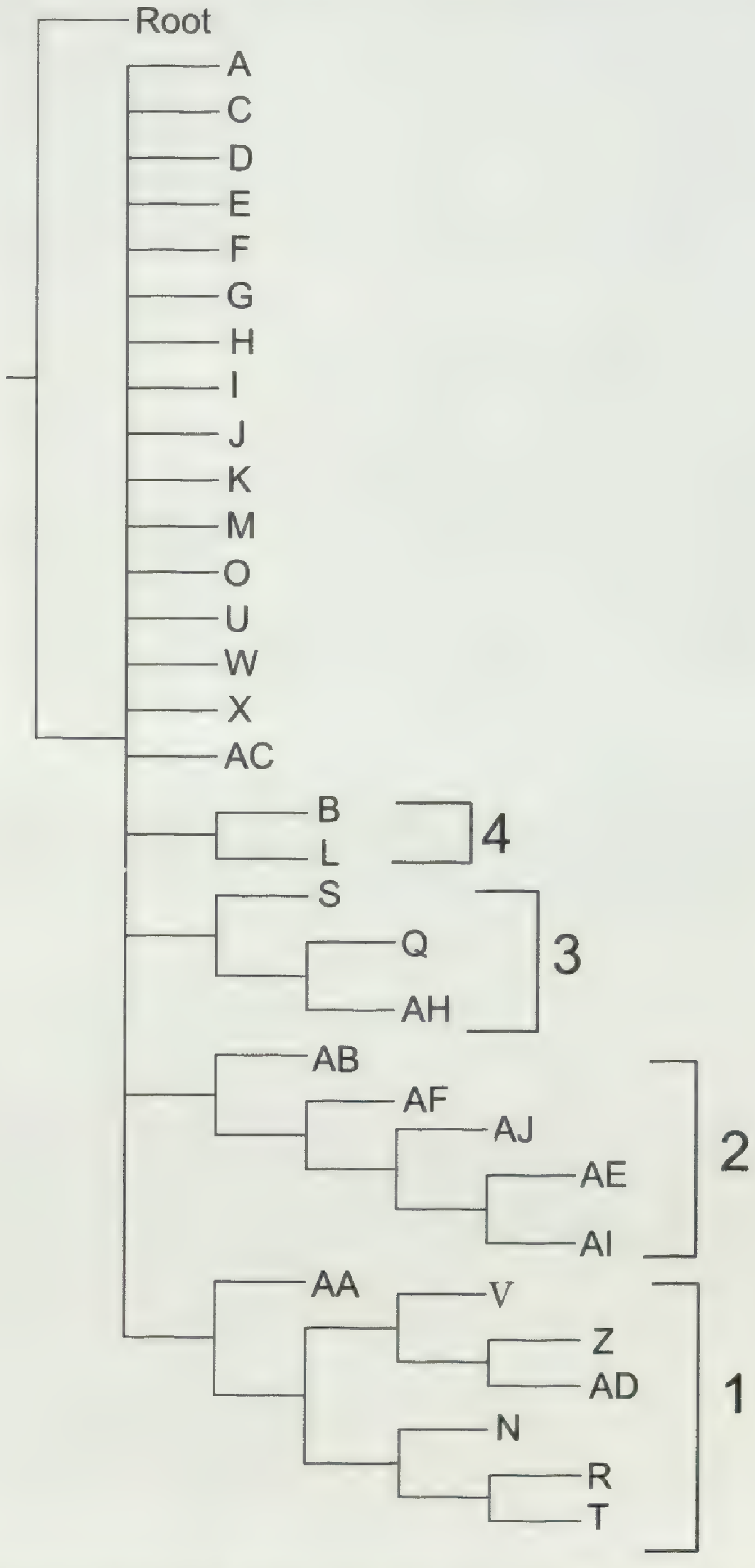

FIG. 3. Strict consensus cladogram obtained with grid-cell analysis. Numbers represent the main groups. The letters corresponds to grid-cells of Fig. 2.

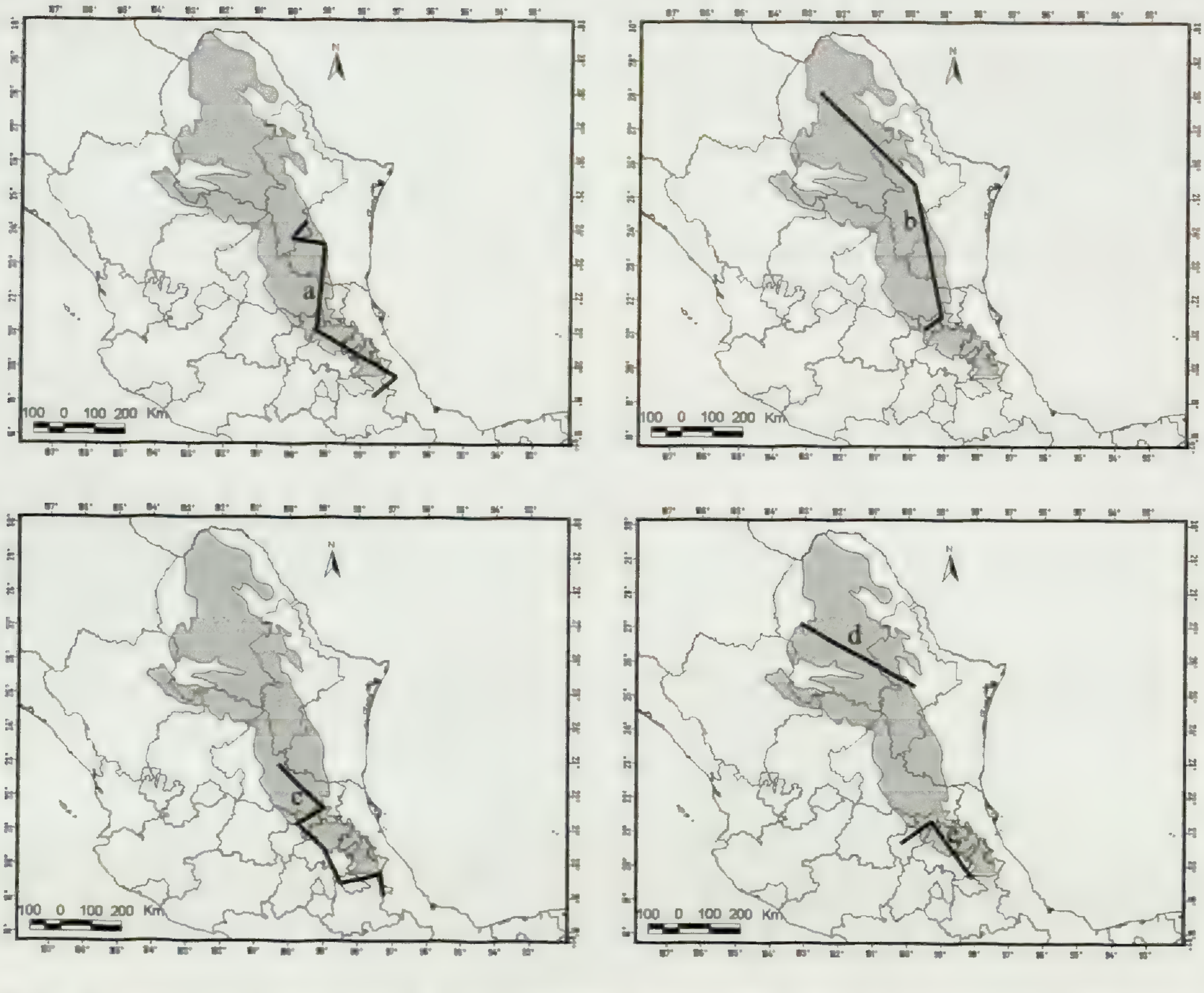

FIG. 4. Generalized tracks found. a) southeastern generalized track; b) northeastern generalized track; c) southern generalized track; d) Sierra Gorda generalized track; e) northern generalized track.

DISCUSSION

Fern species in the SMO are mainly distributed in cloud, oak, pine and mixed forests that develop under temperate climatic conditions, with high rainfalls and humidity. However, some species inhabit other types of vegetation that receive low rainfall, such as arid scrub or tropical thorn forest. Notwithstanding, distributional patterns obtained herein are mainly defined by mountainous landscapes.

The six richest grid-cells with more than 20 fern taxa are located in the southern and central portions of the SMO (grid-cells Z = 34, AE = 28, AI = 26, AD = 24 located in the south mainly in the states of Hidalgo, Querétaro, Puebla, and Veracruz, and grid-cells N = 26 and T = 21 located in the central part in the states of Coahuila, Nuevo León and Tamaulipas). The southern grid-cells coincide with two important areas recognized as terrestrial priority regions for conservation (Fig. 5) or TPR's proposed by CONABIO (Arriaga *et al.*, 2000). Two grid-cells are located at the priority region named as 'Bosques Mesófilos de la Sierra Madre Oriental' (grid-cells AE and AI of Fig. 2), and two more within the 'Reserva de la Biosfera de la Sierra Gorda' (grid-cells Z and AD

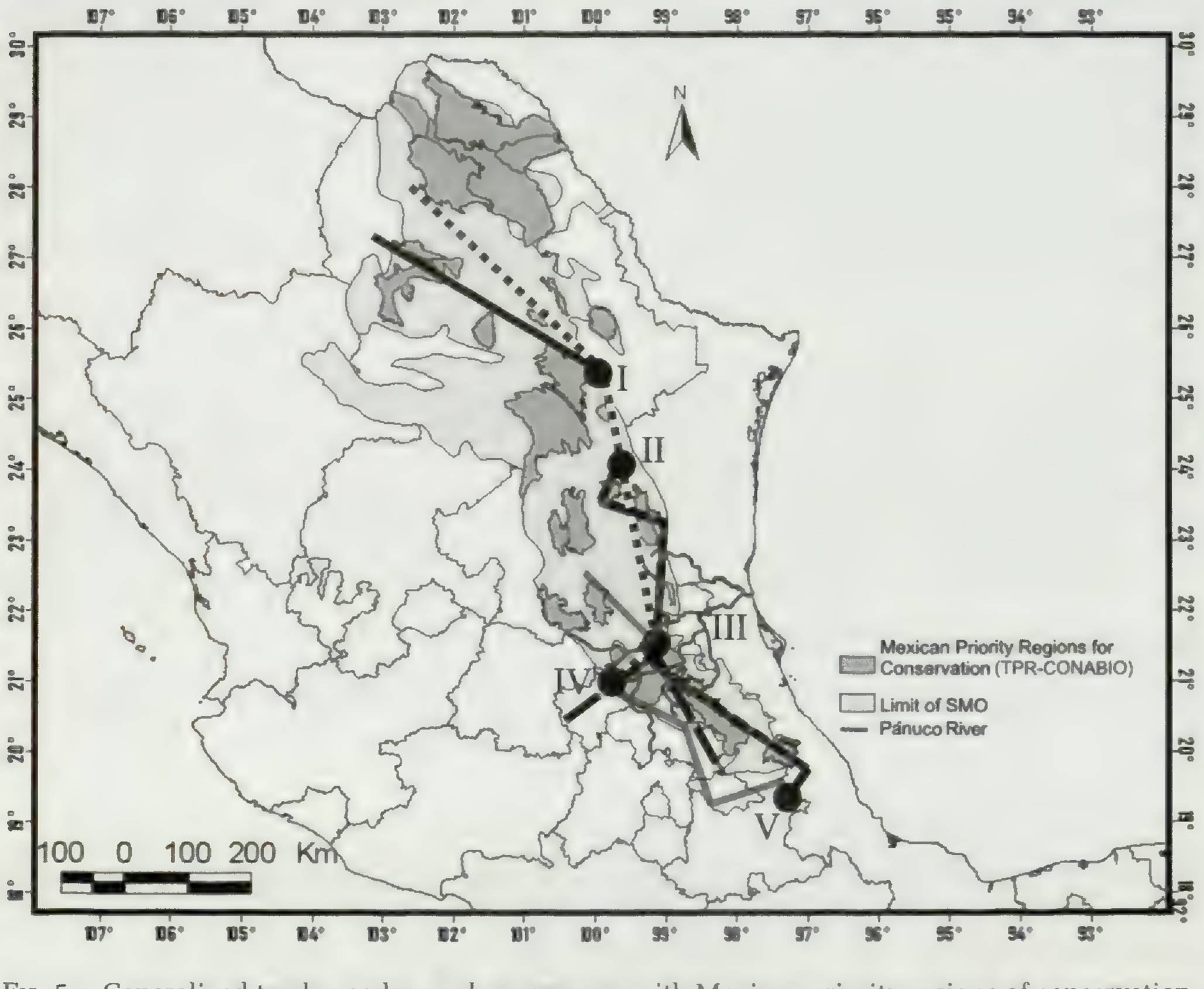

FIG. 5. Generalized tracks, nodes, and congruence with Mexican priority regions of conservation. Nuevo León (I), Tamaulipas (II), Landa (III), Sierra Gorda (IV) and Orizaba (V). Location of the Pánuco River is noted.

of Fig. 2). Two central grid-cells coincide with four TPR's: grid-cell N with El Potosí-Cumbres de Monterrey, and grid-cell T, with three TPR's named Valle de Jaumave, El Cielo and San Antonio-Peña Nevada. Overlap between these grid-cells with TPR's confirm that these areas are important for conservation, because TPR's represent areas with high values of ecosystem and species richness in relation to other areas of Mexico, as well as a functional ecologic integrity where real opportunities for conservation exist (Arriaga *et al.*, 2000). Terrestrial Priority Regions (TPRs) were formulated by an expertise set of Mexican researchers in different fields of biology coordinated by the CONABIO and represent areas with high biodiversity.

Conservation *in situ* could be carried out in protected areas that coincide with those grid-cells containing high fern diversity within the SMO, as in the case of the 'Reserva de la Biosfera Sierra Gorda', which hold native and natural vegetation; a real program of conservation exists in this Mexican Protected Natural Area. Some species studied herein are restricted endemics, and are therefore poorly represented in herbaria; we suggest that conservation strategies are needed for these fern species that inhabit the SMO, because

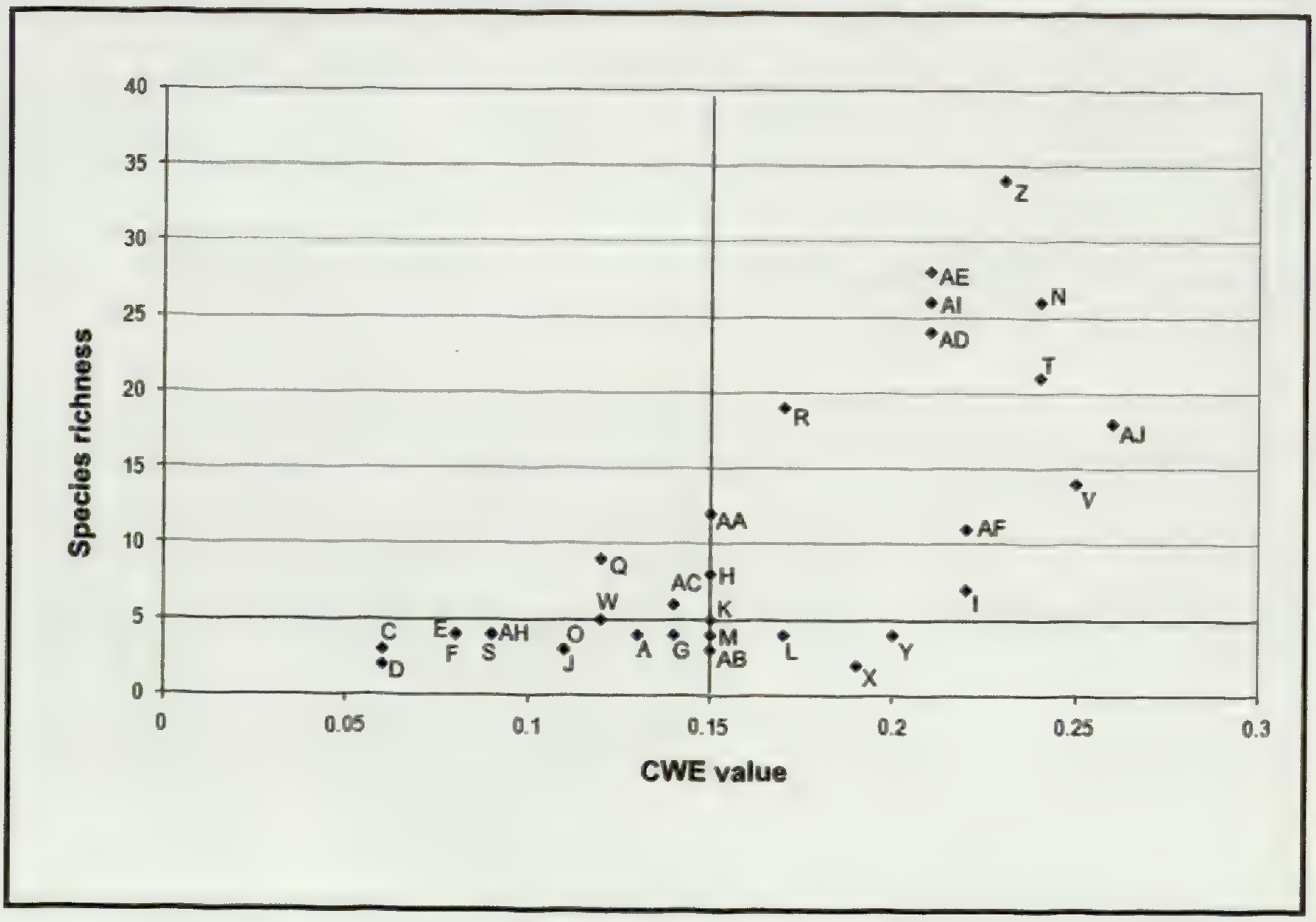

FIG. 6. Olmstead-Tukey corner test of association between richness and corrected weighted endemism (CWE) index. The letters corresponds to grid-cells of Fig. 2. The upper right part of the figure contains the important fern areas of the applied test.

only tree fern species are included in the Norma Oficial Mexicana NOM-059-ECOL-2001 (SEMARNAT, 2002), which is the official document published by the Mexican government that includes native threatened taxa. The following fern species are candidates to be included: *Asplenium dianae, A. semipinnatum, Cheilanthes apiacea, C. chipinquensis, Notholaena brachycaulis, N. leonina,* and *Pellaea ribae*. Demography and population genetics studies with conservation implications are needed for these species; these approaches have been done previously with endangered fern species in other places of the world (Ranker, 1994; Rumsey *et al.*, 1999; Kingston *et al.*, 2004). Genetic diversity is essential for fern species and populations in order to respond to long and short environmental change (Kingston *et al.*, 2004).

In the PAE cladogram, the largest component formed by seven grid-cells (group 1 in Fig. 3) includes areas located at the southern and central portions of the SMO. This component is formed mainly by montane temperate areas, characterizing the mountainous massif of the Sierra Madre Oriental, which might function as a biological corridor for this kind of organisms. This is an important aspect in conservation biology, because natural corridors are considered as useful and as essential components of management of landscapes. They provide connections among patches of habitat and mitigate pernicious effects of landscape fragmentation, also assisting in the mainte-

nance of population and biotic diversity in a region (Inglis and Underwood, 1992; Falcy and Estades, 2007; Williams *et al.*, 2007). Such is the case of the SMO province, because habitat fragmentation is a critical problem mainly in temperate forests due to land use change, especially for coffee, bean and corn plantations and for animal husbandry (Luna-Vega and Alcántara, 2004; Luna-Vega *et al.*, 2006). Interchange of individuals among populations that inhabit in different patches of temperate vegetation may increase local and regional population persistence in order to reduce extinction rates and increasing colonization rates, mainly for small and isolated populations (Rosenberg *et al.*, 1997). This is of particular interest in the case of ferns, especially endemics, but other species with wider distributions can be favored by connections between patches of temperate forests, because neighboring environments are likely to be similar in fern communities, and thus suitable for propagules dispersing short distances, as suggested by Karst *et al.* (2005).

A second and interesting component is located at the southern portion of the SMO (group 2 in Fig. 3), located in the leeward zone of this mountain chain, showing the main role of humidity in the distribution of ferns. Gametophytes need moisture to complete fertilization and most ferns have simple tracheary elements, so is not surprising that fern distribution is strongly linked to water availability (Karst *et al.*, 2005). The abiotic environment at a large scale (as in the present study) may play an important role in determining fern distributions (Karst *et al.*, 2005). The SMO is one of the main physiographic uplifted mountain ranges in Mexico, and wet montane forests cover its lower eastern slopes facing the Gulf of Mexico and give rise to wet pine-oak and cloud forests in a cloud zone above 2,200 m (Mickel and Smith, 2004). This mountain chain extends from the north to the central part of Mexico with peaks 2,000–3,400 m high, with rain and fog nearly every day on the eastern slope, influenced mainly by trade winds (Hernández-Cerda and Carrasco-Anaya, 2004; Mickel and Smith, 2004). These climatic conditions cause an extremely rich fern flora, with tree ferns, filmy ferns, and other groups requiring constant high humidity (Mickel and Smith, 2004), conditions that are reflected in our second component of the PAE cladogram.

The last two groups are smaller and include only two or three grid-cells, respectively (groups 3 and 4). These minor clades possibly represent groups of grid-cells that are supported by widely distributed species (*Astrolepis crassifolia* and *Notholaena aschenborniana*), which share similar habitats and need similar climatic conditions for their growth, and do not necessarily reflect historical relationships.

The largest group in the PAE cladogram (group 1 in Fig. 3) shows that several fern taxa are distributed all along the SMO, mainly in the southern and central portions. This group 1 exemplifies the wide distribution of some species in this region. Long distance dispersal and vicariance have been used as explanations for fern present-day disjunct distributions (Barrington, 1993), but some recent studies have shown that environmental variables can have a dominant role in determining fern distributions, i.e., high humidity, soil moisture and temperate climate, among others (Karst *et al.*, 2005; Ramírez-

Barahona *et al.*, 2011). Unfortunately, vicariance is not detectable in most cases (Wolf *et al.*, 2001), so effective research programs need to be undertaken, especially in the rich fern species areas of Mexico.

The resulting PAE area cladogram contains a polytomy of 17 grid-cells. Polytomies are frequent in this type of analyses, indicating an association with the spatial resolution of grid-cells used (Morrone and Escalante, 2002). Another problem inherent to this method is that the target of parsimony analyses is to find the most parsimonious solution, but biogeographical areas can have more than one history (Morrone and Crisci, 1995; García-Barros *et al.*, 2002).

The generalized tracks and nodes recognized in this work from panbiogeographic analysis reflect a complex biogeographic history of fern taxa involved and the geological and climatic complexity of this mountain region of Mexico. Additionally, they reinforce the idea that the SMO is a component of the Mexican Transition Zone, where Nearctic and Neotropical elements converge (Halffter, 1987). The rise and development of this mountain area has been considered to promote some vicariant events associated with the biotic evolution of the transitional component in northern Mexico (Morrone, 2005), contributing to the complex biogeographic history of this country (Luna-Vega, 2008). From a panbiogeographic perspective, the Pánuco River basin is considered as the main baseline. This river is located in the central part of the SMO (Fig. 5) and divides this mountain chain in two parts: north and south (Smith, 1941; Luna-Vega *et al.*, 1999).

All nodes obtained in this work coincide with those previously obtained with different organisms. From the five nodes obtained, three of them are remarkable. The case of the Nuevo León node (Fig. 5, I) shows the complexity of the area associated to their geological history. The Sierra Gorda and Landa nodes (Fig. 5, III, IV) are interesting in relation to its location as important areas for conservation. They are considered by the Mexican government as natural protected areas under the category of 'Reserva de la Biosfera'; this result supports their category with biogeographic criteria. A third interesting node is located in the southern portion of the SMO bordering with the Trans-Mexican Volcanic Belt (Fig. 5,V), because it represents a complex area where different biogeographic provinces converge, as previously suggested Márquez and Morrone (2004) in their study based on beetles.

The southern portion of the SMO contains high fern diversity in relation to the central and northern portion. The northern portion of the SMO is clearly poor in species and endemism (i.e., grid-cells A, B, C, D, E, F, G, H, J). PAE cladogram shows that the southern-central portion of the SMO includes two different fern components, one related with the leeward region (group 2 in Fig. 3, grid-cells AB, AE, AF, AI and AJ) and another mainly montane (group 1). An idea of a continuous SMO based in the distribution of fern species is based on PAE and CWE index. This represents an important difference with the Luna-Vega *et al.* (1999) study. In the present study, the forests located in the northeastern portion of the state of Querétaro are closely related to those in the central and southern portion of the SMO, and in Luna-Vega *et al.* (1999)

study, they are closely related to the forests of the state of Tamaulipas, located in northern Mexico. Differing from the PAE, the CWE index supports the existence of only one group in the southern-central portion (grid-cells V, Z, AD, AE, AF, AI, AJ). In the panbiogeographic analysis, the Pánuco River basin represents an important physiographic feature dividing the distribution of ferns in the SMO. The slight differences between PAE and CWE index (in relation with grid-cells AA and AB included in PAE and not in CWE) are expected because the areas detected after applying CWE index represent sets of neighbor grid-cells with high values, and are not associated by shared species as those found in the PAE cladogram (Santa Anna del Conde *et al.*, 2009). Our resulting PAE area cladogram represents a hypothesis of area relationship that was contrasted with previous studies for different biological groups and can also be refuted or supported with future studies that include all ferns that inhabit the SMO.

In the northern portion of the SMO, the region of Saltillo-Monterrey (grid-cell N in Fig. 2) appears as an interesting area from a biogeographic point of view, because it shows a convergence of biotas with different origins and relations, based on different analyses with different biological groups (González-Zamora *et al.*, 2007 with Asteraceae; Santa Anna del Conde *et al.*, 2009 with Cactaceae). This grid-cell resulted in panbiogeographic and CWE analyses as an important biogeographic area for ferns in northeastern Mexico; also this area presents a complex geological history (Fischer and Jackson, 1999), reinforcing its inclusion as a node.

The results of the present study suggest that the distributional patterns of endemism of ferns in the SMO are slightly different from those described for other biological groups so we cannot reinforce established generalizations. Ferns have high vagility and can represent an independent valuable test for identification of important biogeographic areas, mainly if they disagree with generalizations about regionalization and biogeographic proposals of Mexican biota. Because dispersal of spores is generally greater than seeds, fern species tend to be more widely distributed and show less endemism than seed plants (Smith, 1993). Another probable explanation to the distributional pattern observed could be the result of climatic differences that does not allow many species to establish, for example, in both sides of the Pánuco River. In this case, we can point to those more tropical southern species that cannot survive in the driest and coldest northern areas, although their spores are still dispersed into these areas. Despite this, ferns sometimes present similar distributional patterns as seed plants, with some narrowly distributed species, which might be considered, with some caution, as a signal of vicariance.

This study analyzes the regional distributional patterns of ferns and establishes an objective way to study the relationship between richness and endemism in non-flowering plants with high vagility. The validity and repeatability of the methods applied in the present study will depend on its application in other parts of the world, mainly in those regions where major geographic centers of fern species are located, such as Central and South America, eastern Africa, Australia, and eastern Asia (Tryon and Tryon, 1984),

and also in other mountainous areas of Mexico. In the SMO, a similar study with widely distributed, nonvascular, seedless plants (such as mosses) is needed. Knowledge of the distributional patterns of the species of ferns, as well as the examination of historical and ecological factors that have determined such patterns, provide additional evidence about the biota that currently occur in the SMO (Luna-Vega *et al.*, 1999; González-Zamora *et al.*, 2007). Clearly, this study has improved our knowledge of the complexity and biogeographic history of this eastern montane part of Mexico. These kinds of study can improve the knowledge of the SMO and of the Mexican biota in general.

As suggested by Luna-Vega (2008), we are in an important moment in the understanding of the biogeography of Mexican plants, where ferns still have much to offer (Wolf *et al.*, 2001), and it is important that their distributional data and biogeographic patterns be incorporated into general statements about Mexican biota and biogeographic history of this highly diverse country.

Acknowledgments

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Literature Cited

Aguilar-Aguilar, R., R. Contreras-Medina and G. Salgado-Maldonado. 2003. Parsimony Analysis of Endemicity (PAE) of Mexican hydrological basins based on helminth parasites of freshwater fishes. J. Biogeogr. 30:1861–1872.

Aguilar-Aguilar, R., G. Salgado-Maldonado, R. Contreras-Medina and A. Martínez-Aquino. 2008. Richness and endemism of helminth parasites of freshwater fishes in Mexico. Biol. J. Linnean Soc. 94:435–444.

Ambrose, A. R. 2004. Water-holding capacity of canopy soil mats and effects on microclimates in an old-growth redwood forest: a report to save the redwoods league. Master in Science Thesis. Humboldt State University, Arcata, California.

Álvarez, E. and J. J. Morrone. 2004. Propuesta de áreas para la conservación de aves en México, empleando herramientas panbiogeográficas e índices de complementariedad. Interciencia 29:112–120.

Arriaga, L., J. M. Espinoza, C. Aguilar, E. Martínez, L. Gómez and E. Loa (coords). 2000. Regiones terrestres prioritarias de México. Escala 1: 1 000 000. CONABIO, México D.F.

Barrington, D. S. 1993. Ecological and historical factors in fern biogeography. J. Biogeogr. 20:275–280.

Canseco-Márquez, L., F. Mendoza-Quijano and M. G. Gutiérrez-Mayén. 2004. Análisis de la distribución de la herpetofauna. Pp. 417–437, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental.* Universidad Nacional Autónoma de México-CONABIO, México D. F.

Cervantes-Zamora, Y., S. L. Cornejo-Olguín, R. Lucero-Márquez, J. M. Espinoza-Rodríguez, E. Miranda-Viquez and A. Pineda-Velázquez. 1990. Clasificación de regiones naturales de México II, IV.10.2. *Atlas Nacional de México.* vol. 2, Escala 1: 4 000000. Instituto de Geografía, Universidad Nacional Autónoma de México. México, D. F.

Christ, H. 1910. Die Geographie der Farne. G. Fischer, Jena.

Cifuentes, J., M. Villegas, R. García-Sandoval, G. Vidal-Gaona, S. Sierra, R. Valenzuela, L. Pérez-Ramírez and E. Morales-Torres. 2004. Distribución de macromicetos: una aproximación al análisis de áreas de endemismos. Pp. 355–374, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental*. Universidad Nacional Autónoma de México-CONABIO, México D. F.

Contreras-Medina, R. 2006. Los métodos de análisis biogeográfico y su aplicación a la distribución de las gimnospermas en México. Interciencia 31:176–182.

Contreras-Medina, R. and I. Luna-Vega. 2007. Species richness, endemism and conservation of Mexican gymnosperms. Biodiv. Conserv. 16:1803–1821.

Contreras-Medina, R., I. Luna-Vega and J. J. Morrone. 2007. Application of parsimony analysis of endemicity (PAE) to Mexican gymnosperm distributions: grid-cells, biogeographic provinces and track analysis. Biol. J. Linnean Soc. 92:405–417.

Craw, R., J. Grehan and M. J. Heads. 1999. *Panbiogeography: Tracking the history of life*. Oxford University Press, New York.

Croizat, L. 1958. *Panbiogeography*. Published by the author, Caracas, Venezuela.

Croizat, L. 1964. *Space, time, and form: the biological synthesis*. Published by the author, Caracas, Venezuela.

Crisp, M. D., S. Laffan, H. P. Linder and A. Monro. 2001. Endemism in the Australian flora. J. Biogeogr. 28:184–198.

Dávila-Aranda, P., S. Arias-Montes, R. Lira-Saade, J. L. Villaseñor and A. Valiente-Banuet. 2002. Phytogeography of the columnar cacti (tribe Pachycereeae) in Mexico: a cladistic approach. Pp. 25–41, *in* T. H. Fleming and A. Valiente-Banuet, eds. *Columnar cacti and their mutualists: Evolution, ecology, and conservation*. The University of Arizona Press, Tucson.

Eguiluz De Antuñano, S., M. Aranda and R. Marret. 2000. Tectónica de la Sierra Madre Oriental, México. Bol. Soc. Geol. Mex. 53:1–26.

Escalante, T. 2003. Avances en el atlas biogeográfico de los mamíferos terrestres de México. Pp. 297–302, *in* J. J. Morrone and J. Llorente, eds. *Una perspectiva latinoamericana de la biogeografía*. CONABIO-Universidad Nacional Autónoma de México, México D. F.

Escalante, T., G. Rodríguez and J. J. Morrone. 2004. The diversification of the Nearctic mammals in the Mexican transition zone: a track analysis. Biol. J. Linnean Soc. 83:327–339.

ESRI. 1999. Arc View GIS ver. 3.2. Environmental Systems Research Institute, Inc., Redlands, USA.

Falcy, M. R. and C. E. Estades. 2007. Effectiveness of corridors relative to enlargement of habitat patches. Conserv. Biol. 21:1341–1346.

Fischer, M. P. and P. B. Jackson. 1999. Stratigraphic controls on deformation patterns in fault-related folds: a detachment fold example from the Sierra Madre Oriental, northeast Mexico. J. Struct. Geol. 21:613–633.

García-Barros, E., P. Gurrea, M. J. Luciañez, J. M. Cano, M. L. Munguira, J. C. Moreno, H. Sainz, M. J. Sanz and J. C. Simón. 2002. Parsimony analysis of endemicity and its application to animal and plant geographical distributions in the Ibero-Balearic region (western Mediterranean). J. Biogeogr. 29:109–124.

García-Mendoza, A. 1995. Riqueza y endemismos de la familia Agavaceae en México. Pp. 51–75, *in* E. Linares, P. Dávila, F. Chiang, R. Bye and T. Elías, eds. *Conservación de plantas en peligro de extinción: diferentes enfoques*. Instituto de Biología, Universidad Nacional Autónoma de México, Mexico D. F.

García-Trejo, E. and A. Navarro. 2004. Patrones biogeográficos de la riqueza de especies y el endemismo de la avifauna en el oeste de México. Acta Zool. Mex. 20:167–185.

Goloboff, P. 1999. Nona, version 2.0 (for Windows). San Miguel de Tucumán, published by the author.

González-Zamora, A., I. Luna-Vega, J. L. Villaseñor and C. Ruiz-Jiménez. 2007. Distributional patterns and conservation of species of Asteraceae (asters etc.) endemic to eastern Mexico: a panbiogeographical approach. Syst. Biodiv. 5:135–144.

Halffter, G. 1987. Biogeography of the montane entomofauna of Mexico and Central America. Ann. Rev. Entomol. 32:95–114.

HASSLER, M. and B. SWALE. 2001. World fern statistics by country. http://homepages.caverock.net.nz/~bj/fern/ferndist.htm (Accessed 06/2009).

HERNÁNDEZ-CERDA, M. and G. CARRASCO-ANAYA. 2004. Climatología. Pp. 63–108, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental*. Universidad Nacional Autónoma de México-CONABIO, México D. F.

HILL, J. D. and J. A. SILANDER-JR. 2001. Distribution and dynamics of two ferns: *Dennstaedtia punctilobula* (Dennstaedtiaceae) and *Thelypteris noveboracensis* (Thelypteridaceae) in a northeast mixed hardwoods-hemlock forest. Amer. J. Bot. 88:894–902.

INGLIS, G. and A. J. UNDERWOOD. 1992. Comments on some designs proposed for experiments on the biological importance of corridors. Conserv. Biol. 6:581–586.

KARST, T., B. GILBERT and M. J. LECHOWICZ. 2005. Fern community assembly: The roles of chance and the environment at local and intermediate scales. Ecology 86:2473–2486.

KATO, M. 1993. Biogeography of ferns: dispersal and vicariance. J. Biogeogr. 20:265–274.

KINGSTON, N., S. WALDREN and N. SMYTH. 2004. Conservation genetics and ecology of *Angiopteris chauliodonta* Copel. (Marattiaceae), a critically endangered fern from Pitcairn Island, South Central Pacific Ocean. Biol. Conserv. 117:309–319.

KOHLMANN, B. and S. SÁNCHEZ. 1984. Estudio areográfico del género *Bursera* Jacq. ex L. (Burseraceae) en México: una síntesis de métodos. Pp. 45–120, *in* B. Kohlmann, ed. *Métodos cuantitativos en la biogeografía*. Instituto de Ecología AC, México D. F.

LEÓN-PANIAGUA, L., E. GARCÍA, J. ARROYO-CABRALES and S. CASTAÑEDA-RICO. 2004. Patrones biogeográficos de la mastofauna. Pp. 469–486, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental*. Universidad Nacional Autónoma de México-CONABIO, Mexico D. F.

LINDER, H. P. 2001. Plant diversity and endemism in sub-Saharan tropical Africa. J. Biogeogr. 28:169–182.

LUNA-VEGA, I. 2008. Aplicaciones de la biogeografía histórica a la distribución de las plantas mexicanas. Rev. Mex. Biodiv. 79:217–241.

LUNA-VEGA, I. and O. ALCÁNTARA. 2004. Florística del bosque mesófilo de montaña de Hidalgo. Pp. 169–191, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental*. Universidad Nacional Autónoma de México-CONABIO, Mexico D. F.

LUNA-VEGA, I., O. ALCÁNTARA, D. ESPINOSA and J. J. MORRONE. 1999. Historical relationships of the Mexican cloud forests: a preliminary vicariance model applying Parsimony Analysis of Endemicity to vascular plant taxa. J. Biogeogr. 26:1299–1305.

LUNA-VEGA, I., O. ALCÁNTARA, D. ESPINOSA and J. J. MORRONE. 2000. Track analysis and conservation priorities in the cloud forests of Hidalgo, Mexico. Divers. Distrib. 6:137–143.

LUNA-VEGA, I., O. ALCÁNTARA and R. CONTRERAS-MEDINA. 2004. Patterns of diversity, endemism and conservation: an example with Mexican species of Ternstroemiaceae Mirb. ex DC. (Tricolpates: Ericales). Biodiv. Conserv. 13:2723–2739.

LUNA-VEGA, I., J. J. MORRONE and F. ESPINOSA. 2004. Introducción. Pp. 1–2, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental*. Universidad Nacional Autónoma de México-CONABIO, México D. F.

LUNA-VEGA, I., O. ALCÁNTARA, C. A. RUIZ-JIMÉNEZ and R. CONTRERAS-MEDINA. 2006. Composition and structure of humid montane oak forests at different sites in Central and Eastern Mexico. Pp. 101–112, *in* M. Kapelle, ed. *Ecology and conservation of Neotropical montane oak forests*. Springer-Verlag Berlin, Heidelberg.

LYELL, K. M. 1870. A geographical handbook of all the known ferns with tables to show their distributions. John Murray, London.

MÁRQUEZ, J. and J. J. MORRONE. 2004. Relaciones biogeográficas basadas en la distribución de Coleoptera (Insecta). Pp. 375–392, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental*. Universidad Nacional Autónoma de México-CONABIO, México D. F.

MÉNDEZ-LARIOS, I., J. L. VILLASEÑOR, R. LIRA, J. J. MORRONE, P. DÁVILA-ARANDA and E. ORTIZ. 2005. Toward the identification of a core zone in the Tehuacán-Cuicatlán biosphere reserve, Mexico, based on Parsimony Analysis of Endemicity of flowering plant species. Interciencia 30:267–274.

MICKEL, J. T. and A. R. SMITH. 2004. *The pteridophytes of Mexico*. New York Botanical Garden, New York.

MORAN, R. C. 2004. *A natural history of ferns*. Timber Press, Portland.

MORRONE, J. J. 2005. Hacia una síntesis biogeográfica de México. Rev. Mex. Biodiv. 76:207–252.

MORRONE, J. J. and J. V. CRISCI. 1995. Historical biogeography: introduction to methods. Ann. Rev. Ecol. Syst. 26:373–401.

MORRONE, J. J. and T. ESCALANTE. 2002. Parsimony Analysis of Endemicity (PAE) of Mexican terrestrial mammals at different area units: when size matters. J. Biogeogr. 29:1095–1104.

MORRONE, J. J. and A. GUTIÉRREZ. 2005. Do fleas (Insecta: Siphonaptera) parallel their mammal host diversification in the Mexican transition zone? J. Biogeogr. 32:1315–1325.

MORRONE, J. J., D. ESPINOSA, C. AGUILAR and J. LLORENTE. 1999. Preliminary classification of the Mexican biogeographic provinces: a Parsimony Analysis of Endemicity based on plant, insect, and bird taxa. Southwest. Natur. 44:507–514.

NAVARRO, A., H. GARZA-TORRES, S. LÓPEZ DE AQUINO, O. ROJAS-SOTO and L. A. SÁNCHEZ-GONZÁLEZ. 2004. Patrones biogeográficos de la avifauna. Pp. 439–467, *in* I. Luna-Vega, J. J. Morrone and D. Espinosa, eds. *Biodiversidad de la Sierra Madre Oriental*. Universidad Nacional Autónoma de México-CONABIO, México D. F.

NIXON, K. C. 2002. WinClada, version 1.00.08. Ithaca, New York, Published by the author.

OCHOA, L. and O. FLORES-VILLELA. 2006. *Áreas de diversidad y endemismo de la herpetofauna mexicana*. Universidad Nacional Autónoma de México-CONABIO, México D. F.

ORTEGA-GUTIÉRREZ, F., R. L. SEDLOCK and R. C. SPEED. 1994. Phanerozoic tectonic evolution of Mexico. Pp. 265–306, *in* R. C. Speed, ed. *Phanerozoic evolution of North American continent-ocean transitions*. The Geological Society of America, Boulder, Colorado.

PACIENCIA, M. L. B. and J. PRADO. 2005. Effects of forest fragmentation on pteridophyte diversity in a tropical rain forest in Brazil. Plant Ecol. 180:87–104.

PAGE, C. N. 1979a. The diversity of ferns: an ecological perspective. Pp. 10–56, *in* A. F. Dyer, ed. *The experimental biology of ferns*. Academic Press, London.

PAGE, C. N. 1979b. Experimental aspects of fern ecology. Pp. 552–589, *in* A. F. Dyer, ed. *The experimental biology of ferns*. Academic Press, London.

PALACIOS-RÍOS, M. and A. GÓMEZ-POMPA. 1997. Phytogeographical analysis of the pteridophytes of Veracruz, Mexico. Pp. 217–234, *in* R. J. Johns, ed. *Holttum Memorial Volume*. Royal Botanic Gardens, Kew, UK.

PORZECANSKI, A. L. and J. CRACRAFT. 2005. Cladistic analysis of distributions and endemism (CADE): using raw distributions of birds to unravel the biogeography of the South American aridlands. J. Biogeogr. 32:261–275.

PRYER, K. M., E. SCHUETTPELZ, P. G. WOLF, H. SCHNEIDER, A. R. SMITH and R. CRANFILL. 2004. Phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. Amer. J. Bot. 91:1582–1598.

PUENTHA, N. 1991. Studies on atmospheric fern spores at Pithorgarh (northwest Himalaya) with particular reference to distribution of ferns in the Himalayas. Pp. 146–161, *in* T. N. Bhardwaj and O. B. Gena, eds. *Perspectives in pteridology: present and future*. Annual Review of Plant Sciences 13. Academic Press, London.

RAMÍREZ-BARAHONA, S., I. LUNA-VEGA and D. TEJERO-DÍEZ. 2010. Species richness, endemism, and conservation of American tree ferns (Cyatheales). Biodivers. Conserv. 20:59–72.

RANKER, T. A. 1994. Evolution of high genetic variability in the rare Hawaiian fern *Adenophorus periens* and implications for conservation management. Biol. Conser. 70:19–24.

ROJAS, C. 2004. Automatización del método de la panbiogeografía. Identificación de centros de biodiversidad del Parque Nacional Iztaccíhuatl, Popocatépetl y anexas. Master in Sciences thesis. Universidad Nacional Autónoma de México, Mexico City.

ROJAS-SOTO, O., O. ALCÁNTARA and A. NAVARRO. 2003. Regionalization of the avifauna of the Baja California Peninsula, Mexico: a Parsimony Analysis of Endemicity and distributional modeling approach. J. Biogeogr. 30:449–461.

ROSEN, B. R. 1988. From fossils to earth history: applied historical biogeography. Pp. 437–481, *in* A. A. Myers and P. S. Giller, eds. *Analytical Biogeography: an integrated approach to the study of animal and plant distributions*. Chapman & Hall, London.

ROSEN, B. R. and A. B. SMITH. 1988. Tectonics from fossils? Analysis of reef-coral and sea-urchin distributions from late Cretaceous to Recent, using a new method. In M. G. Audley-Charles and A. Hallam, eds. Gondwana and Tethys. *Spec. Pub. Geol. Soc. London* 37:275–306.

ROSENBERG, D. K., B. R. NOON and E. C. MESLOW. 1997. Biological corridors: form, function and efficacy. Bioscience 47:677–687.

RUMSEY, F. J., J. C. VOGEL, S. J. RUSSELL, J. A. BARRETT and M. GIBBY. 1999. Population structure and conservation biology of the endangered fern *Trichomanes speciosum* Willd. (Hymenophyllaceae) at its northern distributional limit. Biol. J. Linnean Soc. 66:333–344.

SANTA ANNA DEL CONDE, H., R. CONTRERAS-MEDINA and I. LUNA-VEGA. 2009. Biogeographic analysis of endemic cacti of the Sierra Madre Oriental, Mexico. Biol. J. Linnean Soc. 97:373–389.

SCHNEIDER, H., E. SCHUETTPELZ, K. M. PRYER, R. CRANFILL, S. MAGALLÓN and R. LUPIA. 2004a. Ferns diversified in the shadow of angiosperms. Nature 428:553–557.

SIQUEIROS, M. E. and G. GONZÁLEZ. 2006. *Helechos y plantas afines de Aguascalientes.* Universidad Autónoma de Aguascalientes and CONABIO, México, D. F.

SMITH, H. 1941. Las provincias bióticas de México, según la distribución geográfica de las lagartijas del género *Sceloporus.* Anales Esc. Nal. Cien. Biol. 2:103–110.

SMITH, A. R. 1972. Comparison of fern and flowering plant distributions with some evolutionary interpretations for ferns. Biotropica 4:4–9.

SMITH, A. R. 1993. Phytogeographic principles and their use in understanding fern relationships. J. Biogeogr. 20:255–264.

SMITH, A. R., K. M. PRYER, E. SCHUETTPELZ, E. KORALL, H. SCHNEIDER and P. G. WOLF. 2006. A classification for extant ferns. Taxon 55:705–731.

STEEL, R. G. and J. H. TORRIE. 1980. *Principles and procedures of statistics: a biometrical approach.* McGraw-Hill, Inc., New York.

TEJERO-DÍEZ, D. 1990. Fitogeografía de los helechos y plantas afines presentes en el oeste del Estado de México, México. Abstracts of the XI Congreso Mexicano de Botánica 1:399, Oaxtepec, Morelos, México.

TRYON, R. M. 1970. Development and evolution of fern floras of oceanic islands. Biotropica 2:76–84.

TRYON, R. M. 1972. Endemic areas and geographic speciation in tropical American ferns. Biotropica 4:121–131.

TRYON, R. M. 1985. Fern speciation and biogeography. Proc. Royal Soc. Edinburgh 86B:353–360.

TRYON, R. and A. F. TRYON. 1984. Geography, spores and evolutionary relations in the cheilanthoid ferns. Pp. 145–153, *in* A. C. Jermy, J. A. Crabbe and B. A. Thomas, eds. *The phylogeny and classification of the ferns.* Koeltz Scientific Books, Koenigstein.

WILLIAMS-LINERA, G., A. GUILLÉN, O. GÓMEZ and F. LOREA. 2007. Conservación en el centro de Veracruz, México. El bosque de niebla: ¿reserva archipiélago o corredor biológico? Pp. 303–310, *in* G. Halffter, S. Guevara and A. Melic, eds. *Hacia una cultura de conservación de la diversidad biológica.* SEA, CONABIO, CONANP, CONACYT, INECOL, UNESCO-MAB and Ministerio del Medio Ambiente-Gobierno de España. Monografías Tercer Milenio, Vol. 6, Zaragoza, Spain.

WINKLER, H. 1938. Geographie. Pp. 451–472, *in* F. Verdoorn, ed. *Manual of pteridology.* M. Nijhoff, The Hague.

WOLF, P. G., H. SCHNEIDER and T. A. RANKER. 2001. Geographic distributions of homosporous ferns: does dispersal obscure evidence of vicariance? J. Biogeogr. 28:263–270.

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Endangered Pteridophytes and Their Distribution in Hainan Island, China

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ABSTRACT.—With decreasing population sizes and fragmentation of the original habitats of pteridophytes in Hainan Island, the abundance of most pteridophytes has been dramatically reduced, and some species may be at the brink of extinction. To assess the natural distribution of pteridophytes on Hainan Island, we conducted three analyses: flora, niche, and habitat. Thirty-two species of endangered pteridophytes in 21 families were found in Hainan Island, accounting for 8% of the 400 pteridophytes species there. Floristic studies showed that 9% of these 32 species are tropical, 50% are tropical Asian, 20% are pan-tropical, 3% are from East Asia, 3% from the Southern Hemisphere, and 15% are endemic to Hainan Island. Niche analysis showed that 21 of the 32 species (66%) are epiphytic or semi-epiphytic, while 11 (34%) are terrestrial. About 24 species of endangered pteridophytes are distributed in protected, conservation regions and the others are distributed partly in the conservation and non-conservation regions. In species-poor families (< 6 species), most of the species are endangered.

KEY WORDS.—Hainan Island, pteridophyte, flora, endangered species, conservation

Hainan Island is located in the northern margin of the tropics and has a complex terrain and a tropical climate. Many rare and endangered species of pteridophytes find their home in the diverse environments of the island. Pteridophytes form essential links in the plant ecosystem of Hainan Island and are indicators of plant biodiversity. Many pteridophyte species of the Malaysia floristic region, India floristic region, Eastern Asiatic floristic region, Indo-china Peninsula floristic region and Polynesian floristic region are found on the island (Yang *et al.*, 2007; Dong, 2004). Integration of multiple floras can promote the evolution of new species of pteridophytes (Wulff, 1964). However, with the increase of human populations and rapid economic development, the natural habitats of pteridophytes are becoming small and fragmented with pteridophytes scattered in patches in tropical mountain rainforests. Here we assessed the natural distribution of endangered pteridophytes in Hainan Island with flora, niche, and habitat analyses. The results showed that some pteridophyte populations have dramatically decreased in numbers and are facing extinction, and demonstrate the need for protecting pteridophyte diversity in Hainan Island.

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Materials and Methods

Study Area.—Hainan Island is located along the continental shelf of the southernmost tip of the Chinese continental marginal sea. It is separated from the Leizhou Peninsula of Guangdong by the 18-mile-wide Qiongzhou Strait (Fu, 1954). It is a typical continental island with a total area (satellite islands not included) of 33,900 km^2. Low and flat along its coastline, Hainan Island reaches its highest altitude of 1867.1 meters at the Yinggeling Ridge of Wuzhishan Mountain, located at the center of the island. Connecting the ridge and the coastline are mountains, hills, and plains forming a terraced landscape. The northern part of the island is a broad plateau composed of shallow sediments and basalts, and is 50 meters above sea level. The granite hills of Danzhou, Tunchang, and other areas form a barrier to the cold winds from the north. Hainan has a tropical moist monsoon climate, often with strong winds, frequent tropical storms and typhoons. The sunshine duration throughout the year is 1750 to 2650 hours, with the average annual temperature between 23–25°C and there is no winter season. Most areas of the island have plenty of rainfall with average annual precipitation greater than 1600 mm. The central and eastern coastal areas are humid, especially during the typhoon season. The southeast coast has the highest annual precipitation (2000–2500 mm). The west coastal area has the lowest annual precipitation (800–1000 mm) and the evaporation is 1–10 times greater than the precipitation. Therefore the western side of Hainan Island is the driest place for vegetation. Other regions of the island are sub-humid as shown in Fig. 1 (Ren, 1985).

Background information.—We collected literature on Hainan pteridophytes published in Chinese and English since 1964 (Fu, 1954, 1957; Wu and Qin, 1991; Chen, 1964; Qin *et al.*, 1959, 2001), including reports and supplemental materials on the flora, niche, and habitat. We classified the locations of pteridophytes on Hainan Island into two categories: non-protected and protected areas (nature reserves and forest parks). The distribution of pteridophytes shows a marked discontinuity where the regional abundances of genera and species are distinctly different on either side of a curved line across the island from northwest to southeast. We named it "Lingao-Qiongzhong-Wanning" line as shown in Fig. 2. This line seems to have great significance as it overlaps the "Wangwu-Wenjiao" geologic fault zone. We have carried out preliminary investigations of the causes of the line (Yang *et al.*, 2007).

Evaluation of endangered species.—We used the "IUCN Red List of Threatened Species Categories and Criteria version 3.1" (IUCN, 2001; Fig. 3) as the main criteria to determine the current status of species, determining if they are endangered, critically endangered, threatened, etc. Furthermore, some of the species' data are from "China Plant Red Data Book" (Fu, 1991), "National Protected Plants List (first batch)" (Chinese State Forestry Administration, the Ministry of Agriculture, 1999), "China's rare and endangered plant list" (State Environmental Protection Agency, the CAS Institute of Botany, 1987) and the

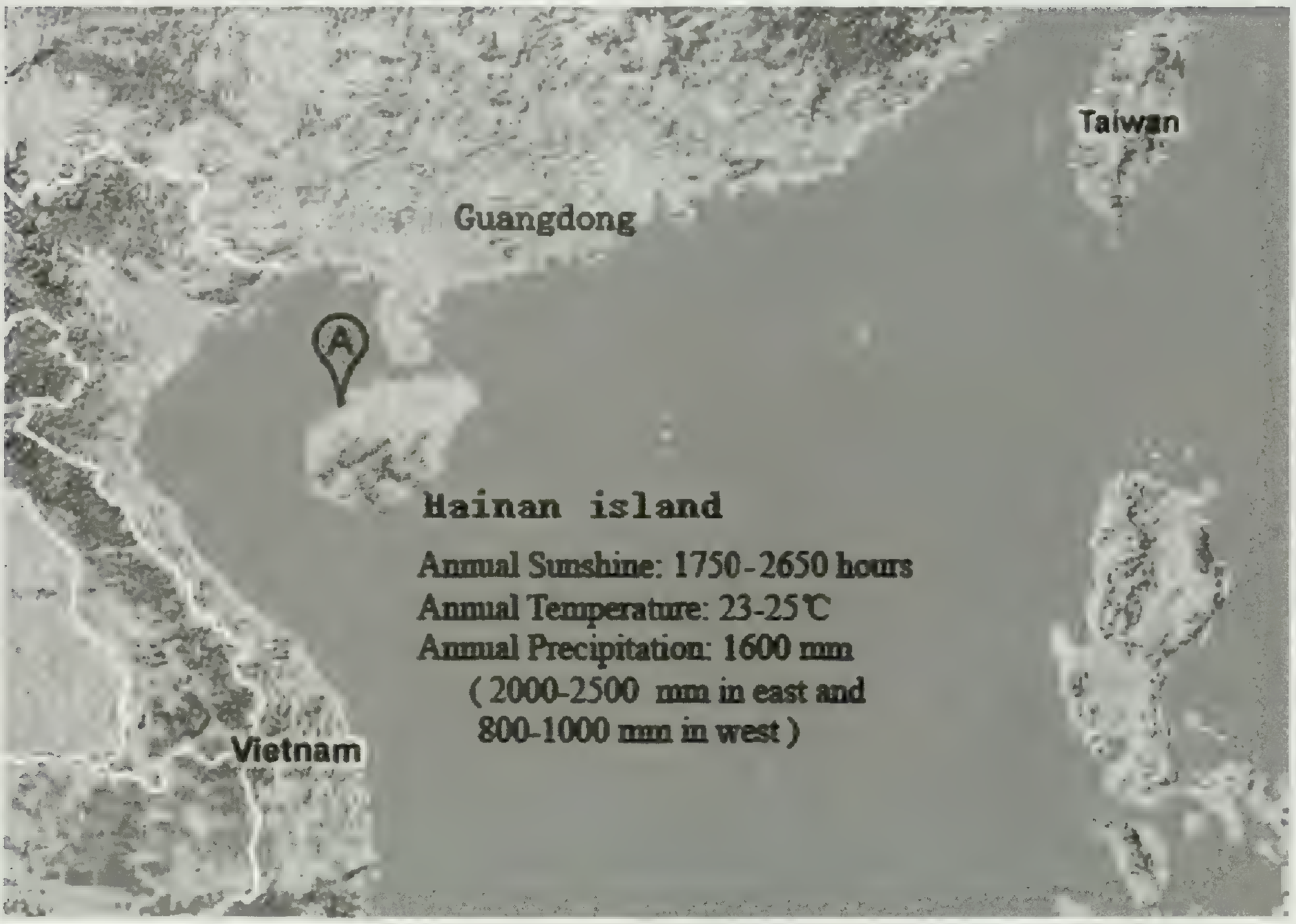

FIG. 1. Overview of Hainan Island.

new version of the "Hainan priority protected terrestrial wild plants directory" (The Hainan Provincial People's Government, China, 2006).

Field investigation.—Field investigations were performed to collect species' information. Data categories included: species name, number of individuals and environment (soil, temperature, humidity, sunlight). We identified and recorded 264 species of wild pteridophytes and took more than 1,000 photos and 700 specimens of local pteridophytes. Some important populations have been periodically re-visited. We conducted field investigations over a five-year period at conservation areas in Wuzhishan National Nature Reserve, Baihualing and Bawangling National Nature Reserve, Jianfengling National Forest Park, and Diaoluoshan Nature Reserve.

Flora and ecotype.—The flora of Hainan Island contains elements of several floras. Floras of neighboring areas like Guangdong, Taiwan, Vietnam, Kalimantan and even Polynesia have some similarities with Hainan Island. Ecological data were collected on niche diversity (epiphytic and semi-epiphytic, ground living) and habitat diversity (wet habitats, high altitude, other habitats including limestone, coastal, mesad, etc.)

RESULTS

Endangered wild pteridophyte species and their distribution on Hainan Island.—Of all the species of pteridophytes on Hainan Island (Fu,

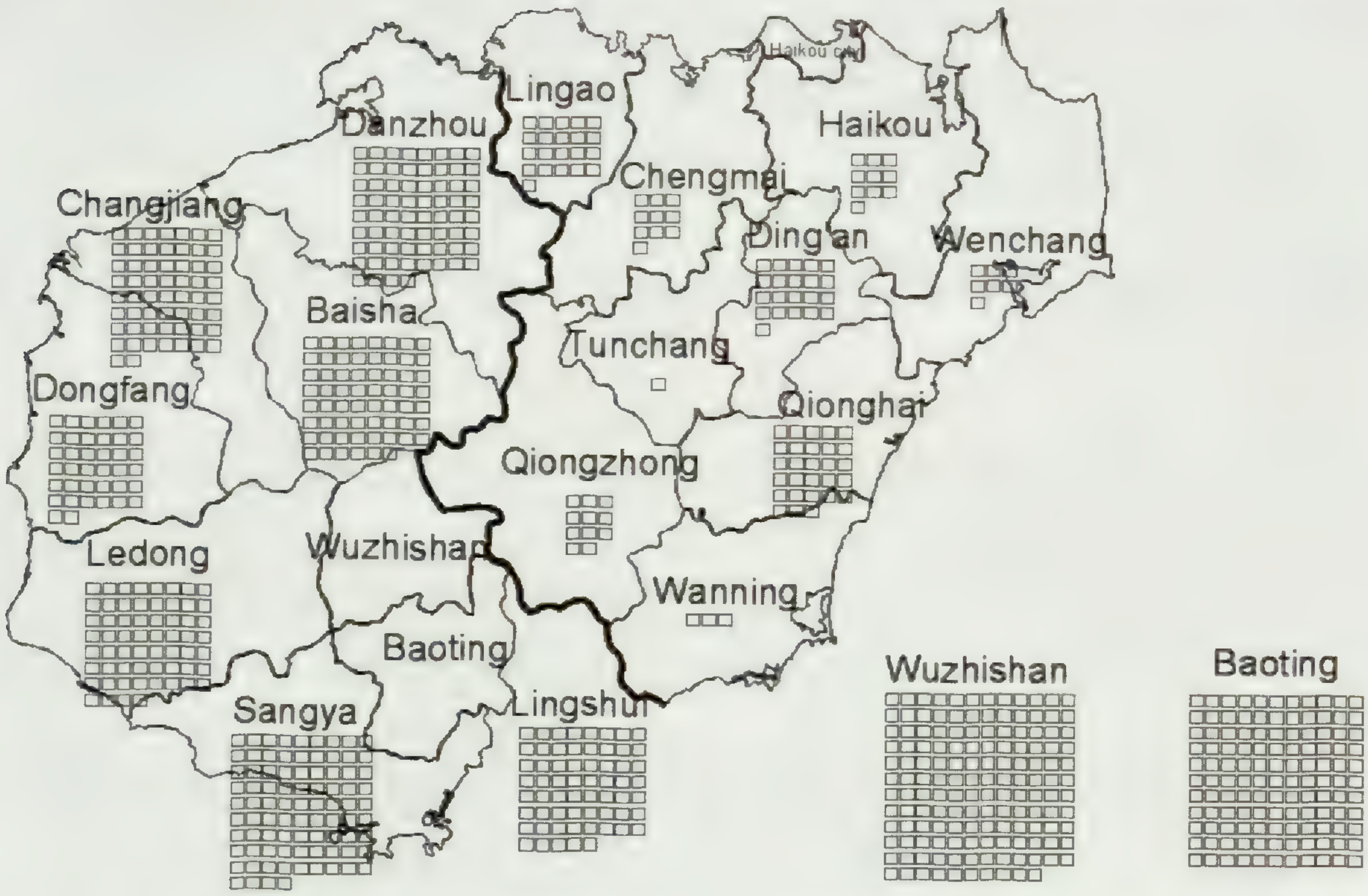

FIG. 2. The distribution of pteridophyte species in Hainan Island (follows the Hainan map of 2004). The thick line is the "Lingao - Qiongzhong – Wanning" line. Each grid indicates a pteridophyte species. The distribution data shows few species in the northeast and more in the southwest in Hainan Island. There is an obvious boundary line; in the nine distributions northeast of this line (Lingao, Chengmai, Qiongzhong, Haikou, Wenchang, Ding'an, Tunchang, Qionghai, Wanning) there are 43 fern genera (36%), and 79 species (17%). In the nine distributions southwest of the line (Danzhou, Changjiang, Baisha, Dongfang, Ledong, Baoting, Sanya, Lingshui, Tongzha) there are 113 fern genera (94%), and 433 species (95%). Some species are located in the northeast and southwest, and are counted in each region.

1954, 1957; Wu, Qin, 1991; Chen, 1964; Qin *et al*, 1959,2001), we identified 32 threatened species (Table 1). Of these, six species were rated critically endangered and 26 were rated endangered, and five species were identified as endemic to Hainan Island.

The endangered pteridophytes of Hainan Island represent a combination of elements from adjacent floras including the Tropics, East Asia, and the Southern hemisphere, and also includes an endemic Hainan element in different proportions (Table 2). Most of the endangered pteridophytes of Hainan Island are from the Tropic flora (almost 79%), while the East Asia and Southern hemisphere elements account for only 6% (relics of paleophytic province) and the Endemics comprise 15% (Yang *et al.*, 2007) of the Hainan pteridophytes.

Tropical pteridophytes have adapted to diverse environmental niches in term of sunlight and humidity, therefore characteristics of niches and habitats of threatened pteridophytes are of great interest. The 32 endangered species have different niches (Table 3). The majority (66%) of the 32 endangered species were epiphytic and semi-epiphytic, while 34% were terrestrial.

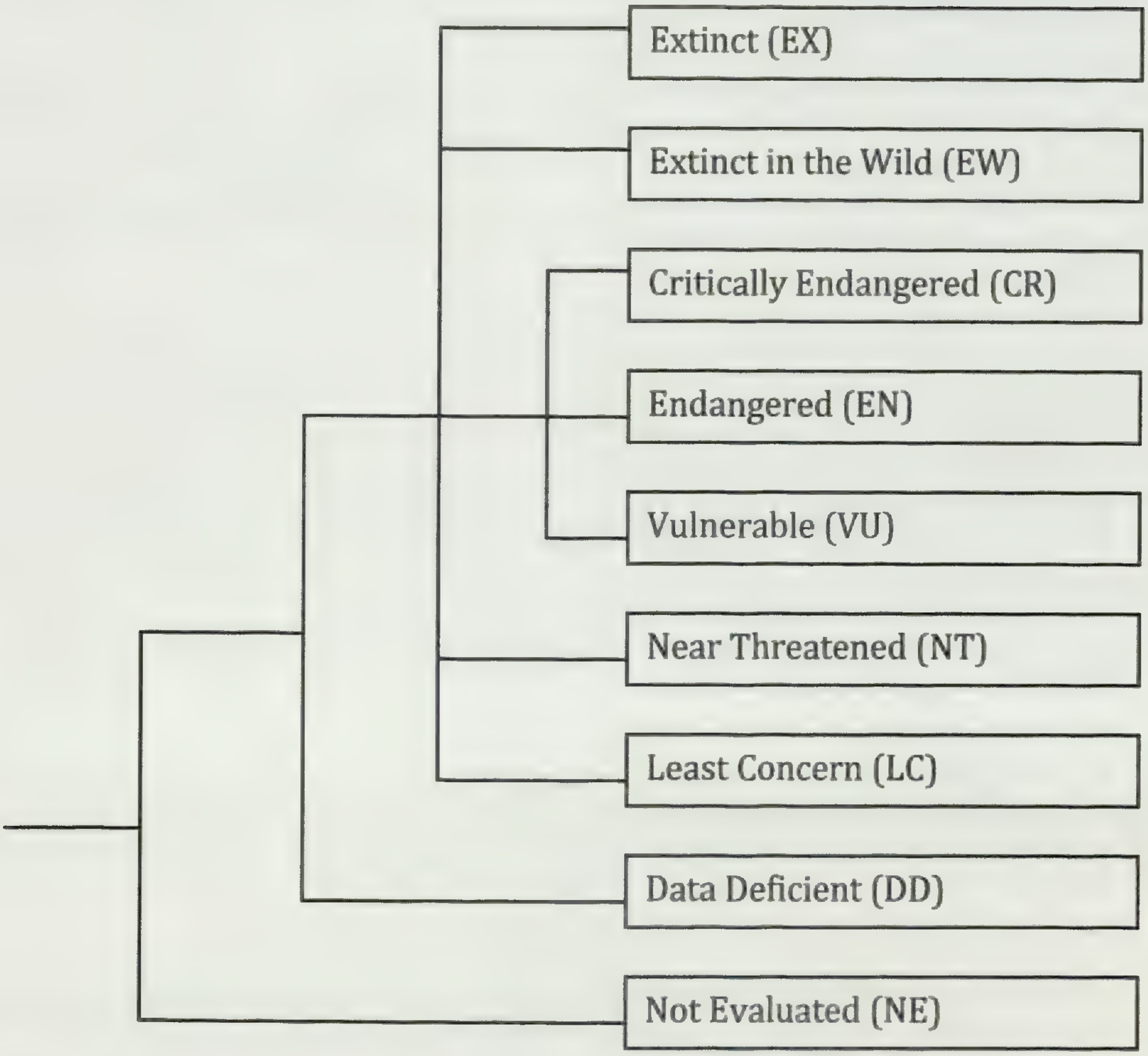

FIG. 3. Structure of the categories at regional level. **EX**: A taxon is Extinct when the last individual has died. No record an individual life form in surveys; **EW**: A taxon is Extinct in the Wild when it is known only to survive in human cultivation; **CR**: A taxon is Critically Endangered when the evidence indicates it is facing an extremely high risk of extinction in the wild; **EN**: A taxon is Endangered when the evidence indicates that it is facing a very high risk of extinction in the wild; **VU**: A taxon is Vulnerable when the evidence indicates that it is facing a high risk of extinction in the wild; **NT**: A taxon is Near Threatened when it is close to qualifying for a threatened category in the near future; **LC**: A taxon is of Least Concern when it has been evaluated against the criteria and does not qualify for Critically Endangered, Endangered, Vulnerable or Near Threatened; **DD**: A taxon is Data Deficient when there is inadequate information to make an assessment; **NE**: A taxon is Not Evaluated when it is has not yet been evaluated against the criteria (IUCN, 2001).

In species-poor families with less than five species, the percentage of species endangered is higher than in species-rich families (Table 4).

DISCUSSION

The endangered pteriodophytes can be divided into three types according to their natural geographical environment and their presence or absence in nature

TABLE 1. Pteridophyte distribution and habitat. CR = critically endangered; EN = endangered; * = endemic species.

Species	Location	Habitat	Level
Psilotum nudum (L.) Beauv.	Bofangling, Ganzhaling, Yajiadaling, Diaoluoshan	Tree trunk or rock aperture. Altitude: 200–500 m	CR
Ophioderma pendula Presl	Diaoluoshan	Tree trunk in rain forest. Altitude: 600–700 m	CR
Schizaea digitata (L.) Sw.	Xinglong, Qionghai, Lehui, Nanshanling	Sandy loam open forest on foothills. Altitude: 2–200 m	CR
Christiopteris tricuspis (Hook.) Christ	Diaoluoshan,Yinggeling	Epiphytic. Altitude: 500–800 m	CR
Helminthostachys zeylanica (Linn.) Hook.	Wanning, Wuzhishan	Under the moist open forest. Altitude: 20–800 m	CR
Gymnogrammitis dareiformis (Hook.) Ching ex Tard.–Balot et C.Chr.	Wuzhishan	Tree trunks or rocks,with bryophyte. altitude: 1500–2700 m	CR
Oleandra undulata (Willd.) Ching	Lingshui, Changjiang, Baoting, Ledong	Rock surface or aperture, hilly. Altitude: 300–1800 m	EN
Oleandra hainanensis Ching	Ledong	Rock surface under forest. Altitude: 300–1800 m	EN*
Hemionitis arifolia (Burm.) Moore	Ledong, Sanya, Luojiang	Wetland under forest, rock aperture and bush. Altitude: 975 m	EN
Diplaziopsis brunoniana (Wall.) W. M. Chu	Nangaoling, Wuzhishan, Bofangling	Undergrowth. Altitude: 1000–1800 m	EN
Dictyodroma hainanense Ching	Nangaoling, Wuzhishan	Valley under forest. Altitude: 800–1000 m	EN*
Boniniella cardiophylla (Hance) Tagawa	Yajiadaling, Bawangling, Baihualing, Wangxia, Wuzhishan, Qionghai	Rocks or sands in forest streamlet. Altitude: 400–600 m	EN*
Brainea insignis (Hook.) J. Sm.	Jianfengling, Qiongzhong	Tailo. Altitude: 450–1700m	EN
Stenochlaena hainanensis Ching et chiu	Wenchang	Valley tree trunks. Altitude: 2–100 m	EN*
Arachniodes hasseltii (Bl.) Cing	Wuzhishan, Diaoluoshan, Jianfengling	Valley under forest Altitude: 900–1200 m	EN
Ctenitopsis sagenioides (Mett.) Ching	Shabaoling, Jianshan	Ravine under rain forest. Altitude: 120–220 m	EN
Lastreopsis subrecedens Ching	Hongmaoshan	Riverside. Altitude: 120–220 m	EN*
Hemigramma decurrens (Hook.) Cop.	Jianfengling, Sanya	Under forest or living on rocks. Altitude: 100–700 m	EN
Lomariopsis spectabilis (Kunze) Mett.	Baishiling, Diaoluoshan, Baoting, Qiongzhong, Wanning	climbing on tree trunks. Altitude: 620–700 m	EN
Acrostichum speciosum Willd.	Qinglan	Coastal wetland. Altitude: 0–35 m	EN

TABLE 1. Continued.

Species	Location	Habitat	Level
Cheiropleuria bicuspis (Blume) C. Presl	Changjiang, Ledong, Baisha	Limestone under forest. Altitude: 1000 m	EN
Dipteris conjugata (Kaulf.) Reinw.	Bawangling	Under forest. Altitude: 600 m	EN
Drynaria rigidula (Sw.) Bedd.	Bawangling, Limushan	Hilly forest or rocks. Altitude: 0–2000 m	EN
Belvisia annanmensis (C. Chr.) Tagawa	Wuzhishan, Jianfengling, Diaoluoshan, Qixianling	Humid tree trunks with bryophyte under forest. Altitude: 800–1000 m	EN
Phynatosorus longissimus (Blume) Pic. Serm.	Danzhou, Ding'an, Baisha	Streamside under open forest. Altitude: 100–240 m	EN
Phymatopteris triloba (Houtt.) Pic. Serm.	Wuzhishan, Diaoluoshan	Tree trunks or wet rocks under open forest. Altitude: 400–1300 m	EN
Schellolepis persicifolia (Desv.) Pic. Serm.	Jianfengling, Diaoluoshan	Tree trunks. Altitude: 700–1000 m	EN
Leptochilus cantoniensis (Baker) Ching	Baoting, Danzhou, Ledong, sanya	Ground or wet rocks under forest. Altitude: 120–280 m	EN
Calymmodon asiaticus Copel.	Wuzhishan, Diaoluoshan	Tree trunks or wet rocks, usually living with bryophytes, 400–1000 m	EN
Grammitis dorsipila (Christ) C. Chr. et Tardieu	Wuzhishan	Under forest or streamside rocks. Altitude: 400–800 m	EN
Prosaptia contigua (G. Forst.) C. Presl	Wuzhishan	Wet rocks. Altitude: 600–1500 m	EN
Seleroglossum pusillum (Blume) Alderw	Wuzhishan	Tree trunks or rocks. Altitude: 800–1000 m	EN

TABLE 2. Floristic element.

Flora type	Ratio	Species
Tropics	9%	*Psilotum nudum, Helminthostachys zeylanica, Ophioderma pendula*
Tropic Asia	50%	*Oleandra undulata, Diplaziopsis brunoniana, Hemionitis arifolia, Brainea insignis, Arachniodes hasseltii, Ctenitopsis sagenioides, Hemigramma decurrens, Lastreopsis spectabilis, Cheiropleuria bicuspis, Dipteris conjugate, Belvisia annanmensis, Christiopteris tricuspis, Phynatosorus longissimus, Phymatopteris triloba, Schellolepis persicifolia, Leptochilus cantoniensis*
Pan-tropics	20%	*Achrostichum speciosum, Drynaria rigidula, Calymmodon asiaticus, Grammitis dorsipila, Prosaptia contigua, Seleroglossum pusillum*
East Asia	3%	*Gymnogrammitis dareiformis*
Southern hemisphere	3%	*Schizaea digitata*
Endemics	15%	*Oleandra hainanensis, Dictyodroma hainanense, Boniniella cardiophylla, Lastreopsis subrecedens, Stenochlaena hainanensis*

reserves. First, some endangered pteridophytes with small populations like *Ophioderma pendula*, *Christiopteris tricuspis*, *Brainea insignis* are only distributed in nature reserves. The possibility of their habitats being destroyed is less likely than if they were outside the preserve. However, it also means the distribution of these pteridophytes is restricted and the population is small, so continued protection is urgent. Secondly, some endangered species, like *Schizaea digitata*, are only found outside of nature reserves. These species are not yet under protection, however the distribution of these species is scattered, and thus they are vulnerable for extinction. These endangered pteridophytes require immediate human intervention for preservation and protection. Thirdly, in contrast to the above, there are some endangered pteridophytes, like *Hemigramma decurrens*, *Drynaria rigidula* and *Leptochilus cantoniensis*, that exist both within and outside the nature reserves but grow well because of their wider distribution and apparently better adaptability. If environmental conditions are appropriate, these species may be eventually removed from the endangered species list. According to the results of our field investigations and information from the literature, we found 24 endangered species which were found within national and provincial nature reserves and national forest parks around the island, accounting for 75% of total endangered species. However, eight endangered pteridophytes are distributed outside nature reserves or only partially distributed in nature reserves, accounting for 25% of total endangered species.

We analyzed the causes of endangered pteridophytes on Hainan Island.

Geological history changes.—The extinction of plant species is a process of interaction between species and the environment. The processes of species

TABLE 3. Niche diversity.

Niche	Ratio	Species
Epiphytic, semi-epiphytic	21 species, 66%	*Psilotum nudum, Ophioderma pendula, Gymnogrammitis dareiformis, Oleandra undulata,Oleandra hainanensis, Hemionitis arifolia, Boniniella cardiophylla, Stenochlaena hainanensis, Hemigramma decurrens, Lastreopsis spectabilis, Cheiropleuria bicuspis, Drynaria rigidula, Belvisia annanmensis, Christiopteris tricuspis, Phymatopteris triloba, Schellolepis persicifolia, Leptochilus cantoniensis, Calymmodon asiaticus, Grammitis dorsipila, Prosaptia contigua, Seleroglossum pusillum*
Ground living	11 species, 34%	*Schizaea digitata, Helminthostachys zeylanica, Diplaziopsis brunoniana, Dictyodroma hainanense, Lastreopsis subrecedens, Brainea insignis, Arachniodes hasseltii, Ctenitopsis sagenioides, Dipteris conjugate, Phynatosorus longissimus, Achrostichum speciosum*

formation, development, endangerment and extinction are determined by a variety of factors, among which genetic variation and dramatic changes in the environment are the most important factors. The genetic variation of a species is defined by a process of accumulation of new mutations and adaptation of species to the changing environment. This evolutionary process takes a long time. Thus, a sudden and drastic change in the environment can endanger the survival of a species of limited amount of genetic variation (Humphries and Parenti, 1999; Craw *et al.*, 1999).

The earth has experienced a number of geological changes in its history – the movement of plates, volcanic eruptions, floods, earthquakes, glaciers, meteorites and other severe environmental rebuilding. Such catastrophic changes wiped out most of the species once flourishing on the planet. Clearly, some species survived and were retained in unique environments or so-called "safe havens", and thus became relic species. After the Cretaceous period, seed plants began flourishing on the earth, whereas the vast majority of spore plants (mainly pteridophytes on land), such as *Rhynia*, *Psilophyton* and *Asteroxylon* of Psilophytina, *Lepidodendron*, *Sigillaria*, *Lepidocarpon* and *Baragwanathia* of Lycopsida, and *Calamite, Hyenia* and *Calamophyton* of Sphenopsida, gradually declined in number and became extinct (Editorial Board of Paleontology Basic Theory, 1983). Of these early genera, only *Psilotum, Lycopodium, Palhinhaea, Lycopodiastrum, Huperzia, Phlegmariurus, Selaginella*, and *Equisetum* remain (Editorial Board of Paleontology Basic Theory, 1983).

Environmental deterioration.—Soil, water and air are the major components of an ecosystem in addition to plants and animals. Industries are booming and the human population is growing in Hainan Island, and development brings along soil contamination, air pollution and acid rain. The island ecosystem is therefore seriously damaged. In addition, logging, local fire, and farm-land expansion accelerate forest destruction. Global warming can cause extreme

TABLE 4. Percentage of endangered species in each family on Hainan Island. The numbers are from Flora of China (Qin *et al.*, 1959, 2001).

Family	Number of species endangered	Total number of species	Percent endangered
Polypodiaceae	6	42	14.2%
Grammitaceae	4	7	57.1%
Oleandraceae	2	2	100%
Hemionitidaceae	1	4	25%
Athyriaceae	2	30	6.6%
Aspleniaceae	2	35	5.7%
Aspidiaceae	2	21	9.5%
Psilotaceae	1	1	100%
Ophioglossaceae	1	2	50%
Schizaeaceae	1	1	100%
Helminthostachiaceae	1	1	100%
Gymnogrammitidaceae	1	1	100%
Thelypteridaceae	1	30	3.3%
Blechnaceae	1	4	25%
Stenochlaenaceae	1	2	50%
Dryopteridaceae	1	25	4%
Lomariopsidaceae	1	2	50%
Acrostichaceae	1	2	50%
Cheiropleuriaceae	1	1	100%
Dipteridaceae	1	1	100%
Drynariaceae	1	4	25%

climate change, which in turn may strongly influence the habitats of vegetation (Chen *et al.*, 2007). The resulting changes in humidity, temperature, sunlight, wind, could also perturb the reproduction and life-cycle of pteridophytes.

Invasions.—In the 1980s, a large number of plant species, including crop species and soil and water conservation plants, were introduced into Hainan Island from Africa and the American continents for the development of local agriculture. Due to the improper management of the introductions and the lack of natural enemies or competitors, these alien plants have proliferated, replacing the native species by producing harmful substances to indigenous plants (Shan, 2006). The allelopathy of alien species can have a devastating impact on the local ecological system, which has been extended to all areas of Hainan Island. *Eupatorium odoratum* L., *Mimosa invisa* Mart. ex Colla, *Eupatorium catarium* Veldkamp, and other invasive plants have been found on the edge of Jianfengling, Bawangling and others have been found in older forest reserves.

Population growth and emigration.—According to Chen (1933) the total human population of Hainan in 1928 was 2,195,600. From 1952 to 1979, up to 330,000 reclamation workers were relocated from the mainland to the island. In 1990, the total population of Hainan was 6,558,100. According to the report of the fifth census, the current population of Hainan is 7,867,500 (Hainan Provincial Bureau of Statistics, 2000). Human population growth and emigration have brought a huge demand for living space, farmland, firewood,

causing destruction of large areas of tropical forests. For example, cultivation of rubber, betel nut and other economic crops that replaced the original vegetation might have led to soil erosion and ecosystem imbalance. In the early 1930s, there were 18 million acres of natural forests on Hainan Island, but by 1984 there were only 3.7 million acres (Situ, 1991).

Hybrid pteridophyte flora.—Hainan Island and the Malaya pteridophyte flora have in common a total of 45 genera, accounting for 35% of all 130 genera of Hainan pteridophytes; the Indian pteridophyte flora has 49 genera in common, accounting for 38%; the Indo-China peninsula pteridophyte flora has 60 genera in common, accounting for 46%; and the East Asia pteridophyte flora has 56 genera in common, accounting for 43% (some genera are like *Lepisorus*, *Colysis* and *Pyrrosia* are distributed in several floras) (Yang *et al.*, 2007; Dong, 2004). The difference of shared genera between Hainan Island and all other regions is not very significant and no single flora component dominates the floristic composition of pteridophytes in Hainan Island (Yang *et al.*, 2007). Therefore, the present pteridophyte flora in Hainan Island is a combination of multiple floras (Wulff, 1964).

The limitations on pteridophyte abundance.—The majority of pteridophytes grow in the mountain rainforests or patchy rainforest valleys. Thus, their habitats are severely restricted. As a result, except for the photophilous pteridophytes and a small fraction of sciophilous pteridophytes, most other pteridophyte populations in Hainan Island are small.

As Hainan Island is at the northern margin of the tropics, it is the southernmost distribution of some pteridophyte species, such as *Gymnogrammitis dareiformis*, which originated from north subtropics or the Himalayas. Such species are distributed only at high altitude as in Wuzhishan (Dong, 2004). Although there are 21 mountains on Hainan Island with a high altitude (above 900 m), these species were only found existing in small population sizes on Wuzhishan Mountain. Since some pteridophyte species originated from the center of equatorial tropics (Editorial Board of Paleontology Basic Theory, 1983), Hainan Island, located at the northernmost boundary, cannot provide the best environments for the survival and reproduction of these species in terms of temperature and sunlight. Thus, these tropical species have a limited range on Hainan Island, and their populations are relatively small. Pteridophytes such as *Ophioderma pendula*, *Schizaea digitata*, *Ctenitopsis sagenioides*, *Schelloiepis persicifolia*, *Drynaria rigidula* have Hainan as their most northern boundary.

In conclusion, flora, niche, and habitat analyses indicated that 8% of the pteridophyte species in Hainan Island are endangered due to a combination of historical and current factors. Some populations are small, not protected by in conservation regions, and are likely facing extinction if not protected. As in many places of the world, the situation is urgent. Our analyses have demonstrated the necessity for protecting pteridophyte diversity in Hainan Island.

Literature Cited

Chen, H. Y. 1964. Hainan Flora. Beijing: Science Press.

Chen, M. S. 1933. Hainan Island chorography. Shanghai: Shenzhouguoguang Press.

Chen, X., Z. L. Tao, Z. X. Wu and Z. D. Zhou. 2007. Analysis of Effects of Climate Changes on Ecological Carrying Capacity in Hainan Island. Journal of South China University of Tropical Agriculture. 13(1):33–37.

Craw, R. C., J. R. Grehan and M. J. Heads. 1999. Panbiogeography - tracking the history of life. Oxford University Press, New York.

Dong, S. Y. 2004. The Classification, Geographical Fauna and Conservation of Ferns on Hainan Island. CAS doctoral dissertation.

Editorial Board of Paleontology Basic Theory (series). 1983. Palaeobiogeographic Provincialism in China. Beijing: Science Press.

Fu, L. G. 1991. China Red Data Book of Plants – Rare and Endangered Plants (Volume 1). Beijing: Science Press.

Fu, S. X. 1954. Chinese Pteridophytes Flora. Beijing: Chinese Academy of Sciences Publishing House.

Fu, S. X. 1957. Introduction and Pictures of China's major plants - Pteridophyta. Beijing: Science Press.

Hainan Provincial Bureau of Statistics. 2000. The Bulletin of fifth population census of Hainan province.

Humphries, C. J. and L. R. Parenti. 1999. Cladistic Biogeography – Interpreting patterns of plant and animal distributions, 2nd ed. Oxford University Press, New York.

IUCN. 2001. IUCN Red List Categories and Criteria version 3.1. Switzerland and Cambridge.

Qin, R. C., G. X. Xing and W. M. Zhu, et al. 1959–2001. Flora of China (Volume 2–6). Beijing: Science Press.

Ren, M. E. 1985. Outline of the Chinese Physical Geography. Beijing: Commercial Press.

Shan, J. L., F. C. Yang and X. Q. Zheng. 2006. Exotic Plants in Hainan Province. Subtropical Plant Science 35(3):39–44.

Situ, S. J. 1991. Historical Land Development Research on Hainan Island. Hainan: Hainan Press.

State Environmental Protection Agency, The CAS Institute of Botany. 1987. The Rare and Endangered Plant List of China. Beijing: Science Press.

State Forestry Administration, the Ministry of Agriculture. 1999. Focal Protection Plant List of China (First batch).

The Hainan Provincial People's Government. 2006. Hainan priority protected terrestrial wild plants directory.

Wu, Z. H. and R. C. Qin. 1991. Families and Genera of Chinese Pteridophytes. Beijing: Science Press.

Wulff, E. B. 1964. Historical Plant Geography. Beijing Science Press.

Yang, F. C., X. W. Hu and L. L. You. 2007. Geographical Distribution and Florisitic Composition of Pteridophytes in Hainan Island. Acta Botanica Ynnanica, 29(2):155–160.

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New and Improved Leaf Terminology for Gleicheniaceae

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Abstract.—The majority of ferns have determinate leaf ontogeny, which makes them suitable for a hierarchal system of leaf terminology to describe dissection and gross morphology. Gleicheniaceae are distinct among fern families because nearly all species have indeterminate and pseudodichotomously forking leaves. Given these two characteristics, the hierarchal system of leaf terminology is inappropriate and cumbersome to use. Therefore, Holttum (1957), Tryon and Tryon (1982), Andersen and Øllgaard (1996), and Lellinger (2002), among others, developed specialized leaf terminology to describe the morphology of Gleicheniaceae leaves. Although each system is sufficient, comparisons among the different systems are cumbersome and confusing. To reduce confusion and simplify, we propose a new leaf terminology system that: 1) is universal to all taxa in Gleicheniaceae, 2) is more useful to apply to partial-leaf herbarium specimens, and 3) clarifies the ambiguity of having multiple leaf terminology systems.

Key Words.—*Gleicheniaceae*, *Dicranopteris*, *Diplopterygium*, *Gleichenella*, *Gleichenia*, *Sticherus*, *Stromatopteris*, leaf terminology, frond terminology, fern

Fern leaf terminology is mostly a hierarchal system that describes a leaf from its base to its tip (Gifford and Foster, 1989; Andersen and Øllgaard, 1996) except for the terms penultimate and antepenultimate which are used to describe the second and third segment basiscopically from the ultimate segment, respectfully. This system works perfectly on the majority of ferns that have determinate leaves (i.e., those that stop growing once they reach maturity). But out of the 37 fern families recognized by Smith *et al.* (2006, 2008), Lygodiaceae and five of the six genera of Gleicheniaceae (i.e., *Dicranopteris, Diplopterygium, Gleichenella, Gleichenia,* and *Sticherus*) have indeterminate leaf growth, due to the repeated breaking of dormancy of the rachis bud, and pseudodichotomously forking pinna (Tryon and Tryon, 1982). Furthermore, under certain conditions, such as damage to or the removal of the rachis bud, some Gleicheniaceae species pinna buds may be reactivated, breaking dormancy and continue to grow (Holttum, 1957). This renders the hierarchal terminology inappropriate because the leaves continue to grow and become more complex over time.

Gleicheniaceae can have considerably large leaves; for example, some species are reported to have leaves that are up to 10 meters long (Gifford and Foster, 1989), with some species growing in dense thickets and scrambling over trees and shrubs (Holttum, 1954), thus making it nearly impossible to collect a whole leaf. Furthermore, botanists usually collect only enough plant material that will fit onto a herbarium sheet, so they will either collect a small portion of a leaf or a small juvenile individual. Both of these sampling techniques lead to incomplete herbarium specimens, which makes it difficult

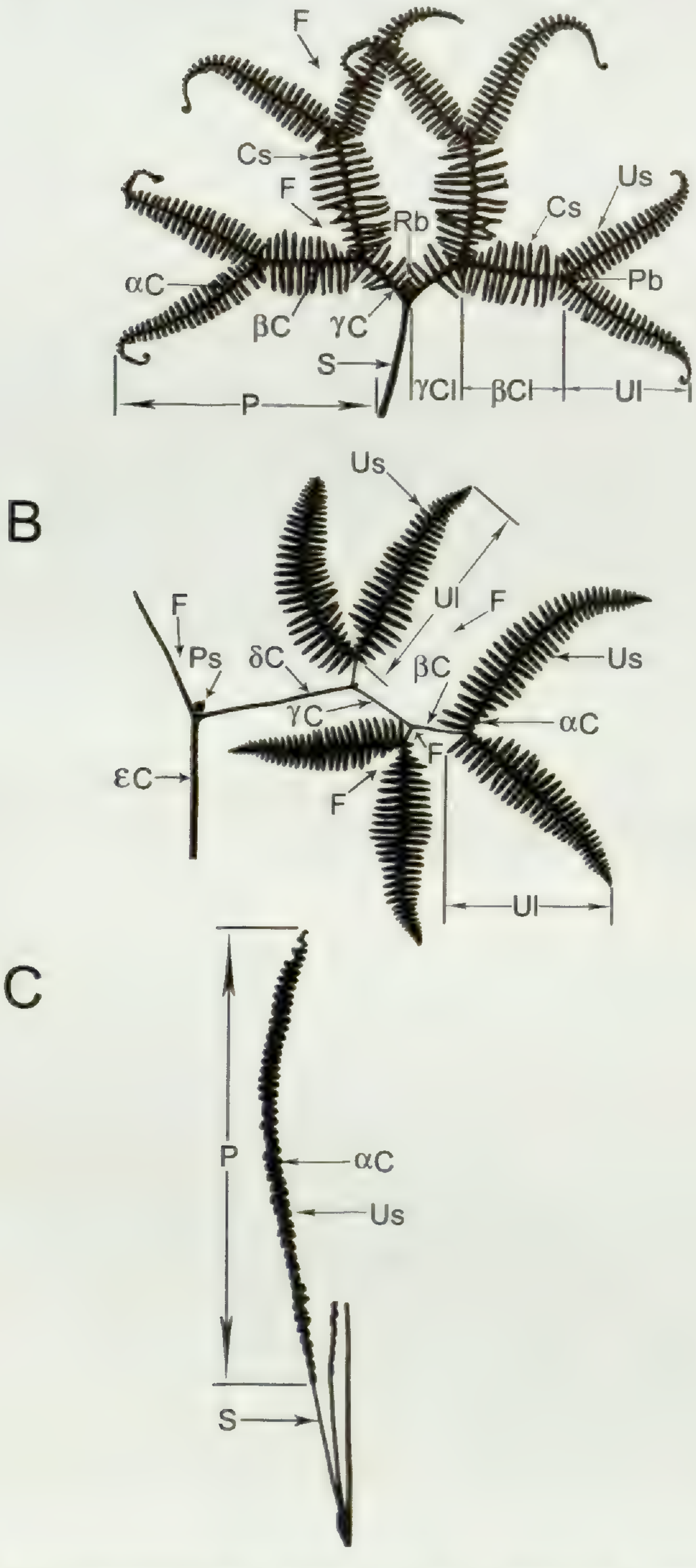

FIG. 1. Illustrations of Gleicheniaceae leaf terminology. A. *Sticherus* B. *Gleichenella* C. *Stromatopteris* D. *Dicranopteris* E. *Diplopterygium* F. *Gleichenia*. **Ac** = Accessory Costa **Al** = Accessory Leaflet, (**α**,**β**,**γ**,**δ**,**ε**) **C** = Costa, (**β**,**γ**) **Cl** = Costal Lamina, **Cs** = Costal Segment, **F** = Fork of pinnae, **P** = Pinna, **Pb** = Pinna Bud, **Ps** = Pseudostipule, **Ul** = Ultimate Leaflet, **Us** = Ultimate Segment, **Rb** = Rachis Bud, **S** = Stipe. Images modified from Smith (1981), Sampson (1985), and Palmer (2003).

D

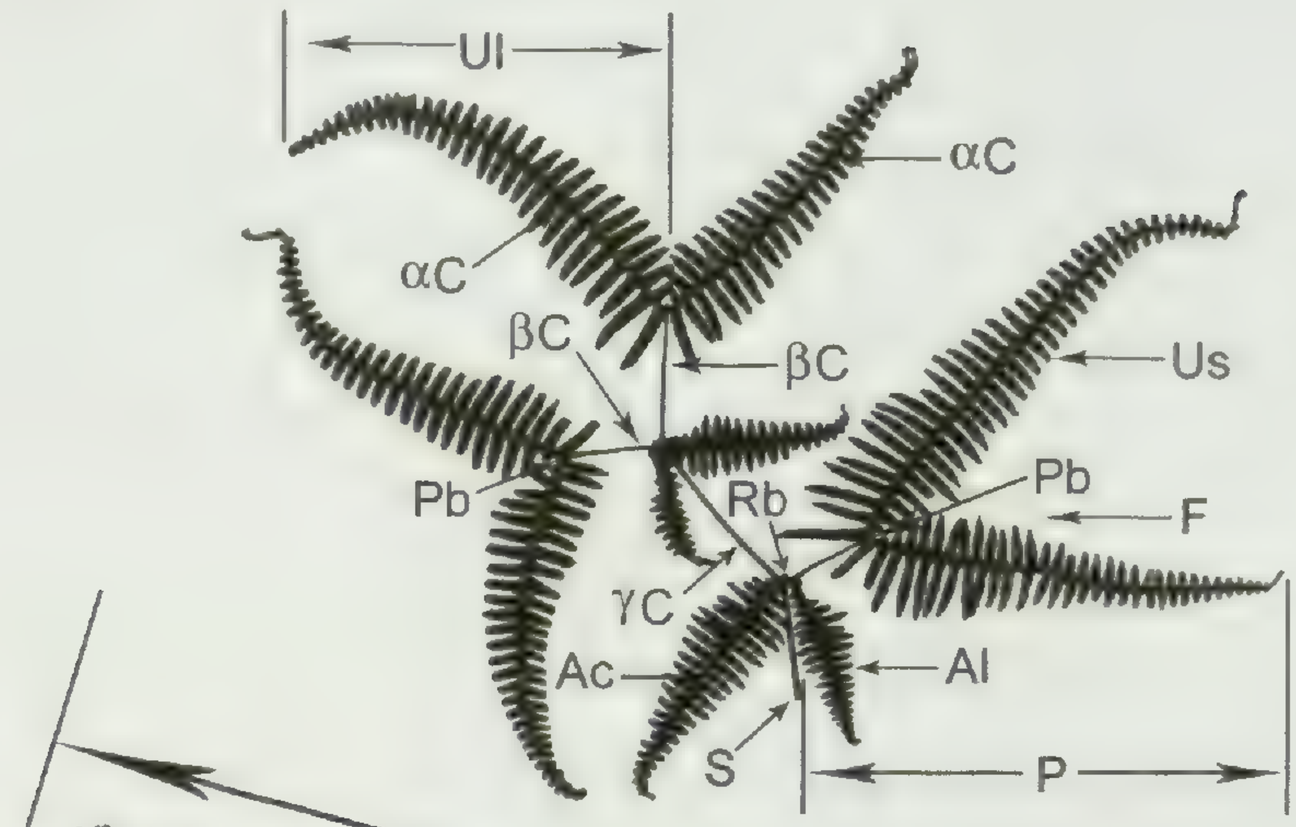

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Fig. 1. Continued.

TABLE 1. Comparisons between Gleicheniaceae leaf terminology systems. Equivalent terms are read across the rows. ***** = Author did not have an equivalent term or did not define the term in their system.

Shaw and Ranker 2011	Lellinger 2002	Andersen and Øllgaard 1996	Tryon and Tryon 1982	Holttum 1957	Nakai 1950
Leaf	Frond	Leaf	Leaf	Frond	Frond
Lamina	Lamina	Lamina	Lamina	Lamina	Pinnule
Stipe	Stipe	*****	Petiole	*****	Stipe, Axis of the frond
Rachis	Rachis	Rachis	Rachis	Rachis, Main rachis, Main axis of the frond	Axis of the frond
Rachis bud	Dormant bud, Latent bud	Rachis bud, Dormant rachis	Rachis bud, Periodically dormant bud, Dormant bud, Leaf bud	Dormant Rachis-apex, Periodic dormancy of the apex of the main rachis, Dormant apex of main rachis, Dormant axis, Leading axis	*****
Pinna	Pinna, Branches	Pinna, Branch pair, Branch	Pinna, Lamina, Primary branch of lamina	Leaves, Primary branches, Frond branches, Primary rachis-branches, Branches of the first order, Lateral branches	Branch, Pinna, Leaves
Pinna bud	Dormant Bud, Latent Bud	Dormant bud, Bud	Periodically dormant bud, Permanently arrested bud, Laminar bud	Permanent dormancy of apices of secondary and lateral branchings, Dormant apex, Permanent dormant apex	Scaly bud
(α,β,γ,δ,ε) Costa	Costa, Costule, Costulet, Penultimate (branch), Antipenultimate (branch)	Costa, Branches that do not bear segments, Branches, Pinnules, Proximal branch orders, Lower levels of branching	Branches, Pinnate branch, Pinna-rachis, naked axis, axes of lower order, Lamina branches, Petiole	Primary rachis-branch, Costa, Primary branches, Secondary, tertiary, fourth order branches, Branches of the first, second, third, fourth, etc., order, Penultimate branches, Lateral branch, Axes of lower order, Branches, axes immediately beyond the forks	Axis of the frond, Rachis, Terminal branch, Costa, Rachides, Branchlets, Right-handed, left-handed, middle branchlet, Terminal branchlet, ultimate branchlets, Segments, Lateral branches

TABLE 1. Continued.

Shaw and Ranker 2011	Lellinger 2002	Andersen and Øllgaard 1996	Tryon and Tryon 1982	Holttum 1957	Nakai 1950
Costal lamina	Lamina, Segment	Segments	Pinnatisect penultimate segments	Leaflet, Axes of lower order that are leafy, Leafy lamina	Pectinate lobes (on axis of frond), basal pinnules
Costal segment	Segment	Segments	Ultimate segment, Penultimate segment	Lobe, Lamina-lobe, Lateral leaflets	Lobe, Pinnules, Leaf-segments
Ultimate leaflet	Ultimate branch	Ultimate branch	Ultimate branch	Leaflet, Lateral branch, Ultimate branch	Terminal pair of pinnae, Terminal branches, pinnae, Ultimate pinnules, Ultimate branchlets
Ultimate segment	Segment	Segment	Ultimate segment, Primary segments	Lobe, Lamina-lobe, Lateral leaflet	Lobe, Ultimate lobe, Pinnules, Leaf-segments
Pseudostipules	Pseudostipules	Pseudostipules	Stipular segments	Lobed leaflet	Aphlebia
Accessory leaflet	Sessile accessory branch	Accessory branch	Accessory branch	Accessory branch, Leaflet	Basal Pinnule
Accessory costa	*****	*****	*****	*****	*****
Midvein	Midvein	Midvein	*****	Midrib, Costule	Midrib, costa
Vein	Vein	Veinlet, Veinlet groups	Vein	Vein	Vein, Veinlet
Fork	Branched, Bifurcate	Pseudodicotomies, Branchings, Dichotomies	Pseudodicotomiesly Branched, fork, Pseudodichotomous	Branching pattern, Branched, Leaf branching, Pseudo-dichotomy, forking, forks	Forked, Dichotomously compound, Frond ladderly compound, Forking
Lobe	Lobe, Lobate	Lobe	*****	Slightly lobed	Lobe

or impossible to compare different taxonomic systems and in some cases apply the hierarchal system of leaf terminology.

Due to the atypical leaf development of Gleicheniaceae, many authors have developed specialized leaf terminology to describe the morphology of Gleicheniaceae leaves. The most cited systems are those of Nakai (1950), Holttum (1957), Tryon and Tryon (1982), Lellinger (1989, 2002), and Andersen and Øllgaard (1996). Although each system describes the morphology of the leaf adequately, each system could use improvement since some of the terms are confusing, especially to non-experts. For example, Nakai (1950), Holttum (1957), and Tryon and Tryon (1982) used terms such as "ultimate branch", "fourth order branches", and "right-handed branchlet" when they described how the pinnae pseudodichotomously split or the number of bifurcations that are in a single pinna.

Another problem occurs when one tries to compare two or more leaf terminology systems. Since each author coined their own terms, they sometimes used a different term for an identical part to which a different term had already been applied by another worker (e.g., Holttum's (1957) lobed leaflet is the same leaf appendage as Tryon and Tryon's (1982) stipular segment). In addition, different authors have used the same term to describe different parts (e.g., a lobe sensu Holttum's (1954) does not equal a lobe sensu Andersen and Øllgaard (1996)). Consequently, comparisons among the different Gleicheniaceae treatments can be unwieldy and perplexing.

Finally, some terminological systems do not apply to all six genera within Gleicheniaceae. For example, when Bierhorst (1971) constructed his leaf terminology system, he believed that *Stromatopteris* should not be assigned to Gleicheniaceae, but should be in its own family (Stromatopteridaceae). Therefore, he did not include *Stromatopteris* in his Gleicheniaceae leaf terminology system and used different terms to describe *Stromatopteris* leaf morphology. Since that time, molecular data have shown that *Stromatopteris* is within the Gleicheniaceae clade (Smith *et al.* 2006, 2008; Schuettpelz and Pryer, 2008) and, thus, it should be included under a Gleicheniaceae leaf terminology system. *Stromatopteris moniliformis* Mett. and *Gleichenia simplex* (Desv.) Hook. both have simple pinnatifid leaves, as well as a few other species that have less complex leaf architecture than normally found within the Gleicheniaceae, but to keep the terminology uniform throughout family, we applied the same terms used throughout, even though the conventional terminology works well on these species.

To address these problems, we propose a new leaf terminology system that is universal to all taxa in Gleicheniaceae and will facilitate working with partial-leaf herbarium specimens.

Unlike all other Gleicheniaceae leaf terminology systems, the one we propose starts from the distal tips of the pinna and continues proximally to the leaf base. Although this direction of description is unconventional, it works satisfactorily on Gleicheniaceae's atypical leaf growth and on incomplete herbarium specimens.

The following is a glossary of the terms we have adopted for this terminology system. Figure 1 illustrates how these terms apply to each genus in

Gleicheniaceae. The terms are a mixture of those assimilated and modified from those of earlier authors (especially Andersen and Øllgaard's (1996) and Lellinger's (2002)) and novel terms that we have coined. Table 1 is a comparison of the major Gleicheniaceae leaf terminology systems over the past 60 years compared to our new system. Each author's leaf term has been aligned in accordance with our new system. This will facilitate quick comparisons among all the different treatments and aid in understanding which part each term represents. The terms we used in our leaf system are based on the following conditions: the terms are functional for all taxa of Gleicheniaceae; they are applicable to incomplete herbarium specimens; and the terms are explicit and precise to simplify Gleicheniaceae leaf terminology.

Glossary

Accessory Costa: the major axis of the accessory leaflet.

Accessory Leaflet: supplementary lamina division that is borne basiscopically near a fork in the **(α,β,γ,δ,ε)** costae, such as the sessile leaflets subtending larger portions of the pinnae in some *Dicranopteris* species.

(α,β,γ,δ,ε) Costa: the major axis of the pinna. Subdivided by forking into equal or unequal sections. Each subsection is designated by a Greek letter starting from the apex (ultimate leaflet) and proceeding proximally towards the rachis/stipe.

Costal Lamina: the expanded portion of a leaf located on β, γ, δ, and/or ε costae, usually consisting of costal segments or lobes. Not always present because β, γ, δ, and/or ε costae may be naked.

Costal Segment: a portion of a costal lamina that is fully adnate to β, γ, δ, and/or ε costae and with deep sinuses on each side and that extends more than fifty percent of the segment length. *Cf.* Lobe.

Fork: a division in the pinna of two equal or unequal sections.

Lobe: a portion of the ultimate segment, costal lamina, accessory leaflet, or pseudostipule that is fully adnate to β, γ, δ, and/or ε costae, and/or accessory costae and with a shallow sinus on either side that extends less than fifty percent of the lobe length. *Cf.* Ultimate segment and Costal segment.

Midvein: the central axis of an ultimate segment or costal segment.

Pinna: the primary division of the leaf, that typically narrows at its base.

Pinna Bud: a bud borne at the apex of a costal axis that is flanked by two younger costal axes. Normally this bud stays dormant.

Pseudostipule: a small, foliaceous, stipule-like structure borne within a fork that subtends and protects a pinna bud.

Rachis: the central axis of a compound leaf.

Rachis Bud: a bud borne at the apex of the rachis that is flanked by two pinnae. This bud may break dormancy allowing the leaf to continue to develop.

Stipe: the central axis of a leaf that connects the base of the lamina to the rhizome.

Ultimate Segment: a portion of an ultimate leaflet, that is fully adnate to an α costa with deep sinuses on each side and that extends more than fifty percent of the segment length. *Cf.* Lobe.

Ultimate Leaflet: the smallest or last order of division of the pinna. Usually borne on a β costa, but in some species can be found on γ, δ, and/or ε costae.
Vein: a strand of vascular tissue, especially one in the laminar tissue of the ultimate segment or costal segment. Usually forked one or more times.

This top-down system simplifies Gleicheniaceae leaf terminology by being applicable to all taxa in Gleicheniaceae and to partial-leaf herbarium specimens. Finally, it reduces the perplexity of having more than one Gleicheniaceae leaf terminology system.

Acknowledgments

We thank the Ranker Lab members (and extended family) for their comments on the leaf terminology system, MO, NY, and UC for specimen loans, and the reviewers for the comments and suggestions. SWS thanks the University of Hawai'i Department of Botany, Westmore Association, and Westmore Ladies Aid Society for financial support.

Literature Cited

Andersen, E. Ø. and B. Øllgaard. 1996. A note on some morphological terms of the leaf in the Gleicheniaceae. Amer. Fern J. 86:52–57.

Bierhorst, D. W. 1971. *Morphology of Vascular Plants.* The Macmillan Co., New York.

Gifford, E. M. and A. S. Foster. 1989. *Morphology and Evolution of Vascular Plants.* Third Edition. W. H. Freeman and Company, New York.

Holttum, R. E. 1954. *A revised Flora of Malaya: an illustrated systematic account of the Malayan flora, including commonly cultivated plants.* Second Edition. Government Print Office, Singapore.

Holttum, R. E. 1957. Morphology, growth-habit, and classification in the family Gleicheniaceae. Phytomorphology 7:168–184.

Lellinger, D. B. 1989. *The Ferns and Fern–allies of Costa Rica, Panama, and the Chocó (Part 1: Psilotaceae through Dicksoniaceae).* American Fern Society, Washington.

Lellinger, D. B. 2002. *A Modern Multilingual Glossary for Taxonomic Pteridology.* American Fern Society, Washington.

Nakai, T. 1950. A new classification of Gleicheniales. Bulletin of the National Science Museum Tokyo 29:1–71.

Palmer, D. D. 2003. *Hawai'i's Ferns and Fern Allies.* University of Hawai'i Press, Honolulu.

Sampson, B. F. 1985. *Early New Zealand Botanical Art.* Reed Methuern Publishers, Auckland.S-Scanned by www.nzetc.org.

Schuettpelz, E. and K. M. Pryer. 2008. Fern Classification. Pp. 395–416, *in*: T. A. Ranker and C. H. Haufler, eds. *Biology and Evolution of Ferns and Lycophytes.* Cambridge University Press, Cambridge.

Smith, A. R. 1981. *Flora of Chiapas. Part 2 Pteridophytes.* California Academy of Science, San Francisco.

Smith, A. R., K. M. Pryer, E. Schuettpelz, P. Korall, H. Schneider and P. G. Wolf. 2006. A classification for extant ferns. Taxon 55:705–731.

Smith, A. R., K. M. Pryer, E. Schuettpelz, P. Korall, H. Schneider, P. G. Wolf and P. 2008. Fern Classification. Pp. 417–467, *in*: T. A. Ranker and C. H. Haufler, eds. *Biology and Evolution of Ferns and Lycophytes.* Cambridge University Press, Cambridge.

Tryon, R. M. and A. F. Tryon. 1982. *Ferns and Allied Plants with Special Reference to Tropical America.* Springer–Verlag, New York.

American Fern Journal 101(2):125–126 (2011)

Shorter Notes

Lectotypification of *Asplenium sellowianun* C. Presl ex Hieron. and Related Names.—During the development of the Project "Flórula del Parque Nacional Mburucuyá", subproject "Flora Pteridofitica", some nomenclatural problems were found, including the need to lectotypify *Asplenium sellowianum* and the names which are included in the synonymy.

The name *Asplenium sellowianum* was first published by Presl (*Tent. Pterid.*:107. 1836). Presl placed this taxon in the subgenus *Asplenium*, within the species group of "frons herbacea". In this work, the author mentions only the specimen *Sellow 46*, but provides neither a description nor a diagnosis to validate the name.

In 1897, Hieronymus validly published two varieties of *Asplenium lunulatum* Sw., *A. lunulatum* var. *tenerrima* and *A. lunulatum* var. *sellowiana* (*Bot. Jahrb. Syst.* 22:359–420. 1897). In the protologue of the var. *sellowiana* description there is no reference to the name published by Presl or to the specimen *Sellow 46*.

Later, in 1919 Hieronymus validly published (*Hedwigia* 60:210–266. 1919) the name *A. sellowianum*, explicitly attributing the name to Presl, and citing *Sellow 46* first among several syntypes. Furthermore, Hieronymus placed his variety *A. lunulatum* var. *sellowiana* as a synonym. These events clearly indicate that Hieronymus believed that there was a single taxon.

In a recent systematic treatment Sylvestre and Ponce (Monogr. Syst. Bot. Missouri Bot. Gard. 107(1):1–8. 2008) treated the authorship of this name as: *Asplenium sellowianum* (Hieron.) C. Presl ex Hieron., with the parentheses indicating that Hieronymus changed the status of the var. *sellowianum* when he transferred it to a species. From my perspective, Hieronymus's intention was to validate Presl's name and not to propose a new combination or a change of status. For this reason, it would not be necessary to use parentheses, because the name *A. lunulatum* var. *sellowiana* is a synonym and not the basonym. In addition, Hieronymus's authorship should not go inside the parenthesis, preceding Presl's abbreviation, since Presl published his name before Hieronymus. According to the I.C.B.N. (Vienna Code) Chapter IV. Effective and Valid Publication, Section 3, Art. 46 about Author Citations, Note 4: *Authors publishing new names and wishing to establish that other persons' names followed by "ex" may precede theirs in authorship citation may adopt the "ex" citation in the protologue* (see ex. 39). For these reasons, in my opinion, the correct authorship citation must be *Asplenium sellowianum* C. Presl ex Hieron., or simply Hieron. would be sufficient.

Lectotypification

Asplenium sellowianum C. Presl ex Hieron., *Hedwigia* 60: 222–3. 1919.

A. lunulatum Sw. var. *sellowiana* Hieron. *Bot. Jahrb. syst.* 22: 377. 1897. *A. ulbrichtii* Rosenst. var. *sellowianum* (Hieron.) Osten and Herter, *Anales Mus. Nac. Montevideo* ser. 2, 1: 349. 1925.—*LECTOTYPUS* (*hic designatus*): [Uruguay], Montevideo, *Sellow d483* (Holotype: B 20 0022113, Photo CTES!, Isotype: B 20 0022109, Photo CTES!)

=*A. lunulatum* Sw. var *tenerrima* Hieron., *Bot. Jahrb. syst.* 22: 377–378. 1897. *A. ulbrichtii* Rosenst.var. *tenerrimum* (Hieron.) Osten and Herter, *Anales Mus. Nac. Montevideo* ser. 2, 1: 349. 1925.—*LECTOTYPUS* (*hic designatus*): Uruguay, Montevideo, february, 1875, *Arechavaleta 403* (B 20 0024537, Photo CTES!).

A. sellowianum C. Presl *nomen nudum, Tent. Pterid.*: 107. 1836.

The isotype of *A. sellowianum. Sellow d483* (B 20 0022109), is glued on a sheet together with other specimens; the isotype is the plant on the right side.

I propose the specimen *Sellow d483* as the lectotype of *A. sellowianum* for several reasons. Most importantly, all of the specimens mentioned by Hieronymus are homogeneous. The specimen chosen here maintains the concept of Hieronymus. Secondly, I have written to several herbaria (B, BAF, BR, G, K, PRC, R, SGO, VT, LYJB, HAL, W, and I reviewed MVFA, MVFQ, MVJB, MVM), asking about the specimen *Sellow 46* cited by Presl (1836) and Hieronymus (1919), but specimen *Sellow 46* could not be found. Third, this specimen, *Sellow d483*, has a complete rhizome, and well preserved fronds. Two of the fronds have proliferous apices, a character that is used by several authors in order to characterize this species and relatives (e.g.: Capurro, 1969. División Pteridofita, in: Cabrera (ed.), *Fl. Prov. Buenos Aires*, Colecc. Ci. Nac. Tecnol. Agropecu. 4(1):123–246.; Sehnem, in Reitz (ed.), *Fl. Il. Catarinense* 1. (ASPL):1–96. 1968). .

Sellow initiated the enumeration of his collections several times, for this reason he used letters as prefix to index the localities of origin. The only specimen found with the number 46 corresponds to a sample of *Varronia multispicata* (Cham.) Borhidi (Boraginaceae) (microfiche Field Museum of Natural History, Chicago (F), F0052394F). This specimen is a type of *Cordia multispicata* Cham. (Feuillet. C. J., *Bot. Res. Inst. Texas* 2:837–842. 2008)

Asplenium sellowianum occurs in southern Brazil, Paraguay, Uruguay, and in Argentina to Buenos Aires province (Sylvestre and Ponce, 2008). The species inhabits forests of the Paranaense Province, in the Amazonian phytogeographical dominion, according to Cabrera (*Bol. Soc. Argent. Bot.* 14:1–42. 1971).

I thank A. Krapovickas and M. M. Arbo for critically reading the manuscript; R. Vogt (B), G. C. Giberti (BAF), and Manuel García (MVM) for providing the phototypes; the managers of herbaria mentioned for answering my questions; and the reviewers for useful comments on the manuscript.—Esteban I. Meza Torres, Instituto de Botánica del Nordeste, Sargento Cabral 2131, C.C. 2009, Corrientes, Argentina, e-mail: mezatorresii@yahoo.com.ar.

American Fern Journal 101(2):127–128 (2011)

Shorter Notes

Distributional Update of *Alsophila cuspidata* (Kunze) Conant from Paraguay, and New Synonymy.—During the palynological studies of the Southern Cone Cyatheaceae (Marquez *et al.*, Rev. Paleobot. Paynol. 156:165–176. 2009), questions arose about the occurrence of *Alsophila cuspidata* (Kunze) D.S.Conant in Paraguay. This species, distributed from Nicaragua to northern Bolivia and through the Andes, has also been reported in Paraguay by Gastony (Gastony Contr. Gray Herb. 203:81–148. 1973), due to the fact that he included *Cyathea hassleriana* H.Christ in its synonymy [sub *Nephelea cuspidata* (Kunze) R. M. Tryon].

Alsophila cuspidata is very similar to *A. sternbergii* (Sternb.) D.S.Conant, which occurs in central-eastern and southern Brazil (Fernandes, Taxonomia e fitogeografia de Cyatheaceae e Dicksoniaceae nas Regiões Sul e Sudeste do Brasil. Doctoral thesis, Universidade de São Paulo. 1997). The species are distinguished by their leaf trichomes, by the scales of the petiole, and by their disjunct geographical distribution. This work verified that the material of these species from Bolivia and Peru corresponded to *A. cuspidata*, and that from Brazil and Paraguay was linked to *A. sternbergii*. Likewise, the material examined in the herbaria determined as *Cyathea hassleriana* coincided with *A. sternbergii*. To verify its identity, the type specimens of *C. hassleriana* and *C. rojasii* H.Christ (= *A. sternbergii* sensu Gastony, 1973), also from Paraguay, were studied. The morphology of trichomes and scales at the base of the petiole are consistent with the diagnostic characteristics of *A. sternbergii*. The trichomes found on the indusium and on the veins of the abaxial surface have several arms contorted, like those of *A. sternbergii* [vs. trichomes with 2–3 (4) arms straight in *A. cuspidata*]. The scales have an elongated apical seta, sometimes a second shorter seta, and just 1–2 marginal spaced setae or even none, while the scales of *A. cuspidata* have several apical setae (shorter) and numerous evenly distributed marginal setae (Tryon and Tryon, Ferns and allied plants with special reference to tropical America. Springer-Verlag, New York. 1982). The shape, the color and the ripening of the indusium are similar in both species. The spores of type material correspond to a typical pattern, which is similar in both species; they are trilete, hemispheric in equatorial view and have short cristate-ridges, distributed randomly on the surface.

Based on this morphological evidence and distribution, *A. cuspidata* is excluded from Paraguay, and *A. sternbergii* is considered to be present there, thus forming the new following synonymy:

Alsophila sternbergii (Sternb.) D.S.Conant. J. Arnold Arbor. 64(3): 371. 1983. *Cyathea sternbergii* Sternb., Fl. von Vorwelt 1: 47. 1820. Type: "Habitat in Brasiliae Capitania Goyaz ad Limoero non procul St. Izidro" *J.B.E.Pohl s. n.* (holotype BR, PCR or W not seen; isotype BR not seen, BM fragment and photographs digital image BM000937587!). *Cyathea hassleriana* H. Christ,

Bull. Herbier Boissier, sér. 2,7: 926. 1907. Type: "Arborea 2–3 m. in silvis humidis pr. Caacupe mens. In September", *E.Hassler 120* (Holotype, not located, isotype S fragment!) ***syn. nov.*** Note: Although the holotype is usually mentioned at P, it is not found in this herbarium (Curator communication). *Cyathea rojasii* H. Christ, Fede Repert. 6: 348. 1901. Type: Paraguay, Amambay, Sierra de Amambay, *E.Hassler & T.Rojas 10 414* (lectotype P not seen, digital image P00642406!, designed by Gastony, 1973).

DESCRIPTION AND ILLUSTRATION.—Gastony 1973: 132–137, Fig 81–87.

GEOGRAPHIC DISTRIBUTION.—Central-eastern and southern Brazil and eastern Paraguay.

STUDIED MATERIAL.—Paraguay. **Canindeyú**, R. N. Mbaracayú, 08.03.1996, *Marín & Jiménez 332* (CTES, PY), idem, 10.05.1996, *Jiménez Marín & GM 217* (CTES, MO, PY), idem, Ao. Vista Alegre, Yerbales, 7.1921, *Rojas 3862* (SI); **Paraguari**, Cerro Leon, 7.1881, *Balansa 2861* (P, CORD, SI).

OBSERVATIONS.—Gastony (1973) cites the specimen of Paraguay, *Balansa 2861*, as one part *A. cuspidata* and the other *A. sternbergii*. We analyzed several duplicates of *Balansa 2861* deposited in the CORD, SI and P (digital images) herbaria, and this study has made it clear that both belong to *A. sternbergii*, present in the Paranaense region.—GONZALO J. MARQUEZ, Cátedras de Palinología y Morfología Vegetal, Facultad de Ciencias Naturales y Museo, UNLP, Paseo de Bosque s/n, 1900, La Plata, Buenos Aires, Argentina, e-mail: cosme@fcnym.unlp.edu.ar, and M. MÓNICA PONCE, Instituto de Botánica Darwinion, Labardén 200, B1642HYD San Isidro, Buenos Aires, Argentina.

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Review

Ferns and Fern-allies of Sikkim, A Pictorial Handbook Part-I, by B. S. Kholia, Botanical Survey of India, Sikkim Himalayan Regional Center, Gangtok. 2010. Published by the Botanical Survey of India and Sikkim State Biodiversity Board. Price: Rs. 750/- & US $ 40. ISBN 978-81-909680-1-0. For information contact Anil Mainra (anil_mainra@yahoo.com) or U. Lachungpa (ulachungpa@gmail.com).

Sikkim is a tiny state (7,096 km^2) located in the northeastern part of India. Its habitats stretch from the alpine areas of the high Himalayas (8500 m) to subtropical valleys (300 m) that receive over 500 cm of rainfall per year. It is one of only three ecological hotspots in India. With so many different habitats, it is not surprising that an area only 0.2% that of all of India harbors 50% of India's 1000 pteridophytes. The Sikkim State Biodiversity Board has begun to document the incredible diversity of this area, and assigned the challenge of producing a pictorial handbook to B. S. Kholia who is a very experienced pteridologist and extremely competent photographer. The volume is Part 1 of the documentation of all of the pteridophytes of the state and includes 150 species and over 600 color photographs.

The guide, which is small enough to carry into the field, begins with a very comprehensive description of the characteristics of lycophytes and ferns. Over 90 excellent photographs illustrate various morphological features of ferns. For example there are 25 close-up photos of rhizomes, illustrating many of the types of phyllotaxy and branching possible. While less attention is given to gametophytes, the life cycle is clearly diagrammed illustrating the standard alternation of generations. There is a key to guide the user to the species names, but this would perhaps be more useful when the full flora of 500 ferns species has been illustrated in additional handbooks in the series. The bulk of the book is devoted to some excellent photos and descriptions of the sporophytes. The sequence is alphabetical within genus and family and for each of the 21 families a page corner is marked with a unique color. The text for each species includes habitat, distribution (including elevation), morphological description (including leaf length, which provides scale for the photos) and other notes. Two to four photos of each species show the leaves and sori as well as other key features such as the vegetative propagules that proliferate on the rachis of *Monochosorum henryi*.

The goal of this handbook is to attract the attention of the population of Sikkim and to make it easy for them to appreciate and value the amazing fern diversity there. With its emphasis on beautiful color photos, it should appeal. However sections on edible ferns and other human uses of ferns as well as the conservation threats appear at the back of the book where they can easily be missed. The rationale for caring about ferns could perhaps have been the starting point for the book. Sikkim has the highest percentage of protected lands of all of the Indian states, with 80% under Forest Department

management. However there are still many threats to ferns and their habitats caused by recent increases in population in the area such as cardamom plantations, ornamental harvesting, clearing of forests and water pollution.

This pictorial guide can have international appeal, particularly because of the photos. The author has used the latest taxonomic concepts in naming the ferns thus the handbook can provide a useful supplement to unillustrated floras. It is especially interesting to note the variety of species in genera that are also well-known in North America. Many of the ferns of Sikkim would be hardy in gardens throughout the world. For example, the fledgling horticultural industry in India could focus on the commercial potential for propagating a plant like *Acrophorus paleolulatus*, a fern with 3–4 pinnate leaves up to 1.5 m long that are bright red as they emerge and expand. The book's accessible format would be a good model for state and local floras anywhere. I would recommend this book to anyone who appreciates the worldwide diversity of ferns.—JOANNE M. SHARPE, Sharplex Services, PO Box 499, Edgecomb ME 04556.

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Review

Ferns and Fern-allies of Sikkim, A Pictorial Handbook Part-I, by B. S. Kholia, Botanical Survey of India, Sikkim Himalayan Regional Center, Gangtok. 2010. Published by the Botanical Survey of India and Sikkim State Biodiversity Board. Price: Rs. 750/- & US $ 40. ISBN 978-81-909680-1-0. For information contact Anil Mainra (anil_mainra@yahoo.com) or U. Lachungpa (ulachungpa@gmail.com).

Ferns and their allied plants are of little economic significance to humankind as compared to other groups, especially Angiosperms; hence these are poorly understood, overlooked or often neglected by the society. None the less, being the major constituent of biodiversity their role in the system of nature cannot be neglected or overlooked in the global changing environmental scenario. Since the time of Beddome (the father of India Pteridology), there have been several books made available either on enumerations or detailed taxonomic accounts of ferns and fern allies in various regions of India. The majority of these are solely taxonomic and technical, based on herbarium specimens, and are mainly useful for academicians, particularly taxonomists. Moreover, most of these are not fully illustrative and few have created great confusion by naming new species based on injudicious taxonomic characters. Insufficient knowledge about the species, species complexes, habitat and study of plants in the field are often not well described. Furthermore, pteridophyte taxonomy has become more complicated due to rapid changes in nomenclature, and there is no general consensus about species and species complexes in India or globally. Due to these complexities most of the published taxonomic works have not attracted the attention of general botanists, nature lovers, nurserymen and society towards this group of plants.

In light of this history, Dr. B. S. Kholia has published *Ferns and Fern-allies of Sikkim, A Pictorial Handbook Part-I* for which he deserves complements. This handy 207-page account is an authoritative and well illustrated manual on Indian Pteridology and was published for the first time in India, providing a brief account of the Pteridophyta, and key characters of species are highlighted along with beautiful live pictures.

Any nature lover cannot overlook the floral diversity around him/her, particularly when he/she comes across such places. In the preface, the author (who was born, grew up, and got his education in fern-rich localities) mentioned how he learned fern diversity. The contents of the handbook beginning from the Introduction to descriptions of species are beautifully illustrated. In the Introduction, the author has precisely given some basic information about ferns and fern allies with the help of suitable pictures which, the reviewers believe will be of immense use for students, amateurs, teachers and others to understand the major morphological attributes of this neglected group of plants. This is followed by a field key completely based on

field characters, which was needed in the field of Pteridophyte taxonomy. In the pictorial description, which is the main part of book, brief descriptions of species starts with genus *Adiantum*. In total, 150 species in this part of the handbook are briefly described. Each species is described in a single page with 3–4 pictures, family name, common name, habitat, distribution and salient attributes. The photographs of the plant, either showing its habitat, or simply a frond or its sori or venation are portrayed attractively, and efforts were made to highlight the diagnostic characters of species with the help of these good quality photographs. Wherever possible, the local names of species are provided in addition to the commercial or trade names of some species of Sikkim having horticultural potential. Distinguishing taxonomic characteristics and brief ecology are also described under 'when and where the Pteridophytes grow'. After the Introduction and Pictorial description, the third chapter of book provides the uses of ferns and fern-allies in Sikkim. This chapter is also illustrated with the help of suitable pictures where the author has documented the pteridophytes used socioeconomically by local inhabitants as food, cattle bed, and in traditional medicine systems along with their local (Nepalese and Limbu) names and uses. In the following chapter information is provided about the ideal methods for collection of live material from nature or for studying them in nature without causing harm to the pteridophyte flora. Towards the end of the book, several natural and manmade threats to biodiversity in general and to pteridophytes in particular are also highlighted. Several conservation measures are also suggested.

Taxonomists may be surprised by the treatment and arrangement of genera and species given in this manual, where instead of following any recognized system of classification the author simply followed the alphabetical arrangement of families followed by the alphabetical ordering of genera and species within each families described in his manual. In a floristic work like this, one should have followed one of many classifications available. However, here author claims that he is writing this book for general public and nurserymen in addition to students of taxonomy. It is hoped that the Part II of the book with remaining known species of Sikkim Himalaya will come out soon.

Overall, it is a commendable attempt where the author has presented a novel idea for writing floristic books. Such types of pictorial work is equally useful for students of taxonomy, morphology and other fields of applied sciences like ecology, molecular biology, cytology, etc. for the correct identification of taxa in nature and at herbaria, as well as for creating awareness among society and stakeholders of biodiversity who can play a key role in the conservation and sustainable use of biodiversity.

Efforts made by the author and the Botanical Survey of India, as well as Sikkim State Biodiversity Board are commended for publishing such a nice handbook in the International Year of Biodiversity. The price of the book is affordable. This book should be a part of bookshelves of teachers, nurserymen, nature lovers, plant enthusiasts, herbaria, gardens, plant scientists and libraries of different teaching and research organizations.—P. B. KHARE, Scientist, National Botanical research Institute, Lucknow (India), N.Punetha, Pithoragarh.

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Equisetum Xylem: SEM Studies and their Implications

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Abstract.—Scanning electron microscope (SEM) studies of xylem of three species of *Equisetum* reveal numerous details not previously reported on the basis of light microscopy. SEM images of thick (ca. 1 mm) sections reveal pit shapes, cell contexts, and microstructure of pit membranes. Pit shapes are remarkably diverse in comparison to those of ferns or conifers. Nodal tracheary elements are isodiametric to fusiform in shape, and have crowded circular (mostly) to elongate prominently bordered pits and uniformly thick secondary walls. Internodal tracheary elements, by contrast, have relatively large circular pits with inconspicuous borders. Secondary walls of internodal tracheids are thin, with thickenings that are annular, looplike, or of some intermediate form. Metaxylem internodal tracheary elements line the inner surface of carinal canals (= proxylem lacunae), and many of the large circular (often crateriform) pits facing the canals may lack pit membranes, especially in *E. giganteum* and *E. myriochaetum*. Because dye experiments show that carinal canals can conduct water in stems of *Equisetum*, the fact that portions of tracheary elements facing the canals may have perforations (many of the pits in *E. myriochaetum* lack pit membranes despite careful handling techniques) is significant. This opens the possibility that internodal tracheary elements may, in some species, be vessel elements that permit conduction from the carinal canals of one internode to those of the next internode (carinal canals are not intercontinuous between internodes), aided by metaxylem tracheids of the nodal plates. Such vessel elements would not be the same as those reported by Bierhorst, who found vessels only a few vessel elements long in rhizomes.

Key Words.—carinal canal, conduction, pit dimorphism, tracheids, vessel elements

Bierhorst (1958a, 1958b) offered careful light microscope observations and figures of *Equisetum* xylem. Fine though that work was, it invites additional observations. He did not study the genus worldwide—he concentrated on temperate North American species. He used thin sections, which, by their nature cannot offer three-dimensional views of tracheary elements, individually and in groups. The contours of secondary wall thickenings can be examined only to a limited extent by light microscopy. The appearance and fine structure of pit membranes can be seen clearly in face view with scanning electron microscopy (SEM), whereas staining and high power light microscopy are limited in what they can show. Bierhorst's observations have not hitherto been supplemented by SEM studies.

SEM reveals a hitherto unappreciated diversity and distribution of pit sizes, shapes, pit border shapes, and secondary wall thickenings. By studying these types with relation to location in the shoot systems, we are able to see distribution of tracheary elements types and pitting that suggest patterns of

water conduction in xylem and in the carinal canals. There is considerable polymorphism of tracheary elements within any given section, a diversity that shows that exploration within a plant body of a species of *Equisetum* is justified at present. Our results can point the way, however, to what genus-wide studies might be useful in the future, and what tracheary element features might deserve intense examination with SEM and with transmission electron microscopy (TEM). The diversity of pitting on *Equisetum* tracheary elements is not only considerable, it exceeds the diversity seen in ferns.

SEM is labor-intensive, and the limited observations of the present study cannot be monographic in focus; they are, rather, an exploration of xylary diversity in *Equisetum* xylem. We have selected three species that are taxonomically not closely related, according to the data of Hauke (1978). Plants of *Equisetum hyemale* L. have unbranched aerial stems with terminal strobili, and are typically a meter or less tall; the species belongs to subgenus *Hippochaete*. *Equisetum myriochaetum* Schlecht & Cham. is one of two species of giant horsetails (the other is *E. giganteum* L.), with plants to five meters tall, in *Equisetum* subgenus *Equisetum*. *Equisetum telmateia* Ehrh. is branched, less than 1 m tall, and belongs to subgenus *Equisetum*. Subgenus *Hippochaete* has strobili at tips of chlorophyllous branches, whereas subgenus *Equisetum* possesses strobili on achlorophyllous branches; the chlorophyllous branches are sterile. These and other distinctions of the subgenera and species are covered in detail by Hauke (1978) and Guillon (2004). We were able to study both aerial and underground stems of *E. hyemale* and *E. telmateia* subsp. *braunii* (Hilde) Hauke. The rhizomes are slender and elongate in *E. hyemale*, whereas in *E. telmateia* they are comparable in thickness to the aerial stems.

Equisetum myriochaetum is distinctive by virtue of the fact that most metaxylem tracheary elements are located on the adaxial or inner surface of the carinal canal (= protoxylem lacuna). Few metaxylem tracheary elements occur within the xylem embedded between the pair of phloem strands. In the other species, the reverse is true: most of the metaxylem elements are flanked by the two phloem strands, and a relatively small number (plus protoxylem) occur lining the carinal canal. The occurrence of metaxylem lining the carinal canal has interesting implications for conduction. Studies testing uptake with the use of dyes have shown that the carinal canals are filled with water and do function in upward conduction (Buchholz, 1921; Bierhorst, 1958b). Because of these observations, the potential role of xylem elements facing the canal becomes a question of importance. The carinal canals of one node are not interconnected with those of the next node above: they alternate in successive nodes, and terminate at the tops and bottoms of nodes. Thus, if the carinal canals play a role in conduction, the metaxylem tracheary elements must be responsible for conduction across nodal regions. The conductive pathways in *Equisetum* stems may thus involve carinal canals, internodal tracheary elements, and nodal metaxylem working in conjunction. This has implications for interpretation of tracheary elements as seen by SEM.

Material and Methods

Aerial stems and rhizomes collected for study were cut into nodal and internodal segments and preserved in 50% ethanol. The collections documenting this study are: *E. hyemale* L.: CA, Santa Barbara Co., ditch adjacent to road, Parma Park on Highway 192 north of Santa Barbara, March 18, 2010, *S. Carlquist & E. L. Schneider s.n.* (SBBG); *E. myriochaetum* Schlecht. & Cham.: CA, Alameda Co., cultivated in University of California Botanical Garden greenhouses, Berkeley, (accession number 78.00364); *E. telmateia* Ehrh. subsp. *braunii* (Hilde) Hauke ("*E. telmateia*" in this paper): CA, cultivated near visitor entrance of Santa Barbara Botanic Garden, and adventive there, 25 March 2010, *S. Carlquist s.n.* (SBBG).

Thin longisections of nodes of *E. hyemale*, made into permanent slides in 1958 by the senior author, and transections of E. *giganteum* prepared by A. W. Haupt were studied with light microscopy. Free-hand longitudinal sections about 1 mm in thickness were prepared by use of single-edged razor blades. These sections were subjected to three changes of distilled water to remove water-soluble substances and to remove loose starch grains and surface contaminants. Sections were then placed between pairs of clean glass slides with pressure applied by clips to assure flatness and air dried at 60° C on a warming table. Sections were sputter coated with gold and examined with a Hitachi S2600N SEM.

Thick sections cut by hand (e.g., Carlquist and Schneider, 2007; Schneider and Carlquist, 2007) have proved preferable to thin sections cut by microtome because damage from handling is minimized. Thick sections also permit one to see three-dimensional structure of tracheary element surfaces and to see cell groupings, such as the relationship of tracheary elements to each other and to carinal canals.

The term "tracheary element" is used here as an inclusive term for both tracheids and vessel elements, because the entirety of a tracheary element was not visible, and thus we could not designate a xylem cell with certainty as either a tracheid or a vessel element. Bierhorst (1958b) reported a few vessel elements in the rhizomes of several species (based on seeing tracheary element end walls in sectional view), but our findings suggest that vessel elements may be present more widely, hence our choice of the more inclusive term "tracheary element."

Results

Internodal tracheary elements.—Pits vary greatly within a stem or rhizome of *Equisetum*, depending mostly on location. Tracheary elements may be divided into nodal and internodal. Internodal metaxylem tracheary elements are elongate, and have secondary walls that appear to have annular thickenings forming bands (note contrast between Figs 1 and 2 and Fig. 3A, B) not unlike those of protoxylem (Fig. 1F). Sometimes the bands are looplike or helical

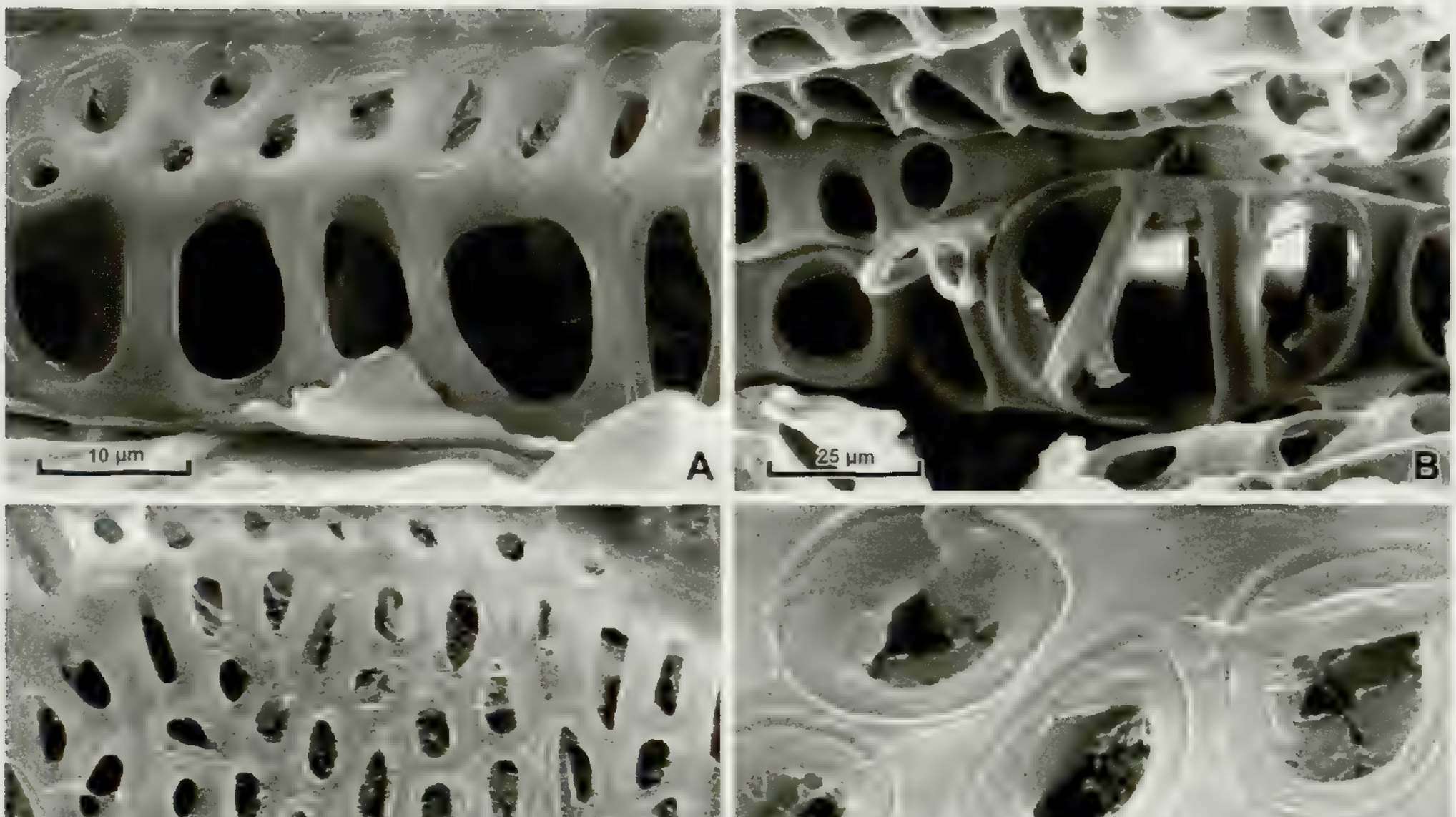

FIG. 1. SEM micrographs of longisections of stems (A–D) and rhizome (E–F) to show tracheids of *Equisetum hyemale*. A) Internodal tracheid, two adjacent facets that show marked difference in size, shape, and border width of pits. B) Portions of several internodal tracheids; pits are markedly unlike in size and shape. C) Tracheid from node; pits are densely placed. D) Pits from tracheid at node; sectioning has scraped away pit membrane material to various extents, so that amorphous material shows in the upper pit, but the cellulosic reticulum is revealed on the lower pits; note wide borders. E) Metaxylem tracheid portion from node; the densely-placed pits have minute depressions in pit membranes. F) Protoxylem tracheid from node; sectioning has removed part of the primary wall between the annuli. Scales, 10 µm (A, C); 25 µm (B); 3 µm (D–F).

rather than annular (Fig. 3A–C). Pits were observed to lie between such bands. Pits have narrow, but perceptible borders.

Large circular pits, often crateriform in shape, may be found along the surfaces of internodal metaxylem tracheary elements that face carinal canals (Fig. 2A–D, Fig. 3B–D). The three-dimensional shape of these pits not been

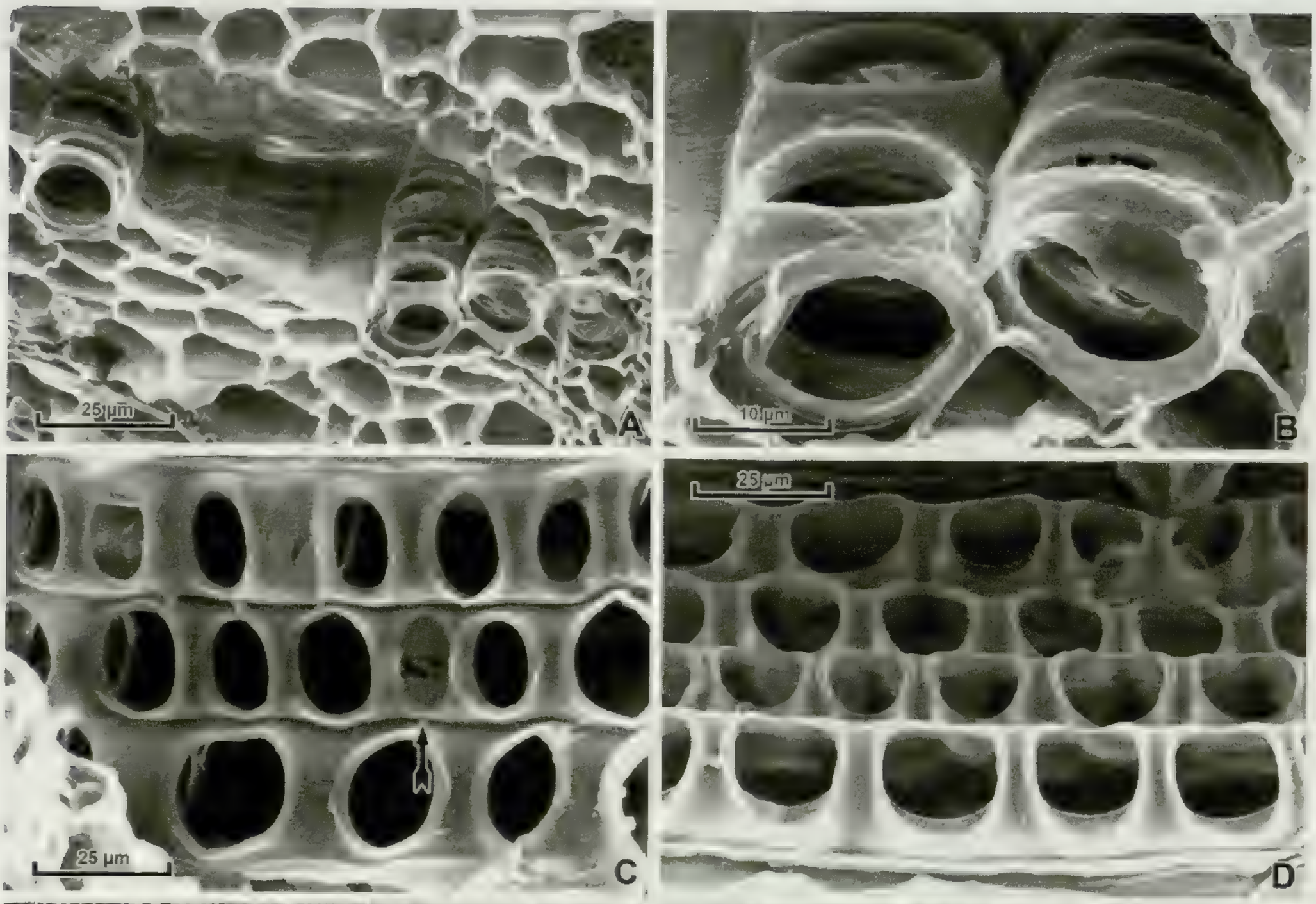

FIG. 2. SEM micrographs of internodal (A–D) and nodal (E–F) metaxylem tracheids from stem longisections of *Equisetum myriochaetum*. A) Oblique view of carinal canal from end of section; portions of three tracheids (one at center left, two at lower right) face the canal. B) Photo at higher magnification of pits from A, to show crateriform shape of pits (above) and wall thickness of tracheids (below). C) View of the surface of tracheids that face a carinal canal; most pits lack pit membranes, but a few smaller pits (e.g., arrow) retain pit membranes. D) Somewhat oblique view of the portions of several tracheids, facing a carinal canal; all pits shown lack pit membranes. E) Tracheids from nodal region; pits are densely placed. F) Portions of adjacent nodal tracheid; the cellulosic reticulum of the pit membranes is revealed by the sectioning process. Scales, 25 µm (A, C); 10 µm; 100 µm (E); 2 µm (F).

described hitherto, probably because it is obviously only when imaged by means of SEM. The crateriform pits mostly lacked pit membranes in our preparations, except for those depicted in Fig. 3C. These pits should not have been affected by sectioning, because they lie within the carinal canals, and

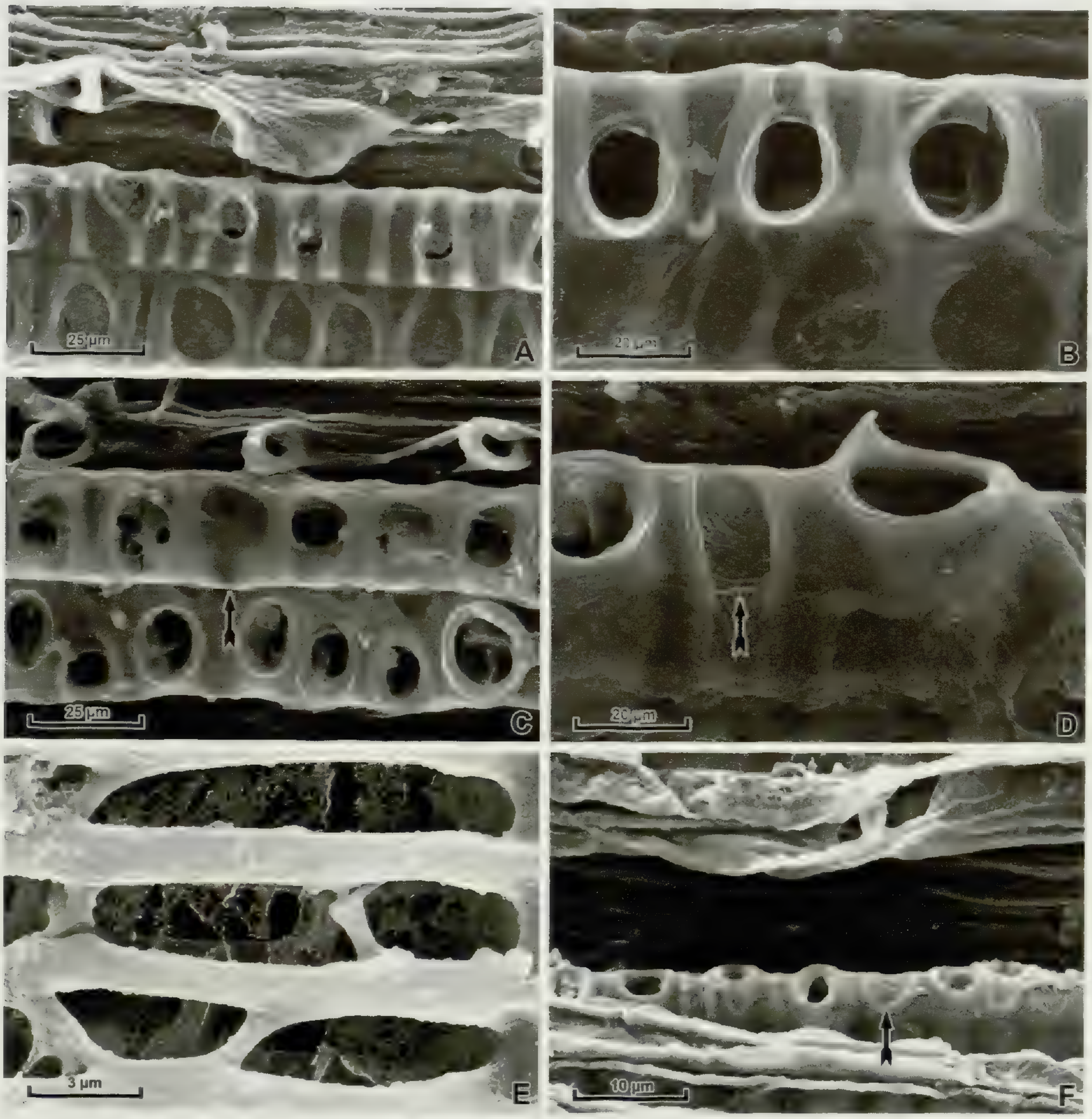

FIG. 3. SEM micrographs of internodal (A–D, F) and nodal (E) tracheids from stem (A–E) and rhizome (F) longisections of *Equisetum telmateia*. The tracheid surfaces depicted in A–E and F face carinal canals. A) Portions of two adjacent tracheids; pits are smaller, and lie between wall annuli in the upper tracheid. B) Three pits lacking pit membranes, located between annular wall thickenings. C) A protoxylem tracheid (top) and, below it, two metaxylem tracheids; Pit membrane portions are present in the metaxylem tracheids, only the pit indicated by the arrow has a pit membrane. D) Two crateriform pits lacking pit membranes (= perforations?) and, between them, a non-crateriform pit (arrow) with an intact pit membrane. E) Pits from cut-away surface of nodal tracheid; reticulate cellulosic pit membrane portions are revealed. F) Portion of internodal tracheid that faces a carinal canal (the canal, center of photograph, is dark gray). A non-crateriform pit with an intact pit membrane (arrow) and four crateriform pits that lack pit membranes (= perforations?) are shown. Scales, 25 µm (A, C); 20 µm; 3 µm (E); 10 µm (F).

were exposed to view but not touched by the sectioning process. Somewhat narrow, non-crateriform pits were observed occasionally on the surfaces of internodal tracheary elements that face carinal canals. These non-crateriform pits were observed to retain pit membranes (Fig. 2C, 3C, 3D, 3F, as indicated by arrows). The pit membranes of the non-crateriform pits appeared to be thinner and more easily ruptured than the secondary wall portions that lie between the annular thickenings.

The precise location of particular tracheid types in the nodal-internodal continuum is best studied with light microscopy and was not a goal of the present study.

Nodal tracheary elements.—Nodal metaxylem tracheary elements have more densely placed pits, and the pits tend to be polygonal to elongate, with wide borders (Fig. 1C–D, 2E–F). Strips of secondary wall material were observed to traverse or subdivide some elongate pits (Fig. 3E).

Pit membranes.—Pit membranes of metaxylem tracheary elements that are not affected by sectioning appear to be uniform in thickness and non-porose, except for the metaxylem tracheary elements depicted in Fig. 1E and Fig. 3E. However, sectioning often did separate, remove, or partially scrape away layers of the pit membranes. Where that happened, the amorphous layer between adjacent tracheids was partly or wholly lacking, and a reticulum of cellulosic fibrils was revealed (Fig. 1C, 1D, 2F). The pits at upper left in Figure 1D retain the amorphous materials (fracturing in the pit membrane is an artifact).

Stretched primary walls were observable on protoxylem tracheary elements (Fig. 1F, 3C, top). In both of these instances, the front half of the primary wall has been sectioned away, affording a view of the inside of the primary wall. In neither instance was any cellulosic network exposed, even on the sectioned edge of the primary wall (Fig. 1F, above).

Discussion and Conclusions

Pit polymorphism.—All of the species of *Equisetum* studied, despite their differing habits, share the basic plan, in both aerial stems and underground stems (rhizomes) of two distinctive types of metaxylem tracheary elements: internodal and nodal. The former are markedly elongate, with narrowly bordered circular pits and walls thickened with annular bands or variations thereof. Nodal tracheary elements, by contrast, are isodiametric to moderately elongate and have uniformly thick walls and densely arranged pits (smaller than those of the internodal tracheids) angular to circular in shape, with wide borders (Bierhorst, 1958a, 1958b). Tracheary elements with intermediate characteristics may be found at the juncture between nodal and internodal xylem. The dimorphism in tracheary element types is striking, because one does not, for example, find such dimorphism in angiosperms with basal meristem growth, such as bamboo (Carlquist and Schneider, 2011).

The dimorphism of metaxylem tracheary elements may be related to conductive patterns. The nodal tracheary elements are placed so as to

interconnect xylem of the node above with xylem of the node below and with the xylem of lateral branches, if any are present. Barratt (1920), Jeffrey (1899) and Eames (1936, p. 97) give helpful three-dimensional reconstructions of these xylem patterns. Nodal tracheary elements are relatively strong and undergo virtually no elongation, whereas the secondary walls of internodal metaxylem tracheary elements are relatively thin. Protoxylem tracheary elements, like metaxylem tracheary elements, have continuous and intact primary wall cover. Xylem is not, however, of primary significance in the mechanical strength of *Equisetum* stems, because sclerenchyma is relatively abundant (Jeffrey, 1899; Hauke, 1978). Bierhorst (1958a) covered the range of pitting types in *Equisetum* well from the standpoint of light microscopy. The three dimensional views afforded by SEM provide a valuable supplement, particularly in the case of the large circular pits of metaxylem tracheary elements on the surfaces of carinal canals (Figs. 3A–D).

Microstructure of pit membranes of nodal metaxylem pits was well revealed by sections. The razor-blade sectioning method employed shaved away portions of pit membranes. Amorphous material was present in some pit membranes, whereas in others, that material was scraped away, so that a cellulosic network is revealed. Such a cellulosic network is common in pit membranes of tracheary elements of ferns (Carlquist and Schneider, 2007), cycads (Schneider and Carlquist, 2007) and angiosperms (Carlquist and Schneider, 2010). This cellulosic network is embedded within the amorphous (non-cellulosic) substances of the pit membrane, and is not evident unless exposed by sectioning. We observed such a reticulum only in pit membranes of internodal tracheids, not in the nodal tracheids in *Equisetum*. In ferns (Carlquist and Schneider 2007) and monocots (Carlquist and Schneider 2010), pits of end walls of tracheary elements may bear pores that clearly interconnect one tracheary element with another in a series. Those may be considered pre-vessels, or even vessels (if one were to expand the definition of vessel). We did not observe such end walls in tracheary elements of *Equisetum*.

Vessel elements.—Bierhorst (1958b) reported vessel elements in the nodal metaxylem of stolons of a few species of *Equisetum* His report was based on absence of pit membranes between files of two to three tracheary elements. There is no reason to question these observations. However, such short vessels, uncommon in occurrence, would have limited significance at best with respect to conduction.

In the present study, we encountered, on surfaces of internodal tracheary elements that face carinal canals, a large number of crateriform circular pits that lack pit membranes. Because most of these tracheary elements were below the cut surface of sections, we are reluctant to attribute the absence of pit membranes to sectioning. Indeed, scattered among the crateriform pits are occasional non-crateriform pits in which pit membranes are intact. The consistent presence of pit membranes on these non-crateriform pits also suggests that ageing or the action of micro-organisms is not responsible for absence of pit membranes on the crateriform pits. We therefore entertain the possibility that absence of pit membranes on crateriform pits may not be an

artifact. The possible conductive nature of the carinal canals has been mentioned by Westermaier (1884), Sykes (1906), and Barratt (1920), and the dye experiments of Buchholz (1921) and Bierhorst (1958b) reinforce this possibility. Bierhorst (1958b) further enhances this possibility by mentioning "The very intimate connection between the nodal metaxylem and the carinal canals...". If carinal canals do indeed function in conduction, the water column pathway may lead from one carinal canal upward to metaxylem tracheary elements and thence to a carinal canal in the next internode above through the crateriform pits of the internodal tracheary elements. We open the possibility, based on our observations of large numbers of crateriform pits without pit membranes, that at least many of the crateriform pits may be perforations, and that at least some of the internodal tracheary elements may be vessel elements. This possibility seems most prominent in *E. myriochaetum*. The occurrence of such possible perforations facing carinal canals and their extent within the genus need confirmation and further study.

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LITERATURE CITED

BARRATT, K. 1920. A contribution to our knowledge of the vascular system of *Equisetum*. Ann. Bot. 34:201–235.

BIERHORST, D. W. 1958a. The tracheary elements of *Equisetum* with observations on the ontogeny of the internodal xylem. Bull. Torrey Bot. Club 85:416–43.

BIERHORST, D. W. 1958b. Vessels in *Equisetum*. Am. J. Bot. 534–537.

BUCHHOLZ, M. 1921. Über die Wasserleitbahnen in den interkalaren Wachstumszonen monocotyler Sprosse. Flora 114:119–186.

CARLQUIST, S. and E. L. SCHNEIDER. 2007. Tracheary elements in ferns: new techniques, observations, and concepts. Am. Fern J. 97:199–211.

CARLQUIST, S. and E. L. SCHNEIDER. 2010. Origins and nature of vessels in monocotyledons. 12. Pit membrane microstructure in the tracheary elements of *Astelia*. Pac. Sci. 54:607–618.

CARLQUIST, S. and E. L. SCHNEIDER. 2011. Origins and nature of vessels in monocotyledons. 13. Scanning electron microscope studies of xylem in large grasses. Int. J. Plant. Sci. 172(3):345–351.

EAMES, A. J. 1936. *Morphology of vascular plants. Lower groups*. McGraw Hill Book Co., Inc., New York and London.

GUILLON, J.-M. 2004. Phylogeny of horsetails (*Equisetum*) based on the chloroplast *rps4* gene and adjacent noncoding sequences. Syst. Bot. 29:251–259.

HAUKE, R. L. 1978. A taxonomic monograph of *Equisetum* subgenus *Equisetum*. Nova Hedwigia 30:385–455.

JEFFREY, E. C. 1899. The development, structure, and affinities of the genus *Equisetum*. Mem. Boston Soc. Nat. Hist. 5:155–190.

SCHNEIDER, E. L. and S. CARLQUIST. 2007. Scanning electron microscope studies of cycad tracheids. S. Afr. J. Bot. 73:512–517.

SYKES, M. G. 1906. Tracheids in the nodal region of *Equisetum maximum*. New Phytol. 5:129–132.

WESTERMAIER, M. 1884. Untersuchungen über die Bedeutung todter Röhren und lebender Zellen für die Wasserbewegung in der Pflanze. Situngsber. Köngl. Preus. Akad. Wissensch. 1105–1117.

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Phylogenetic Positions of the Enigmatic Asiatic Fern Genera *Diplaziopsis* and *Rhachidosorus* from Analyses of Four Plastid Genes

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Abstract.—Nucleotide sequences from four plastid genes (*rbcL, atpB, atpA, rps4*) were used to infer relationships of *Diplaziopsis* and *Rhachidosorus*. The phylogenetic positions of these two Asian fern genera have been debated, and neither had been included in the most recent global molecular systematic studies of ferns. Our four plastid gene sequence analyses supported a sister relationship between *Diplaziopsis*, *Rhachidosorus* and the North American *Homalosorus*, the monophyletic group of the newly-examined genera is an early diverging lineage of Woodsiaceae, and far away from athyrioid ferns. The inferred relationships of *Diplaziopsis* and *Rhachidosorus* are not consistent with most recent treatments, while, some synapomorphic characteristics are shared with these two genera. Further studies on more morphological characters and gametophytes of these two genera are needed to test these relationship hypotheses.

Key Words.—*Diplaziopsis, Rhachidosorus*, Molecular systematics, *rbcL, atpB, atpA, rps4*

The fern family Woodsiaceae, as circumscribed in the most recent familial classification (Smith *et al.*, 2006), comprises about 15 genera and more than 700 species distributed mainly from tropical America to Old World temperate, which is characterized by monomorphic or nearly monomorphic leaves and vascular anatomy (Tryon and Tryon, 1982). The family exhibits an extensive dysploid series of base chromosome numbers, ranging from 31 to 42, mostly $x = 40$, 41, also 31 (*Hemidictyum*), 33, 38, 39 (*Woodsia*), and 42 (*Cystopteris*) (Smith *et al.*, 2006). The monophyly of Woodsiaceae of Smith *et al.* (2006) is lacking in all broad analyses (Hasebe *et al.*, 1995; Sano *et al.*, 2000; Schneider *et al.*, 2004; Schuettpelz and Pryer, 2007). The more recent fern global phylogenetic analyses showed Woodsiaceae of Smith *et al.* (2006) consists of four well-supported clades: together, *Cystopteris* and *Gymnocarpium* are sister to the rest of eupolypods II; *Hemidictyum* is sister to the asplenioid ferns; and *Woodsia* is sister to a large clade of onocleoid, blechnoid, and athyrioid ferns (Schuettpelz and Pryer, 2007). This is the most inclusive analysis of leptosporangiate fern relationships conducted to date, in which three plastid genes (*rbcL, atpA, atpB*) from 400 leptosporangiate fern species were utilized. However, the taxomically problematic genera *Diplaziopsis*, *Homalosorus* and

Rhachidosorus were not included in this groundbreaking study, and the phylogenetic affinities of these taxa are unclear. Smith *et al.* (2006) tentatively placed these genera in the Woodsiaceae and suggested, "further sampling will likely shed additional light on this subject, and the recognition of several additional families may be warranted."

Diplaziopsis is a problematic genus, which has undergone many systematic changes. Bower (1928) treated it provisionally along with his "Asplenoid ferns", while Christensen (1938) revised his opinion to give it a generic status and treats as a group of ferns with areolate veins under *Diplazium* Sw.. Copeland (1947) defined *Diplaziopsis* by its pinnate leaves, thin lamina texture, anastomosing veins, and sausage-like sori, comprising two species (*D. javanica* and *D. cavaleriana*). Ching (1964a) added two new species from China to *Diplaziopsis*, which were later emended into three species in the flora of China (i.e., *D. javanica*, *D. cavaleriana*, and *D. brunoniana*) (Chu *et al.*, 1999). In geographical distribution, *Diplaziopsis* is essentially an Old World genus mainly from Eastern and Southeastern Asia (Ching, 1964a).

The monotypic genus *Homalosorus* was established by Pichi Sermolli (1973) with *H. pycnocarpos* distributed only in the temperate eastern North America, but other authors have included it in the genus *Athyrium* Roth (Kramer and Kato, 1990) or the genus *Diplazium* (Tryon and Tryon, 1982; Kato and Iwatsuki, 1983). A relationship between *H. pycnocarpos* and *Diplaziopsis* (Tryon and Tryon, 1973; Kato and Iwatsuki, 1983) has been suggested from their similar pinna shape, rachis-grooving, indusia, and spores, although they have different lamina apex, venation, and chromosome numbers (Kato and Darnaedi, 1988; Price, 1990). The sister relationship between *H. pycnocarpos* and *Diplaziopsis* has been supported by *rbcL* (Sano *et al.*, 2000) and *rbcL+rps4* trees (Wei *et al.*, 2010), while a previous *trnL-F* study lent support to the placement of *Diplaziopsis cavaleriana* in *Diplazium* (Wang *et al.*, 2003).

Previously, plants here recognized as *Rhachidosorus* have been included in either *Athyrium* (Tagawa, 1936) or *Diplazium* (Kato, 1977; Kramer and Kato, 1990), but Ching (1964b) later separated those plants into the genus *Rhachidosorus* from South-east Asia, and determined that the genus consists of eight species. The genus *Rhachidosorus* differs from both *Athyrium* and *Diplazium* (or rather *Allantodia* R. Br.; most *Diplazium* species in China have been placed in *Allantodia*) in having thick creeping rhizomes, the scales near the base of stipe and, above all, in the narrow semilunate sori and indusia of the asplenioid type, which are never diplazioid nor athyrioid, and in the spore morphology (Ching, 1964b). Based on previous *rbcL* (Sano *et al.*, 2000) and *trnL-F* analyses (Wang *et al.*, 2003), *Rhachidosorus* does not cluster with either *Athyrium* or *Diplazium* but occupies a position isolated from the other taxa in the eupolypods II; such a conclusion was suggested by Ching (1964b; 1978).

In this study, we use more DNA sequence data from four plastid genes (*rbcL*, *atpB*, *atpA*, *rps4*) to make comparisons with the previous studies, to investigate the phylogenetic relationships of *Diplaziopsis*, *Homalosorus*, and *Rhachidosorus* to other ferns of eupolypods II, and specifically to address whether they belong within athyrioid ferns.

Materials and Methods

Taxon Sampling.—In order to make comparisons with other studies (Sano *et al.*, 2000; Schuettpelz and Pryer, 2007), we assembled three data matrices (Table 2), all of which included newly generated sequences and sequences obtained from GenBank. A total of 98 new sequences were generated for this study, the corresponding voucher specimens have been deposited in the Herbarium of the Yunnan University (PYU). Taxa, vouchers, and accession numbers are provided in Table 1. The first data matrix consisted of 59 *rbcL* sequences, of which 24 were newly generated. The second matrix comprised *rbcL*, *atpB* and *atpA* sequences of 59 taxa, which included 22 *atpB* and 20 *atpA* sequences newly generated in this study plus additional sequences from GenBank. The third matrix comprised *rbcL*, *atpB*, *atpA* and *rps4* sequences of 59 taxa, which included the three sequences of 59 taxa from the second matrix and 32 *rps4* newly generated in this study plus additional sequences from GenBank. Those taxa with incomplete sequences were included in the analyses of the combined data, and the unsequenced fragments were coded as missing data. In order to investigate the phylogenetic relationships of *Diplaziopsis*, *Homalosorus,* and *Rhachidosorus* to other genera, our sampling included 14 of 15 recognized genera in Woodsiaceae as treated by Smith *et al.* (2006). The two previously unincluded Asian genera in the study of Schuettpelz and Pryer (2007), *Diplaziopsis* and *Rhachidosorus*, are represented by two or more species, with each species represented by one or more specimens. In addition, we examined species of Aspleniaceae, Blechnaceae, Onocleaceae, and Thelypteridaceae, which were all included with the Woodsiaceae in the eupolypods II clade of Smith *et al.* (2006). Following the previously published molecular systematic studies of leptosporangiate ferns (Schuettpelz and Pryer, 2007), in which it is well established that eupolypods II is most closely related to eupolypods I, we selected *Drynaria rigidula*, *Dryopteris uniformis* and *Polypodium vulgare* as outgroups.

DNA extraction, gene amplification, and sequencing.—Total genomic DNA was extracted from 2 g of fresh or 1 g of silica gel dried leaves using the CTAB procedure (Doyle and Doyle, 1987). The selected DNA regions were amplified with standard polymerase chain reaction (PCR). The protocols used to amplify four genes were identical and followed Li *et al.* (2004). For information on amplification and sequencing primers, see Table 3.

Sequence analysis.—The obtained sequences have been assigned GenBank accession numbers (Table 1). Alignments of all sequences were performed using Clustal X (Thompson *et al.*, 1997) and subsequently edited manually in BioEdit (Hall, 1999). There were no insertions or deletions (indels) in the protein-coding sequence alignments. Indels were introduced into the alignment of *rps4–trnS* spacer region, in which ambiguously aligned regions were excluded from all analyses. Phylogenetic analyses were investigated by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods in PAUP* 4.0b10 (Swofford, 2002), PHYML 2. 4. 3 (Guindon and Gascuel, 2003), and MrBayes 3. 1. 2 (Ronquist and Huelsenbeck, 2003). For MP

analysis, unweighted analyses were performed by heuristic searches with tree-bisection-reconnection (TBR) branch swapping, the MulTrees in effect, steepest descent off using 1000 random taxon-addition replicates, and one tree held at each step during stepwise addition. Bootstrap analyses (Felsenstein, 1985) were conducted to examine the relative level of support for individual clades on the cladograms of each search (MPBS), using 500 bootstrap replicates and the same tree search procedure as described above. For the ML and BI analyses, the best-fitting model of sequence evolution for each data was identified with the Akaike Information Criterion in Modeltest 3.07 (Posada and Crandall, 1998). The SYM+I+G model was selected for the *rbcL* data set, and the GTR+I+G model was selected for the combined data sets (Table 2). Once the best sequence evolution model was determined, the ML analysis was performed for each data set, the parameters such as base-composition, Gamma-shape, and ratio of invariable sites were also estimated during each ML analysis. Nodal robustness on the ML tree was estimated by the nonparametric bootstrap (500 replicates, MLBS). BI was conducted using MrBayes 3.1.2 with appropriate evolutionary models determined as described above and the default priors. We ran two concurrent analyses, each with four chains of the Markov chain Monte Carlo, sampling one tree every 100 generations of 2 × 1,000,000 generations, starting with a random tree. The first 25% of the samples (5000 trees) were discarded as "burn-in". At this point, the standard deviation of split frequencies was <0.01, indicating that convergence to a stationary distribution had been achieved. The posterior probability (PP) was used to estimate nodal robustness.

Results

The alignment length and the number of included characters for the three data sets are presented in Table 2. The aligned *rbcL* matrix contained 1308 characters, of which 417 were variable. MP, ML and BI analyses of *rbcL* matrix resulted in nearly identical topologies, with several minor differences at the genus level (results not shown). Strong support was lacking along the backbone of the *rbcL* tree. The 50% majority-rule consensus tree revealed that eupolypods II fall into nine lineages (Fig. 1): athyrioids (Woodsiaceae I), Blechnaceae + Onocleaceae, Woodsiaceae II (*Woodsia*, *Prowoodsia* and *Cheilanthopsis*), Thelypterdiaceae, Woodsiaceae III (*Cystopteris*, *Acystopteris* and *Gymnocarpium*), Woodsiaceae IV (*Diplaziopsis* and *Homalosorus*, in shadow in Fig. 1), Woodsiaceae V (*Rhachidosorus*, in shadow in Fig. 1), Aspleniaceae, and Woodsiaceae VI (*Hemidictyum*). In the *rbcL* tree, all four *Rhachidosorus* specimens are united in a single clade; two species of *Diplaziopsis* form another monophyletic clade with *Homalosorus*; the three genera of *Woodsia*, *Prowoodsia* and *Cheilanthopsis* (Woodsiaceae II) are united in a single clade; and the three genera of *Cystopteris*, *Acystopteris* and *Gymnocarpium* (Woodsiaceae III) are united in another one. All four clades are isolated from other genera in the family. *Hemidictyum* in Woodsiaceae is sister to Aspleniaceae with low support (PP = 0.90 and MLBS, MPBS < 50%); the

TABLE 1. Taxa Examined and GenBank Accession Numbers.

Family and Species	*rbcL*	*atpB*	*atpA*	*rps4*[a]	Locality and Voucher[b]
Outgroups					
Drynaria rigidula (Sw.) Bedd.	EF463247	EF463493	EF463811	AY529188	
Dryopteris uniformis (Makino) Makino	EF463183	EF463395	EF463679	**JN168069**	Zhejiang, SG LU/FH44
Polypodium vulgare L.	AB044899	EF463510	EF463841	EF551081	
Aspleniaceae					
Asplenium auritum Sw.	EF463146	EF463327	EF463591	AY549759	
Asplenium normale D. Don	EF463152	EF463337	EF463601	AY549784	
Asplenium trichomanes L.	EF463157	EF463349	EF463613	EF645629	
Hymenasplenium cheilosorum (Kunze ex Mett.) Tagawa	AB014704	EF463350	EF463614	AY549757	
Hymenasplenium unilaterale (Lam.) Hayata	EF452140	EF452020	EF452078	AY459170	
Blechnaceae					
Blechnum gracile Kaulf.	EF463158	EF463351	EF463615	AF313606	
Blechnum occidentale L.	U05910	U93838	EF452080	AF533868	
Brainea insignis (Hook.) J. Sm.	AF533870	**JN168027**		**JN168070**	Yunnan, SG LU/YY4
Struthiopteris eburnea (Christ) Ching	**JN168003**	**JN168028**		**JN168071**	Hubei, SG LU/Z1
Woodwardia prolifera Hook. et Arn.	AY137666	**JN168029**	**JN168049**	**JN168072**	Fujiang, SG LU/WY17
Woodwardia virginica (L.) Sm.	AY137660	EF463359	EF463623	AF533857	
Onocleaceae					
Onoclea sensibilis L.	U62034	EF463488	EF463793	AF425159	
Thelypteridaceae					
Macrothelypteris torresiana (Gaud.) Ching	EF463277	EF463533	EF463873	AF425172	
Pseudophegopteris tibetana Ching et S. K. Wu	**JN168004**	**JN68030**	**JN168050**	**JN168073**	Xizang, SG LU/XZ72
Thelypteris abrupta (Desv.) Proctor	EF463280	EF463536	EF463876		
Thelypteris palustris Schott	U05947	AY612713	EF452127	AF425189	

TABLE 1. Continued.

Family and Species	*rbcL*	*atpB*	*atpA*	*rps4*[a]	Locality and Voucher[b]
Woodsiaceae					
Acystopteris japonica (Luerss.) Nakai	**JN168005**	**JN168031**	**JN168051**	**JN168074**	Sichuan, SG LU/X7
Acystopteris tenuisecta (Bl.) Tagawa	**JN168006**	**JN168032**	**JN168052**	**JN168075**	Yunnan, SG LU/DL2
Athyrium distentifolium Tausch ex Opiz	EF463304	EF463560	EF463901		
Athyrium filix-femina (L.) Roth	U05908	EF463561	EF463902	AF425152	
Athyrium otophorum (Miq.) Koidz.	EF463305	EF463563	EF463904	**JN168076**	Sichuan, SG LU/X8
Athyrium niponicum (Mett.) Hance	D43891	EF463562	EF463903	**JN168077**	Zhejiang, SG LU/FH25
Athyrium yokoscense (Franch. & Sav.) Christ	D43893	EF463564	EF463905	**JN168078**	Jiangxi, SG LU/JX51
				JN168003	
Cheilanthopsis elongata (Hook.) Cop.	**JN168007**	**JN168033**	**JN168053**	**JN168079**	Yunnan, SG LU/JU33
Cheilanthopsis indusiosa (Christ) Ching	**JN168008**	**JN168034**	**JN168054**	**JN168080**	Yunnan, SG LU/JU34
				JN168003	
Cornopteris decurrenti-alata (Hook.) Nakai	D43897	EF463565	EF463906	**JN168081**	Zhejiang, SG LU/P9
Cystopteris fragilis (L.) Bernh.	**JN168009**	**JN168035**	**JN168055**	**JN168082**	Xizang, SG LU/XZ88
Cystopteris kansuana C. Chr.	**JN168010**	**JN168036**		**JN168083**	Xizang, SG LU/XZ89
Cystopteris moupinensis Franch.	**JN168011**			**JN168084**	Yunnan, SG LU/JC18
Cystopteris pellucida (Franch.) Ching ex C.Chr.	**JN168012**	**JN168037**	**JN168056**	**JN168085**	Yunnan, SG LU/JU29
Deparia bonincola (Nakai) M. Kato	D43899	EF463566	EF463907		
Deparia lancea (Thunb.) Fraser-Jenk	EF463306	EF463567	EF463908	AF425153	
Deparia petersenii (Kunze) M. Kato	**JN168013**	EF463568	EF463909	**JN168086**	Hainan, SG LU/V23
Deparia unifurcata (Baker) M. Kato	EF463307	EF463569	EF463910	**JN168087**	Sichuan, SG LU/CQ11
Diplaziopsis brunoniana (Wall.) W. M. Chu	**JN168014**	**JN168038**	**JN168057**	**JN168088**	Yunnan, SG LU/BN10
Diplaziopsis cavaleriana (Christ) C. Chr.	**JN168015**	**JN168039**	**JN168058**		Sichuan, SG LU/CQ1
Diplaziopsis cavaleriana (Christ) C. Chr.	**JN168016**		**JN168059**	**JN168089**	Yunnan, SG LU/XCB13
Diplazium bombonasae Rosenst.	EF463308	EF463570	EF463911		
Diplazium centripetale (Baker) Maxon	EF463309	EF463571	EF463912		
Diplazium conterminum H. Christ	**JN168017**	**JN168040**	**JN168060**	**JN168090**	Sichuan, SG LU/X37
Diplazium cristatum (Desr.) Alston	EF463310	EF463572	EF463913		

TABLE 1. Continued.

Family and Species	*rbcL*	*atpB*	*atpA*	*rps4*[a]	Locality and Voucher[b]
Diplazium dilatatum Blume	EF463311	EF463573	EF463914	**JN168091**	Yunnan, SG LU/B49
Diplazium hachijoense Nakai	EF463312	EF463574	EF463915	**JN168092**	Zhejiang, SG LU/P14
Diplazium legalloi Proctor	EF463313	EF463575	EF463916		
Diplazium plantaginifolium (L.) Urb.	EF463314	EF463576	EF463917		
Diplazium proliferum (Lam.) Thouars	EF463315	EF463577	EF463918		
Diplazium wichurae (Mett.) Diels	**JN168018**	EF463579	EF463920	**JN168093**	Guangxi, SG LU/GX56
Gymnocarpium jessoense (Koidz.) Koidz.	**JN168019**	**JN168041**	**JN168061**	**JN168094**	Yunnan, SG LU/K18
Gymnocarpium oyamense (Baker) Ching	**JN168020**	**JN168042**	**JN168062**	**JN168095**	Hubei, SG LU/Z7
Hemidictyum marginatum (L.) C. Presl	EF463318	EF463581	EF463922		
Homalosorus pycnocarpos (Spreng.) Pic. Ser.	AB021722			AF425154	
Protowoodsia manchuriensis (Hook.) Ching	**JN168021**	**JN168043**	**JN168063**	**JN168096**	Zhejiang, SG LU/P28
Rhachidosorus blotianus Ching	**JN168022**	**JN168044**	**JN168064**	**JN168097**	Yunnan, SG LU/B46
Rhachidosorus consimilis Ching	**JN168023**	**JN168045**	**JN168065**	**JN168098**	Yunnan, SG LU/J21
Rhachidosorus consimilis Ching	**JN168024**	**JN168046**	**JN168066**	**JN168099**	Yunnan, SG LU/MLP25
Rhachidosorus consimilis Ching	**JN168025**	**JN168047**	**JN168067**	**JN168100**	Yunnan, SG LU/YY33
Woodsia polystichoides Eaton	**JN168026**	**JN168048**	**JN168068**	AF425147	Guizhou, SG LU/FJS5

Note. Circumscription of family and genera follows Smith *et al.* (2006). Accession numbers in boldface type for newly generated sequences.

[a] In the table, *rps4* and *rps4-trnS* IGS are given together because they are submitted to GenBank as a continuous sequence.

[b] Voucher information for newly generated sequences.

TABLE 2. Statistics for the Three Data Sets Analyzed in This Study.

Data set	Included taxa	Alignment length	Variable characters	Best-fitting model[a]
rbcL	59	1308	417	SYM+I+G
rbcL+atpB+ atpA	52	4092	1045	GTR+I+G
rbcL+atpB+ atpA+ rps4	53	5192	2155	GTR+I+G

[a] As identified with the Akaike Information Criterion in Modeltest.

athyrioids clade is resolved in *rbcL* trees, but support for this relationship is very low (PP = 0.53 and MLBS, MPBS < 50%).

The combined *rbcL*, *atpB* and *atpA* data matrix included 4092 characters, with 1045 characters that were variable. The results from the three combined sequences showed better resolved and supported inter- and intra-familial relationships than that of *rbcL* tree (results not shown), especially, Woodsiaceae IV (*Diplaziopsis* and *Homalosorus*, shown in shadow in Fig. 1) and Woodsiaceae V (*Rhachidosorus*, shown in shadow in Fig. 1), the focus of our study, are united in a single clade (PP = 1.00, MLBS=65%, MPBS=71%), so the trees reveal that eupolypods II fall into eight lineages.

The four combined data matrix (*rbcL*, *atpB*, *atpA* and *rps4*) included 5227 characters, with 2127 characters that were variable. MP, ML and BI analyses from the four combined sequences resulted in nearly identical topologies, with most differences at the statistical support values. Because the resultant topologies for relationships of eupolypods II from each of the datasets were not in conflict with one another, the phylogenetic relationships presented here are based on analyses of the four combined data set. The 50% majority-rule consensus tree resulting from MP, ML and BI analyses of the four combined sequences data set is shown in Fig. 1. These analyses yielded an almost robust phylogeny with the exception of a few nodes. Together, *Hemidictyum* and Aspleniaceae are sister to the rest of eupolypods II, *Hemidictyum* is sister to

TABLE 3. Primers Used for Amplifying and Sequencing DNA of This Study.

DNA region	Primer	5′-3′ Primers sequence	Primer source
rbcL	rbcLF1	ATG TCA CCA CAA ACG GAG AC	Li *et al.*, 2004
rbcL	rbcLF631	CCA TTC ATG CGY TGG AGA G	This study
rbcL	rbcLR631	CTC TCC ARC GCA TGA ATG G	This study
rbcL	rbcLR1369	GGA CTC CAC TTA CWA GCT TC	This study
atpB	atpBFwood	ATG AGT GCC ACA GAC GG	This study
atpB	atpBRwood	CCA GGA AGA ATC ATT TG	This study
atpA	AtpARF200	GAA TCK GAT AAT GTT GGG	This study
atpA	AtpAR1140	CAG CCA CCT GTT TCA TAG C	This study
rps4	Rps4F	ATG TCC CGT TAT CGA GGA CC	Li *et al.*, 2006
rps4	trnSR	TAC CGA GGG TTC GAA TC	Souza-Chies *et al.*, 1997 1997 al.,1997
rps4	rps4F	CGA GAG TAA TAC TCA ACA AC	This study
rps4	rps4R	ATG AAT TRT TAG TTG TTG AG	This study

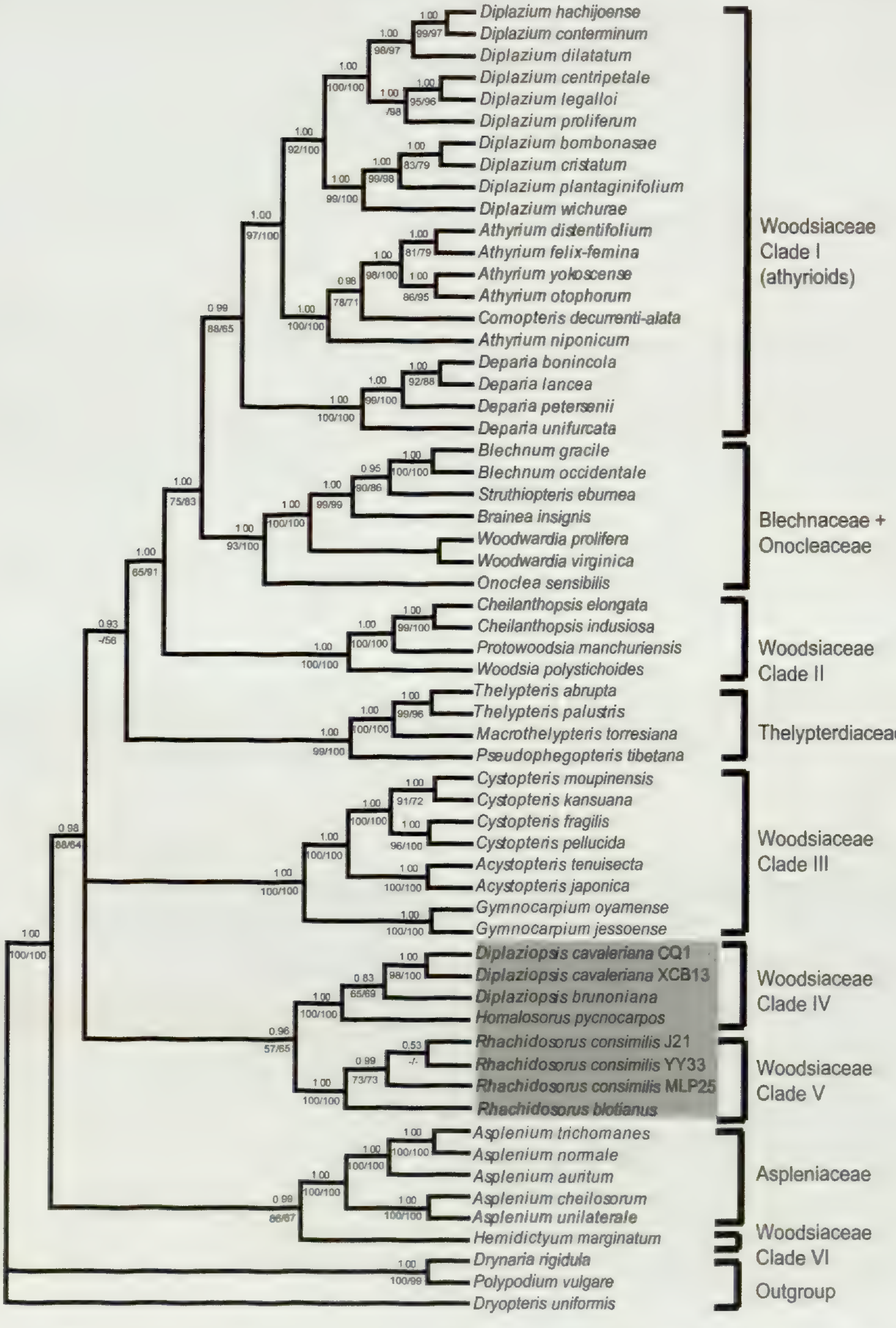

FIG. 1. Fifty-percent majority-rule consensus tree from Bayesian inference (BI) of the 53-taxon phylogeny based on the combined *rbcL*, *atpB*, *atpA*, and *rps4* sequence data. Values above branches are posterior probability from BI; values below branches are bootstrap percentage ≥ 50% from maximum parsimony and maximum likelihood analyses. Six clades within Woodsiaceae are marked in the phylogenetic tree. Taxon names in shadow represent the clade that is newly examined. Family circumscription follows Smith *et al.* (2006).

the asplenioid ferns; then the clade of Woodsiaceae IV and Woodsiaceae V (*Rhachidosorus*, *Diplaziopsis* and *Homalosorus*, in shadow in Fig. 1); the clade of Woodsiaceae III; and the large clade of athyrioids, Blechnaceae + Onocleaceae, Woodsiaceae II, and Thelypterdiaceae.

Discussion

Phylogenetic relationships of eupolypods II, comparisons with previous studies.—Generally, our phylogenetic results are compatible with previous studies on the relationships among fern genera in eupolypods II. The overall eupolypods II relationships shown in Fig. 1 are not in conflict with the results of Sano *et al.* (2000). Our phylogenetic analyses of multiple chloroplast genes confirmed those results and showed better resolved and supported inter- and intra-familial relationships than that of the *rbcL* tree. With more extensive sampling of Chinese Woodsiaceae, a sister relationship between *Diplaziopsis*, *Rhachidosorus* and the North American *Homalosorus* was moderately supported by the four chloroplast gene data, and the three genera of *Acystopteris*, *Cystopteris*, and *Gymnocarpium* were resolved as a monophyletic lineage with strong statistical support. Both were early diverging lineages in eupolypods II, and far away from athyrioid ferns. We mapped the three enigmatic genera (*Diplaziopsis*, *Rhachidosorus*, and *Homalosorus*) onto the Schuettpelz and Pryer (2007) global fern phylogenetic framework. However, there is a major point of difference between our study and theirs; their phylogeny found the clade of *Cystopteris* and *Gymnocarpium* sister to the rest of eupolypods II, while we found the asplenioid clade (including *Hemidictyum*) sister to the rest of eupolypods II, i.e., the most basal-most lineage of eupolypods II is the clade of asplenioid ferns. It is possible that the sampling of different markers or their combinations caused the topological difference. Plastid DNA is inherited as an intact unit, and differences between trees constructed from separate regions can be due to functional constraints and evolution rates (Wendel and Doyle, 1998). We can correct for both factors by directly combining these separate regions, because combined analyses confidently resolved the conflicts between the single gene analyses, enhanced phylogenetic resolution, and were better supported by morphological information (Gontcharov *et al.*, 2004).

Phylogenetic relationships of Diplaziopsis and Homalosorus.—Cladistic analysis of four plastid gene (*rbcL, atpA, atpB* and *rps4*) sequences provided strong evidence that *Diplaziopsis* and *Homalosorus* form a monophyletic lineage and are clearly separated from *Diplazium*. The relationship agrees with the results of *rbcL* analyses (Sano *et al.* 2000) and a recent study based on *rbcL+rps4* analyses (Wei *et al.*, 2010). *Diplaziopsis* and *Homalosorus* were formerly treated as members of *Diplazium* (Christensen, 1938; Kato, 1977; Kato and Iwatsuki, 1983), with which they shared features such as linear sori, similar stipe base and frond axes (Kato, 1977). *Diplaziopsis* and *Homalosorus* differ from *Diplazium* by lamina simple to once-pinnate, veins anastomosing

with numerous areoles (but not goniopteroid), rachis groove V-shaped, rhizome and roots not black and sclerified (Price, 1990).

In China, Ching had a central role in interpreting the delineation of diplazioid genera (Ching, 1964a, b). He regarded *Diplaziopsis* as one younger offshoot from the great stock of diplazioid ferns, while our four plastid gene sequences analyses revealed that the monophyletic lineage of *Diplaziopsis*, *Rhachidosorus*, and *Homalosorus* diverged earlier than other diplazioid genera, indicating that the lineage may not be a direct derivative from diplazioid ferns as Ching assumed. Consequently, *Diplaziopsis* and *Homalosorus* are morphologically well-defined and should be treated as a separate genus from *Diplazium* as proposed by Sano *et al.* (2000). While living materials of the type species, *Diplaziopsis javanica* (Bl.) C. Chr., and the monotype genus, *Homalosorus pyconcarpos,* are currently unavailable, increased sampling is needed to resolve generic relationships within the clade with more accuracy. With living materials, the morphological and developmental characteristics of the clade can be evaluated in more detail, and then the taxonomic status of this clade and can be revised.

Phytogeography of Diplaziopsis and Homalosorus.—Geographically, *Diplaziopsis* is essentially eastern Asian, while that of *Homalosorus* is eastern North American. The disjunct distributions between eastern North America and eastern Asia are not only demonstrated by many flowering plants (reviewed in Wen, 1999), but also by some ferns (e.g., Tryon and Tryon, 1973; Kato and Iwatsuki, 1983). *Homalosorus pycnocarpos* and species of *Diplaziopsis* have been cited as examples of this by Tryon and Tryon (1973), Kato and Iwatsuki (1983), and Kato and Darnaedi (1988). A vicariance scenario for the disjunct distribution is possible as suggested by Barrington (1993) and Kato (1993). Estimates of divergence times using molecular and palaeontological data to test this hypothesis are currently being performed.

Diplaziopsis, Rhachidosorus, and Homalosorus.—Cladistic analysis of four plastid gene (*rbcL, atpA, atpB* and *rps4*) sequences provide moderate statistical support that *Diplaziopsis*, *Rhachidosorus*, and *Homalosorus* form a monophyletic lineage (Fig. 1), which has not been recovered in previous single DNA fragment analyses (Sano *et al.*, 2000; Wang *et al.*, 2003). All these three genera share some morphological characteristics with the athyrioid ferns, yet no obvious morphological characters have been identified to support their sister relationship. Morphologically, *Diplaziopsis* and *Homalosorus* have once-pinnate leaves, whereas *Rhachidosorus* has highly divided (bipinnate to tripinnate) blades. *Diplaziopsis* is with reticulated venation, while *Rhachidosorus* and *Homalosorus* with free venation. Above all, the genera differ in their indusium types: *Diplaziopsis* and *Homalosorus* are of very typical diplazioid type and *Rhachidosorus* of the asplenioid one. Some characters of these genera, such as the swelled mature indusium and the basic chromosome number of x=41 of *Rhachidosorus* and *Diplaziopsis* (Kato *et al.*, 1992; Nakato *et al.*, 1995), showed some hints for the relationship. Herein, we did not provide strong evidence for their systematic relationships; more studies on morphological and developmental characteristics of these genera are required

that we may be able to identify additional morphological character changes supporting these relationship hypotheses.

The family Woodsiaceae has been variously circumscribed, and its limits are still uncertain (Hasebe *et al.*, 1995; Sano *et al.*, 2000; Schneider *et al.*, 2004; Schuettpelz and Pryer, 2007). Wang *et al.* (2004) divided the Athyriaceae (excluding woodsioid ferns, in their circumscription), by far the largest component in the family, into five subfamilies: Cystopteroideae, Athyrioideae, Deparioideae, Diplazioideae, and Rhachidosoroideae. Because the three enigmatic genera (*Diplaziopsis*, *Homalosorus*, and *Rhachidosorus*) were not included in the most inclusive analysis of leptosporangiate fern relationships conducted to date (Schuettpelz and Pryer, 2007), and the other two previous studies (Sano *et al.*, 2000; Wang *et al.*, 2003) showed different phylogenetic positions for *Diplaziopsis*, we added the taxa of *Diplaziopsis* and *Rhachidosorus* to our four-gene dataset. As delimited by Smith *et al.* (2006), the monophyly of Woodsiaceae is lacking, because of this uncertainty, Smith *et al.* (2006) believe that further sampling will likely shed additional light on this subject, and the recognition of several additional families may be warranted. Our analyses revealed another lineage in Woodsiaceae of Smith *et al.* (2006), i.e., the *Diplaziopsis-Homalosorus-Rhachidosorus* lineage, which is clearly helpful for Woodsiaceae realignments within the next few years. Because the overall topology of eupolypods II is not yet well-resolved (Schuettpelz and Pryer, 2007) and the clade of *Diplaziopsis-Homalosorus-Rhachidosorus* in this study is only moderately supported, we do not advocate major taxonomic realignments at this time. Within the next few years we expect that increased taxon sampling, combined with additional morphological and molecular studies, will result in a phylogenetically accurate scheme for a better classification of Woodsiaceae.

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Literature Cited

Barrington, D. S. 1993. Ecological and historical factors in fern biogeography. J Biogeogr. 20:275–279.

Bower, F. O. 1928. The ferns (Filicales), Vols III. London, Cambridge University Press, Pp. 1–306.

Ching, R. C. 1964a. On the genus *Diplaziopsis* C. Chr.. Acta Phytotax Sin. 9:31–36.

Ching, R. C. 1964b. On some confused genera of the family Athyriaceae. Acta Phytotax Sin. 9:41–84.

Ching, R. C. 1978. The Chinese fern families and genera: systematic arrangement and historical origin. Acta Phytotax Sin. 16(3):1–19; 16(4):16–37.

CHRISTENSEN, C. 1938. Filicinae. Pp. 522–550, *In:* F. Verdoorn, ed. *Manual of Pteridology*. The Hague, Martinus Nijhof.

CHU, W. M., Z. R. WANG, X. C. ZHANG, Z. R. HE and Y. T. HSIEH. 1999. *Flora Reipublicae (3)*. Beijing, Popularis Sinicae and Science Press, Pp. 32–511.

COPELAND, E. B. 1947. Genera filicum. Pp. 100–154, *In:* F. Verdoorn, ed. *Annales cryptogamici et phytopathologici, Vol 5 "The genera of ferns"*. Massachusetts, Chronica Botanica Company.

DOYLE, J. J. and J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull. 19:11–15.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39:783–791.

GONTCHAROV, A. A., B. MARIN and M. MELKONIAN. 2004. Are combined analyses better than single gene phylogenies? A case study using SSU rDNA and rbcL sequence comparisons in the Zygnematophyceae (Streptophyta). Mol Biol Evol. 21:612–624.

GUINDON, S. and O. GASCUEL. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 52:696–704.

HASEBE, M., P. G. WOLF, K. M. PRYER, K. UEDA, M. ITO, R. SANO, G. J. GASTONY, J. YOKOYAMA, J. R. MANHART, N. MURAKAMI, E. H. CRANE, C. H. HAUFLER and W. D. HAUK. 1995. Fern phylogeny based on *rbcL* nucleotide sequences. Am Fern J. 85:134–181.

HALL, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41:95–98.

KATO, M. 1977. Classification of Athyrium and allied genera of Japan. Bot Mag Tokyo. 90:23–40.

KATO, M. and K. IWATSUKI. 1983. Phytogeographic relationships of pteridophytes between temperate north America and Japan. Ann Mo Bot Gard. 70:724–733.

KATO, M. and D. DARNAEDI. 1988. Taxonomic and phytogeographic relationships of *Diplazium flavoviride*, *D. pycnocarpon*, and *Diplaziopsis*. Am Fern J. 78:77–85.

KATO, M., N. NAKATO and X. CHENG. 1992. A cytotaxonomic study of ferns of Yunnan, southwestern China. Bot Mag Tokyo 105:105–124.

KATO, M. 1993. Biogeography of ferns: dispersal and vicariance. J Biogeogr. 20:265–274.

KRAMER, K. U. and M. KATO. 1990. Dryopteridaceae subfamily Athyrioideae. Pp. 130–144, *In:* K. U. Kramer and P. S. Green, eds. *Pteridophytes and gymnosperms*. Berlin: Springer-Verlag.

LI, C. X., S. G. LU and Q. YANG. 2004. Asian origin for *Polystichum* (Dryopteridaceae) based on *rbcL* sequences. Chin Sci Bull. 49:1146–1150.

NAKATO, N., M. KATO and B. D. LIU. 1995. A cytotaxonomic study of some ferns from Jiangsu and Zhejiang provinces, China. J Jap Bot. 70:194–204.

PICHI SERMOLLI, R. E. G. 1973. Fragmenta Pteridologiae, IV. Webbia. 28:445–477.

POSADA, D. and K. A. CRANDALL. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

PRICE, M. G. 1990. Philippine fern notes. Contr Univ Mich Herb. 17:267–278.

RONQUIST, F. and J. P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19:1572–1574.

SANO, R., M. TAKAMIYA, M. ITO, S. KURITA and M. HASEBE. 2000. Phylogeny of the lady fern group, tribe Physematieae (Dryopteridaceae), based on chloroplast *rbcL* gene sequences. Mol Phylogenet Evol. 15:403–413.

SCHNEIDER, H., E. SCHUETTPELZ, K. PRYER, R. CRANFILL, S. MAGALLÓN and R. LUPIA. 2004. Ferns diversified in the shadow of angiosperms. Nature. 428:553–557.

SCHUETTPELZ, E. and K. M. PRYER. 2007. Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. Taxon. 56:1037–1050.

SMITH, A. R., K. M. PRYER, E. SCHUETTPELZ, P. KORALL, H. SCHNEIDER and P. G. WOLF. 2006. A classification for extant ferns. Taxon. 55:705–731.

SOUZA-CHIES, T. T., G. BITTAR and S. NADOT. 1997. Phylogenetic analysis of Iridaceae with parsimony and distance methods using the plastid gene *rps4*. Plant Syst Evol 204:109–123.

SWOFFORD, D. L. 2002. PAUP* Phylogenetic Analysis Using Parsimony (* and other methods) Version 4.0b10. Sinauer Associates, Sunderland.

TAGAWA, M. 1936. Spicigelium Pteridographiae Asiae Orientalis 11. Acta Phytotax Geobot. 5:189–197.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN and D. G. HIGGINS. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876–4882.

TRYON, R. M. and A. F. TRYON. 1973. Thelypteris in northeastern North America. Am Fern J. 63:65–76.

TRYON, R. M. and A. F. TRYON. 1982. Ferns and allied plants, with special reference to tropical America. New York, Springer, Pp. 454–627.

WANG, M. L., Y. T. HSIEH and G. F. ZHAO. 2004. A revised subdivision of the Athyriaceae. Acta Phytotax Sin. 42:524–527.

WANG, M. L., Z. D. CHEN, X. C. ZHANG, S. G. LU and G. F. ZHAO. 2003. Phylogeny of the Athyriaceae: evidence from chloroplast *trnL-F* region sequences. Acta Phytotax Sin. 41:416–426.

WEI, R., X. C. ZHANG and X. P. QI. 2010. Phylogeny of *Diplaziopsis* and *Homalosorus* based on two chloroplast DNA sequences: *rbcL* and *rps4+rps4-trnS* IGS. Acta Bot Yunnan. Suppl. XVII:46–54.

WEN, J. 1999. Evolution of eastern Asian and eastern North American disjunct distributions in flowering plants. Annu Rev Ecol Syst. 30:421–455.

WENDEL, J. F. and J. J. DOYLE. 1998. Phylogenetic incongruence: window into genome history and molecular evolution. Pp. 265–296, *In:* D. E. Soltis, P. S. Soltis and J. J. Doyle, eds. *Molecular systematics of plants II: DNA sequencing.* Kluwer, Boston.

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Taxonomic Studies on *Asplenium* sect. *Thamnopteris* (Aspleniaceae) I: Cytological Observations

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ABSTRACT.—The section *Thamnopteris* is morphologically distinct among the large fern genus *Asplenium*, but the species recognition within this section is very difficult. To understand the species concept of this section from a cytological view, I examined chromosome numbers of fifteen samples representing eight taxa (species and intraspecific taxa). As result, seven taxa were determined to be sexual tetraploids with chromosome number 2n = 144 and *Asplenium humbertii* is a sexual hexaploid with 2n = 216. Along with the chromosome numbers reported, notes on nomenclature, diagnostic characters, and geographical distribution for the eight taxa are given. Cytological data available so far indicate that *Asplenium* sect. *Thamnopteris* is not a monophyletic group.

KEY WORDS.—Bird-nest fern, chromosome, polyploid, taxonomy

Asplenium L. is a natural genus with about 700 or more species in various regions all over the world (Kramer and Viane, 1990; Bellefroid *et al.*, 2010). Though widely accepted as monophyletic, *Asplenium*, with so many species, is difficult to subdivide because of a lack of suitable distinguishing characters at the intrageneric level. *Asplenium* sect. *Thamnopteris* C. Presl (1836), or treated as a separate genus *Neottopteris* (Smith, 1841; Wu, 1999), however, is one of the very few groups which can be clearly recognized. It is characterized by simple, entire fronds and intramarginal veins in morphology, is usually epiphytic on tree trunks or rocks in forests with bird-nest-like appearance, and is distributed in tropics and subtropics of the Old World.

Despite being readily recognized as a morphologically distinct group, the circumscription of species is confusing and the identification is very difficult within *Asplenium* sect. *Thamnopteris*. Since Linnaeus's *A. nidus* (1753), the first member in this group, 33 taxa have been published (Tardieu-Blot, 1933; Ching and Wang, 1964; Holttum, 1974; Miao, 1980; Jones, 1988; Wu, 1989; Murakami *et al.*, 1999). The establishment of these taxa was mostly based on a minor variation of one or two characters, especially the shape of fronds, which results in the obscure delimitation of species. Though Holttum (1974) presented a taxonomic revision on the section *Thamnopteris*, his treatment was based on herbarium specimens at his time and many problems in nomenclature and taxonomy still remained. After Holttum (1974), no taxonomic work was carried out on this group other than that of Seto (1979) and Murakami *et al.* (1999) who studied the morphology and the molecular systematics of *Thamnopteris* from Japan, respectively. Lacking a comprehensive comparison of characters, the taxonomic confusion of this section is partly reflected by the present situation that many specimens were named as

"*Asplenium nidus* L. s.l." or "*A. phyllitidis* sensu Holttum" and more than 20 new species were proposed but not formally published based on materials in various herbaria.

Aiming to revise the taxonomy of this difficult group, the present author undertook examining type collections, studying materials in large herbaria of southeastern Asia and Europe, field surveying in China, Philippines and Indonesia, chromosome counting, and gametophyte observations in last six years. Cytological data are necessary and important to understand the concepts and relationships of species and to date only *A. antiquum* Makino, *A. australasicum*, *A. nidus*, and *A. phyllitidis* D. Don have been documented with chromosome numbers n = 72 and / or 2n = 144 (Bir, 1960; Abraham *et al.*, 1962; Kawakami, 1970, 1997; Tsai and Shieh, 1983; Kato and Nakato, 1999; Yatabe *et al.*, 2001; Tindale and Roy, 2002). These cytological observations are reported as the first part of my serial studies on the taxonomy of *Asplenium* sect. *Thamnopteris*.

Material and Methods

Gross morphology and identification.—In order to correctly name the living materials involved in this research, gross morphology was studied based on herbarium specimens and wild and cultivated living plants. The present author examined specimens of *Asplenium* sect. *Thamnopteris* deposited in the following herbaria: BM, BO, GAUA, HITBC, IBK, IBSC, K, KUN, KYO, L, P, PE, PNH, PYU, and SING before the year 2009. The specimens of the section of *Thamnopteris* being examined in above herbaria amount to about 2500 collections. To understand the living state and variation of morphological characters, I made field observations in 17 trips to southern and southwestern China since 2005, one trip to Mindanao, Philippines in 2007, one trip to Java, Indonesia in 2009, and one trip to West Papua and Kalimantan, Indonesia in 2010. In each field trip, the state of following characters was determined or measured: distribution, shape, color, and size of scales; outline, size, and basal shape of fronds; prominent extent and transaction of frond midribs; distribution, length, and density of sori; color of stipes; spreading angle of fronds; and habitat. In addition, one or two living plants were introduced to my green house in South China Botanical Garden, Guangzhou, for morphological observation and chromosome counts.

Cytology.—Root tips of sporophytes or prothalli in gametophyte phase were used for chromosome counts. Living plants (sporophytes) or fresh spores were collected by me from natural populations in various localities or from Chinese gardens (Table 1) and cultivated in my green house in South China Botanical Garden. Fresh root tips or immature prothalli (nearly cordate in shape, before sex organs formed) raised from spores were pretreated for 3–6 hours with 0.002 mol/L 8-hydroxyquinoline at about 25°C, then fixed for 1 hour in Carnoy's fluid (1 volume of pure acetic acid and 3 volumes of 95% ethanol) at about 4°C. The tips were hydrolyzed for 10 minutes in 1 N HCL at 60°C, macerated for 10 min in 45% acetic acid, and then stained in 2% aceto-orcein

TABLE 1. Chromosome counts for 15 samples representing eight taxa in *Asplenium* sect. *Thamnopteris*.

Taxon	Cytological material	Chromosome count (ploidy level)	Collection locality and voucher	Figure
A. antiquum	Prothalli	n = 72 (4×)	South China Botanical Garden (in cultivation), China; *Dong 3360* (IBSC)	1
	Root tips	2n = 144 (4×)	Xiamen Botanical Garden (in cultivation), Fujian, China; *Dong 2894* (IBSC)	-
A. antrophyoides	Root tips	2n = 144 (4×)	Huanjiang, Guangxi, China; *Dong 1976* (IBSC)	2
A. australasicum	Root tips and prothalli	2n = 144, n = c 72 (4×)	South China Botanical Garden (in cultivation), Guangdong, China; *Dong 3400* (IBSC)	3
A. cymbifolium f. *lingganum*	Root tips and prothalli	2n = 144, n = c 72 (4×)	Marilog, Davao, Mindanao, Philippines; *Dong 2610* (IBSC)	4
A. humbertii	Root tips	2n = 216 (6×)	Longzhou, Guangxi, China; *Dong 2287B* (IBSC)	5
	Root tips	2n = c 216 (6×)	Mt Exianling, Hainan, China; *Dong 1234* (IBSC)	-
A. nidus	Root tips	2n = c 144 (4×)	Gongshan, Yunnan, China; *Dong 3402* (IBSC)	-
	Root tips	2n = c 144 (4×)	Mt Yinggeling, Hainan, China; *Dong 1667* (IBSC)	-
	Root tips	2n = 144 (4×)	Napo, Guangxi, China; *Dong 2939* (IBSC)	6
	Prothalli	n = c 72 (4×)	South China Botanical Garden, China; *Dong 3401* (IBSC)	-
A. phyllitidis subsp. *malesicum*	Root tips and prothalli	2n = 144, n = c 72 (4×)	Mt Yinggeling, Hainan, China; *Dong 1645* (IBSC)	7
	Root tips and prothalli	2n = 144, n = c 72 (4×)	Marilog, Davao, Mindanao, Philippines; *Dong 2606* (IBSC)	-
	Root tips	2n = 144 (4×)	South China Botanical Garden (in cultivation), China; *Dong 3360* (IBSC)	8
A. simonsianum	Root tips	2n = 144 (4×)	Mengla, Yunnan, China; *Dong 2768* (IBSC)	9

for 1-2 hours. Finally, the root tips or prothalli were squashed in 2% aceto-orcein. The chromosomes were counted and photographed using a light microscope (Olympus BX41).

Reproductive characteristics.—The number of spores in a sporangium indicates the possible reproduction mode for most leptosporangiate ferns, i.e., ferns with 64 spores per sporangium reproduce sexually while those with 32 spores per sporangium reproduce apogamously (Manton, 1950; Lovis, 1977;

Walker, 1979; Kato and Nakato, 1999). When checking the number of spores, a well-developed, intact sporangium was selected and moved to a drop of water on a microscope slide with a needle. Using the needle to break the sporangium, spores were freed from the sporangium, and then the number of spores was counted with a light microscope (Olympus BX41). For one given specimen, the spores of at least five sporangia were counted.

The data on morphology and distribution given in the following section is entirely based on herbarium specimens examined and recent collections gathered by the present author.

Results and Discussion

As shown in Table 1, the somatic chromosome number is 216 for *Asplenium humbertii* and 144 for other species. As the basal number of chromosome (x) is 36 in *Asplenium*, *A. humbertii* is hexaploid and others are all tetraploid. In addition, my examination showed there are 64 spores in each sporangium and the spores can normally develop and produce new sporophytes for the all specimens examined cytologically in this study. Therefore, the eight species with chromosomes counted here are of sexual reproduction mode.

Chromosome number, nomenclature, identification, and distribution

Asplenium antiquum Makino in J. Jap. Bot. 6: 32. 1929. *Neottopteris antiqua* (Makino) Masam. in Trans. Nat. Hist. Soc. Taiwan. 22: 215. 1932. TYPE.—Japan. Kagoshima: Yakusima, 30 Jul 1961, *H. Ito 289* (neotype! designated here, SING).

Cytology.—The plants of this species cultivated in two botanical gardens of China were examined and determined to be tetraploid with n = 72 (Fig. 1) and 2n = 144. The origin of the plants examined is unknown. Previously, a population of this species from Tokunoshima Isl., Japan, was reported with the same chromosome number (2n = 144) (Kawakami, 1970).

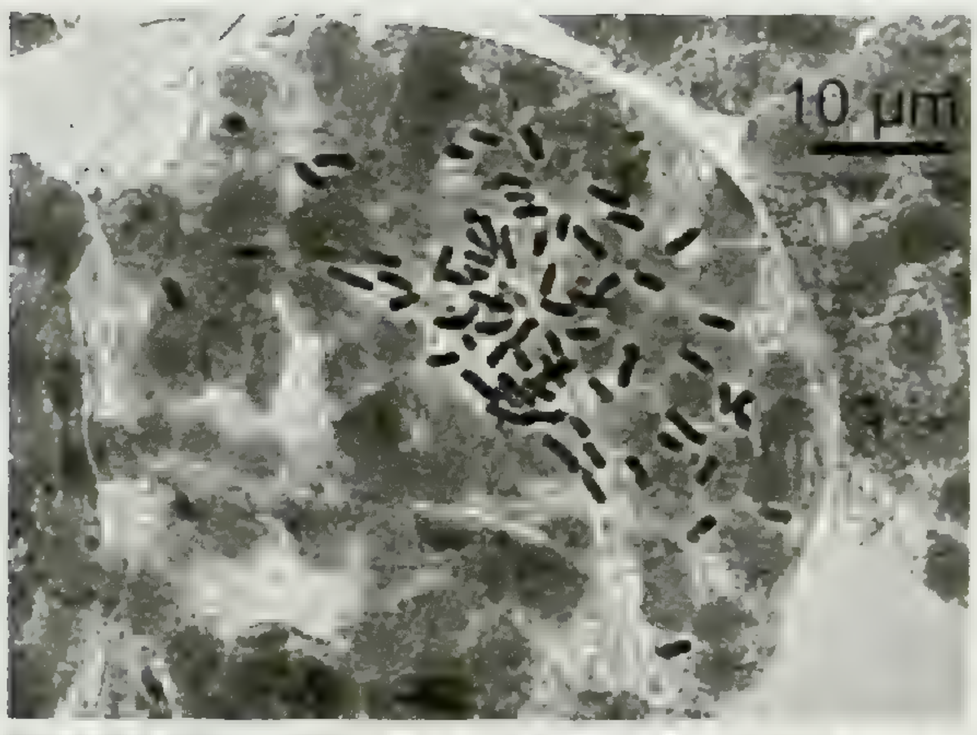
Diagnostic characters.—Stipe scales broadly lanceolate, 15–30 × 2–6 (8) mm; fronds narrowly lanceolate; midribs slightly prominent on both surfaces; sori long and sparse, occupying 2/3–3/4 length of veins, 6–10 sori every 2 cm length along midribs.

Distribution.—China (Fujian), Japan (common in southern islands), Korea (Cheju Island). The misreport of this species in Hainan Island, China (Wu, 1999), is due to the misidentification of *A. phyllitidis* subsp. *malesicum* Holttum.

Representatives of 32 specimens examined.—China. **Fujian**: Luoyuan, 25°07′N, 123°32′E, 430 m, 12 Jul 2010, *Dong 3403* (IBSC); without specific locality, 20 Aug 1943, *Lin s.n.* (IBSC, herb no. 295306). **Taiwan**: Nantou, 1300 m, 28 Aug 2002, *Lu s.n.* (PYU); Taitung, 23 Jan 1940, *Tagawa 3071* (L).

Korea. **Cheju**: without specific locality, 29 May 1908, *Taquet 2369* (P).

Japan. **Hizen**: without specific locality, 16 Jun 1909, *Mistuo s.n.* (KYO). **Kagoshima**: Cape Sata, 19 Oct 1924, *Tashiro s.n.* (KYO); Oosumi, 26 Jul 1913,



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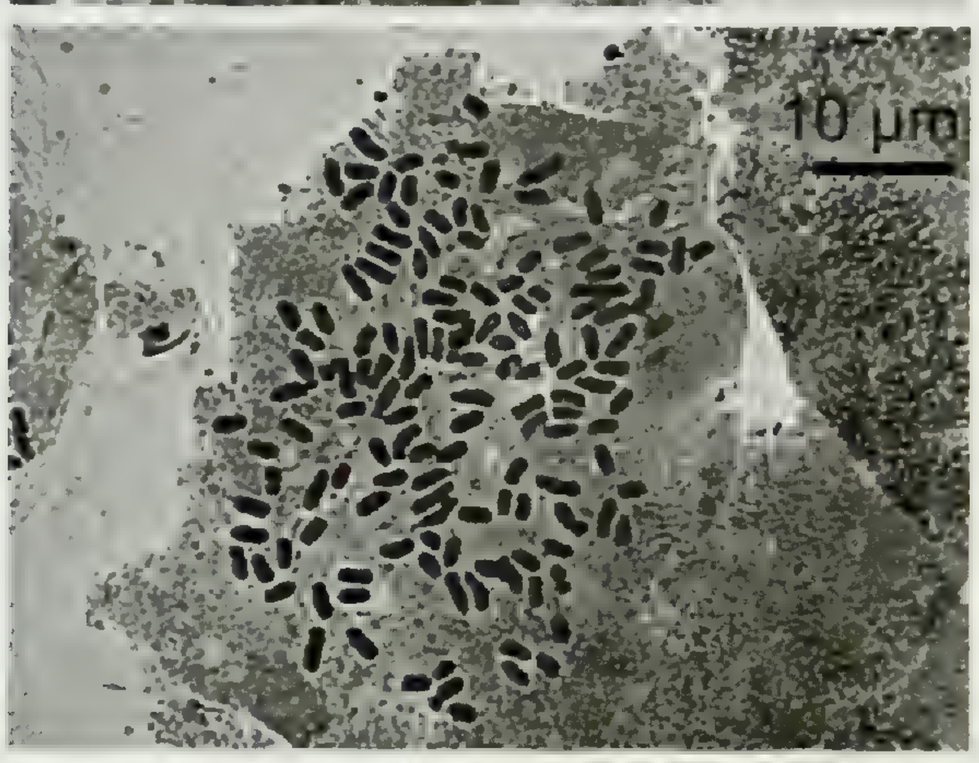

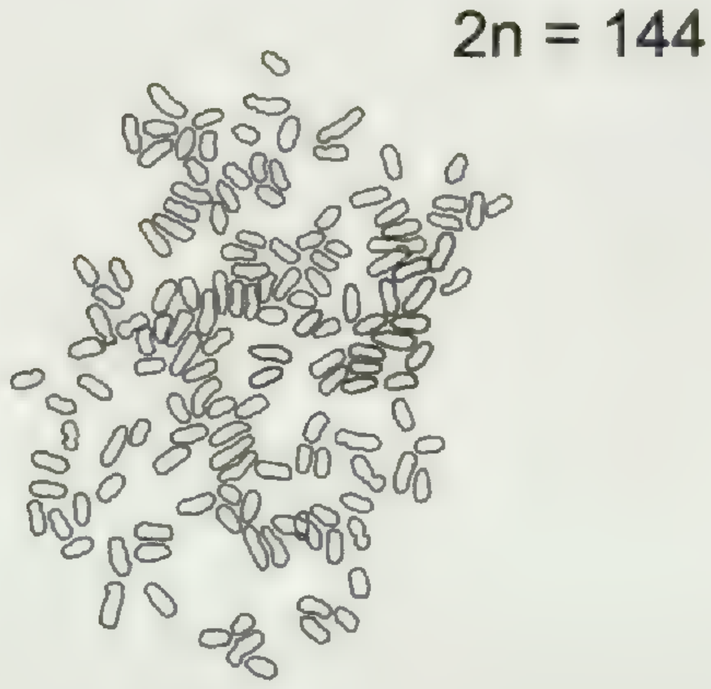

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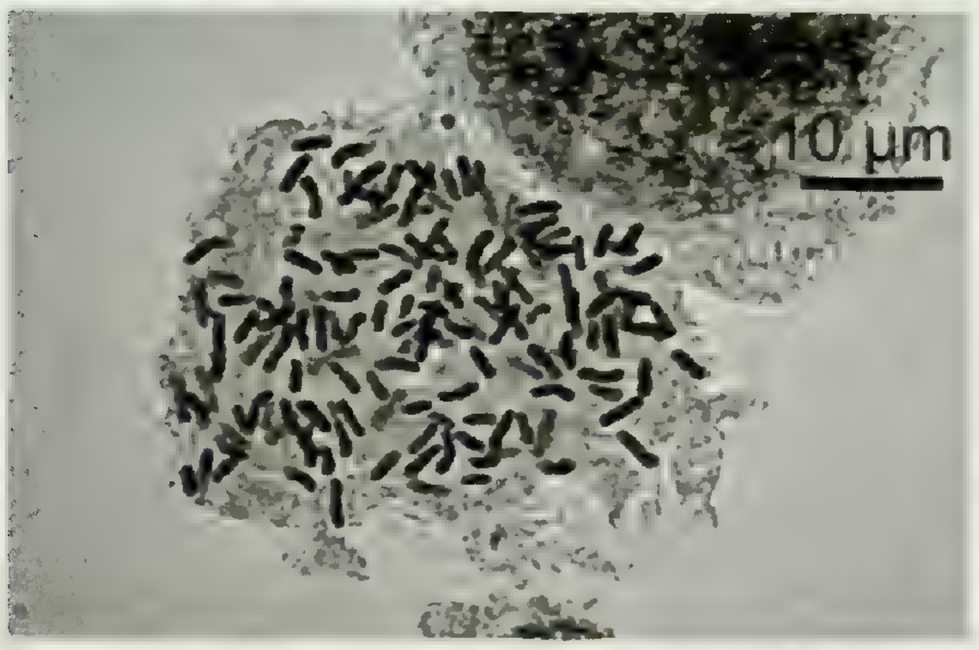

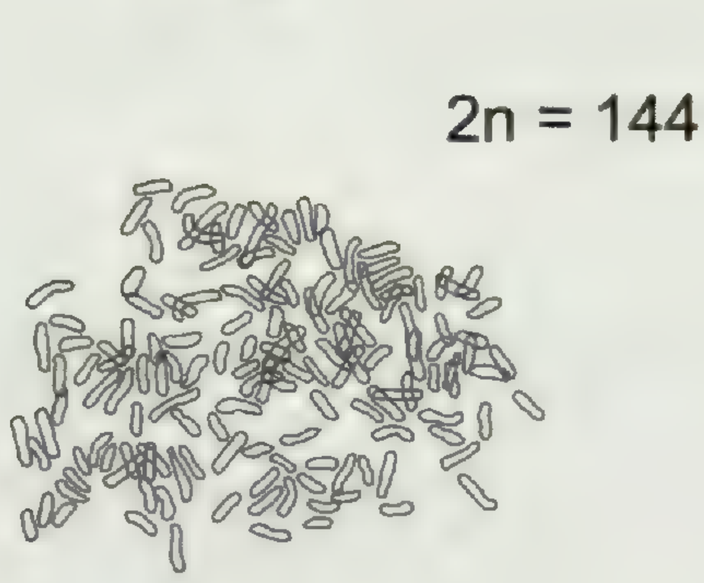

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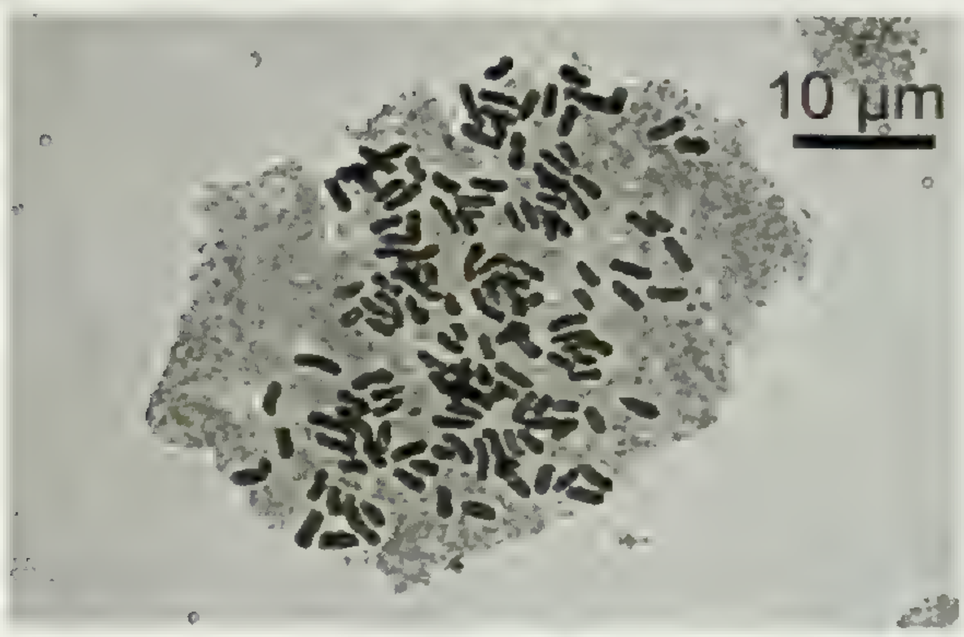
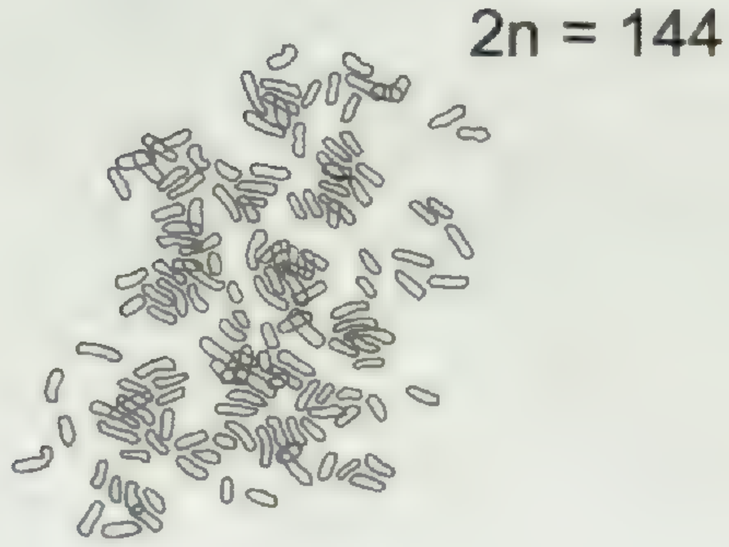




4

FIGS. 1–4. Photomicrographs (left) and explanatory diagrams (right) of chromosomes at mitosis phase. 1. *Asplenium antiquum* cultivated in Guangzhou, China (*Dong 3360*); 2. *A. antrophyoides* from Guangxi, China (*Dong 1976*); 3. *A. australasicum* cultivated in Guangzhou, China (*Dong 3400*); 4. *A. cymbifolium* f. *lingganum* from Mindanao, Philippines (*Dong 2610*).

Tashiro s.n. (KYO); ibid., 12 Aug 1916, *Tashiro s.n.* (KYO); ibid., 100 m, 24 Jan 1965, *Togashi et al. 10071* (L); Yakushima, Aug 1907, *Kudo 5* (KYO); ibid., Sep 1921, *Koidzumi s.n.* (KYO, PE); ibid., 13 Dec 1956, *Togasi s.n.* (P); ibid., 30–50 m, 14 Nov 1983, *Mitsuta & Nagamasu 275* (PYU); ibid., 30–100 m, 16 Jul 1979, *Yamazaki et al. 2330* (KUN); 30 Jul 1961, *Ito 289* (SING). **Nagasaki**: Naru, 28 Sep 1910, *Tashiro s.n.* (KYO). **Nagono**: without specific location, 1866–74, *Savatier 1551* (P). **Okinawa**: Iriomote, 200 m, 19 Mar 1982, *Saiki 2050* (PYU). **Wakayama**: Susami, 10 Aug 1913. *Ui s.n.* (KYO); Katsuura, 5 Jun 1951, *Kodama s.n.* (PE); Wakayama, Aug 1901, *Kinashi s.n.* (KYO). **Without specific locality**: 11 Nov 1881, *Dickins 1877* (P).

In nomenclature, *Asplenium antiquum* was invalidly published by Makino (1929) because no type was designated. I tried to access the original material studied by Makino but failed. I designated a neotype for this species to validate the name *Asplenium antiquum* Makino.

Asplenium antrophyoides H. Christ in Bull. Acad. Int. Geogr. Bot. 20: 170. 1909. *Neottopteris antrophyoides* (H. Christ) Ching in Bull. Fan Mem. Inst. Biol., Bot. 10: 7. 1940. TYPE.—China. Guizhou: Lofu, Sep 1907, *Cavalerie 1877* (holotype, P!; isotype, BM!).

Cytology.—The somatic chromosome number is 144 for a population from Guangxi, China (Fig. 2). This is the first cytological record for *A. antrophyoides.*

Diagnostic characters.—Stipe scales broadly lanceolate, 15–20 × 3–5 mm; fronds more or less spathulate with the upper part the broadest; midribs obviously keeled on abaxial surface with the transection deltoid; sori long and usually sparse, occupying 2/3–3/4 length of veins, 7–10 (12) sori every 2 cm length along midribs.

Distribution.—China (Guangdong, Guangxi, Guizhou, Hunan, Sichuan, Yunnan), Thailand (Chiang Mai), Vietnam (Tonkin).

Representatives of ca. 160 specimens examined.—China. **Guangdong**: Liannan, 150 m, 16 Jan 2005, *Wang et al. 725* (IBSC); Lianxian, 21 Nov 1930, *Ko 50962* (PE). **Guangxi**: Huangjiang, 25°07′N, 107°58′E, 600 m, 13 Aug 2005, *Dong 1976* (IBSC); Lingyun, 11 Dec 1933, *Steward & Cheo 7* (PE); Longzhou (formerly Lungchow), 1901, *Morse 63* (K); Napo, 23°00′N, 105°51′E, 1000 m, 13 Jun 2009, *Dong 2956* (IBSC); Tianlin, 24°29′N, 106°21′E, 1150 m, 20 Jun 2009, *Dong 3001* (IBSC). **Guizhou**: Libo, 25°17′N, 107°56′E, 600 m, 30 Jul 2010, *Dong et al. GZE52* (IBSC); Pingchow, 350 m, 15 Sep 1930, *Tsiang 7141* (BM, P). **Hunan**: Xinning, 370 m, 14 Nov 1962, *Liu 15545* (IBSC, KUN). **Sichuan**: Qianwei, 380 m, 12 Aug 1984, *Xing et al. 05235* (PE). **Yunnan**: Hekou, 22°40′N, 103°56′E, 150 m, 6 Oct 2009, *Song 116* (IBSC); Jingdong, 1350 m, 7 Dec 1961, *Li 3740* (KUN); Jinping, 850 m, 17 Jan 1959, *Chu 4704* (PYU); Maguan, 650 m, 23 Nov 1982, *Chu et al. 15340* (PYU); Mengla, 700–800 m, 25 Jul 1984, *Sino-Japan Exped. 104* (KUN); Yanshan, 1100 m, 15 Nov 1939, *Wang 84955* (KUN, PE).

Thailand. **Chiang Mai**: Chiang Dao, 19°25′N, 98°55′E, 1500–1900 m, 7 Dec 1965, *Hennipman 3256* (L); ibid., 1600–2100 m, 4 Jan 1966, *Tagawa et al. T4210* (IBSC, L); ibid., 1600 m, 2 Mar 1995, *Maxwell 95–176* (L)

VIETNAM. **Cao Bang**: Nguyen Binh, 22°39′N, 105°57′E, 500–550 m, 21 Apr 1999, *Loc et al. 1609* (P); without specific locality, Feb 1925, *Colani 1982* (P). **Lai Chau**: Ngai Thau, 1000–1200 m, 7 Apr 1936, *Poilane 25554* (P); without specific locality, 800–900 m, 6 Jan 1938, *Poilane 27030* (P). **Thanhoa**: Lung Van, 1000–1200 m, 26 Jan 1931, *Poilane 18889* (P).

Asplenium australasicum (J. Sm) Hook., Fil. Exot. t. 88. 1859. *Neottopteris australasica* J. Sm., Cult. Ferns 49. 1857. TYPE.—Cult. Hort. Bot. Kew., origin Australia (lectotype designated by Holttum in 1974, BM? isolectotype, K!).

Cytology.—Cultivated plants from South China Botanical Garden were examined. A total of 144 chromosomes were counted for sporophytes (Fig. 3) and about 72 for gametophytes. Before my examination, this species was reported to be tetraploid with 144 chromosomes based on root-tips from Middle Brother and Lord Howe Island, Australia (Tindale and Roy, 2002).

Diagnostic characters.—Stipe scales narrowly lanceolate, 10–20 × 1–1.5 mm; fronds lanceolate; midribs distinctly keeled on abaxial surface with the transection deltoid; sori short and dense, occupying less than 1/2 length of veins, (10)13–18 sori every 2 cm length along midribs.

Distribution.—Australia (Queensland, New South Wales, Lord Howe Island), Fiji, Samoa, Tonga, Vanuatu (New Hebrides), Polynesia (Tahiti) and other Pacific islands.

REPRESENTATIVES OF CA. 110 SPECIMENS EXAMINED.—AUSTRALIA. **Lord Howe Island**: without specific locality, Sep 1853, *Horald 696* (K); ibid., Dec 1869 (?), *More 76* (K). **New South Wales**: Coffs Harbour, 150 m, 2 May 1956, *Constable P7404* (K); Newell Falls Creek, 3000 ft, *Werner 18* (K). **Queensland**: Cape York, 420 m, 17 Aug 1948, *Brass 19879* (K); Mt Lewis, 16°29′S, 145°14′W, 21 Sep 1975, *Coveny 7242* (K, L); Mt Mistake, 24 Nov 1930, *Hubbard 5202* (K); Richmond River, 1885, *Anonymous s.n.* (SING, herb no. 0079510).

PACIFIC ISLANDS. **Christmas**: 1°52′N, 157°20′W, 4 Dec 1987, *Maclean s.n.* (K). **Fiji**: Ovalau, 17°42′S, 178°48′W, 100–300 m, 8–11 May 1953, *Smith 7322* (K, L); Viti Levu, 17°58′S, 178°06′W, 150–250 m, 17 Oct 1953, *Smith 8602* (K, L). **Guam**: Finegayan, 29 Jul 1987, *Flis 15398* (K). **Henderson**: North end, 24°22′S, 128°19′W, 5 m, 17 June 1934, *John & Fosberg 15088* (K). **Mangaia**: 21°55′S, 157°55′W, Oct 1972, *Dickie 25* (K). **New Caledonia**: 21°26′S, 165°54′W, Nov 1911, *Anonymous 147* (K); without specific locality, Mar 1889, *Hennecart s.n.* (K). **Niue**: 19°01′S, 169°55′W, 20 m, 11 Jan 1940, *Yuncher 9590* (K). **Oeno**: 23°56′S, 130°44′W, 1921(?), *Quayle 409* (K). **Samoa**: Laugapapa, Sep 1877, *Powell 164* (K); without specific locality, Jun 1872(?), *Powell 164* (K). **Tahiti**: Mt Marau, 17°37′S, 149°32′W, 1250 m, 30 May 1985, *Fosberg 64558* (K). **Tonga**: Tavau, 18°40′S, 174°00′W, *Crosby 277* (K). **Vanuatu**: New Hebrides, 17°31′S, 168°21′W, 580 ft, 2 Jul 1971, *Braithwaite 2002* (K).

This species is morphologically characterized by having fronds ascending steeply and midribs distinctly keeled on abaxial surface. Nakato (1987) reported a population of "*A. australasicum*" from Iriomotejima, Japan, with 144 somatic chromosomes. However, *A. australasicum* is known so far to be restricted to Queensland and nearby Pacific islands. The so-called *A.*

australasicum from southern Japan proved to be *A. setoi* N. Murak. & Seriz. based on molecular data (Murakami *et al.*, 1999).

Asplenium cymbifolium f. ***lingganum*** Alderw. in Bull. Jard. Bot. Buitenzorg III, 5: 184. 1922. TYPE.—INDONESIA. Lingga Arch., 8 Aug 1919, *Bunnemeijer 7388* (holotype, K!, isotype, L).

Cytology.—Both the root tips and prothalli originally from a Philippine population were cytologically examined. The chromosome number is 144 for root tips (Fig. 4) and ca. 72 for young prothalli. This taxon is confirmed to be a sexual tetraploid for the first time.

Diagnostic characters.—Stipe scales narrowly lanceolate, ca. 10 × 1–2 mm; fronds lanceolate and widened at base; midribs prominent on abaxial surface with the transection elliptic; sori long and sparse, occupying 2/3–3/4 length of veins, 4–7 (9) sori every 2 cm length along midribs.

Distribution.—Indonesia (Lingga Arch., Sumatra), Papua New Guinea, Philippines (Mindanao).

ADDITIONAL SPECIMENS EXAMINED.—INDONESIA. **Jappen-Biak**: Wasabori near Seroei, 12 Aug 1939, *Aet & Idjam 400* (BO). **Sumatra**: Sikundar Nature Conservations, 100–250 m, 14 Apr 1971, *Iwatsuki et al. S361* (K). **Ditschi**: without specific locality, 1200 m, Jun 1928, *Mayr 183a* (BO).

PAPUA NEW GUINEA. **East Sepik**: Mt Samai, 4°29′S, 142°41′E, 450 m, 27 Apr 1991, *Takeuchi 6216A* (K).

PHILIPPINES. **Mindanao**: Marilog, Davao, 1200 m, 17 Nov 2007, *Dong 2610* (IBSC).

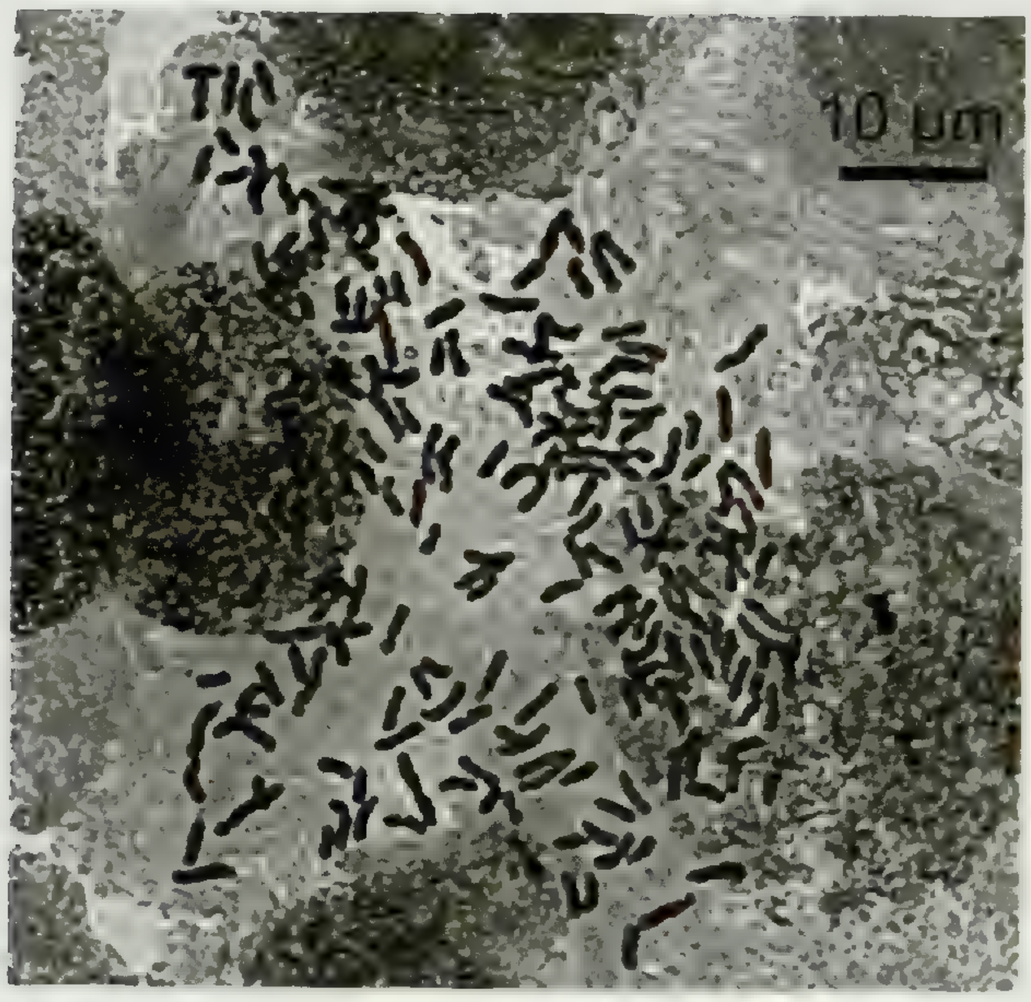
In comparison with *A. cymbifolium* with wide fronds (18–20 cm), the form *lingganum* was characterized by much smaller fronds (6 cm wide). Besides the remarkable difference in frond size the form *lingganum* differs from the form *cymbifolium* in lacking dense scales on abaxial surface of midrib towards the base. So it seems more suitable to treat the form *lingganum* as a variety or a separate species from *A. cymbifolium*.

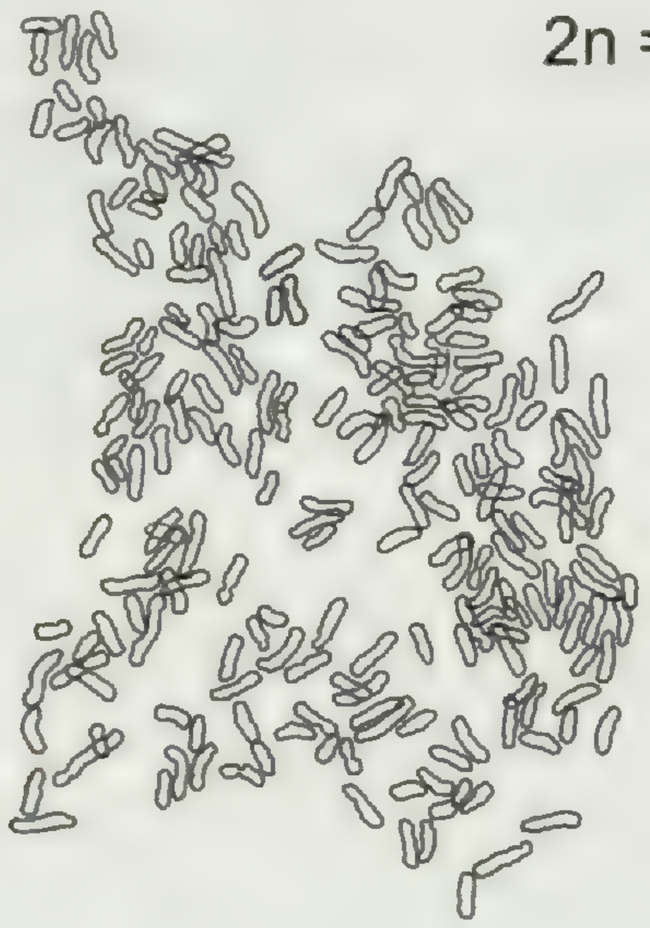
Asplenium humbertii Tardieu, Asplen. Tonkin 25, pl. 2, f. 1–2. 1932. *Neottopteris humbertii* (Tardieu) Tagawa in J. Jap. Bot. 22: 161. 1948. TYPE.—VIETNAM. Tonkin, Jan 1886, *Balansa 68* (holotype, P!).

Cytology.—A population from Hainan Island and other one from Guangxi, China proved to be sexual hexaploids with chromosome number 2n = 216 (Fig. 5). This species is the only hexaploid so far found in *Asplenium* sect. *Thamnopteris*.

Diagnostic characters.—Stipe scales broadly lanceolate, 8–10 × 2–3 mm; fronds more or less spathulate with long and narrow stipes; midribs slightly prominent with the transection elliptic; sori long and usually sparse, occupying more than 2/3 length of veins, 8–13 sori every 2 cm length along midribs.

Distribution.—China (Hainan, Guangxi, Yunnan), Laos (Cammon), Thailand (Chiang Mai), Vietnam (Tonkin). *Asplenium humbertii* only occurs in limestone within these regions.





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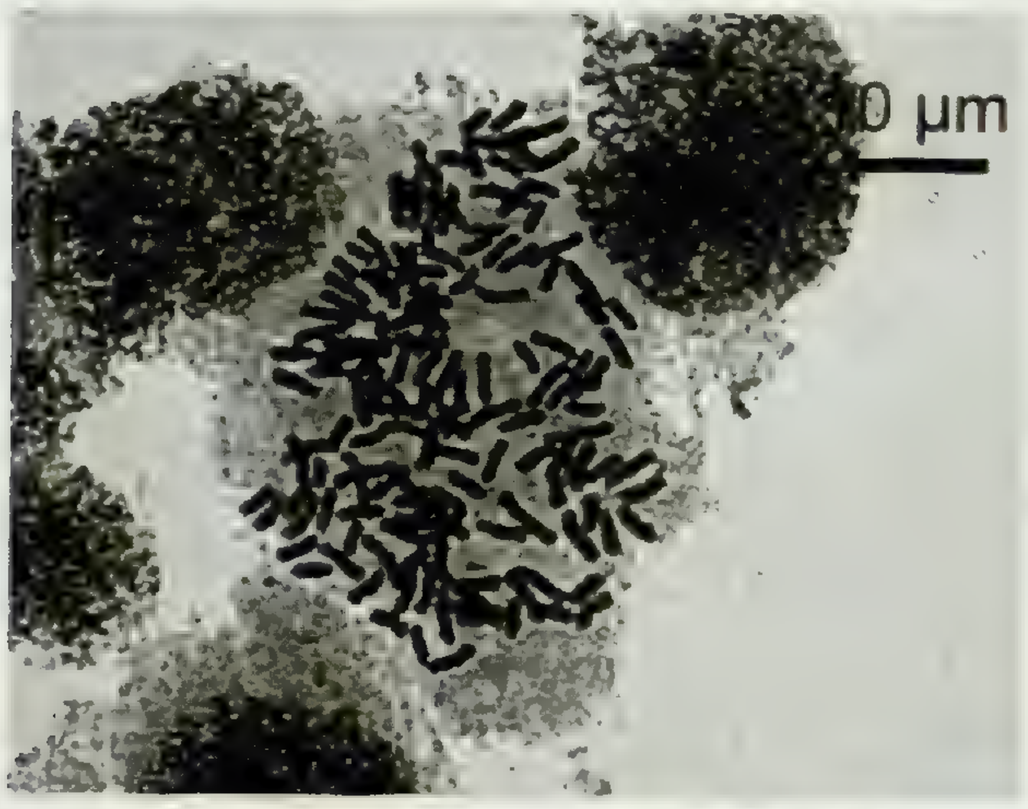

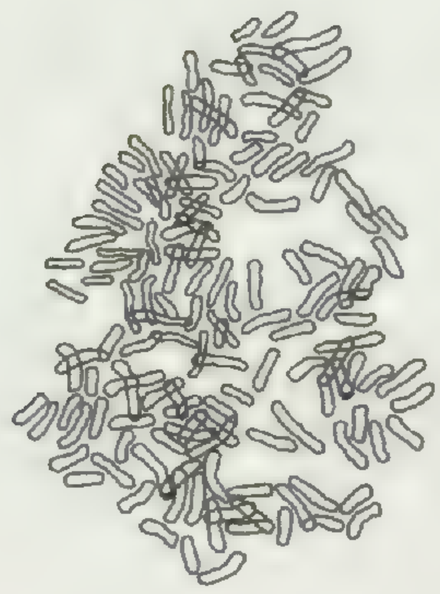

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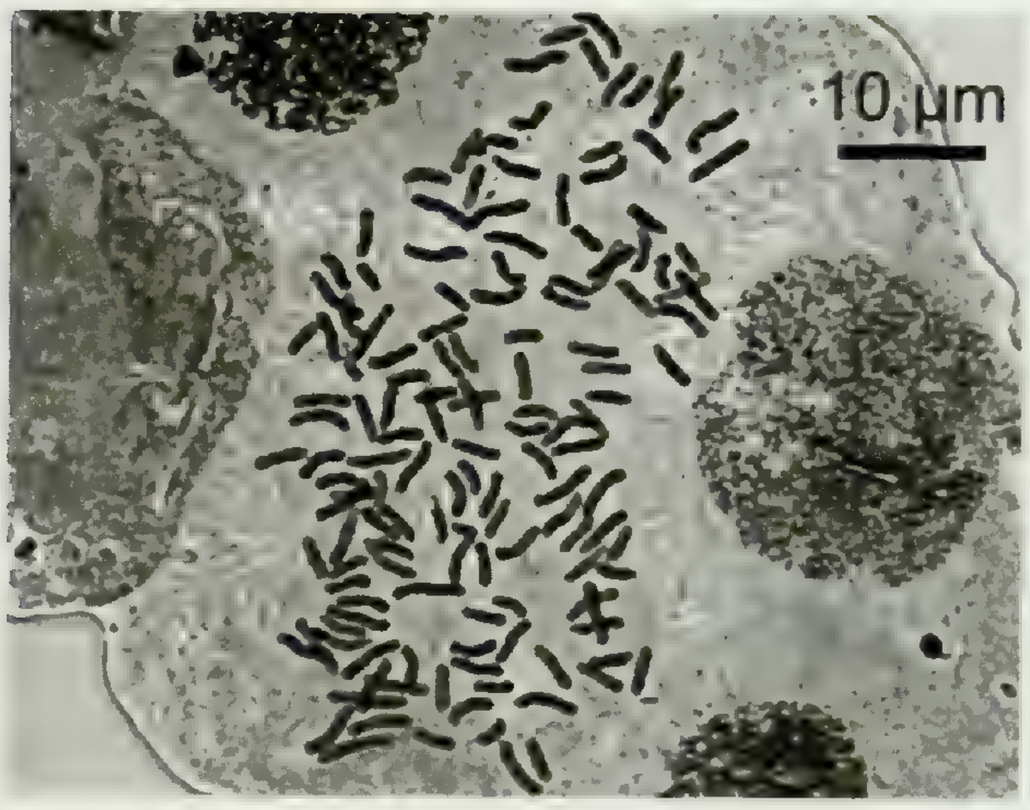
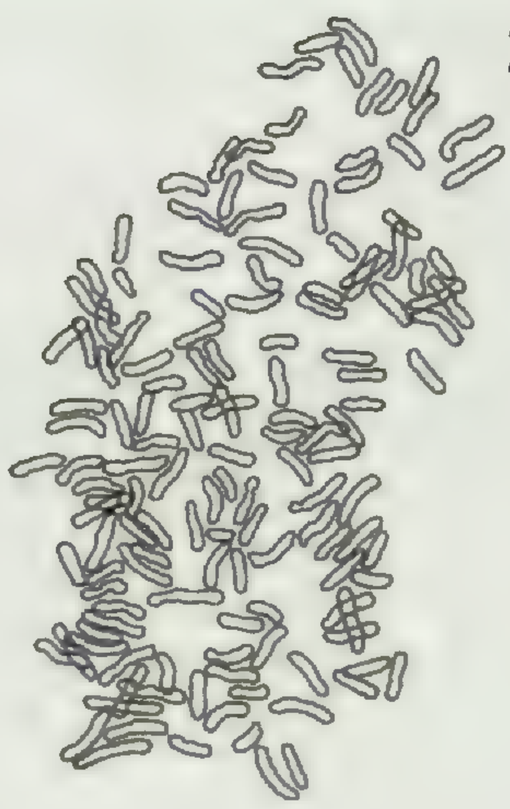




7

FIGS. 5–7. Photomicrographs (left) and explanatory diagrams (right) of chromosomes at mitosis phase. 5. *Asplenium humbertii* from Guangxi, China (*Dong 2287B*); 6. *A. nidus* from Guangxi, China (*Dong 2939*); 7. *A. phyllitidis* subsp. *malesicum* from Hainan, China (*Dong 1645*).

ADDITIONAL SPECIMENS EXAMINED.—CHINA. **Hainan**: Changjiang, 19°01′N, 109°06′E, 1000 m, 20 Oct 2004, *Dong 1234* (IBSC); ibid. 5 Apr 1987, *Chen 703* (IBSC). **Guangxi**: Longzhou, 22°34′N, 106°48′E, 200 m, 14 Mar 2007, *Dong 2287B* (IBSC). **Yunnan**: Hekou, 22°40′N, 103°56′E, 150 m, 11 Aug 2010, *Dong 3248* (IBSC); ibid., 400 m, *Song 119* (IBSC); ibid., 400 m, Feb 1959, *Chu 3922* (PYU). Jinping, 23°02′N, 103°24′E, 500 m, 12 Aug 2010, *Dong 3432* (IBSC); ibid., 600 m, 10 Oct 2009, *Song 133* (IBSC).

LAOS. **Cammon**: Tham, 160 m, Nov 1930, *Colani 4098* (P); Thok, 160 m, Nov 1930, *Colani 4091* (P).

THAILAND. **Chiang Mai:** Chiang Dao, 1100 m, 16 Feb 1958/59, *Sorensen et al. 1237* (K); ibid., 550 m, 15 Jan 1989, *Maxwell 89–59* (L). **Chiang Rai**: local name unknown, 500 m, 27 May 1926, *Garrett 289* (K). **Muang Sing**: Kwai Noi Basin, 150 m, 3 Jun 1946, *Hoed 920* (L).

VIETNAM. **Bac Giang**: Lang Met, May 1929, *Colani 1982* (P). **Lang Son**: Lang Mac, Feb 1929, *Colani s.n.* (P). Without specific locality, 260–390 m, 22 Dec 1964, *Sino-Vietnam Exped. 699* (PE).

Asplenium nidus L., Sp. Pl. 1079. 1753. *Neottopteris nidus* (L.) J. Sm. in J. Bot. 3: 409. 1841. TYPE.—INDONESIA. Java, *Osbeck 49* (lectotype designated by Holttum in 1974, L).

Cytology.—Wild plants of this species from Hainan, Yunnan, Guangxi, or cultivated plants (Table 1) proved to be sexual tetraploid with chromosome number 2n = 144 (Fig. 6) or n = 72. This species has been reported as tetraploid from northern India (Bir, 1960), southern India (Abraham *et al.*, 1962), Kagoshima, Japan (Kawakami, 1970, 1997), Taiwan (Tsai and Hsieh, 1983), Hainan Island, China (Kato and Nakato, 1999), and from Java, Indonesia (Yatabe *et al.*, 2001).

Diagnostic characters.—Stipe scales narrowly lanceolate, 15–20 × 1–2 mm; fronds narrowly lanceolate; midribs obviously prominent on adaxial surface and nearly flat on abaxial surface; sori short and dense, occupying usually less than 1/2 length of veins, (10) 12–18 sori every 2 cm length along midribs.

Distribution.—The whole range of *Asplenium* sect. *Thamnopteris*, mainly on tropical Asia and Pacific islands, west to Africa and northeast to Hawaii.

REPRESENTATIVES OF CA. 1220 SPECIMENS EXAMINED.—AUSTRALIA. **Queensland**: Iron Range, Cape York Peninsula, 20 m, 6 Jun 1948, *Brass 19049* (K).

CHINA. **Xizang**: Motuo, 800 m, 11 Aug 1974, *Qinghai-Xizang Exped. 74–4215* (KUN, PE). **Hainan**: Mt Yinggeling, 680 m, 3 Dec 2005, *Dong 1689* (IBSC). **Guangxi**: Napo, 23°00′N, 105°51′E, 1000 m, 13 Jun 2009, *Dong 2939* (IBSC). **Yunnan**. Malipo, 23°01′N, 104°46′E, 780 m, 17 Jun 2009, *Dong 2980* (IBSC).

INDIA. **Andamans**: Mt Harriet, 3 Jan 1998, *Balachandra 0879* (K).

INDONESIA. **Kalimantan**: Balikapapan, 01°09′ S, 116°50′ E, 40 m, 16 Dec 2010, *Dong 3470* (IBSC); Samarinda, 01°07′ S, 117°12′ E, 20 m, 18 Dec 2010, *Dong 3474* (IBSC). **Irian Jaya**: Manokwari, 00°59′ S, 133°58′ E, 40 m, 12 Dec 2010, *Dong 3455 & 3456* (IBSC). **Java**: Bogor, 280 m, 25 Oct 2009, *Dong 3362* (IBSC). **Seram**: Manusela National Park, 3°08–09′S, 129°29′E, 1290–2000 m, 3 Jan 1985, *Kato et al. C350bis* (BO).

JAPAN. **Bonin**: Hahajima, 3 Jun 1971, *Sohma et al. 715352* (KYO); **Kagoshima**: Yakushima, 12 Aug 1957, *Iwatsuki 3285* (KYO).

KENYA. **Kwale**: Kaya Chombo, 04°08′S, 39°29′E, 240 m, 1 Sep 1999, *Luke & Mbinda 5972* (K).

MALASIA. **Malay Peninsula**: Perak, 2500–3000 ft, Jul 1884, *King's Collector 6347* (SING).

MYANMAR. **Bhamo**: Palin to Nampa, 500 ft, 15 Dec 1908, *Lace 4495* (K).

PACIFIC ISLANDS. **Hawaii**: Wai'anae Mts, O'ahu, 21°33′N, 158°11′W, 1550 ft, 11 Aug 1973, *Herbst 3090* (K). **Tonga**: Eua Island, 21°22′S, 174°56′W, 200–300 m, Jun 1926, *Parks 16258* (K). **Vanuatu**: Tegua, 220 m, 28 Oct 1993, *Curry 1364* (K).

PHILIPPINES. **Luzon**: Mt Sicapoo, 800 m, 6 Dec 1975, *Iwatsuki et al. P890* (K).

SIKKIM. **Ratongchu**, 1000 m, 15 May 1960, *Hara et al. 2416* (BM, K).

SRI LANKA. **Sabaragamuwa**: Ratnapura, 6°35′N, 80°43′E, 580–600 m, 30 Dec 1976, *R.B. & Faden 653* (K). **Opanake**: without specific locality, 3 Jan 1951, *Ballard 1372* (K).

THAILAND. **Udawn**: Mt Luang, 17°25′N, 101°25′E, 750 m, 7 Jan 1966, *Hennipman 3522* (BM).

UKUNDA. **Longomwagandi**: Shimb Hills, 04°14′S, 39°25′E, 380 m, 12 Jun 1996, *Luke er al. 4512* (K).

VIETNAM. **Bac Can**: Na Ri, 22°17′S, 106°03′E, 550–600 m, 20 Oct 1999, *Hiep et al. 3746* (K).

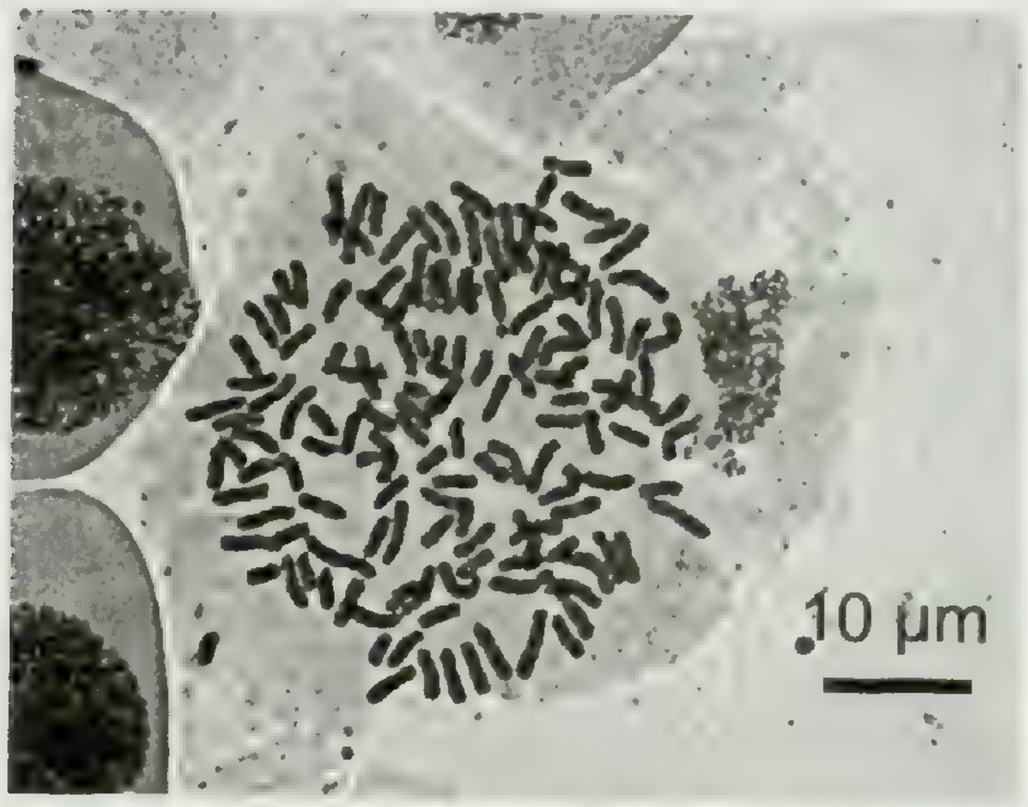
Molecular data indicate *A. nidus* is a complex containing several cryptic species (Murakami *et al.*, 1999; Yatabe *et al.*, 2001). The data of spore morphology have showed that the perispores are fenestrate and alately folded in populations of *A. nidus* from South China but imperforate and costately folded in those from Java, Indonesia (pers. obs.), which supports the *A. nidus* in present description is a complex. A taxonomic treatment on this complex is needed.

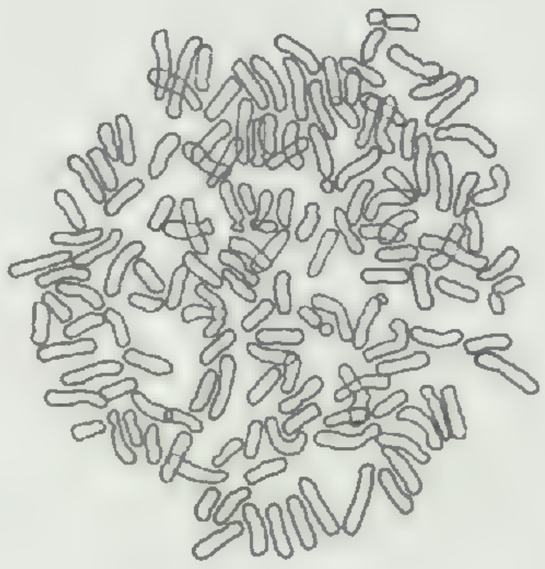
Asplenium phyllitidis subsp. ***malesicum*** Holttum in Gard. Bull. Singapore 27: 153. 1974. TYPE.—PHILIPPINES. Samar, *Cuming 319* (holotype, K!).

Cytology.—Both root tips and young prothalli were examined for a population from Hainan Island, China (Fig. 7), and one from Mindanao, Philippines. The chromosome number, 2n = 144 and n = ca. 72, were found in both populations. In addition, a cultivated plant with the origin unknown was confirmed to be a tetraploid (Fig. 8). This is the first cytological examination for this taxon.

Diagnostic characters.—Stipe scales narrowly lanceolate, 10–15 × 1–2 mm; fronds narrowly lanceolate; midribs obviously prominent on abaxial surface with the transection bluntly deltoid; sori long and sparse, occupying usually more than 3/4 length of veins, 7–9 sori every 2 cm length along midribs.

Distribution.—Throughout Malesia (Singapore, Malaysia, Indonesia, New Guinea, Philippines, Caroline Islands), Society Islands, Indo-China Peninsular (Thailand, Vietnam), southwestern China (including Hainan Island).





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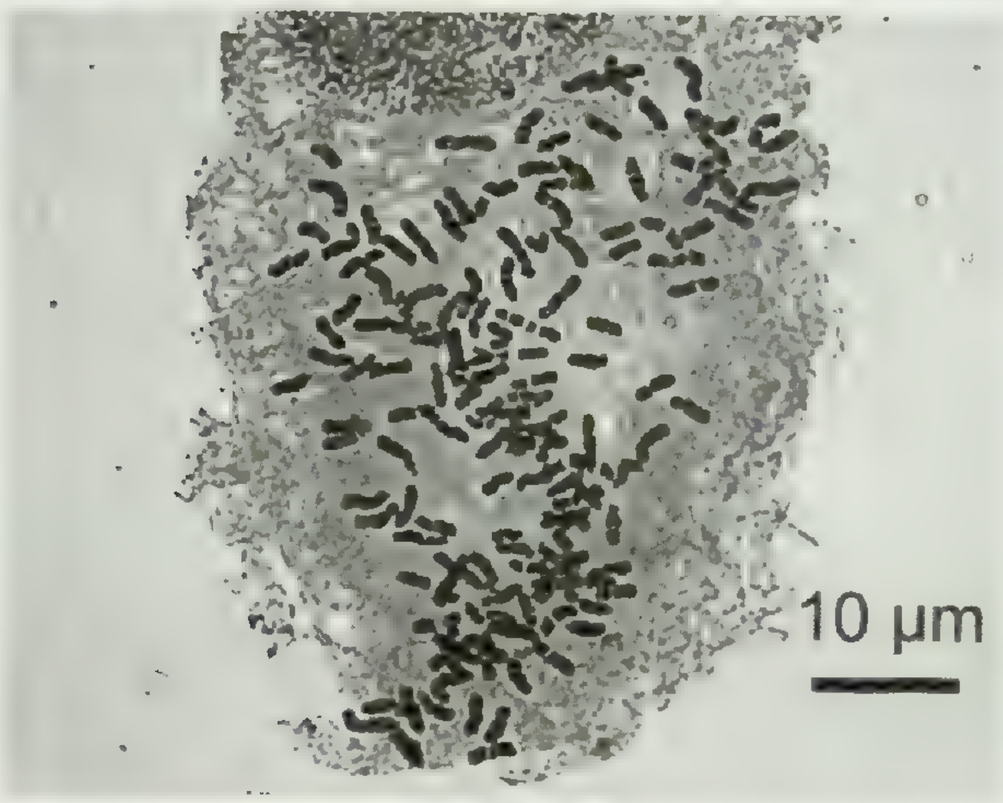

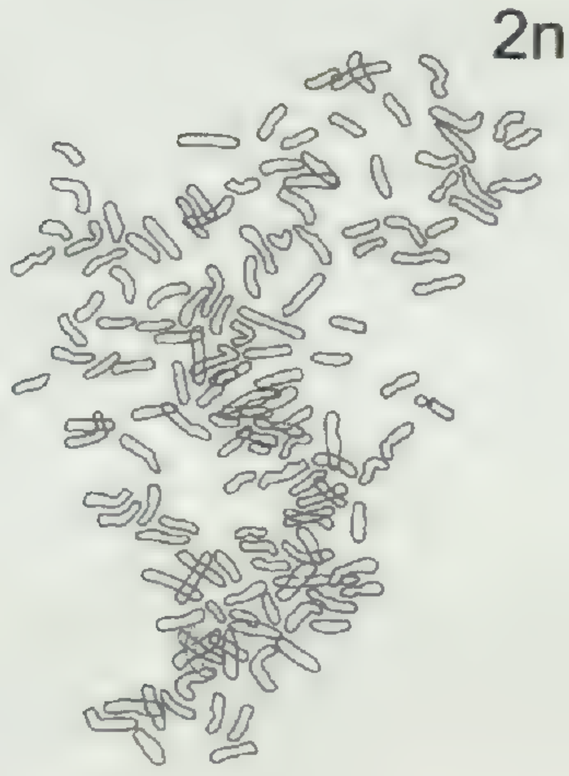

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FIGS. 8–9. Photomicrographs (left) and explanatory diagrams (right) of chromosomes at mitosis phase. 8. *Asplenium phyllitidis* subsp. *malesicum* cultivated in Guangzhou, China (*Dong 3360*); 9. *A. simonsianum* from Yunnan, China (*Dong 2768*).

REPRESENTATIVES OF CA. 600 SPECIMENS EXAMINED.—CHINA. **Guangxi**: Tianlin, 16 Jun 1958, *Li 600812* (IBSC, KUN). **Hainan**: Mt Yinggeling, 18°57′N, 109°24′, 1170 m, 27 Nov 2005, *Dong 1625* (IBSC). **Yunnan**: Mengla, 21°36′N, 101°35′, 700 m, 28 Apr 2008, *Dong 2762* (IBSC); Pingbian, 1400 m, 17 Jun 1934, *Tsai 60226* (KUN, P, PE).

INDONESIA. **Kalimantan**: Balikpapan, 01°09′S, 116°50′E, 40 m, 16 Dec 2010, *Dong 3469* (IBSC). **Sumatra**: Asahan, Nov 1932, *Krukoff 4305* (SING). **Ternate**: Castela, 4 Aug 1954, *Alston 16661* (BO). **Java**: Bodogol, 06°46′S, 106°51′E, 820 m, 1 Nov 2009, *Dong 3384* (IBSC).

MALAYSIA. **Pahang**: Kuantan, 130 m, 6 Jun 1968, *Ogata 10469* (L). **Sarawak**: Pulan Bruit, 12 ft, 14 Jun 1957, *Anderson 7939* (L).

PACIFIC ISLANDS. **Bismarck**: Mandiuh Lake, Namatanai, 4°28′S, 153°3′E, 650 m, 7 Oct 1975, *Croft 68298* (L). **Caroline**: Urukthapel, 1–10 m, 17–19 Mar 1950, *Fosberg* (L). **New Britain**: Talasea, 5°21′S, 149°58′E, 600 m, 23 Oct 1974, *Barker & Vinas 66659* (L). **New Ireland**: Tamai River, 600 m, 14 Oct 1975, *Croft 254* (L). **Society**: Guadalcanal, 2000 ft, 5 Nov 1962, *Whitmore & Womersley 1045* (L); Raiatea Island, 160 m, 5 Oct 1926, *Moore 171A* (L).

PAPUA NEW GUINEA. **Kaisenik**, 07°20′ S, 146°40′ E, 2200 m, 17 Feb 1978, *Unkau 025* (K, L). **Maneau**: Mt Dayman, Maneau, 16 Jul 1953, *Brass 23439* (L).

PHILIPPINES. **Laguna**: Los Banos, 14°08′N, 121°12′E, Jun–Jul 1917, *Elmer 17976* (K). **Leyte**: Palo, 11°10′N, 124°59′E, Jan 1906, *Elmer 7037* (K). **Mindanao**: Davao, 1200 m, 16 Nov 2007, *Dong 2606* (IBSC). **Mindoro**: Luzon, 13°17′N, 121°00′E, 1200 m, 15 Mar 1997, *Argent et al. 20104* (L). **Sorsogon**: Irosin, 12°46′N, 124°03′E, Sep 1916, *Elmer 17312* K).

THAILAND. **Trat**: Klawng Mayom, 100 m, 16 Feb 1955, *T.S. 2177* (L). **Narathiwat**: Tak Bai, 1 Sep 1987, *Niyomdham & Sriboonma 1475* (K).

VIETNAM. **Quang Ninh**: Mong Cai, 27–30 Sep 1936, *Tsang 26918* (IBSC, K).

This taxon is very widespread in forest area of tropical Asia and Pacific islands but herbarium materials are too often misidentified as *A. phyllitidis* subsp. *phyllitidis* or *A. nidus*. As being distinct from other members of *Thamnopteris* in bluntly deltoid midribs in transection, long and sparse sori, and exclusively spinulate perispores, *A. phyllitidis* subsp. *malesicum* is suggested to be a separate species but not a subspecies. A detailed discussion and taxonomic treatment are being prepared by the present author in another paper.

Asplenium simonsianum Hook., Icon. Pl. 10: t. 925. 1854. *Neottopteris simonsiana* (Hook.) J. Sm., Hist. Fil. 330. 1875. TYPE.—INDIA. Khasia, *Simons 232* (err. 432) (holotype, K!).

Cytology.—The chromosome number carried out to be 2n = 144 for a population of this species from Yunnan, China for the first time (Fig. 9). It is a sexual tetraploid.

Diagnostic characters.—Stipe scales ovate or broadly lanceolate, 2–3 × 1 mm or 5–8 × 1.5–2 mm; fronds narrowly lanceolate; midribs slightly prominent on both surfaces; sori long, dense or sometimes sparse, occupying usually 2/3–3/4 length of veins, 10–13 sori every 2 cm length along midribs.

Distribution.—Southwestern China (Yunnan, Xizang), northeastern India (Khasia, Assam), Nepal, Bhutan, Sikkim, Myanmar, Thailand (Lampang).

REPRESENTATIVES OF CA. 160 SPECIMENS EXAMINED.—BANGLADESH. **Sylhet**, *Wallich 723* (BM). "Ham. Hanopokhri", 18 Jul 1914, *Ghose 11* (P).

BHUTAN. **Gelephu** (formly Surelakha), 4000 ft, 1 Oct 1937, *Ludlow & Sherriff 2932* (BM); ibid., 3000 ft, 29 Mar 1949, *Ludlow et al. 18727* (BM).

CHINA. **Guangxi**: Fusui, 350 m, 26 Apr 1957, *Chun 12136* (IBSC, KUN, PE). **Yunnan**: Fohai, 25 Jun 1956, *Yunnan Univ Exped. 579* (PE); Jinghong, 960 m, Jan 1958, *Yunnan Univ Exped. 682* (PE); Jinping, 27 Apr 1956, *Sino-Russia Exped. 302* (IBSC, PE); Lvchun, 1800 m, 11 Jul 1973, *Tao 742* (IBSC, KUN); Maguan, 19 Feb 1990, *Min 276* (KUN); Mengla, 21°36′N, 101°35′E, 700 m, 29 Apr 2008, *Dong 2768* (IBSC); Mengzi, Apr 1953, *Cai 280* (PE); Nabang, 8 Sep 1980, *Dong 792* (PE). **Xizang**: Motuo, 19 Aug 1980, *Chen 14290* (PE); unknown, 28°00′N, 97°45′E, 4000 ft, 1926, *Ward s.n.* (K).

INDIA. **Assam**: Digboi, 450–700 ft, 2 Mar 1936, *Barnard 43A* (BM); Garrow Hills, 1880, *Day s.n.* (K); Gauhati Hills, *Simons s.n.* (BM); Naga Hills, 5000 ft, 8

Feb 1946, *Bor 44* (BM). **West Bengal**: Darjeeling, 1871, *Anonymous s.n.* (P, "Syn. Fil. no. 1").

NEPAL. **Pokhara**: Bakhri Kharka, 4500 ft, 23 Apr 1954, *Stainton et al. 5005* (BM).

THAILAND. **Lampang**: without specific locality, 450 m, *Smith(?) 895 or 1682* (K). **Kamphaeng Phet**: Hual Krasa, 16°05′N, 99°09′E, 900 m, 19 Mar 1968, *Hansen & Smitinand 12972* (K). **Phetchabun**: Phu Miang, 1200–1300 m, 2 Oct 1967, *Shimizu et al. T11378* (L).

KEY TO THE SPECIES AND INTRASPECIFIC TAXA OF ASPLENIUM SECT. THAMNOPTERIS RECORDED HERE

1. Stipe scales narrowly lanceolate, the ratio of length to width more than 10
   2. Frond midribs flat or slightly rounded on abaxial side . . . . . *A. nidus*
   2. Frond midribs obviously prominent, keeled or nearly so on abaxial side
      3. Frond midribs not typically keeled on abaxial side, bluntly triangular in transection; perispores echinate . . . . . *A. phyllitidis* subsp. *malesicum*
      3. Frond midribs strongly keeled on abaxial side, sharply triangular in transection; perispores folded . . . . . *A. australasicum*
1. Stipe scales ovate or broadly lanceolate, the ratio of length to width usually being 2 to 5
   4. Frond midribs strongly keeled on abaxial side, sharply triangular in transection . . . . . *A. antrophyoides*
   4. Frond midribs rounded on abaxial side, ellipse or nearly so in transection
      5. Fronds rounded at base; wingless stipes lacking . . . . . *A. cymbifolium* f. *lingganum*
      5. Fronds cuneate at base; wingless stipes present
         6. Stipes with very narrow wings along either side, the same long or a bit shorter than laminae; chromosome number 2n = 216; restricted to limestone area. . . . *A. humbertii*
         6. Stipes very short, less than 1/20 the length of laminae; chromosome number 2n = 144; not occurring on limestone area
            7. Fronds 8–12 cm wide; stipe scales 15–30 mm long . . . . . *A. antiquum*
            7. Fronds 3–6 cm wide; stipe scales less than 8 mm long . . . . . *A. simonsianum*

PHYLOGENETIC SIGNIFICANCE OF THE CYTOLOGICAL DATA

The eight species recorded here are widely accepted species which constitute the majority of *Asplenium* sect. *Thamnopteris*. Observations in this study and those by previous authors (Bir, 1960; Abraham *et al.*, 1962; Kawakami, 1970, 1997; Kato and Nakato, 1999; Yatabe *et al.*, 2001; Tindale and Roy, 2002) showed that the major members of *Thamnopteris*, several with more than one population from various regions examined, are sexual tetraploids and *A. humbertii* is the only hexaploid so far found. It is readily presumed that these tetraploids originated from diploids via some genetic mechanism which resulted in doubled chromosome sets. *Asplenium humbertii* with six chromosome sets is presumed to have a hybrid origin in that a diploid ancestor crossed with a tetraploid and subsequently, the triploid hybrid changed to sexual by doubling chromosome sets. Before this study, no diploid species was known in the section *Thamnopteris*, which suggests this group probably originated from a diploid *Asplenium* species outside of section *Thamnopteris*.

Acknowledgments

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Literature Cited

Abraham, A., C. A. Ninan and P. M. Mathew. 1962. Studies on the cytology and phylogeny of the pteridophytes. VII. Observations on one hundred species of south Indian ferns. J. Indian Bot. Soc. 41:339–421.

Bellefroid, E., S. K. Rambe, O. Leroux and R. L. L. Viane. 2010. The base number of 'loxoscaphoid' Asplenium species and its implication for cytoevolution in Aspleniaceae. Ann. Bot. 106:157–171.

Bir, S. S. 1960. Cytological observations on the East Himalayan member of *Asplenium* L. Curr. Sci. 29:445–447.

Ching, R. C. and C. H. Wang. 1964. Additional materials for the pteridophytic flora of Hainan. Acta Phytotax. Sin. 9:345–373.

Holttum, R. E. 1974. *Asplenium* L. sect. *Thamnopteris* Presl. Gard. Bull. Singapore 27:143–154.

Jones, D. L. 1988. New fern species from northern Australia. Austrobailey 2:469–480.

Kato, M. and N. Nakato. 1999. A cytotaxonomic study of Hainan (S China) pteridophytes with notes on polypoidy and apogamy of Chinese species. Pp. 1–19, *In:* X. C. Zhang and K. H. Shing, eds. *Ching Memorial Volume*. Beijing: Chinese Forestry Publishing House.

Kawakami, S. 1970. Karyological studies on Aspleniaceae II. Bot. Mag. (Tokyo) 83:74–81.

Kawakami, S. 1997. Induction of apogamy in twelve fern species and the study of their somatic chromosomes. Chromosome Science 1:89–96.

Kramer, K. U. and R. L. L. Viane. 1990. Aspleniaceae. Pp. 52–56, *In:* K. U. Kramer and P. S. Green, eds. *Pteridophytes and gymnosperms*. Berlin: Springer-Verlag.

Linnaeus, C. 1753. Species Plantarum, Stockholm.

Lovis, J. D. 1977. Evolutionary patterns and processes in ferns. Adv. Bot. Res. 4:229–451.

Makino, T. 1929. A contribution to the knowledge of the Flora of Japan. J. Jap. Bot. 6:29–32.

Manton, I. 1950. Problems of cytology and evolution in the Pteridopyhta. Cambridge: Cambridge University Press.

Miao, R. H. 1980. Additions to the pteridophytic flora of Hainan. Acta Sci. Nat. Univ. Sunyatseni 1:99–100.

Murakami, N., M. Watanabe, J. Yokoyama, Y. Yatabe, H. Iwasaki and S. Serizawa. 1999. Molecular taxonomic study and revision of the three Japanese species of *Asplenium* sect. *Thamnopteris*. J. Pl. Res. 112:15–25.

Nakato, N. 1987. Notes on chromosomes of Japanese pteridophytes (1). J. Jap. Bot. 62:261–267.

Presl, C. B. 1836. Tentamen Pteridographiae, seu Genera Finicacearum. Typis Filiorum Theophili Haase, Prague.

Seto, K. 1979. Notes on the bird's nest ferns in Japan. Nankiseibutu 21:6–10.

Smith, J. 1841. Genera Filicum, an arrangement and definition of the genera of ferns with observations on the affinities of each genus. Hooker's J. Bot. Kew Gard. Misc. 3:409.

Tardieu-Blot, M. 1933. Contribution a l' étude des Aspléniées de l'Ido-Chine I, *Asplenium*. Bull. Mus. Natl. Hist. Nat. 5:480–487.

Tindale, M. D. and S. K. Roy. 2002. A cytotaxonomic survey of the Pteridophyta of Australia. Aus. Sys. Bot. 15:839–937.

Tsai, J. L. and W. Q. Shieh. 1983. A cytotaxonomic survey of the pteridophytes in Taiwan. J. Sci. Engin. 20:137–158.

WALKER, T. G. 1979. The cytogenetics of ferns. Pp. 87–132, *In:* A. F. Dyer, ed. *The Experimental Biology of Ferns*. London: Academic Press.

WU, S. H. 1989. Materials of Chinese Aspleniaceae II. Guihaia 9:289–292.

WU, S. H. 1999. *Flora Reipublicae Popularis Sinicae 4*(2). Science Press, Beijing.

YATABE, Y., S. MASUYAMA, D. DARNAEDI and N. MURAKAMI. 2001. Molecular systematics of the *Asplenium nidus* complex from Mt. Halimun National Parks, Indonesia: evidence for reproductive isolation among three sympatric *rbc*L sequence types. Amer. J. Bot. 88:1517–1522.

American Fern Journal 101(3):172–181 (2011)

Development of the Epidermal Cells of the Pinnules of *Adiantum raddianum* C.Presl (Pteridaceae): Environmental Adaptive Plastidial Characteristics

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Abstract.—Plants have different strategies for adapting to environmental conditions, such as characteristics that allow them to be more efficient in shady or sunny environments. Those that grow in dimly lit environments often have a photosynthetic epidermis. However, *Adiantum raddianum*, known as the maidenhair fern, is a species found in sunny environments that has this characteristic. Within the abaxial and adaxial epidermal cells of the leaf pinnules of this species, there are arm-like projections where chloroplasts agglomerate. The goal of this study was to describe the development of the epidermal cells of *A. raddianum*, describe the morphological characteristics of the chloroplasts in these cells, and interpret any cytological characteristics that this species might have as a result of an adaptive survival strategy. The study found that the arm-like projections within the epidermis develop when the leaves are young and still exhibit circinate vernation. Cytological observations revealed a plastidial dimorphism, where there was variation in the arrangement of the thylakoid system, and the presence of stromules, which may help establish a connection among chloroplasts and between these organelles and mitochondria and peroxisomes. Descriptions of the stromules and plastidial dimorphism, made in this study, can be included with other known epidermal adaptive strategies (e.g., plastidial movement and mucilage secretion), which help this plant to survive under different environmental conditions.

Key Words.—*Adiantum*, chloroplasts, dimorphism, stromules, adaptation

Within a natural environment, light varies in duration, amount, and spectral quality. Many plants have developed adaptations in response to these variations, for example, within their leaves (Vogelmann and Martin, 1993). Leaves that grow predominantly in the shade often vary in thickness and size and have variable stomata and vein density when compared to those that grow in the sun. In addition, the leaf mesophyll, which is usually the main photosynthetic stratum, may vary in the number of palisade and spongy parenchyma layers and in the compression of the cells that form the tissue (Evans, 1999; Dickinson, 2000).

According to Esau (1965), the functions of the aerial epidermis are transpiration restriction, protection, gas exchange via stomata, and the storage of water and metabolic products. However, Esau also stated that in some cases the epidermis might assume additional functions that could cause it to have

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atypical characteristics, such as the ability to photosynthesize or secrete metabolic products. The presence of chloroplasts in the leaf epidermis is common in pteridophytes (mainly in filmy ferns), aquatic plants, and some terrestrial gymnosperms and angiosperms, especially those that grow in the shade (Esau, 1965; Mickel, 1972; Dickinson, 2000).

In members of the leptosporangiate *Adiantum*, the leaf pinnules have an epidermis that acts as a photosynthetic stratum, in addition to participating in gas exchange and the transportation of metabolites (through its veins). The importance of the epidermal cells in this genus is greater in some species, especially when the mesophyll of the species contains sparsely distributed arm-like cells that form projections on the internal periclinal walls where chloroplasts agglomerate (Wylie, 1948). This characteristic is found in the abaxial and adaxial epidermal cells of *Adiantum raddianum* C. Presl (popularly known as the maidenhair fern), which is a common species in South and Central America, Mexico and the Old World (Wylie, 1948; Ogura, 1972; Davidse *et al.*, 1995; Gómez and Arbeláez, 2009). Although a photosynthetic epidermis is generally associated with shade plants, *A. raddianum* grows in semi-shady to sunny environments, such as on cliffs along roadsides, on the banks of watercourses (Sehnem, 1972), and along the edges of forests (Senna and Kasmirczak, 1997).

Silva *et al.* (2007) observed the accumulation of vacuolar mucilage and the motion of chloroplasts within the epidermis of *A. raddianum*. The motion of the chloroplasts was observed on the external periclinal walls of immature leaves, and was restricted to projections corresponding to the internal periclinal walls when the leaves were mature. These characteristics were interpreted as environmental adaptations.

The goal of this study was to describe the development of the epidermal cells of *A. raddianum*, describe the morphological characteristics of the chloroplasts in these cells, and interpret any cytological characteristics that *A. raddianum* might have as a result of an adaptive survival strategy.

Material and Methods

Collection site and stages of development.—The material was collected at Morro Santana, in Porto Alegre, Rio Grande do Sul, Brazil (30°02′00″S to 30°04′40″S and 51°06′30′W to 51°09′00″W). The plants were collected on a cliff with exposed soil, which received six to eight hours of sunlight per day. For this study, four developmental stages were identified based on morphological and anatomical differences. Pinnules of all stages were always collected at the same time. Three stages of pinnule development were collected from leaves with circinate vernation and the fourth stage collected was of leaves that were fully expanded.

Optical microscopy.—Median portions of sterile pinnules were prepared using a fixative of 1% glutaraldehyde and 4% formaldehyde, in a 0.1 M sodium phosphate buffer, at pH 7.2 (McDowell and Trump, 1976). They were then dehydrated in a graded ethanol series and placed in an ethanol-

chloroform graded series (Purvis *et al.*, 1964). The material was then embedded in hydroxyethylmetacrylate resin (Gerrits and Smid, 1983). Transverse and longitudinal sections (2 μm thick) were cut using a rotary microtome (Microm HM 340) with a glass knife. The sections were stained with Toluidine Blue O (C.I. 52040), at pH 4.4 (Feder and O'Brien, 1958). In addition, a histochemical test using Lugol's iodine (Sass, 1951) was performed in order to detect starch grains. The sections were observed using a bright field Leica DM R microscope.

Transmission electron microscopy (TEM).—The median region of sterile pinnules was fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M sodium phosphate buffer, at pH 7.2 (Roland and Vian, 1991). The material was postfixed in a solution of 2% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide ($K_3Fe(CN)_6$) in a 0.2 M sodium phosphate buffer, at pH 7.2 (Weber, 1992). Subsequently, the material was dehydrated in an acetone series and embedded in low-viscosity epoxy resin (Spurr, 1969). Transverse, ultrathin sections were obtained using a Leica Ultracut UCT ultramicrotome with a diamond knife. The sections were adhered to copper grids and stained with a 2% aqueous uranyl acetate (Bozzola and Russel, 1999) and 5% lead citrate solution (Hanaichi *et al.*, 1986). The analyses were performed using a Jeol 1200 ExII transmission electron microscope.

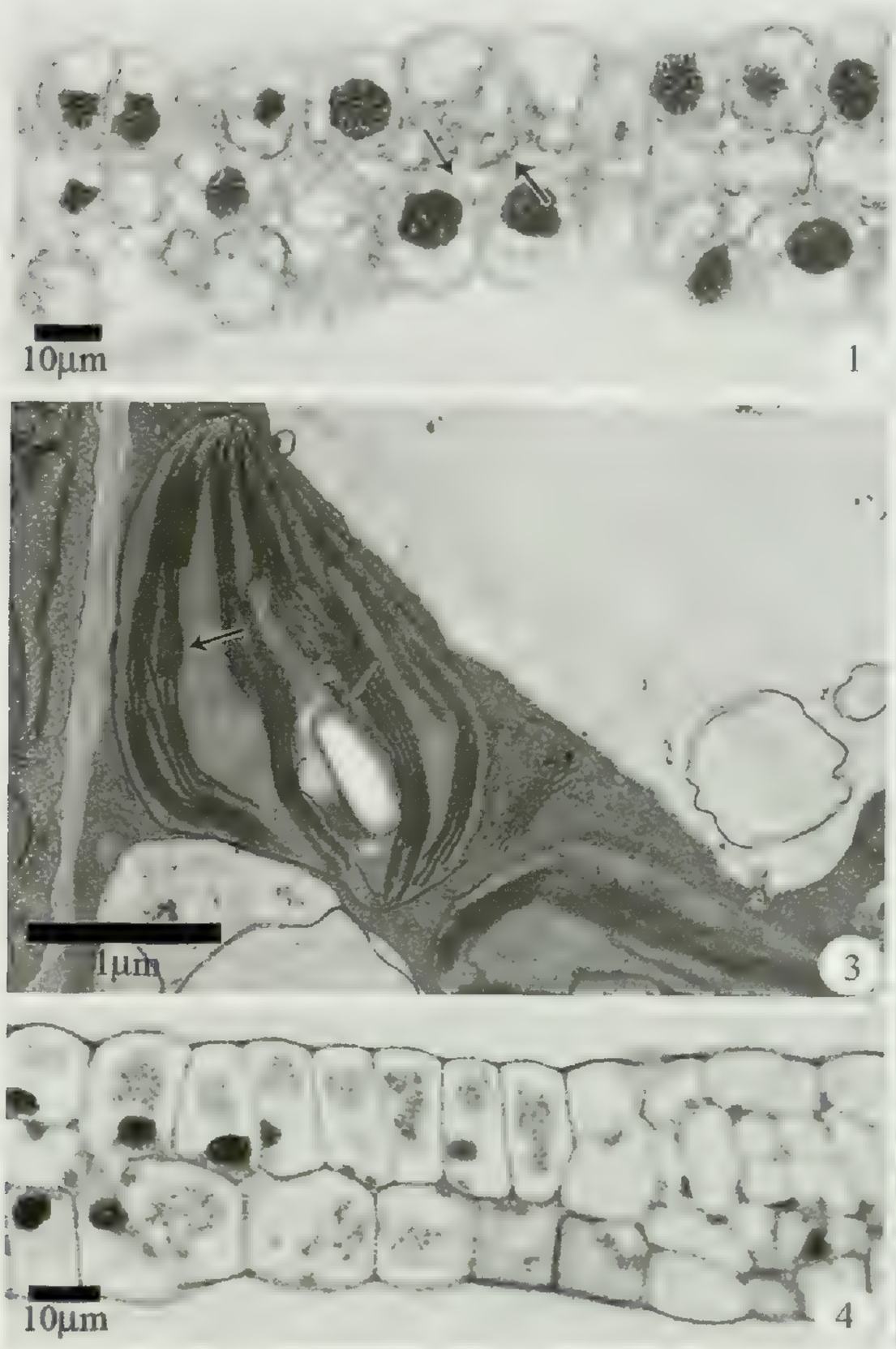
Confocal laser scanning microscopy.—In order to analyze the morphology of the chloroplasts in the epidermis, epidermal peels were made using fully expanded pinnules. The fresh material, in paradermal view, was incubated with Calcofluor (Pacini *et al.*, 1999) and mounted in immersion oil. The slides and coverslips were sealed with nail polish. The material was analyzed using an Olympus FV1000 confocal microscope using an ×60 oil immersion objective. Excitation filters were used to analyze the Calcofluor test (at band width 330–385 nm) and chlorophyll autofluorescence (at band width 530–550 nm). Z-series profiles of 20 optical sections were collected at 1μm using the Olympus FV1000 software.

Results

The most undifferentiated pinnules were represented by those of "stage one", where all epidermal cells were rectangular and lacked large intercellular spaces (Fig. 1). In this stage, contact among epidermal cells was observed (Fig. 1, arrows). These cells contained dense cytoplasm and a large vacuole, and many mitochondria were observed near the chloroplasts (Fig. 2, arrows). A smooth endoplasmic reticulum was observed next to the other organelles. The epidermal chloroplasts had starch granules (Fig. 3, asterisk), thylakoids, and were starting to form grana (Fig. 3, arrow).

In the second stage, the epidermal cells were more anticlinally elongated and there were no intercellular spaces (Fig. 4). Another relevant characteristic observed was the formation of protrusions of the plastidial membrane, which were free of thylakoids (Fig. 5, asterisks).

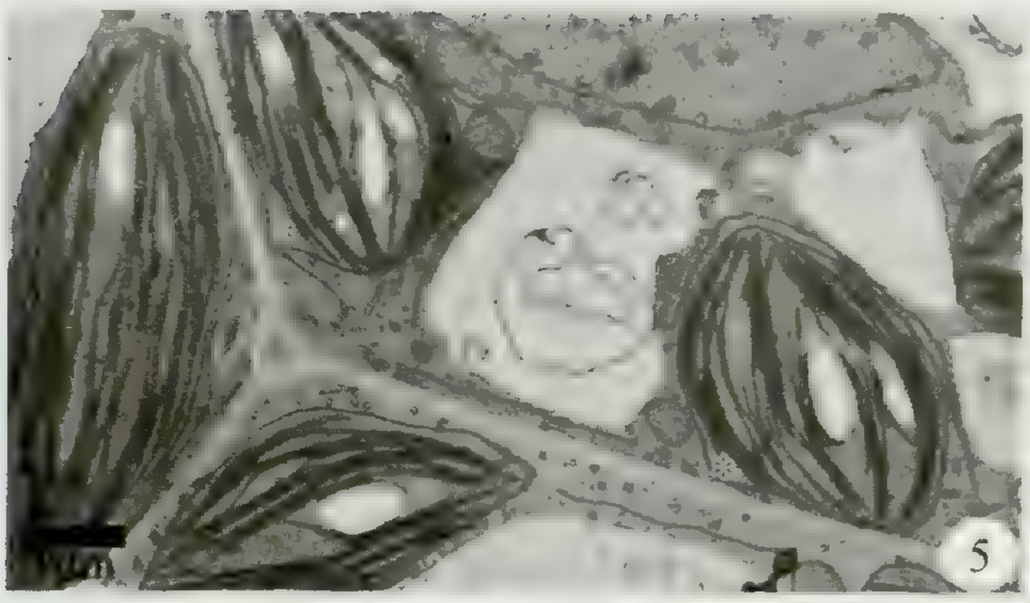
The third and final developmental stage collected while the leaves had circinate vernation, showed the initial development of the arm-like projections

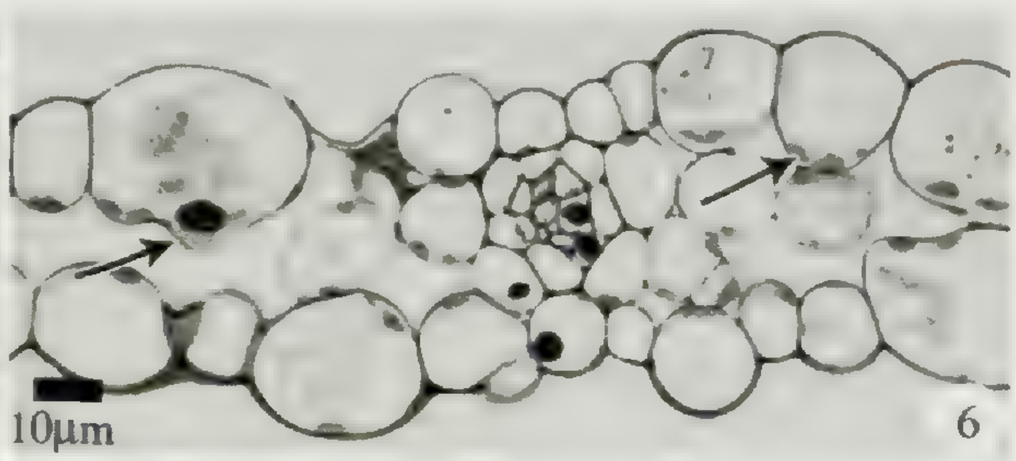


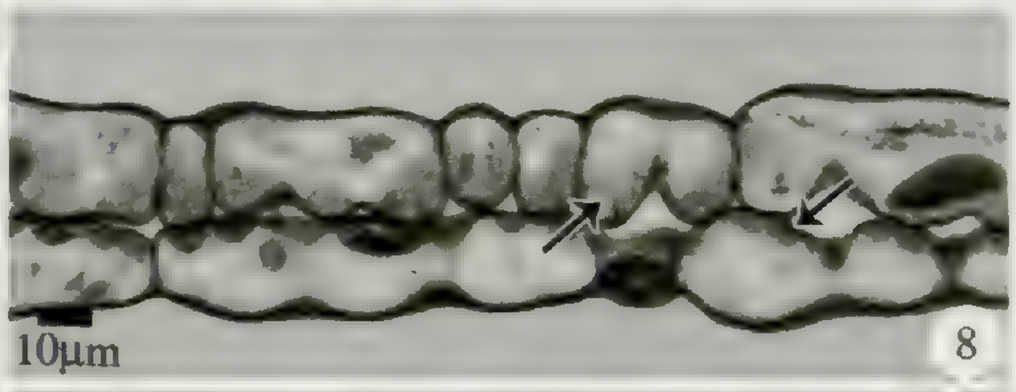
FIGS. 1–4. Photomicrographs (1 and 4) and electron micrographs (2 and 3) of transverse sections of pinnules of *Adiantum raddianum*. **1)** Stage one, general aspect of the epidermal cells and contact between the surfaces of the epidermis (arrows). **2)** Stage one, montage of two electron micrographs of the general aspect of the cell with large vacuole (v) and the association of chloroplasts and mitochondria (arrows). **3)** Stage one, chloroplasts with thylakoids and initial formation of grana. **4)** Stage two, elongated epidermal cells without intercellular spaces.

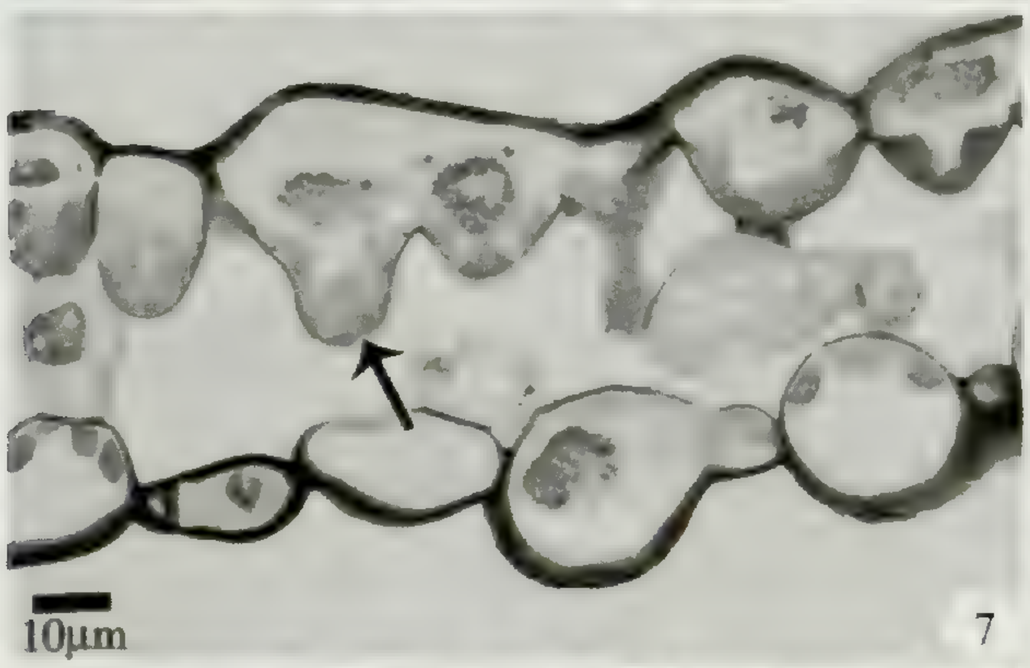
(in both epidermal surfaces) where the chloroplasts gather (Fig. 6, arrows). Few mesophyll cells were present, which resulted in wide intercellular spaces during leaf expansion.

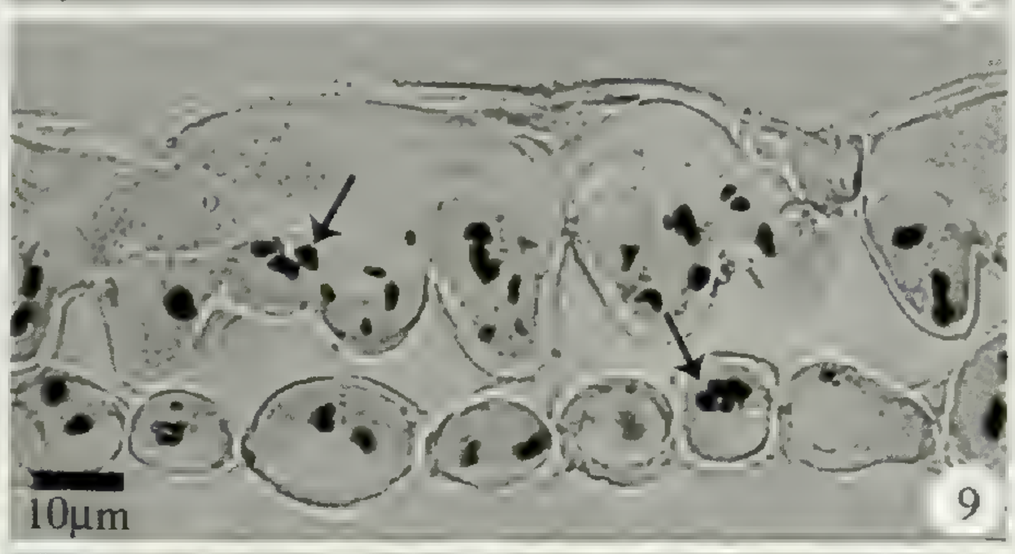
In the fourth stage, which was of pinnules that were completely expanded, the arm-like projections within the adaxial and abaxial epidermal cells were fully developed, establishing the region where the chloroplasts gather (Fig. 7). In longitudinal section, the arm-like projections of the adaxial surface were more prominent than those of the abaxial surface (Fig. 8). The starch granules were visible in the chloroplasts of both epidermal surfaces (Fig. 9, arrows), and were not noticeably different in size or quantity when compared to those observed in the initial stages. However, the analysis of transversal TEM sections revealed a plastidial dimorphism related to the orientation of the thylakoids and the grana system (Fig. 10). Chloroplasts showed two types of membrane organization, one with the thylakoids oriented in all directions

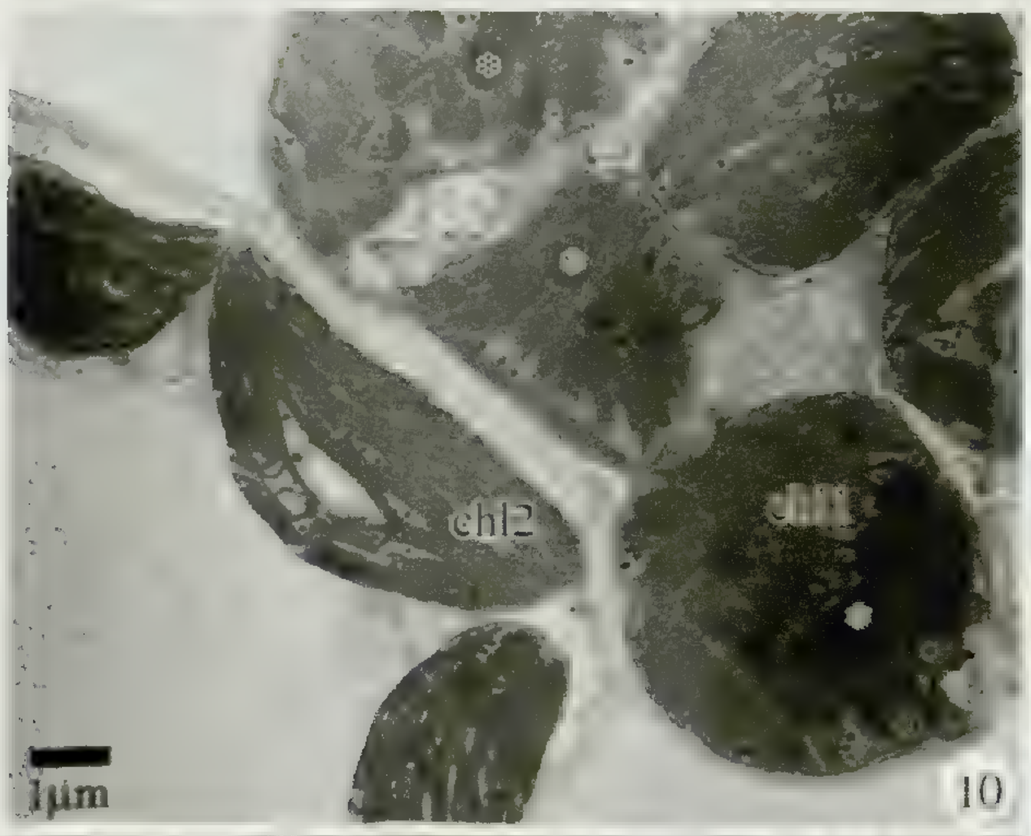


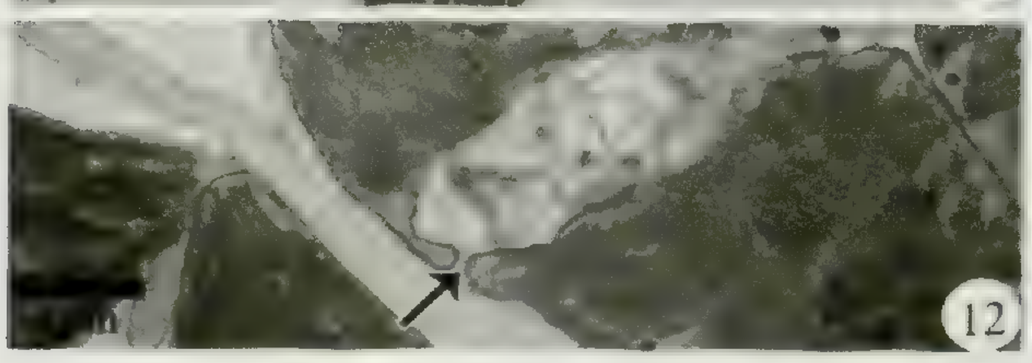












FIGS. 5–9. Photomicrographs (6, 7, 8, 9, 11) and electron micrographs (5 and 10, 12, 13) of transversal and longitudinal sections of pinnules of *A. raddianum*. **5)** Stage two, initial development of the membrane projections free from thylakoids (asterisks). **6)** Stage three, development of the arm-like projections (arrows) in adaxial epidermal cells and the presence of intercellular spaces. **7)** Stage four, general aspect of the epidermal cells with chloroplasts accumulated in the arm-like projections. **8)** Stage four, longitudinal section, elongated epidermal cells and arm-like projections in both surfaces (arrows). **9)** Stage four, detection of starch granules in the chloroplasts of adaxial and abaxial epidermal cells (arrows). **10)** Stage four. Clear dimorphism between chloroplasts, type Chl1 and Chl2, grana in frontal view (asterisks). **11)** Stage four, discoid-shaped chloroplasts in the cells of the adaxial surface of the epidermis (arrows). **12)** Stage four, contact between chloroplasts through the stromules. **13)** Stage four, contact between the projection of the plastidial membrane and mitochondria (arrow).

(Fig. 10, Chl1) and the other with this system of membranes oriented in only one direction (Fig. 10, Chl2). Both types were observed in the same leaf surface (data not shown).This type of dimorphism was based on the internal structure of the chloroplasts, but was not reflected in the external morphology; analysis of the plastidial morphology using confocal microscopy showed a morphological resemblance between the chloroplasts, all of which were discoid to slightly elliptic (Fig. 11).

In both types, the protrusion of the plastidial membrane, called a stromule, was evident. When the leaves were mature (the forth stage), the stromules were more elongated, allowing them to contact neighboring chloroplasts (Fig. 12, arrow), or mitochondria (Fig. 13, arrow) and peroxisomes (not shown).

Discussion

Light assimilation by leaves depends on their anatomical structure, and is associated with the number and distribution of chloroplasts per cell and the amount of chlorophyll pigment (Vogelmann and Martin, 1993). The development of arm-like projections within the adaxial and abaxial epidermal cells begins when the leaves are immature and still exhibit circinate vernation. Wylie (1948) stated that besides the chloroplasts being more efficiently located to properly capture light (resembling palisade parenchyma), such cells could facilitate the transport of metabolites among the veins.

In an anatomical study with species of the Pteridaceae, including some species of *Adiantum*, Graçano *et al.* (2001) observed the presence of chlorophyllous epidermal cells with arm-like projections in certain leaf blades. This characteristic was observed in plants that grew in shady environments. However, differently from the species described by Graçano *et al.* (2001), *A. raddianum* is a species that grows in sunny environments and has arm-like projections that orient the chloroplasts in a way that will not damage the photosynthetic apparatus when capturing light.

Besides the quick development of the arm-like projections as an adaptative characteristic to help direct light capture, Silva *et al.* (2007) observed the accumulation of vacuolar mucilage and the movement of chloroplasts in *A. raddianum*, which, when the pinnules were immature, were positioned on the external periclinal walls and, when mature, were restricted to the projections corresponding to the internal periclinal walls. These characteristics were interpreted as environmental adaptations.

The structure of the chloroplasts and the relative amounts of plastidial components are clear expressions of adaptation to environmental conditions (Bonatti and Fornasiero, 1990). Depending on the environmental conditions, the chloroplasts can be morphologically round or flat (Esau, 1965). The polymorphism of chloroplasts has been reported for some plants, and in some cases more than seven forms have been found inside a single leaf (Fisher and Evert, 1982). Besides this, there are reports about differentiated zones in relation to the organization of plastidial membranes inside a single chloroplast, where there is polarization among thylakoids with and without

the formation of grana, as described by Machado *et al.* (1986), and in bizonoplast, as described by Sheue *et al.* (2007). All of these characteristics denote the extreme flexibility of these organelles to different environmental conditions, and the chloroplast characteristics described in leaf blade of *A. raddianum* also reveals this environmental plasticity.

A. raddianum has a leaf blade composed mainly of epidermis and manifests structural variations among the chloroplasts. Similarly, in *Teratophyllum rotundifoliatum* (Bonap.) Holttum, a pteridophyte that grows in extremely shady environments, there is a dimorphism among the chloroplasts within the adaxial and abaxial epidermal cells, which is also the predominant leaf tissue in this species (Nasrulhaq-Boyce and Duckett, 1991). Jagels (1970) also verified a dimorphism among the chloroplasts occurring in both faces of the epidermis in the microphylls in five species of *Selaginella*, in which mesophyll is practically absent. However unlike previous studies, our study found that both types of chloroplasts occur within the same leaf surface.

The orientation of the grana lamellae in all directions in pteridophytes (such as in *Selaginella* [Jagels, 1970] and *T. rotundifoliatum* [Nasrulhaq-Boyce and Duckett, 1991]), Cycadaceae (Bonatti and Fornasiero, 1990), and angiosperms that grow in the shade (Anderson *et al.*, 1973) could be associated with an increase in the absorption of weak and diffuse solar radiation that reaches the forest floor (Nasrulhaq-Boyce and Duckett, 1991). *Adiantum raddianum*, however, occurs in sunny environments, and has the same characteristic of chloroplasts in the epidermis and plastidial dimorphism of the thylakoids' arrangement as the species analyzed by the aforementioned authors.

In plants with C4 metabolism, the dimorphism of the chloroplasts is related to the size of the chloroplast, the architecture of the thylakoid, and the starch content. This dimorphism can be observed between the bundle-sheath and the mesophyll cells and is associated with different biochemical functions. Nasrulhaq-Boyce and Duckett (1991) associated the plastidial dimorphism, in the pteridophyte *T. rotundifoliatum*, to the presence of metabolic differences, which resembled a plant with a C4 metabolism. Therefore, it is possible that in *A. raddianum*, each plastidial type could have a particular metabolic specificity.

Besides photosynthesis, chloroplasts are necessary for the biosynthesis of starch, amino acids, fatty acids, (Neuhaus and Emes, 2000), carotenes, purines, and pyrimidines (Köhler *et al.*, 1997). For this reason, it is presumed that the physical interaction between chloroplasts and the other organelles would turn the transference of metabolites among cellular compartments into a more efficient process (Kwok and Hanson, 2004). The external surface of the chloroplasts has the ability to form tubules filled with stroma, called stromules (Köhler and Hanson, 2000). Similar structures were observed in the chloroplasts of *A. raddianum*, from the youngest stage to the oldest, in both types of chloroplasts. Stromules have the ability to exchange metabolites with other organelles (Köhler and Hanson, 2000), with the cytoplasm (Natesan *et al.*, 2005), and even with the nucleus (Kwok and Hanson, 2004). Such structures have been observed before in species exposed to saline and high

temperatures (Holzinger *et al.*, 2007) and plants from alpine environments, which are naturally exposed to intense solar radiation (Lütz and Engel, 2007), and they could be considered an additional adaptation developed by plants to face certain climate conditions. Although, *A. raddianum* is a plant that is structurally similar to plants that live in shade, the occurrence of stromules indicates a possible adaptation to environments with high amounts of light. This is the first time that these structures are described for a species with a phenotype that is similar to a shade plant (with a photosynthetic epidermis), but grows in a sunny environment.

In conclusion, the plastidial characteristics observed during the development of the epidermal cells of the pinnules of *A. raddianum* seem to be associated with the adaptive ability of this species. The discrimination of the arm-like projections in immature pinnules, the presence of both forms of chloroplasts, and the presence of stromules in the epidermal chloroplasts can be added to characteristics (plastidial movement and the secretion of mucilage) already described by Silva *et al.* (2007) for the epidermis of *A. raddianum*, forming a set of adaptive strategies at several structural levels that this species uses to cope with particular environmental conditions.

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Literature Cited

Anderson, J. M., D. J. Goodchild and N. K. Boardman. 1973. Composition of photosystems and chloroplast structure in extreme shade plants. Bioch. Biophys. Acta 325(3):573–585.

Bonatti, P. M. and R. B. Fornasiero. 1990. Developmental pattern and structural organization of leaf chloroplasts in *Lepidozamia peroffskyana*. Aust. J. Bot. 38(1):53–62.

Bozzola, J. J. and L. D. Russel. 1998. *Electron microscopy. Principles and Techniques for Biologists*. Jones and Bartlett Publishers, Boston.

Davidse, G. S., S. Sousa, S. M. Knapp and S. M., (eds.) 1995. *Flora Mesoamericana. Volumen 1. Psilotaceae a Salviniaceae*. Universidad Nacional Autónoma de México, México. D.F.

Dyckinson, W. C. 2000. Integrative Plant Anatomy: Harcourt, San Diego.

Esau, K. 1965. *Plant anatomy. 2nd ed.* John Wiley & Sons Inc., New York.

Evans, J. R. 1999. Leaf anatomy enables more equal access to light and CO_2 between chloroplasts. New Phytol. 143:93–104.

Feder, N. and T. P. O'Brien. 1958. Plant microtechnique. Some principles and new methods. Amer. J. Bot. 55:123–142.

Fisher, D. G. and R. F. Evert. 1982. Studies on the leaf of *Amaranthus retroflexus* (Amaranthaceae): chloroplast polymorphism. Bot. Gaz. 143(2):146–155.

Gerrits, P. O. and L. Smid. 1983. A new, less polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. J. Microsc. 132:81–85.

Gómez, L. D. and A. L. Arbeláez. 2009. *Flora de Nicaragua. Helechos (Tomo IV).* Stevens, W. D., O. M. Montiel and A. Pool, eds. Monographs in systematic botany from the Missouri Botanical Garden, volume 116. Missouri Botanical Garden Press, St. Louis.

Graçano, D. A., A. A. Azevedo and J. Prado. 2001. Anatomia foliar das espécies de Pteridaceae do Parque Estadual do Rio Doce (PERD)-MG. Rev. Bras. Bot 24:337–347.

Hanaichi, T., T. Sato, T. Iwamoto, J. Malavasi-Yamashiro, M. Hoshiro and N. Mizuno. 1986. A stable lead by modification of Sato's method. J. Elect. Microsc. 35:304–306.

Holzinger, A., O. Buchner, C. Lütz and M. R. Hanson. 2007. Temperature-sensitive formation of chloroplast protrusions and stromules in mesophyll cells of *Arabidopsis thaliana*. Protoplasma 230:23–30.

Jagels, R. 1970. Photosynthetic apparatus in *Selaginella*. 2. Changes in plastid ultrastructure and pigment content under different light and temperature regimes. Can. J. Bot. 48(10): 1853–1870.

Köhler, R. H., J. Cao, R. Z. Warren, W. W. Webb and M. R. Hanson. 1997. Exchange of protein molecules through connections between higher plant plastids. Science 276:2039–2042.

Köhler, R. H. and M. R. Hanson. 2000. Plastids tubules of higher plants are tissue-specific and developmentally regulated. J. Cell Sci. 113:81–89.

Kwok, E. Y. and M. R. Hanson. 2004. Plastids and stromules interact with the nucleus and cell membrane in vascular plants. Plant Cell Rep. 23:188–195.

Lütz, C. and L. Engel. 2007. Changes in chloroplast ultrastructure in some high-alpine plants: adaptation to metabolic demands and climate? Protoplasma 230(3–4):183–192.

Machado, R. D., W. R. Sant'Anna and M. Benchimol. 1986. Internal dimorphism of chloroplasts in the leaves of *Peperomia pellucida* (L.) H.B.K. Protoplasma 134(2–3):73–77.

McDowell, E. M. and B. R. Trump. 1976. Histological fixatives for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. 1000:405–414.

Mickel, J. T. 1972. A "Film Fern" in the genus *Cystopteris*. Am. Fern. J. 62(3):93–95.

Nasrulhaq-Boyce, A. and J. G. Duckett. 1991. Dimorphic epidermal cell chloroplasts in the mesophyll-less leaves of an extreme-shade tropical fern, *Teratophyllum rotundifoliatum* (R. Bonap.) Holtt.: a light and electron microscope study. New Phytol. 119:433–444.

Natesan, S. K. A., J. A. Sullivan and J. C. Gray. 2005. Stromules: a characteristic cell-specific feature of plastid morphology. J. Exp. Bot. 56(413):787–797.

Neuhaus, H. E. and M. J. Emes. 2000. Nonphotosynthetic metabolism in plastids. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:111–140.

Ogura, Y. 1972. *Comparative anatomy of vegetative organs of the pteridophytes.* Gebrüger Borntraeger, Berlin.

Pacini, E., G. G. Franchi and M. Ripaccioli. 1999. Ripe pollen and histochemistry of some gymnosperms. Plant Syst. Evol. 217:81–99.

Purvis, M. J., Collier, D. C. and Walls, D.. 1964. *Laboratory techniques in botany.* Butterworths, London.

Roland, J. C. and B. Vian. 1991. General preparation and staining of thin sections. *In*: Hall, J. L. and E. Hawes, eds. *Electron Microscopy of Plant Cells.* Academic Press, London.

Sass, J. E. 1951. *Botanical microtechinique.* Iowa State University Press, Ames.

Sehnem, A. S. J. 1972. Pteridáceas. *In:* Reitz, R. ed. *Flora Ilustrada Catarinense.* Tipografia e Livraria Blumenauense; Itajaí.

Senna, R. M. and C. Kasmirckaz. 1997. Pteridófitas de um remanescente florestal no Morro da Extrema, Porto Alegre, RS. Rev. Fac. Zoo. Vet. Agro. 4(1):33–48.

Sheue, C.-H., V. Sarafis, R. Kiew, H.-Y. Liu, A. Salino, L.-L. Kuo-Hang, Y.-P. Yang, C.-C. Tsai, C.-H. Lin, J. W. H. Young and M. S. B. Ku. 2007. Bizonoplast, a unique chloroplast in the epidermal cells of microphylls in the shade plant *Selaginella erythropus* (Selaginellaceae). Am. J. Bot. 94(12):1922–1929.

Silva, F. S., A. A. Mastroberti and J. E. A. Mariath. 2007. Aspectos anatômico-funcionais das células epidérmicas de pínulas de *Adiantum raddianum* Presl. (Pteridaceae). Rev. Bras. Bio. 5(supl.1):831–833.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrast. Res. 26:31–34.

VOGELMANN, T. C. and G. MARTIN. 1993. The functional significance of palisade tissue: penetration of directional versus diffuse light. Plant Cell Environ. 16:65–72.

WEBER, M. 1992. The formation of pollenkitt in *Apium nodiflorum* (Apiaceae). Ann. Bot. 70:573–577.

WYLIE, R. B. 1948. The dominant role of the epidermis in leaves of *Adiantum*. Amer. J. Bot. 35:465–473.

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An Efficient Regeneration Pattern via Gemmae for *Huperzia serrata* (Thunb. ex Murray) Trev. in Hainan Province, China

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Abstract.—In the present study, we investigated a natural population of *H. serrata* in the Bawangling Nature Reserve of Hainan Province, South China. In field sampling, we examined the number of adult plants, gemmlings and sporelings as well as the gemma number of plants at different ages. A significant difference was observed between the numbers of gemmlings and sporelings. Most seedlings derived from gemmae, which critically influenced the population regeneration. The reproductive ability of gemmae became stronger from the 4th year of gemma growth. In the cultivation test, gemmae were planted in three different soil media, i.e., habitat soil, sand and humus. No significant difference was found in the gemmation rate among the three media, but the survival rate in sand was significantly lower than in the other two media. We also investigated the morphology of the gemma and gemmling growth pattern of *H. serrata*. The results may reveal the contributing role of gemmae in reproductive strategies, and be helpful to the resource protection and cultivation of *H. serrata*.

Key Words.—*Huperzia serrata* (Thunb. ex Murray) Trevis., Gemma, Gemmling, Sporeling, Regeneration pattern

Huperzia serrata (Thunb.ex Murray) Trevis. (formerly *Lycopodium serratum* Trevis., Huperziaceae) is a club moss, and commonly named Qiancengta in Chinese. *Huperzia serrata*, a perennial evergreen herb of 10–30 cm in height, grows well in forest, especially in shadowy, wet and humus-rich habitats. It is widely distributed from temperate East Asia to tropical Southeast Asia, even through Pacific islands to Central America. In China, this plant is distributed throughout the southern area (Ching, 1981; Zhang *et al.*, 2000). Since Liu *et al.* (1986 a, b) first extracted and isolated Huperzine A (Hup A) from *H. serrata*, this plant has attracted great attention. As a potent, reversible and selective acetylcholinesterase inhibitor capable of improving memory ability, Hup A is used in the treatment of Alzheimer's disease (AD) (Tang *et al.*, 1996). However, the great medicinal value of Hup A has caused its over-exploitation in recent years, and has greatly depleted stored material (Ma *et al.*, 2006).

The spatial distribution patterns of plant populations are determined by processes of growth, mortality and regeneration of individuals within the

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population (Ehrlen and Eriksson, 2000). Although *H. serrata* is distributed widely in temperate and tropical areas, these populations are usually small and scattered (Wu *et al.*, 2005). Its population size and structure are generally restricted to its propagation pattern and ecological factors. Its gametophyte and sporophyte usually grow slowly in habitats with relatively high humidity and rich organic matter. Spore propagation in other species of *Huperzia,* and the related genus, *Lycopodium,* has long been studied by botanists (Primack, 1973; Whittier *et al.,* 1986, 2006, 2007), but little is known about the process of spore germination and the morphology of gametophyte of *H. serrata.* Besides the spores and shoot culture, the specific vegetative propagules, which are called gemmae or bulbils, play an important role in dispersal and regeneration of *Huperzia* (Øllgaard, 1987; Reutter, 1987; Gola, 2008). Wang *et al.* (2007) studied the gemma morphology and seedlings of *H. javanica* (Sw.) Fraser-Jenk., a species closely related species to *H. serrata*, which can also produce gemmae. In Hainan populations, the adult plants produce new leaves, sporangia and gemmae from February to March, and usually the sporangia crack open to release spores in December and the following January. The gemmae fall down during the period from August to October. However, whether gemmae or spores are the main regeneration process of the natural population in Hainan Province remains unknown.

A lack of knowledge on the lifecycle and population structure of *H. serrata* results in difficulty in cultivating and propagating this plant. To solve the above problem, we investigated the natural population of *H. serrata* in the Bawangling Nature Reserve of Hainan Province from 2008 to 2009. We focused on the potential value of gemmae for propagation during the survey. Meanwhile, we observed the gemma structure of this plant both in field and in lab. Some mature gemmae were brought back to the botanical garden of our institute for cultivation tests in different soil media. Here we report the natural population structure, the gemma morphology and the survival rate of gemmlings of *H. serrata* in different media.

Materials and Methods

Study site.—This study took place in the tropical montane evergreen dwarf forest (alt.1300 m) in the Bawangling Nature Reserve of Hainan Province, South China. The climate is tropical monsoon, with annual precipitation of 1750 mm. Most of the precipitation occurs between May and October (Zang *et al.*, 2005). The parent material is granite and the soil is latosols (Lu *et al.*, 1986). The main concomitant species in the arbor layer include *Lithocarpus howii, Ternstroemia gymnaanthera, Symplocos chunii, Rhododendron klossii, R. moulmeinense,* and *Pinus fenzeliana.* The herbanceous layer consists of some species of ferns and mosses, such as *Abacopteris* sp., *Selaginella* sp., *Dumortiera* sp., *Frullania* sp. and *Radula* sp.

Materials.—The voucher (Zhu2008001) was deposited in the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical

Sciences (IMD). The gemmae for the cultivation test were all collected from this population.

Investigation methods in field.—We investigated for the first time the natural population of *H. serrata* in August, 2008. Since then we have continually observed the development of the population and we collected experimental materials more than ten times in other seasons.

In the field investigation, 36 quadrates were randomly set up (0.25 m^2 each quadrate) in a 10 m × 10 m area. The investigated items for each quadrat included plant number, seedling (including the gemmlings and sporelings) number, plant height, and gemma number.

Observation of gemma and cultivation experiments.—Mature gemmae were brought back for observation of gemma structure and cultivation experiment. Observation and photography of dissected gemmae were conducted with a light microscope (Nikon SMZ1500). A drawing of an adult sporophyte was made to explain the relationship between the gemma layer and the morphology of the plant.

Mature gemmae were collected from the above-mentioned natural population of *H. serrata* and cultivated in a sunlight-proof shed in December, 2008. Although gemmae shed from August to October, the gemmae survived for 2–3 months at 4°C after they were collected. The media adopted were as follows: the habitat soil (from the 5 cm topsoil of the *H. serrata* habitat, which contained the humus of mosses and leaves of conifers, fagaceous and theaceous trees), sand (from the Medicinal Botanic Garden of the Hainan Branch of the Institute of Medicinal Plant Development), and humus (from the 5 cm surface plantation soil of the above garden). For each treatment, we gathered enough soil for 3 repeats, mixed it well and put it into pots. The pots, 16 cm in diameter and 20 cm in depth, were placed in a sunlight-proof shed with 20% transmittance. The gemma bases were buried downward in the medium, at the depth of 2/3 gemma length and 20 gemmae per pot. The gemmation rate was calculated after 30 days cultivation, and the gemmling survival rate was calculated after 120 days cultivation. The cultivation was carried out at temperature of 18–30 °C and humidity of above 80%. From January to April in 2009, the gemmation rate of gemmae and the survival rate of gemmlings in different media were recorded. With the above planting method, we specifically planted some gemmae in other pots for the physiological observation of gemmlings. The observation procedure is as follows: we pulled the gemmae from the soil and cleaned them with flowing water. Then we observed the growth of young roots and shoots, and replanted them after observation. We pulled out different gemmae each time.

Data collection.—All plants sampled in the natural population were divided into three groups. Group1 (G1): the plants with gemmae or sporangia; Group 2 (G2): the plants without gemmae or sporangia, but with remnants of gemmae and modified leaves at the stem base; Group 3 (G3): the plants without gemmae and sporangia, but with modified leaves at the stem base. The plants in G1 were considered as adult plants, while those in G2 and G3 covered all young plants, gemmlings and sporelings.

In natural populations, most plants produce gemmae and gemmiphores,once a year. The gemmae shed from mother plants in the same year after gemmae come into being, but the gemmiphores remain on stems for several years, even until plant death. Accordingly, we divided the adult plants into several groups in order of gemmiphore layers. The adult plants with gemmae were subdivided into six groups according to their ages, i.e. G_{n+1}, G_{n+2}, ..., G_{n+6} , because gemmae usually form one layer annually. Here n represents the growth years before gemmae appeared, and 1–6 indicate the layer numbers of each plant, respectively. The sixth group included the plants with 6 layers and >6 layers, which is very rare in all plants.

Statistical analyses.—We used one-way ANOVA to examine the main pattern of population regeneration, the gemma number per plant per year and the more suitable matrix to cultivate gemmae.

We counted the number of sporlings and gemmlings in the natural population and calculated their respective proportion out of the total number of plants. Then we compared the difference between sporelings and gemmlings proportions by one-way ANOVA to make clear the main regeneration pattern of this population. We also counted the gemma number per year in those plants with different gemma layers. We tested and compared the difference between the gemma numbers by one-way ANOVA and Least Significant Difference (LSD) to determine the changing trends of gemma number along with the increase of gemma layers in those plants at different ages.

In the cultivation experiment, we calculated the gemmation rate of gemmae and the survival rate of gemmlings in different cultivation media. The gemmation rate and survival rate are calculated as follows:

$$Rg = ng/Nt \times 100\%,$$

$$Rs = ns/Nt \times 100\%,$$

Where Rg is the gemmation rate of gemmae, Rs is the survival rate of gemmlings, ng is the gemmation number after 30 days cultivation, Rs is the gemmling number after 120 days cultivation, and Nt is the total number of cultivated gemmae. By homogeneity of variance and LSD, we analyzed the difference between different treatments to choose the better media for gemma cultivation.

Results

Gross morphology of H. serrata.—Fig. 1 shows an adult plant of *H. serrata* with three grades (I, II, and III) of branches. The plant is dichotomous with the small leaves (sl) and the big leaves (bl) arranged alternatively along the branches. Sporangia (s) exist in axillae of most leaves, and gemmae (ge) or gemmiphores (gp) appear above every layer of big leaves. Gemmae are generally present from February to March, and fall down from August to

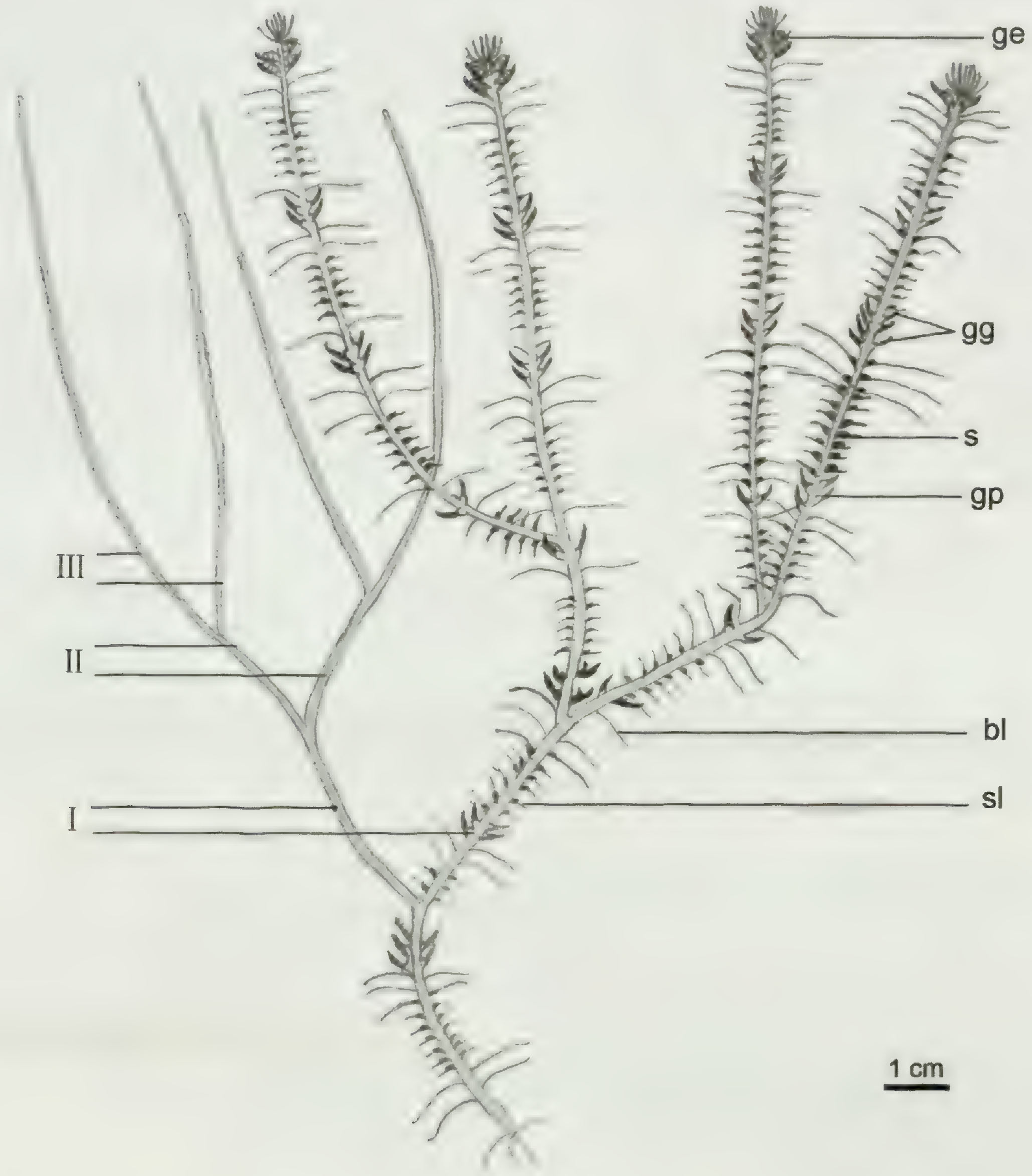

FIG. 1. The pattern figure of *H. serrata* sporophyte. (I) The first branch; (II) The second branch; (III) The third branch. sl: small leaf; bl: big leaf; s: sporangium; gp: gemmiphore; gg: a grade of gemmiphore; ge: gemma on the gemmiphore. Scale bar =1 cm.

October, usually once a year. The layers of gemmae or gemmiphores may be used as indicators to judge the plant ages.

Structure and number of gemmae.—As shown in Fig. 2, the gemmiphore with five modified leaves encloses a gemma (Fig. 2A, E). The gemma with the dorsal-ventral pattern is composed of one bud and six gemma leaves (Fig. 2A,

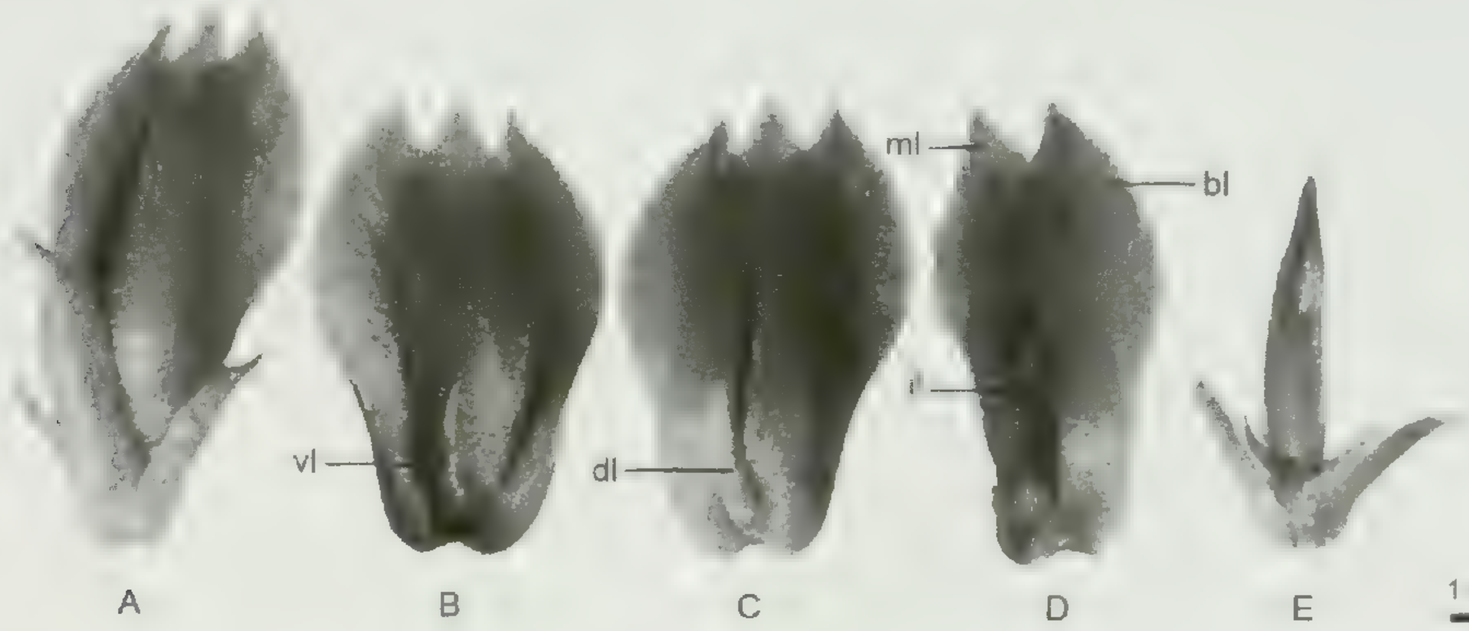

FIG. 2. Gemmiphore and the gemma structure. (A) Gemma and gemmiphore; (B) ventral view of the gemma (vl: ventral small leaf of the gemma); (C) dorsal view of the gemma (dl: dorsal small leaf of gemma); (D) leaves of the gemma (il: inner gemma leaf, bl: big gemma leaf; ml: middle big gemma leaf)(dorsal view); (E) gemmiphore. Scale bar (A, B, C, D, E)=1 mm.

B, C, D). These structures are succulent and dark green. Three big gemma leaves and one small gemma leaf are on the dorsal side (Fig. 2C), and the small one is on the ventral side (Fig. 2B). The bud is on the inner side of the middle gemma leaves (ml and il) (Fig. 2D). This structure is similar to that of a *H. javanica* gemma (Wang *et al.*, 2007).

Gemmae usually form one layer annually, and each layer includes one to several dozens of gemmae. The mean gemma number of G_{n+1} was 6.3. The number usually increased along with plant ages, reaching 30.0 in G_{n+4}, and then decreased slightly in G_{n+5} and G_{n+6} (Fig. 3). One-way ANOVA analysis showed that there was significant difference between G_{n+2} and G_{n+3} ($p<0.01$). The average gemma number was over 20 for the plants with more than three layers.

Population structure.—The investigated population covered a total area of 150 m^2. Most plants concentrated at a high density in over 100 m^2. Most seedlings derived from the fall-off gemmae in this population. Green, light-yellow or colorless gemma leaves remained at the base of the seedling stems for more than one year, which was considered as the evidence for the plants derived from gemmae.

We sampled a total of 605 plants in 36 quadrats, including 80 adult plants (G1) all producing gemmae, 315 plants (G2) developed from gemmae and 210 plants (G3) from spores. Those gemmlings accounted for 60% of the total of young plants, sporlings and gemmlings, while the sporelings accounted for 40% (Fig. 4). Significant difference was observed between gemmlings and sporelings ($p<0.01$).

The gemmation rate and survival rate of gemmae under cultivation conditions.—The cultivated gemmae germinated accompanied by splaying of surrounding modified leaves after 3 or 4 days; at the same time, very short roots ($\approx$ 0.1 mm) appeared at the base of gemmae. Seven days later, gemmlings grew out one or two entire leaves, which were narrow and bright green.

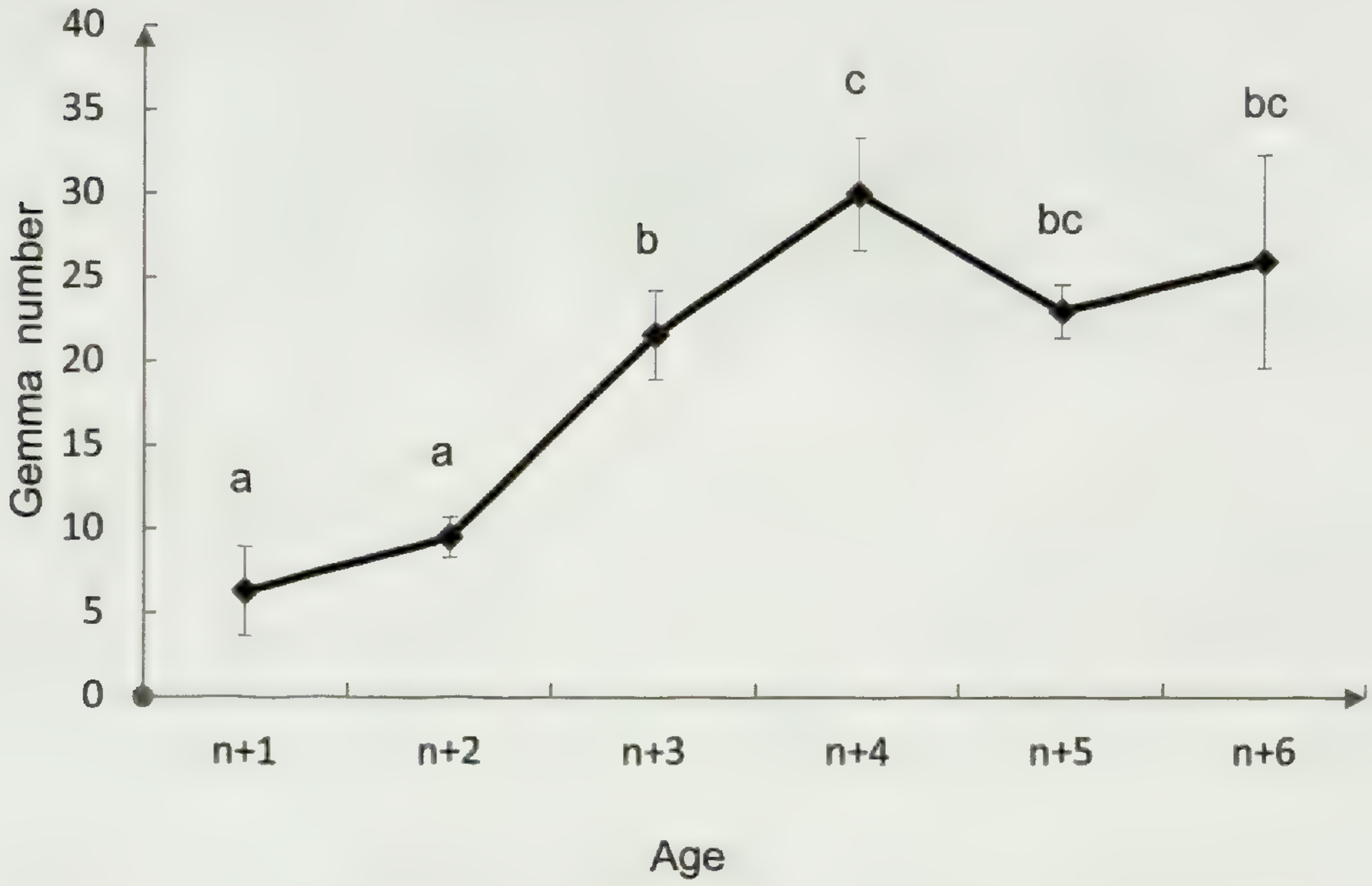

FIG. 3. Gemma numbers of plants at different ages.

Subsequently the leaves in circular shape changed gradually into an ovate shape, and the color of modified gemma leaves at the base of stems faded from green into light yellow. Three months later, the modified gemma leaves became colorless, membranaceous and transparent, then the gemmling was able to live independently. The gemmling height reached about 2 cm after 120 days (Fig. 5). Leaves with serrate margin formed gradually after 120 days.

The effects of different soil matrixes were not significantly different on the gemma gemmation rate, but were significantly different on the seedling survival rate (Fig. 6). For the cultivation in the habitat soil, sand and humus, the average gemmation rate was respectively 95%, 96.7% and 91.7%, with no significant difference ($p>0.05$); the average survival rate of gemmlings was

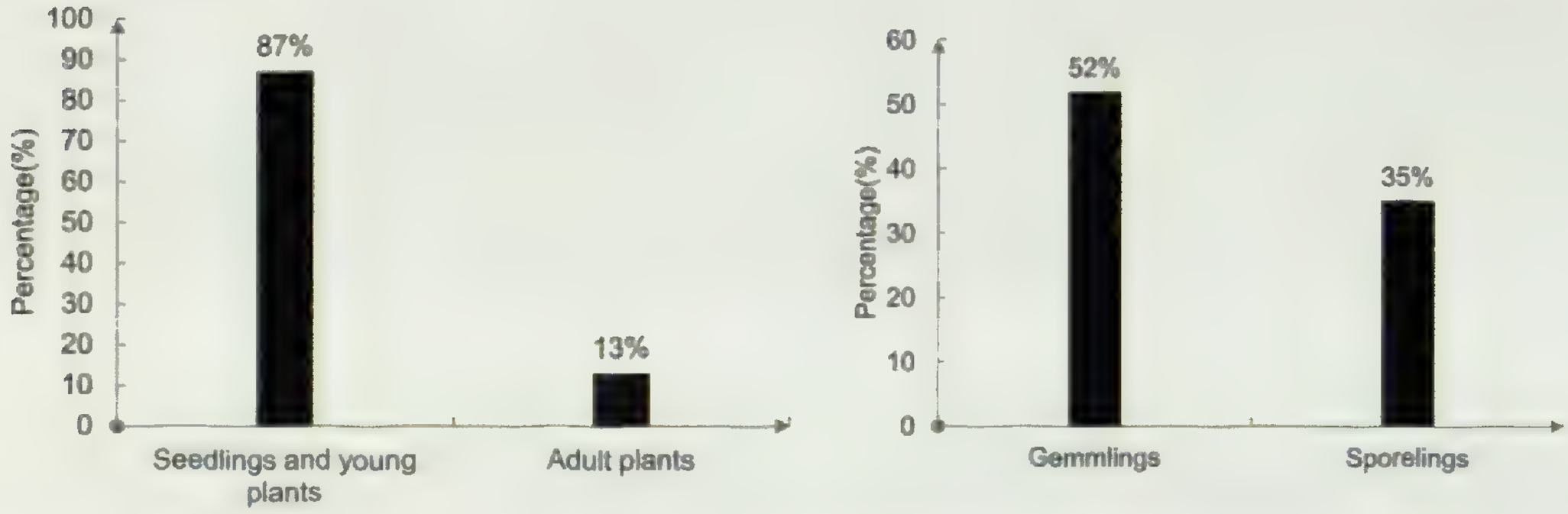

FIG. 4. Population structure: Proportion of adult plants, seedlings (gemmlings and sporelings) and young plants (Left); Proportion of gemmlings and sporelings (Right).

FIG. 5. The gemmlings at different ages.

respectively 91.7%, 48.3% and 88.3%, with the value of the sand medium significantly lower than those of the other two media ($p < 0.01$).

DISCUSSION

The gemma, as a vegetative propagule, has long drawn attention in *Huperzia*. Smith (1920) studied the gemma structure and origin in *Lycopodium lucidulum* Michx., which is included in *Huperzia* now, and he believed that gemmae could germinate into new plants. Reutter (1987) described the growth pattern of *L. lucidulum* from gemma to gemmling. Gola (2008) emphasized again the importance of gemmae in reproductive strategies of *Huperzia* by comparing the gemmae differences of *H. lucidula* and *H. selago* (L.) Bernh. ex Schrank & Mart. between mountain and lowland habitats. Wang *et al.* (2007) studied the gemma structure and the gemmlings of *H. javanica*,

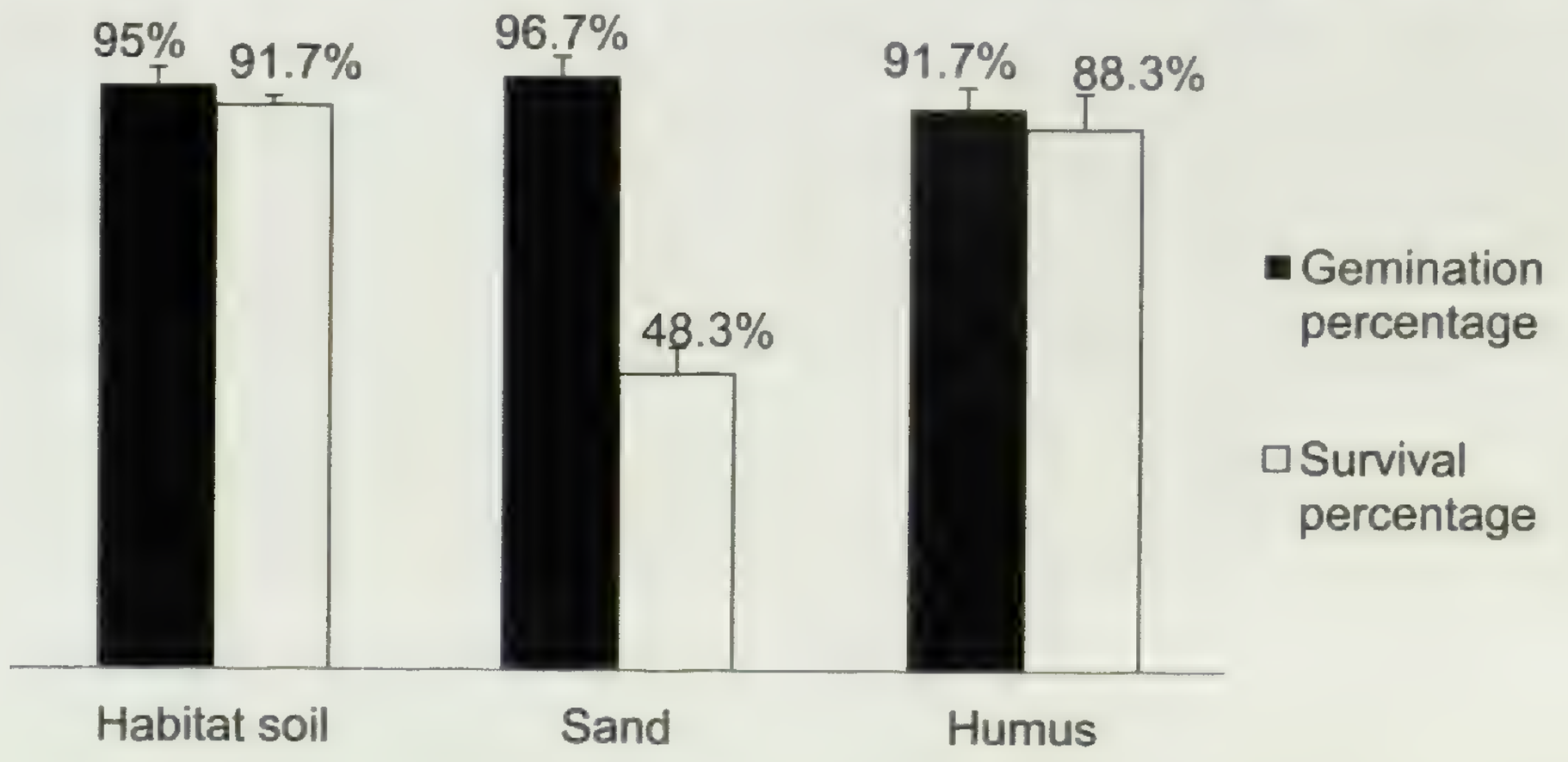

FIG. 6. The percentages of gemmae gemmation and survival of gemmlings in different media of the cultivation experiment.

and noticed that some seedlings developed from gemmae in natural populations. Although most researchers believe that gemmae play an important role in plant dispersal and propagation of *Huperzia*, little is known about the extent to which they act during the regeneration of natural populations.

From 2008 to 2009, we continually observed two growth seasons of a natural population in the Bawangling Nature Reserve of Hainan Province, and surveyed the population structure by field sampling. We found that this was a young population, in which seedlings (gemmlings and sporelings) and young plants accounted for 86.8%, and the gemmlings accounted for 60% of the total seedlings. In gemmlings and sporelings, we deemed that some of plants called sporelings might derive from gemmae because their gemma modified leaves perished. Considering that we did not calculate those gemmlings losing modified leaves at their stem bases, gemmlings could make up a much higher proportion. There was a significant difference between the number of gemmlings and that of sporelings, showing that regeneration of the population mainly depended on gemmae. We also found that the perennial plants of *H. serrata* with over three gemma layers generated more than 20 gemmae per year on average, and that there existed a significant difference between the plants $\geq$ n+3 years old and those $<$n+3 years old. This shows that the reproductive ability of gemmae became stronger after the third year of gemmae growth.

To further confirm the reproductive ability of the gemmae in *H. serrata*, mature gemmae were collected and used for breeding experiments in habitat soil, sand and humus. These gemmae germinated easily and grew fast, and plant height reached 2 cm in 120 days. The gemmation rate was respectively 95%, 96.7% and 91.7% in the three media, with no significant difference, but the survival rate in sand (48%) was significantly lower than in the other two media (91.7% and 88.3%). Under the cultivation conditions, *H. serrata* gemmae easily germinated using its own stored nutrients, with no special requirements for the cultivation soil. During the gemmling development process, good growth could be expected if the soil provided relatively rich nutrients after the stored nutrients were used. The sand medium did not have enough nutrients to support a high survival of gemmlings, while humus was suitable for gemmling growth as in the habitat soil. This indicates the possibility to quickly establish a cultivation base of *H. serrata* through gemma propagation, thus relieving the resource crisis of *H. serrata*.

Previous studies have showed that *Huperzia* spores take 2–5 years to develop into sporophyte (Bruchmann, 1910; Whittier, 2007). The plants of *H. serrata* grow more slowly, normally requiring 15–20 years of growth from spore germination to maturity (Ma *et al.*, 2006). Up to now, spore germination techniques in soil or in axenic culture have not been established. Despite the success in culturing the shoot of *Huperzia selago* (Szyputa *et al.*, 2005), it has been difficult to culture *H. serrata* shoots because of the endophytic fungi (Shen *et al.*, 2002). The cutting propagation of *H. serrata* has also been reported (Sheng *et al.*, 2000). As stem tips are used as cuttings in this technique, large amounts of wild resources are consumed. Because of the shortage of the

species in the wild, it is almost impossible to make large-scale production. Compared with spore collection and cutting, gemmae have the advantages of being abundant, high propagation rate and fast growth. Our results may not only reveal the important role of gemmae in reproductive strategies, but also be helpful to resource protection and cultivation of *H. serrata*.

Acknowledgments

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Literature Cited

Bruchmann, H. 1910. Die Keimung der sporen und die entwickelung der prothallien von *Lycopodium clavatum, L. annotinum*, and *L. selago*. Flora 101:220–267.

Ching, R. C. 1981. The taxonomy of Chinese *Lycopodiaceae* (sen. lat.)(I). Acta. Bot. Yunnan 3:1–9.

Editorial board of China Herb, China Herb. 1999. *Editorial board of China Herb*, Shanghai, *Shanghai Scientific and Technical Press*, Shanghai. 2, 29 PP.

Ehrlen, J. and O. Eriksson. 2000. Dispersal limitation and patch occupancy in forest herbs. Eco. 81:1667–1674.

Gola, E. M. 2008. *Reproductive strategies of Huperzia*. Polish botany of society, Institute of plant biology, University of Wroclaw, 5–14 PP.

Øllgaard, B. 1987. A revised classification of the *Lycopodiaceae* s. lat. Opera Botanica 92:153,178.

Liu, J. S., C. M. Yu, Y. Z. Zhou, Y. Y. Han, F. W. Wu, B. F. Qi and Y. L. Zhu. 1986a. Study on the chemistry of huperzine-A and huperzine-B. Acta Chimica Sinica 44:1035–1040.

Liu, J. S., Y. L. Zhu, C. M. Yu, Y. Z. Zhou, Y. Y. Han, F. W. Wu and B. F. Qi. 1986b. The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. Canadian Journal of Chemistry 64:837–839.

Lu, Y., M. G. Li, Y. W. Huang, Z. H. Chen and Y. J. Hu. 1986. Vegetation of Bawangling gibbon natural reserve, in Hainan Island. Acta Phytoecologica et Geobotanica Sinica 10:106–110.

Ma, X. Q., C. H. Tan, D. Y. Zhu and D. R. Gang. 2006. A survey of potential huperzine A natural resources in China: The Huperziaceae. Journal of Ethnopharmacology 104:54–67.

Reutter, U. 1987. Growth Patterns of Gemmlings of *Lycopodium lucidulum*. Amer. Fern J. 77:50–57.

Primack, R. B. 1973. Growth patterns of five species of *Lycopodium*. Amer. Fern J. 63:3–7.

Sheng, S. J., J. F. Xu, Z. A. Wang and X. P. Yu, X.p. 2000. Studies on the cottage propagation of *Huperzia* serratum. Resource Development and Market 16:268–269; 293.

Shen, X. X., X. P. Yu and S. J. Sheng. 2002. The study on the sterilization methods in vitro culture of *Huperzia serrata* shoots. China journal of Chinese Meteria Medica 27:458–459.

Smith, R. W. 1920. Bulbils of *Lycopodium lucidulum*. Bot Cazette 69:426–437.

Szypula, W., A. Pietrosiuk, O. Olszowska, M. Furmanowa and O. Kaximierska. 2005. Somatic *embryogenesis* and in vitro culture of *Huperzia selago* shoots as a potential source of huperzine A. Plant science 168:1443–1452.

Tang, X. C. 1996. Huperzine A (Shuangyiping): a promising drug for Alzheimer's disease. Acta Pharmaclolgica Sinica 17:481–484.

Whittier, D. P. and T. R. Webster. 1986. Gametophytes of *Lycopodium lucidulum* form axenic culture. Amer. Fern J. 76:48–55.

Whittier, D. P. 2006. Gametophytes of Four Tropical, Terrestrial *Huperzia* Species (Lycopodiaceae). Amer. Fern J. 96:54–61.

Whittier, D. P. and H. Storchova. 2007. The gametophyte of *Huperzia selago* in culture. Amer. Fern J. 97:149–154.

Wang, X. L., W. K. Li, J. Y. Zhou and Z. B. Hu. 2007. Morphological study on gemmae and gemmalings of *Huperzia javanica* (Huperzaiceae). Acta Botanica Yunnanica 29:521–526.

Wu, H., P. Zhuang, Z. B. Feng and C. Zhang, C. 2005. Resource Investigation and Journal of natural resources. Journal of natural resources 20:59–67.

Zang, R. G., J. Tao and C. Li. 2005. Within community patch dynamics in a tropical montane rain forest of Hainan Island, South China. Acta Oecologica 28:39–48.

Zhang, L. B. and X. X. Kong. 2000. Taxonomy of the genus *Huperzia Bernh.* (sen. Str.) sect. Serratae (Rothm.) Holub in China. Acta Phytotaxonomica Sinica 38:1–22.

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Ethnopteridology of the Guaranís of Misiones Province, Argentina

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Abstract.—An ethnobotanical study was performed of the ferns and lycophytes used by the Guaraní of Misiones Province, Argentina. It was determined that fifty species are used, and details of the uses and the Guaraní names and nomenclature are given and discussed. Fern and lycophytes are used for medicines, crafts, in magic rituals, and marketing of the plants. The most important traditional use of ferns is for medicine and the most important modern use is commercialization for use in horticulture.

Key Words.—Guaraní communities, ethnobotany, ferns and lycophytes, Paraná forest

Economic botanists have frequently concentrated on ferns as the focus of their studies, especially their medicinal properties and to a lesser extent their use as foods (Copeland, 1942; Looser and Rodríguez, 2004; Molina *et al.*, 2009, Ortega and Díaz, 1993; Ruiz López, 1805; Turner *et al.*, 1992). Ethnobotanical studies of ferns and lycophytes have been carried out in various part of the world, for example in Bolivia with the ethnopteridological study of the Chácobo (Boom, 1985), the comparative study of ferns and lycophytes used by the Huaorani in Ecuador and the Tacana of Bolivia (Macia, 2004), and in Nigeria in a study of various ethnic groups by Nwosu (2002). Precusors of this type of study in Argentina are limited to the work of Hurrel and de la Sota (1996) who studied the ethnobotany of ferns in a high altitude pasture in the Province of Salta.

The Province of Misiones is the center of diversity of ferns and lycophytes of Argentina (Ponce *et al.*, 2002) where there are 1,123,000 hectares of subtropical, semideciduous Paraná forest and Alto Paraná Atlantic rainforest (Placci and Di Bitteti, 2005). The catalogue of vascular plants of Argentina cited 158 species of monilophytes and lycophytes for the Province of Misiones (Ponce, 1996), but there have been many recent additions (Marquez *et al.*, 2006; Martínez, and de la Sota, 2005; Meza Torres *et al.*, 2006, 2008, 2010, Ponce, 2001; Tressens *et al.*, 2008) bringing the total up to 180 species. This shows the increasing knowledge about the botanical richness of the extreme northeast of the country. The diversity of ferns and lycophytes is also high at the local level. In a reserve of 5340 hectares (about 0.18% of the area of the Province) 80 taxa of these groups were found which represents 43.23% of the total fern flora of the Province (Tressens *et al.*, 2008). This diversity of species

in an area that can be studied in a few days means that they are readily available for use by local peoples who depend on the resources of the flora for their livelihood, especially the indigenous communities that have lived in the area for thousands of years.

Misiones has about one hundred Guaraní communities of the Mbya and Ava Chiripa. Up to present day these groups have maintained much of their traditional life including aspects of cosmology, religion, methods of subsistence, swidden agriculture, ways of hunting and fishing and the gathering of natural products from the forest. However, the fragmentation of their original habitat has obliged them to adopt various new strategies for survival as well as adapting customs of the surrounding global society, such as engaging in temporary employment and the commercialization of various natural products such as ornamental plants and crafts, especially baskets. For the Guaraní, the native vegetation is one of the most important sources of materials for their traditional way of life and also of prime materials for selling to a wider audience. In this paper we analyze the importance of ferns and lycophytes to the indigenous population of Misiones, identifying the species, the Guaraní names, their uses and significance.

Material and Methods

The fieldwork was carried out during an ethnobotanical program that took place between 2000 and 2008 in eleven Guaraní villages in the Departments of Concepción (1), Eldorado (1), Guaraní (4), Lib. Gral. San Martín (1), Montecarlo (1), San Ignacio (1) and San Pedro (2). Eighty four members of the Mbya clan and five members of the Ava Chiripa were interviewed (informants). We interviewed persons of both sexes and of different ages including both old people (more than sixty years of age) and children (less than twelve years of age). During this time we used various ethnographic methods such as participant observations and structured and semi-structured interviews. In some cases herbarium vouchers were collected during walks with informants and in other cases the herbarium material was shown to community members to ask them about names and uses of the plants. This material is deposited in the Instituto Botánica del Nordeste, Corrientes, Argentina (CTES) with duplicates distributed to various other herbaria in Argentina and other countries (ASU, B, BA, CANB, CESJ, ESA, GH, LIL, LP, MEXU, MO, NY, PC, SI). Part of the ethnobotanical work was carried out in a village that is in the Guarani Multiple-use Reserve and is where Tressens *et al.* (2008) carried out an exhaustive floristic inventory and so some of the herbarium vouchers are from that study. The literature studied delimits the ferns and lycophytes families in various ways and here we have followed the nomenclature of de la Sota *et al.* (1998) and Mickel and Smith (2004) both of whom presented their results at the generic and species level without assignment to family.

Results and Discussion

A total of 50 species were indicated as useful by the communities studied (Table 1). These belong to 32 genera and represent 28% of the fern flora of the Province. Regarding the categories of use (Fig. 1), 38 species (76%) were indicated as medicinal, 19 species (38%) are sold commercially as ornamentals or as physical supports for growing ferns and orchids, 15 species (30%) are used in magic, mainly as talismans, 4 species (8%) are ecological indicators, 3 species (6%) are used in crafts (necklaces), and a single species (2%) is used as food. The use of tree ferns to make arrow points is mentioned in the literature but was not found to be in use today.

Folk nomenclature.—The general term for ferns in Guaraní is *amambái* and this includes those species in the class Polypodiopsida. They do not consider tree ferns or those generically known as *chachi* (various ferns with entire fronds) as *amambái*. The Guaraní plant names usually describe a morphological or organoleptic character of the plant. For example, *amambái taka* (bifurcate or branched fern) refers to the fertile fronds that are several times divided of *Doryopteris nobilis*. Because of its sturdy structure *Pteridium arachnoideum* is called *amambái rata* (= hard fern). *Pecluma pectinatiformis* is named *amambái e'e* (= sweet fern) because of the sweet taste of its fronds. Other species of this genus such as *P. sicca* are called *amambái piru* (= dry fern) because their leaves shrivel up in dry periods and then return to normal once humid conditions return. It is interesting to note that the specific epithet of this species "siccum' (= dry) also refers to this same quality. Other names are associated with animals because of some morphological similarity. For example, *mborevi po* (tapir paw) is the name of *Doryopteris nobilis* whose sterile fronds look like the footprint of a tapir (Fig. 2A). Names can sometimes be associated with the habitat of animals, as in *jakare ka'a* (= caiman herb) for *Thelypteris riograndensis*, which, like caimans, lives beside water sources. This name is similarly applied to various ferns by the Tupi-guaraní of Amazonia (Balée, 1994).

Some names refer to other plants, for example species of *Selaginella* are called *koto jaryi* (= false moss) and *Adiatopsis chlorophylla* is called *kurunjy u miri* (small specimen of the tree *kurunjy u*). Some species have bilingual names, as for *Huperzia mandiocana* which is called *pino tyre'i* (epiphytic pine). Other names are derived from the Spanish as is the case for *Adiantum* called *kurantrijo* (derived from culantrillo: *Adiantum capillus-veneris* L., widely distributed in Europe) or from the Quichua language as in *karaguára* (calaguala) that refers to the genera *Asplenium* L. (*A. balansae* and *A. brasiliense*), *Campyloneuron* and *Niphidium*. Finally, various names refer to their use, such as *Pleopeltis pleopeltifolia* being called *memby ja* (giver of children) which is taken by women to increase their fertility.

Medicines.—Medicinal plants are generally used by the Guaraní in the fresh state preferably on the day they are collected. The storing of medicines is confined to plants located far from the village or of short duration. The most frequent method of use is in decoctions of macerated plant material in water at

TABLE 1. List of the ferns and fern allies used by the Guaraní of Misiones, Argentina.

SPECIES	GUARANÍ NAME	USES	PARTS USED	VOUCHERS
Adiantopsis chlorophylla (Sw.) Fée	*kurunjy u miri* (small tree of *Trema micrantha*)	- Necklace beads - Expectorant, treatment of heart problems, stomach refresher	- Fronds	*Keller 2787*
Adiantopsis radiata (L.) Fée	*amambái ũ* (black fern)	- Febrifuge, treatment of nosebleed	- Fronds	*Keller 1057*
Adiantum pseudotinctum Hieron.	*kurantrijo* (from the Spanish "culantrillo", = small cilantro")	- Treatment of headaches and nausea, post partum washing, nosebleed - Necklace beads	- Petioles - Fronds	*Tressens et al. 6469*
Adiantum raddianum C. Presl.	*kurantrijo* (from the Spanish "culantrillo", = "small cilantro"	- Treatment of headache and nausea, febrifuge, nosebleed, diarrhea	- Fronds	*Keller 1371*
Alsophila setosa Kaulf.	*chachĩ rakua* (tree fern with spines)	- Stands for ornamental plants - Formerly used for arrow points - Indicator that soil not suitable for agriculture - Treatment of *Herpes sp.*	- Stems - Sclerenchyma strands - Exudate from petiole	*Tressens et al. 4719*
Anemia phyllitidis (L.) Sw.	*nachĩ'ũ rã* (similar to a mosquito), *typycha ovy* (blue brush)	- Male charm to attract opposite sex - Treatment of sinusitis, expectorant, antidepressant, stomach refresher, treatment of heart infections	- Fertile fronds - Fronds	*Keller 2979*
Anemia simplicior (Christ) Mickel	*jakare ka'a* (caiman plant)	- Male charm to attract opposite sex	- Fronds	*Keller 829*
Anemia tomentosa (Sav.) Sw.	*jakare ka'a* (caiman plant)	- Male charm to attract opposite sex - Muscular tonic, prevention of illness	- Fronds	*Keller & Gatti 1693*
Asplenium balansae (Baker) Sylvestre	*karaguara yvy reegua* (calaguala of the earth)	- Sold as an ornamental - Contraceptive, menstrual analgesic	- Whole plant	*Keller 5629*
Asplenium brasiliense Sw	*karaguara yvy reegua* (calaguala of the earth)	- Sold as an ornamental - Contraceptive, menstrual analgesic	- Whole plant	*Keller 5628*

TABLE 1. Continued.

SPECIES	GUARANÍ NAME	USES	PARTS USED	VOUCHERS
Asplenium scandicinum Kaulf.	*kuña manje'a* (for women)	- Sold as an ornamental - Male charm to attract the opposite sex	- Whole plant - Fronds	*Keller et al. 1939*
Blechnum australe L. subsp. *auriculatum* (Cav.) de la Sota	*amambái* (fern)	- Female contraceptive, treatment of headache	- Whole plant, fronds	*Keller 3599*
Blechnum austrobrasilianum de la Sota	*amambái* (fern)	- Sold as an ornamental	- Whole plant	*Keller 773*
Blechnum brasiliense Desv.	*amambái* (fern)	- Sold as an ornamental	- Whole plant	*Keller 1072*
Campyloneurum lapathifolium (Poir.) Ching	*karaguara ita reegua* (growing on rocks)	- Sold as an ornamental - Menstrual analgesic, treatment of gastritis	- Whole plant - Rhizomes	*Fernández et al. 98*
Campyloneurum nitidum (Kaulf.) C.Presl	*karaguara ita reegua* (growing on rocks), *mburika ka'a* (donkey herb), *jagua ka'a* (dog herb)	- Sold as an ornamental - Treatment of nausea, epilepsy, muscular tonic, blood purifier, female contraceptive, abortive, menstrual analgesic, to facilitate child birth, post-partum washing, treatment of gastritis, asthma, lumbago and kidney infections.	- Whole plant - Rhizomes	*Keller 1081*
Dicksonia sellowiana Hook.	*chachĩ raviju* (woody tree fern), *kereke*	- Stands for ornamental plants - Formerly used for arrow points - Treatment of burns and measles	- Stems - Sclerenchyma strands - Exudate of petiole	*Tressens et al. 4631*
Didymochlaena truncatula (Sw.) J. Sm.	*amambái* (fern)	- Stands for ornamental plants - Sold as an ornamental	- Stems -Whole plant	*Keller 1106.*

TABLE 1. Continued.

SPECIES	GUARANÍ NAME	USES	PARTS USED	VOUCHERS
Doryopteris nobilis (Moore) C.Chr.	*mborevi po* (tapir pawr); *amambái taka* (fern with bifurcate fronds)	- Sold as an ornamental - Necklace beads - Male charm to attract opposite sex - Treatment of colds, headaches, cardiac infections, diarrhea, menstrual analgesic.	- Whole plant - Petioles - Propagules - Fronds	*Keller 1368*
Elaphoglossum pachydermum (Fée) T. Moore	*karaguara ita reegua* (growing on rocks)	- Female contraceptive, abortive, menstrual analgesic	Whole plant	*Keller 7462*
Equisetum giganteum L.	*kavaju ruguái* (horse tail)	- Treatment of head-aches, epilepsy and kidney infections	- Shoots	*Keller 3282*
Hemionitis tomentosa (Lam.) Raddi	*rorarija* (from spanish "doradilla", because the ferruginous indumentum)	- Sold as an ornamental - Used in a procedure to gain power, - Treatment of heart and kidney infections, menstrual analgesic, treatment of female sterility, for healing childs navel, blodd purifier	- Whole plant - Whole plant - Fronds	*Keller & Gatti 1858*
Huperzia mandiocana (Raddi) Trevis.	*pino tyre'i* (epiphytic pine)	- Sold as an ornamental	- Whole plant	*Keller et al. 1941*
Lastreopsis effusa (Sw.) Tindale	*amambái tyre'i* (orphan fern)	- Male charm to attract opposite sex	- Propagules	*Keller 5624*
Lycopodiella cernua (L.) Pic. Serm.	*urukure'a ka'a* (owl herb)	- Male charm to attract opposite sex	- Whole plant	*Keller 1994*
Lycopodium clavatum L.	*urukure'a ka'a* (owl herb)	- Male charm to attract opposite sex	- Whole plant	*Keller 49*
Lygodium volubile Sw	*jakare ka'a* (caiman plant)	- Male charm to attract opposite sex	- Fronds	*Keller & Franco 5814*
Microgramma lindbergii (Kuhn) de la Sota	*ambere ka'a* (small lizard plant)	- Treatment of kidney infections and deafness, menstrual analgesic	- Whole plant	*Keller 5678*

TABLE 1. Continued.

SPECIES	GUARANÍ NAME	USES	PARTS USED	VOUCHERS
Microgramma squamulosa (Kaulf.) de la Sota	*ambere mbói* (small lizard-snake), *anguja ruguái* (rat tail)	- Slimming, menstrual analgesic, female contraceptive, post partum washing, treatment of lumbago.	- Whole plant	*Keller 1080*
Microgramma vacciniifolia (Langsd. & Fisch.) Copel.	*ambere ka'a, ambere mby* (small lizard plant)	- Treatment of kidney infections and deafness, menstrual analgesic	- Whole plant, fronds	*Keller 7541*
Niphidium crassifolium (L.) Lellinger	*karaguara yvyra reegua* (that which grows on trees)	- Sold as an ornamental - Indicator of cardinal points - Muscular toner, menstrual analgesic, treatment to ease child birth, post partum washing	- Whole plant - Rhizomes	*Keller 1889*
Ophioglossum reticulatum L.	*kochĩ apia'i* (peccary penis)	- For colds	- Whole plant	*Keller 3065*
Osmunda regalis L.	*ñachi'ũ rã guachu* (large *Anemia phyllitidis*)	- Treatment of sore throats - Male charm to attract opposite sex	- Whole plant - Fertile fronds	*Keller 1058*
Pecluma filicula (Kaulf.) M. G. Price	*amambái piru* (dry fern)	- Sold as an ornamental - Treatment of female sterility	- Whole plant - Fronds	*Keller 797*
Pecluma pectinatiformis (Lindm.) M. G. Price	*amambái re'ẽ* (sweet fern)	- Chewed as a sweet - Sold as an ornamental - Treatment of epilepsy, blood purifier	- Fronds - Whole plant - Rhizomes	*Keller et al. 3096*
Pecluma sicca (Lindm.) M.G. Price	*amambái piru* (dry fern)	- Sold as an ornamental - Treatment of female sterility	- Whole plant - Fronds	*Tressens 4942*
Pecluma singeri (de la Sota) M.G. Price	*amambái piru* (dry fern)	- Sold as an ornamental - Treatment of female sterility	- Whole plant - Fronds	*Keller 5594*
Phlebodium areolatum (Willd.) J. Sm.	*karaguara* (from quichua "Calaguala")	- Menstrual analgesic	- Rhizomes	*Keller & Paredes 7465*
Pleopeltis pleopeltifolia (Raddi) Alston	*memby ja* (giver of children)	- Menstrual analgesic, treatment of excessive menstruation and female sterility	- Whole plant, fronds	*Keller 776*

TABLE 1. Continued.

SPECIES	GUARANÍ NAME	USES	PARTS USED	VOUCHERS
Pleopeltis squalida (Vell.) de la Sota	*teko'a porã ja* (owner of good customs)	- Indicator of cardinal points - Menstrual analgesic, treatment of excessive menstruation and female sterility	- Whole plant, fronds	*Keller 1891*
Pteridium arachnoideum (Kaulf.) Maxon.	*amambái ratã* (hard fern)	- Menstrual analgesic	- Tender fronds	*Keller & Benitez 2727*
Pteris deflexa Link	*amambái* (fern)	- Indicator of places with an abundance of ticks - Used in a magic process to forget an ex wife	- Fronds	*Tressens et al. 6750*
Pteris denticulata Sw.	*ñachĩ'ũ rã rã* (similar to *Anemia phyllitidis*)	- Treatment of sore throat, antidepressant	- Fronds	*Keller 1892*
Selaginella muscosa Spring	*guaimi ragué* (old lady's hair), *ygau jaryi* (false moss)	- Female contraceptive, washing wounds	- Whole plant	*Tressens et al. 4635*
Selaginella sulcata (Poir.) Mart.	*koto jaryí* (false moss)	- Female contraceptive	- Whole plant	*Keller 1163*
Serpocaulon latipes (Langsd. & Fisch.) A. R. Sm.	*karaguara* (calaguala)	- Menstrual analgesic	- Rhizomes	*Keller & Franco 5827*
Thelypteris recumbens (Rosent.) C. F. Reed	*amambái tyre'i* (orphan fern)	- Male charm to attract opposite sex - Tranquilizer for children	- Propagules	*Tressens et al. 6845*
Thelypteris riograndensis (Lindm.) C. F. Reed	*jakare ka'a* (caiman herb)	- Male charm to attract opposite sex - Antidepressant	- Fronds - Whole plant	*Keller 2975*
Thelypteris scabra (C. Presl)Lellinger	*amambái tyre'i* (orphan fern)	- Male charm to attract opposite sex	- Propagules	*Keller & Gatti 1861*
Vittaria lineata (L.) Sm.	*avukuja guachu* (great owner of long hair)	- Sold as an ornamental - Treatment to make hair grow	- Whole plant - Fronds	*Keller 2409*

room temperature. It is also common to mix the medicinal material in mate water (the infusion of leaves of *Ilex paraguariensis* A. St.-Hil. in the Aquifoliaceae) taken on a daily basis. Many species are used to treat infections of the reproductive system and this use accounts of the most uses reported

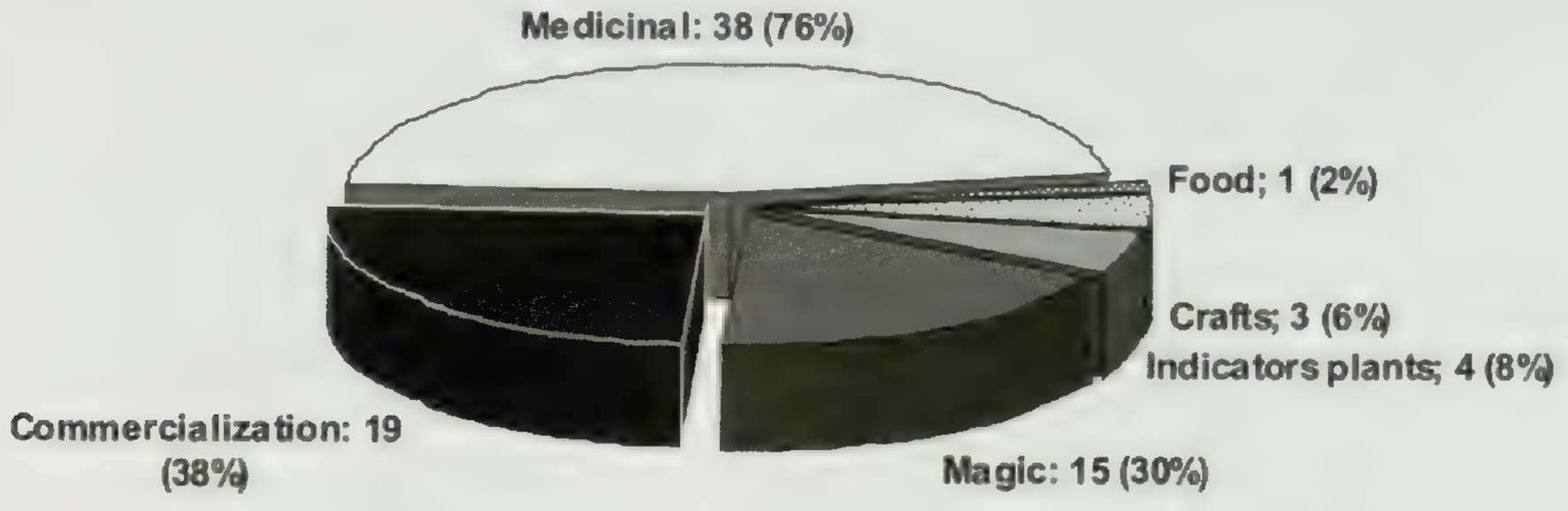

FIG. 1. Species in each category of use.

here (46%). This agrees with the findings of an ethnobotanical study of the Guaraní communities of Pai'i tavytera in Amambay Department of Paraguay (Basualdo and Soria, 2002) where of the three species cited two were used to treat female fertility. Other medicinal use categories that stand out are: treatment of infections of the respiratory (18%), digestive (16%), circulatory (12%) and nervous systems (12%).

Many plants used by the Guaraní of Misiones have their origins from the doctrine of signatures (Keller, 2007). Women who want to have a large family eat ferns of the genera *Pleopeltis* and *Pecluma* that are characterized by their prolific production of small fronds. Tapirs (*Tapirus terrestris*) sleep on their backs with their hooves pressed against their chests and the Guaraní maintain that in this way they cure heart problems. For this reason they attribute heart-healing properties to *Doryopteris nobilis* (*mborevi po* or tapir hooves) whose sterile fronds resemble the tracks of this animal.

Commercialization.—The sale of ornamental plants is the second most important use of ferns and their allies in the communities studied. Ornamental species are sold as single plants or on frames or wooden supports, and others are used to add to groups of epiphytic orchids, which they sell in stands beside the highways (Fig. 2B). One of the most sought after species from the roadside stands is *Huperzia mandioccana*, which is not a common plant. The commercial use of this species could threaten the future of its natural populations. The stems of the tree fern *Dicksonia sellowiana*, a rare species in the region, are cut and sectioned for sale (Fig. 2C). This is a substrate widely used by nurseries as a support for orchids and other epiphytes. The bases of other ferns with a robust stem such as *Alsophylla setosa* are also sometimes used in the same way.

Magic.—Most of the plants used for magic by the Guaraní have names associated with animals and they are usually aromatic plants. They term them *vy'aja* (givers of happiness) or *irû porã* (good friends) to their personal charms. They frequently carry fragments of leaves and other plant materials in pouches in order to have good results form various events especially in their declarations of love. The most used ferns in this category are species of the genus *Anemia* Sw. whose fronds are aromatic and are used in various procedures to attract members of the opposite sex. Sometimes they use these

FIG. 2. A: sterile fronds of *Doryopteris nobilis*, similar to tapir footprints. B: Guarani stand for sale of crafts and ornamental plants. C: part of the stem of *Dicksonia sellowiana* for sale. D: propagules of *Doryopteris nobilis*. E: ferns and other epiphytic plants growing on the north facing side of a tree. F: dense population of *Alsophylla setosa*.

plants as a perfume, rubbing the fragrant material on their cheeks. At other times they place fragments of the fern in the bowl of their pipes and blow the smoke in the direction of the person they hope to conquer. The propagules of fern fronds with gemmae are also frequently used as charms (Fig. 2D).

Ecological indicator plants.—Various small ferns grow on tree trunks and often, together with mosses and lichens, form a living carpet along the branches. The Guaraní have noted that some of these small plants are more abundant on the north-facing side of the host tree (Fig. 2E) because this side does not receive as much direct sunlight, and this is particularly so on trees of

large diameter. During their long treks through the forest at night or on cloudy days it is possible to estimate the probable compass points from the location of the carpets of epiphytes on a tree. This is especially true on large, straight-trunked trees.

There are various edaphic characteristics of the deep red soils of Misiones that make them hard to cultivate, such as low fertility, high acidity, high aluminum content, and susceptibility to erosion (Ligier *et al.*, 1990). The Guaraní identify where this type of soil occurs in the forest by the presence of tree ferns (Fig. 2F), specifically *Alsophylla setosa*, and so they avoid establishing their slash and burn agriculture on these sites.

Some large ferns, such as *Pteris deflexa*, form dense clumps on the edge of or in the forest. The Guaraní say that it wise to avoid these areas because of the large number of ticks that occur there. In addition they say that the small deer *Mazama nana* (Cervidae) has the habit of hiding under the fronds of this fern and so they call the deer "amambái guy'i", which translated means "he who is under the fern."

Crafts.—The Guaraní make many crafts from nature such as baskets and carvings either for their own personal use or to sell. They often make bead necklaces to sell to tourists or for themselves for use by either men or women. Amongst the materials used to make beads we have observed the use of the shiny black petioles of *Adiantopsis chlorophylla, Adiantum pseudotinctum* and *Doryopteris nobilis*.

Arrow points.—The construction of arrow points involving the use of the cord-like sclerenchimatous tissue of tree fern petioles by the indigenous people of Misiones was mentioned by Queirel (1897). The mythology of the guayakíes of Paraguay refers to "arrows of ferns" (Fernández, 1992). We have not been able to verify this use from contemporary Guaranis.

Conclusions.—The Guaraní of Misiones use a considerable part of the fern flora of the Province. Ferns and lycophytes provide a variety of resources to maintain their traditional methods of subsistence and their more modern commercial life. The conservation of the biological diversity of Misiones Province undoubtedly has helped to avoid erosion of the cultural diversity of the region as well.

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Literature Cited

Balée, W. 1994. *Footprints of the Forest. Ka'apor Ethnobotany, The Historical Ecology of Plant Utilization by an Amazonian People.* Ed. Columbia University Press, New York.

Basualdo, I. and N. Soria. 2002. Etnobotánica de la parcialidad Pa'î Tavyterã. Suplemento Antropológico de la Universidad Católica de Asunción XXXVII(1):173–272.

Boom, B. M. 1985. Ethnopteridology of the Chácobo Indians in Amazonian Bolivia. Amer. Fern J. 75:19–21.

COPELAND, E. B. 1942. Edible ferns. Amer. Fern J. 32:121–126.

DE LA SOTA, E. R., M. M. PONCE, M. A. MORBELLI and L. CASSÁ DE PAZOS. 1998. Pteridophyta in M. N. Correa: (ed.), Fl. Patagónica, Colecc. Ci. Inst. Nac. Tecnol. Agropecu. 8(1):282–369.

FERNÁNDEZ, F. 1992. *Antropología Cultural y medicina indígena de América y arte rupestre argentino. Tomo II.* Ed. Galerna, Buenos Aires. 326 PP.

HURREL, J. A. and E. R. DE LA SOTA. 1996. Etnobotánica de las Pteridofitas de los Pastizales de altura de Santa Victoria (Salta, Argentina). Revista Mus La Plata (NS). Botánica 15(105):353–364.

KELLER, H. A. 2007. Notas sobre medicina y magia entre los guaraníes de Misiones, Argentina, un enfoque etnobotánico. Suplemento Antropológico de la Universidad Católica de Asunción XLII(2):345–384.

LIGIER, H. D., H. R. MATTEIO, H. L. POLO and J. R. ROSSO. 1990. *Atlas de suelos de la República Argentina. Tomo II.* Secretaría de Agricultura, Ganadería y Pesca. Proyecto PNUD Argentina 85/019, Buenos Aires. 676 PP.

LOOSER, G. and R. RODRÍGUEZ. 2004. Los helechos medicinales de Chile y sus nombres vulgares. Gayana Bot. 61(1):1–5.

MACÍA, M. J. 2004. A comparison of useful Pteridophytes between two amerindian groups from Amazonian Bolivia and Ecuador. Amer. Fern J. 94(1):39–46.

MÁRQUEZ, G., G. E. GIUDICE and M. PONCE. 2006. Pteridofitas de la Reserva "Valle del Arroyo Cuñá Pirú"(Misiones, Argentina). Darwiniana 44(1):108–126.

MARTÍNEZ, O. G. and E. R. DE LA SOTA. 2005. Sobre la presencia y distribución de Pteris brasiliensis (Pteridaceae-pteridophyta) en la Argentina. Hickenia 3(56):239–242.

MEZA TORRES, E. I., H. A. KELLER and C. A. BRUSSA. 2010. New Records to Pteridophyte Flora of Northeastern Argentina and Uruguay. Amer. Fern J. 100(3):183–187.

MEZA TORRES, E. I., M. S. FERRUCI, H. A. KELLER and G. MARQUEZ. 2006. Presencia de *Doryopteris lomaricea* (Pteridaceae-Pteridophyta) en Argentina. Bonplandia 15(3–4):143–148.

MEZA TORRES, E. I., G. J. MARQUEZ, E. R. DE LA SOTA and M. S. FERRUCCI. 2008. Nuevas citas en Argyrochosma y Vittaria (Pteridophyta) del NE Argentino. Darwiniana 46(2):360–366.

MICKEL, J. T. and A. R. SMITH. 2004. The pteridophytes of Mexico. Memoirs of the The New York Botanical Garden 88:1–1055.

MOLINA, M., V. REYES-GARCÍA and M. PARDO DE SANTAYANA. 2009. Local Knowledge and Management of the Royal Fern (Osmunda regalis L.) in Northern Spain: Implications for Biodiversity Conservation. Amer. Fern J. 99(1):45–55.

NWOSU, M. O. 2002. Ethnobotanical studies on some Pteridophytes of southern Nigeria. Econ. Bot. 56:255–259.

ORTEGA, F. and W. DIAZ. 1993. Ethnopharmacological notes on two Venezuelan Asplenium. Amer. Fern J. 83:71.

PLACCI, G. and M. di BITETTI. 2006. La situación ambiental en la eco región del bosque atlántico del Alto Paraná (Selva Paranaense). In Brown, A., U. Martinez Ortiz, M. Acerbi and J. Corcuera (eds.) *La situación ambiental Argentina 2005.* Fundacion Vida Silvestre Argentina, Buenos Aires. Pp. 195–210.

PONCE, M. M. 1996. Pteridophyta. In F. O. Zuloaga and O. Morrone, eds. Catálogo de las Plantas Vasculares de la República Argentina I. Monogr. Syst. Bot. Missouri Bot. Gard. 60:1–79.

PONCE, M. M. 2001. Additions and corrections to the pteridophyte flora of Northeastern Argentina. Amer. Fern J. 91(2):70–72.

PONCE, M. M., K. MEHLTRETER and E. R. DE LA SOTA. 2002. Análisis biogeográfico de la diversidad pteridofítica en Argentina y Chile continental Biogeographical analysis of the pteridophyte diversity of continental Argentina and Chile. Rev. Chil. Hist. Nat. 75(4):703–717.

QUEIREL, J. 1897. Misiones. Taller tipográfico de la Penitenciaría Nacional, Buenos Aires. p. 163.

RUIZ LÓPEZ, H. 1805. Memoria sobre la legitima Calaguala y otras dos raices que con el mismo nombre nos vienen de la America meridional. imprenta de D. José del Collado, Madrid. 60 p.

TRESSENS, S. G., H. A. KELLER and V. REVILLA. 2008. Las plantas vasculares de la Reserva de Uso Multiple Guaraní, Misiones. Bol. Soc. argent. Bot. 43(3–4):273–293.

TURNER, N. J., L. M. JOHNSON GOTTESFELD, H. V. KUHNLEIN and A. CESKA. 1992. Edible wood fern rootstocks of Western North América: solving an ethnobotanical puzzle. J. Ethnobiol. 12(1):1–34.

American Fern Journal 101(3):205 (2011)

Shorter Notes

First Record of Cyatheaceae in Uruguay.—The family Cyatheaceae comprises about 500 species (Korall *et al.*, Mol. Phylogenet. Evol. 39: 830–845. 2006) classified in four genera: *Alsophila*, *Cyathea*, *Cnemidaria* and *Sphaeropteris* (Lellinger, Amer. Fern J. 77 (3): 90–94. 1987). Among the tree ferns, Cyatheaceae is characterized by the presence of abaxial sori and scales at the petiole bases (Tryon and Tryon, *Ferns and allied plants with special reference to tropical America*, Springer-Verlag, New York. 1982).

To date, Dicksoniaceae has been reported as the only family of tree ferns to grow in Uruguay, with the presence of *Dicksonia sellowiana* Hook. in the Departments of Cerro Largo, Tacuarembó and Treinta y Tres Orientales (Lehnert *et al.*, Dicksoniaceae, Catálogo de las Plantas Vasculares del Cono Sur, Vol1, Missouri Botanical Garden, 2008). Recent collections of *Cyathea atrovirens* (Langsd. & Fisch.) Domin in Rivera and Tacuarembó Departments represent the first records of the Cyatheaceae from Uruguay. *Cyathea atrovirens* is a tree fern distributed in northeastern Argentina, western Paraguay, and eastern and southern Brazil (Marquez *et al.*, Bol. Soc. Argent. Bot. 41:313–315. 2006). This species is distinguished from other *Cyathea* species in these areas by the presence of single veins, free sori and spore surfaces without perforations. It grows at elevations up to 1500 m on roadsides, gullies, abandoned crops, swamps and secondary forests.

Specimens examined.—Uruguay. **Rivera**, Establecimiento Dutra, Ruta 27 al Este, próximo a Paso Zerpa, 03.01.2001, *Brussa & Grela s/n* (MVFA 29485); ídem, 15.02.2001, *Brussa & Grela s/n* (MVFA 29632); ídem, Establecimiento El Tajamar, Ruta 5 al Oeste, 4 Km al Sur del empalme con Ruta 27, 14.12.2005, *Brussa & Grela s/n* (MVJB 24568). **Tacuarembó**, Establecimiento La Corona, Ruta 5 próximo a Cuchilla de la Palma, 5.5.2010, *Rossado & Masciadri s/n* (MVJB 27495).—Gonzalo J. Marquez, Cátedras de Palinología y Morfología Vegetal, Facultad de Ciencias Naturales y Museo, UNLP, Paseo de Bosque s/n, 1900, La Plata, Buenos Aires, Argentina, e-mail: cosme@fcnym.unlp.edu.ar, and Carlos A. Brussa, Museo y Jardín Botánico "Prof. Atilio Lombardo", Av. 19 de Abril 1181, Montevideo, Uruguay; Facultad de Agronomía, UDELAR, Av. Garzón 780, Montevideo, Uruguay.

American Fern Journal 101(3):206–207 (2011)

Shorter Notes

The Identity of *Asplenium macilentum* Kunze ex Klotzsch.—*Asplenium macilentum* was first recognized by Kunze and validly published by Klotzsch (Linnaea 20: 351. 1847), citing six syntypes. Morton and Lellinger (Mem. New York Bot. Gard. 15:18. 1966) designated a specimen of unknown collector and locality (Herb. Willd. 19890, B) as lectotype. This specimen is consistent with the protologue in the described elements for the identification of the species. Moore (Ind. Fil.: 115. 1859) regarded the epithet *macilentum* as a variety of *Asplenium auritum* Sw.

In modern Floras for the Neotropics, this species has been considered as a synonym of a variety of *A. auritum* (Morton and Lellinger, Mem. New York Bot. Gard. 15:18. 1966) or as a distinct species (Proctor, *Ferns of Jamaica*: 381. 1985; Adams, Flora Mesoamericana: 290–324. 1995). Smith (*Flora of Chiapas* 2:48. 1981) affirmed that some plants identified in herbaria as *A. macilentum* greatly resemble *Asplenium monodon* Liebm. He also introduced the criteria of number of spores per sporangium and morphology of spores to differentiate *A. auritum* and *A. monodon*; 64 reniform spores per sporangium characterize *A. auritum*, while 32 globose spores per sporangium typify *A. monodon*. After a revision of the isosyntype (*Moritz 183*, from Venezuela, UC), Smith (1981) reported large globose spores for this specimen, which probably should be referred to *A. monodon*. He also found that another isosyntype (*Schomburgk 1168*, from Guyana, UC), has small reniform spores, consequently referable to *A. auritum s.s.* Mickel and Smith (Mem. New York Bot. Gard. 88:107. 2004) further discussed the identity of *A. macilentum* and stated that since the spore morphology of the lectotype had still not been studied, it was "imprudent to place the name".

Spores from the lectotype (Herb. Willd. 19890, B) were examined with the Scanning Electron Microscope Phillips SEM 515 (15kw), in the Botanical Garden and Botanical Museum of Berlin-Dahlem. Spores from this specimen proved to have the same morphological ornamentation of *A. auritum* (Tryon and Lugardon, *Spores of Pteridophyta*: 545, Fig. 212. 22. 1991; Regalado and Sánchez, Grana 41:111, Fig. 3 A, E. 2003): a psilo-lophate perispore with thin ridges limiting lacunae of foveolate or perforate surface (Fig. 1). Mean values of spore length measured along the major equatorial diameter of this specimen were (29–) 33.2 (–37.4) µm, falling within the range ((30–) 35.6 (–47.5) µm) measured from nine Cuban specimens of *Asplenium auritum* (unpublished data). The removed sporangium contained 44 spores, but the sporangium had already opened. For this reason, *Asplenium macilentum* should be treated under the synonymy of *A. auritum* and not under *A. monodon*.

Asplenium auritum Sw., J. Bot. (Schrader) 1800 (2): 52. 1801. TYPE: [specimen].—JAMAICA, *Swartz s. n.* (lectotype selected by Morton and

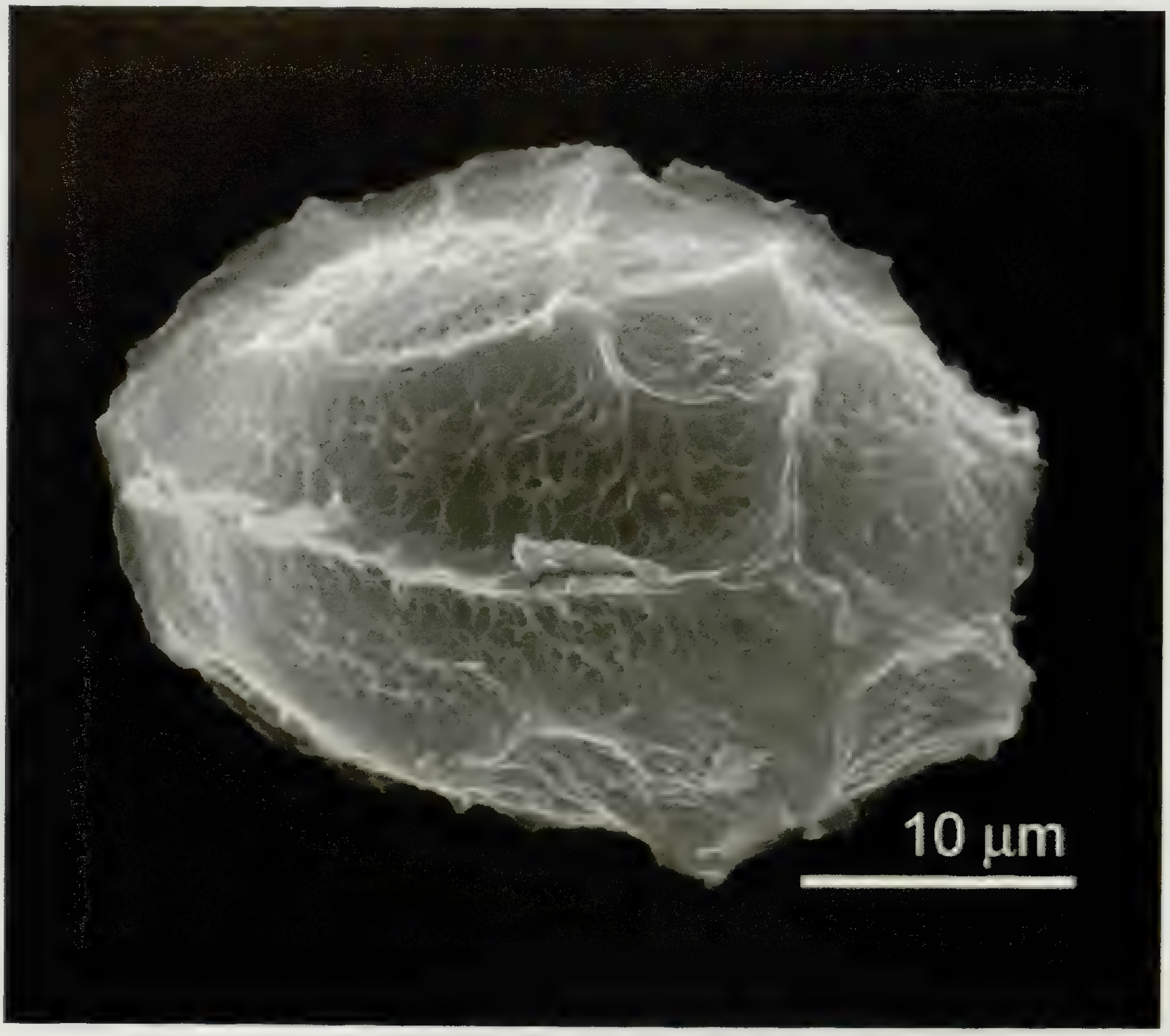

FIG. 1. Spore morphology of the lectotype of *Asplenium macilentum* Kunze ex Klotzsch (19890 B).

Lellinger 1966:18, R-450 S!; isolectotypes: UPS Herb. Thunberg 24781, 24782).

= *Asplenium macilentum* Kunze ex Klotzsch, Linnaea 20: 351. 1847. TYPE: [specimen].—Collector and locality not stated (Lectotype selected by Morton and Lellinger, 1966: 18, B-W 19890!).

= *Asplenium auritum var. macilentum* (Kunze ex Klotzsch) T. Moore Ind. Fil. 115. 1859.

I am grateful to Prof. Dr. Brigitte Zimmer and Ms. Monica Lüchow, from the Botanical Garden and Botanical Museum of Berlin-Dahlem and to Dr. Rosa Rankin, from the Jardín Botánico Nacional, La Habana, Cuba, for the pictures and counts of spores from the lectotype of *Asplenium macilentum*. I also extend my appreciation to Dr. Alan R. Smith for his critical review of the manuscript.—LEDIS REGALADO GABANCHO, Instituto de Ecología y Sistemática, Carretera de Varona km 3½, Capdevila, Boyeros, La Habana 19, CP 11900, La Habana, Cuba, e-mail: ledisregalado@ecologia.cu.

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Shorter Notes

Mericlinal Chimeras in the Gametophyte of *Dryopteris thelypteris* (L.) Gray.—The typical fern gametophyte is a heart-shaped monolayer of cells with an apical cell as the meristem. All cells are derived from an apical cell lineage with each consecutive apical cell dividing into a daughter apical cell and a vegetative cell with the latter proliferating into a group of cells that Douin (Rev Gen. Bot. 38:487–508. 1924) termed a merophyte (Fig. 1); Gifford (Ann. Rev. Plant Physiol. 34:419–440. 1983) specifically notes that a merophyte is to include the original sister cell as well as all its derivatives. Later Korn (Acta Biotheoretica 41:175–189. 1993) described a merophyte as a clone. Klekowski (Evolution 38: 417–426. 1984) raised the question of whether an apical-celled organ can express a chimera by noting that a mutation in the apical cell will lead to all subsequent cells as mutant and therefore a persistent mericlinal chimera is not possible. A mericlinal chimera in angiosperms includes a region of one layer within either L1, L2 or L3 and in a fern gametophyte it would include one transient subclone of a merophyte clone. The question is then can chimeras occur in fern gametophytes? This note reports a mutant of the fern *Dryopteris thelypteris* (L.) Gray that produces not one but a number of mericlinal chlorophyll chimeras in a gametophyte, that is, it appears to be an eversporting chimera (Hejnowicz , Recent Adv. Bot. 2:146–148. 1951).

Sterilized spores were plated in agar dishes and periodically observed for morphology of subsequent gametophytes (Korn, Bot. J. Linn. Soc. 68:63–171.1974). Merophytes can be easily identified in *D. thelypteris* as a papilla forms on the most anterior, marginal cell of each merophyte (Korn, Bot. J. Linn. Soc. 68: 63–171. 1974) (Figs. 1 and 2). About 1,100 spores were seeded in each of three plates and 16 chimeric gametophytes (Fig. 3) were found for a frequency of 16/3300, or 0.0048, gametophytes with chimeras.

Where in the parental sporophyte the mutation occurred can be calculated as follows. First, the frequency of gametophytes or spores with the mutation of 0.0048 is converted to the frequency heterozygous diploid cells of the leaf, or 0.0048 becomes 0.0096, or approximately 0.01. Next, the fraction of a leaf with the patch of heterozygous cells is determined. Each sporangium has about 22.7 $\pm$ 3.3 (n = 25) spores which come from 22.7/4, or about 5.7 sporocytes, a sorus has an average of 30.6 $\pm$ 6.0 (n=25) sporangia per sorus, a secondary pinnae has an average of 12.6 $\pm$ 1.9 (n = 25) sori, a primary pinna has 45.8 $\pm$ 6.3 secondary pinnae and a leaf has 45.7 $\pm$ 9.5 (n= 5) primary pinnae for a total count of 5.7 $\times$ 30.6 $\times$ 12.6 $\times$ 45.8 $\times$ 45.7, or there are about 4,599,901 sporocytes per leaf. If 0.01 sporocytes are heterozygous then there were 4,599,901 $\times$ 0.01, or 45,999 heterozygous sporocytes per leaf. With about 45,999 heterozygous sporocytes per leaf and with 5.7 $\times$ 30.6 $\times$ 12.6, or 2,198 sporocytes per secondary pinna, there were 45,999/2,198, or 20.9 secondary pinna with heterozgygous sporocytes. With 45.8 secondary pinnae per primary

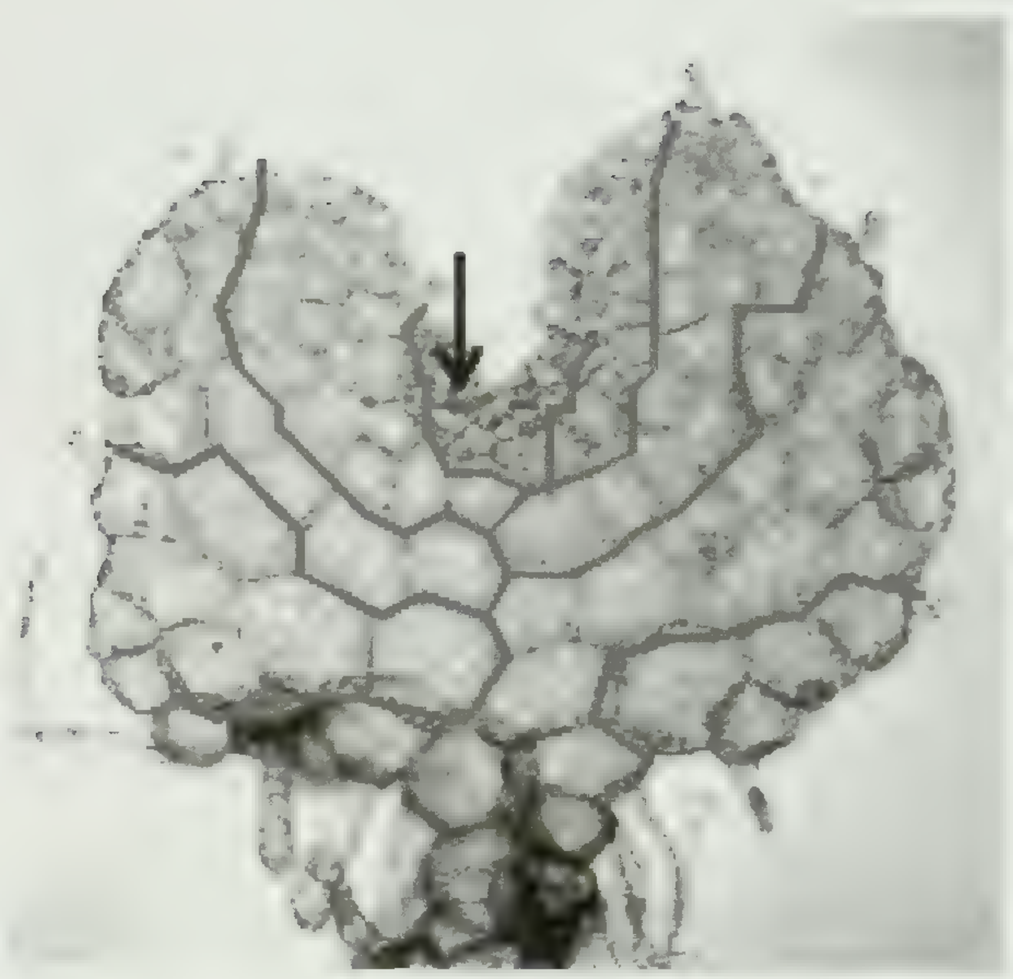

FIG. 1. Wild type gametophyte with merophyte boundaries drawn in red and arrow points to location of the apical cell.

pinnae then 20.9/45.8, or about a region of 46% of a primary pinna, or (1/45.7) × 0.46 or 0.01 of leaf area had this mutation. Third, the distributions of sporocytes in a sporangium, sporangia in a sorus, sori in a secondary pinna, secondary pinnae in a primary pinna and primary pinnae in a leaf are regular, namely, ordered, which permits fractions of leaf area to be given in any of these units, such as 0.01 of a leaf based on sporocyte calculations.

The above calculations are based on the assumption that there was a single mutation in some leaf that was passed on to spores from that portion of the leaf where the mutation occurred. It is also possible that the entire leaf and all other leaves of the mother sporophyte were heterozygous because one of two possible gametophytic parents was mutant. This is an unlikely explanation because gametophytes studied here came from only one leaf while spores from over eight other leaves from this sporophyte over a period of ten years never produced chimeric gametophytes.

Three gametophytes where all clones could be clearly recognized were analyzed in detail for the number of cells per albino subclone (Fig. 4) with the lowest value of one cell as the most frequent (20) and the largest subclone of 35 cells as the most infrequent (1). The data set on number of cells in a subclone was tested to see if it follows a geometric series, $P(n) = Nq^{n-1}$p (Meyer, Introductory Probability and Statistical Applications, Addison Wesley. 1965), where N is the number of merophytes scored in the observed data, or 58, n is

FIG. 2. A marginal papillar cell.

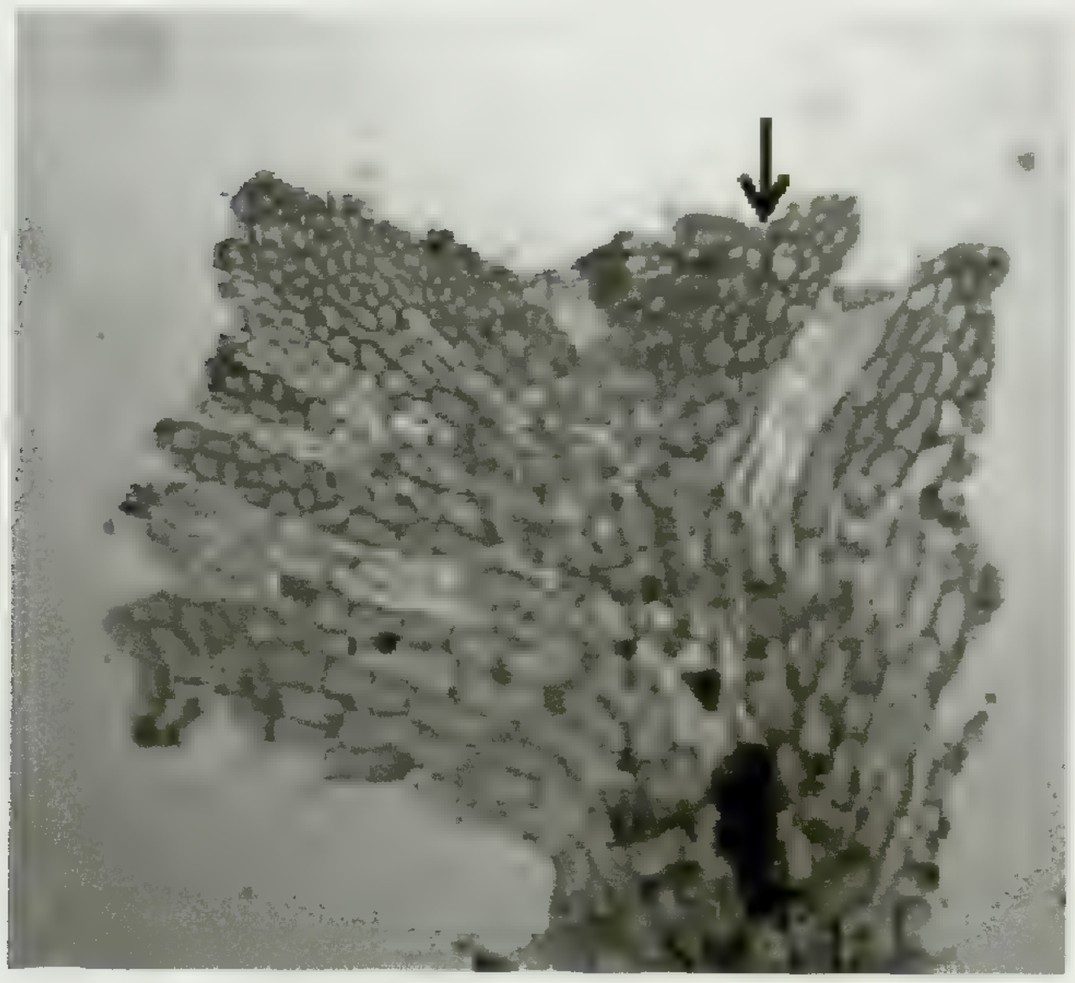

FIG. 3. Mutant gametophyte with various sizes of albino subclones and arrow points to apical cell.

the number of cell cycles, or cell generations, q is the probability of subclonal cells dividing at n^{th} generation and q is the probability that subclonal cells cease dividing at the n^{th} generation (Fig. 4). The value of q was determined by averaging the ratios of $n/n+1$ and p is 1-q. A χ^2 probability of this equation generating data like the observed is >90%. The good fit of expected to obtained data indicates albino clones arise at any time during merophyte formation, more and smaller subclones form as a merophyte increases in cell number.

A second study of these 58 merophytes was to determine the number of albino subclones per merophyte (Fig. 5). The data has a sharp peak at two and does not follow any tested probability distribution. It is assumed that an early subclone proliferates and therefore limits the number of more, small subclones later in a merophyte. A computer program was written whereby there were 58 merophyte inital cells and the probability of an albino subclone forming is

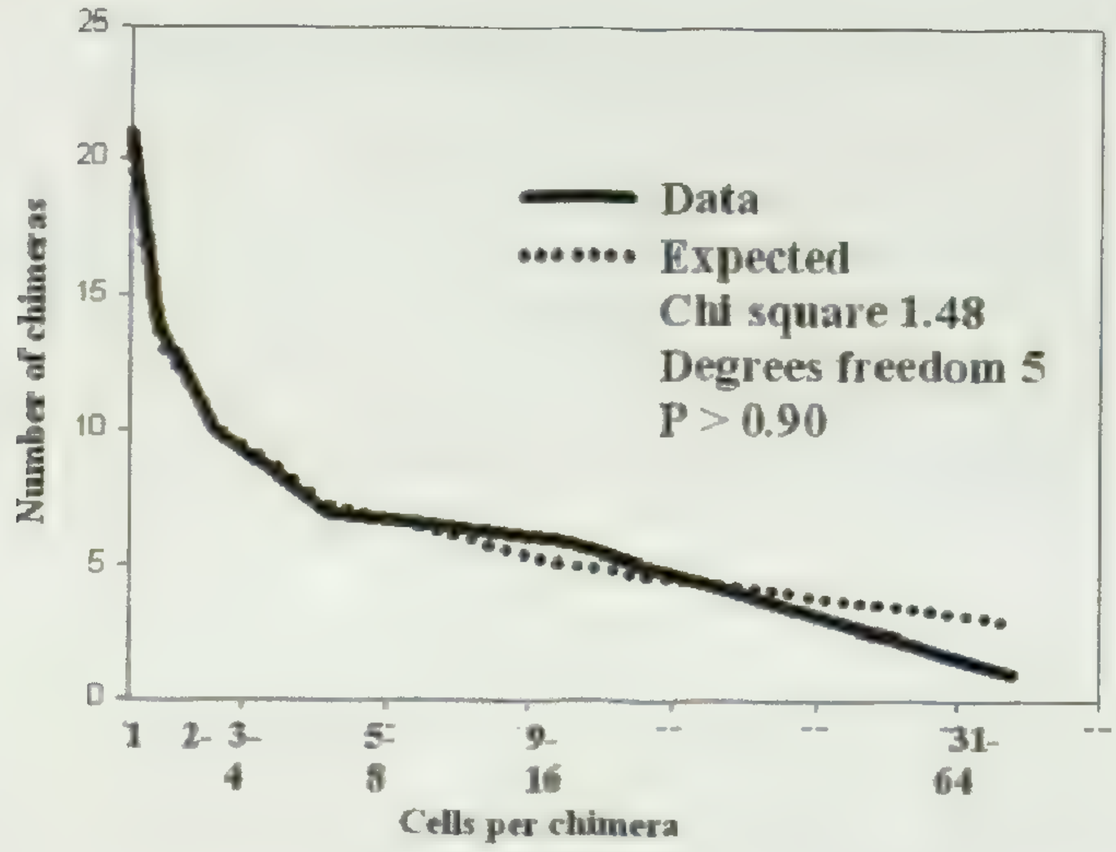

FIG. 4. Cells per albino subclone vs number of such cases.

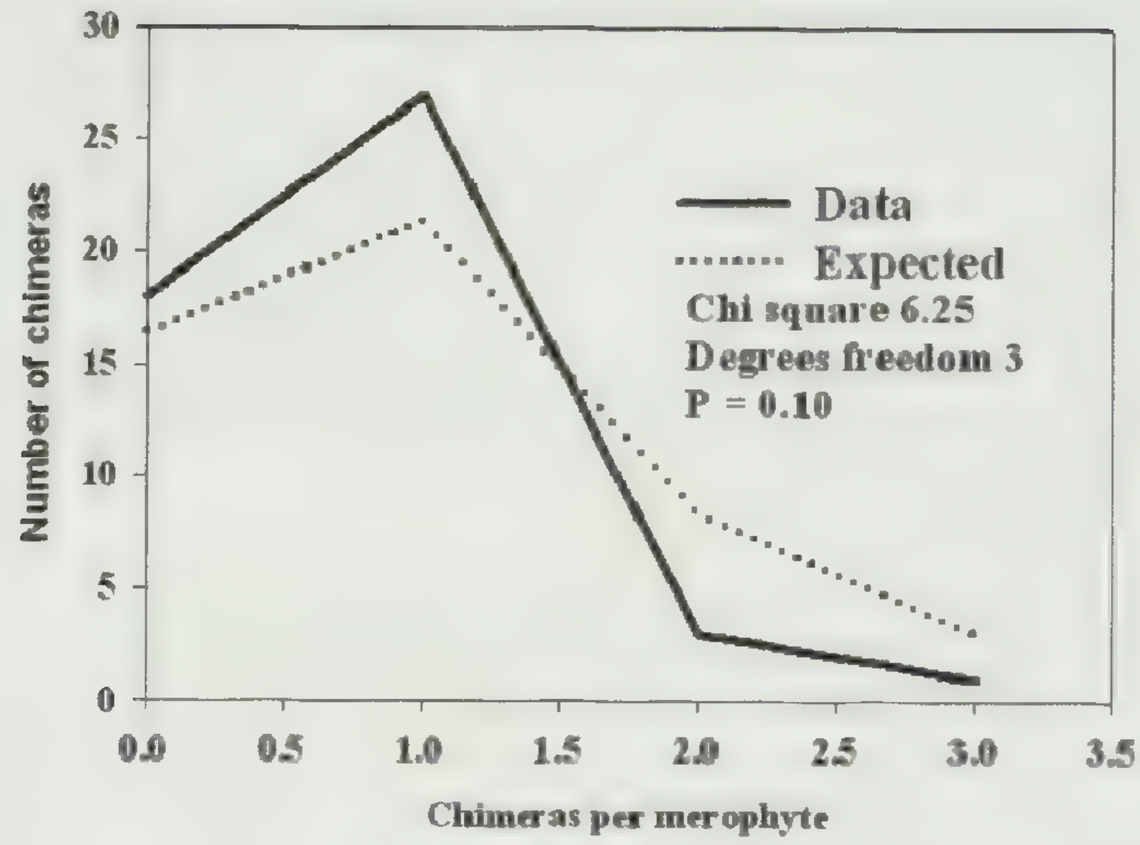

FIG. 5. Number of albino subclones per merophyte vs number of such cases.

arbitrarily taken as 0.05 at each cell generation with the initial cells passing through five cell cycles, or an increase of 2^5-1, or 31 cells. The resultant data from five runs gave a χ^2 probability of 0.10, a marginally good fit (Fig. 5). It is suggested from this statistical result that subclones arise independently of other subclones, a feature of clones demonstrated earlier for plants in general (Korn, Cell Prol. 41:691–708. 2008).

These two studies together indicate subclones form randomly among cells during the development of a merophyte. It would thus seem that all cells of the gametophyte have an unstable mutant gene for green plastids that either further spontaneously mutates or causes a non-allelic gene to mutate to an albino state. Since the gametophyte is haploid mutant genes would be expressed directly. If the plastid is the unit of expression one-celled pure subclones are not possible by immediate segregation during a cell division involving segregation of one mutant and about 15 wild type plastids randomly into daughter cells. It is not clear how the second mutation leads immediately to an albino cell but once that cell appears it proliferates into a clone of albino cells.

One unexpected feature of these 16 gametophytes is the lack of sex organ production, hence, sporophytes could not be produced. This is not unlike morphological mutations in the desmid *Cosmarium turpinii* (Korn, Genetics 65:41–49. 1970) where of 17 shape mutations 13 were sterile and only four were sexual. One interesting result is that albino subclones are smaller than green areas (Fig. 3) indicating photosynthate of cells contributes mostly to their own growth, that is, much of growth is not a property of an organism but is a local phenomenon. It is also possible that mutant subclones have too little reserves for the plant to be sexual.

Chimeras in *Dryopteris* parallels those in various species of juniper (Tilney-Bassett, Plant Chimeras, Edward Arnold. 1986; Korn, Amer. J. Bot. 88:1945–1952. 2001). In juniper the first chimera is a periclinal sandwich one with a white tunica ,WLI, and green corpus, GL2, and the second chimera is from a periclinal replacement division in L1 where one daughter is in L2 to gives rise

to a mericlinal WL2 chimera. Repetition of this type of division in the same plant generates many periclinal chimeras which are termed eversporting (Hejnowicz, 1951). Here in *Dryopteris* the first step is an unstable mutant state in all cells of the gametophyte inherited from the spore and the second step is a mutation to an albino phenotype in a cell that proliferates into an albino subclone. Repetition of this second step produces an eversporting case of mericlinal chimeras. This type of chimera involving an unstable state is too peculiar to have been anticipated by Klekowski (1984) but explains how chimeras are possible in fern gametophytes.—Robert W. Korn, Bellarmine University, 2001 Newburg Rd., Louisville, Kentucky, U.S.A.

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Broad-Scale Integrity and Local Divergence in the Fiddlehead Fern *Matteuccia struthiopteris* (L.) Todaro (Onocleaceae)

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ABSTRACT.—*Matteuccia struthiopteris* (Onocleaceae) has a present-day distribution across much of the north-temperate and boreal regions of the world. Much of its current North American and European distribution was covered in ice or uninhabitable tundra during the Pleistocene. Here we use DNA sequences and AFLP data to investigate the genetic variation of the fiddlehead fern at two geographic scales to infer the historical biogeography of the species. *Matteuccia struthiopteris* segregates globally into minimally divergent (0.3%) Eurasian and American lineages. These two clades have little to no variation even at large geographic scales. Within hemisphere, patterned genetic variation was evident only in the AFLP data and only locally. Genetic variation within Vermont was greater within the westward-trending Winooski River watershed than in the Passumpsic River watershed, which drains east into the Connecticut River. We suggest that historical factors have created this pattern; a Mississippi Valley Pleistocene refugium for the American lineage of the species seems plausible.

KEY WORDS.—*Matteuccia*, Onocleaceae, Pleistocene, Biogeography, AFLP, *PgiC*

Matteuccia struthiopteris (L.) Todaro (Onocleaceae) has a present-day distribution across much of the north-temperate and boreal regions of the world. It is commonly known as the fiddlehead fern in the northeast of North America because of the resemblance of its elegant croziers to their namesake (Kato, 1993). *Matteuccia struthiopteris* is found most commonly on river banks and flood plains. However, the species can also be found in uplands anywhere there are rich alluvial soils (Smith, 1993). Though robust in full sun, we have found that the ferns are more common in either full or partial shade, sheltered by broadleaf deciduous trees. They often form large colonies on wooded floodplains via prolific spread by stolons. The fronds are dimorphic; large (0.3 to greater than 1.5 m) sterile fronds produced in the spring are followed by smaller (0.3 to 0.7 m) fertile fronds produced in late summer, which have their sporangia rolled up inside of the pinnules (Prange and von Aderkas, 1985). The sterile fronds are oblanceolate, tapering gradually at the base and abruptly at the tip; the widest part of the frond is subapical (Prange and von Aderkas, 1985). Sterile fronds present two morphological variants that we call *downy* and *smooth*. In the downy variant the petioles of young fronds are covered with a minute (less than 0.5 mm),

colorless, non-laminar indument. In the smooth variant the petiole lacks this indument. This downy indument dissipates in early summer, so its presence cannot be scored later in the growing season.

Matteuccia struthiopteris is distributed throughout much of the northern hemisphere in boreal deciduous forests (Lloyd, 1971); it is collected as a seasonal edible green in New England and the eastern provinces of Canada (von Aderkas, 1984), as well as in Japan (Miyazawa *et al.*, 2007). Fiddlehead fern collection is documented for several Native American groups, including primarily the Abenaki Indians of New England and Malecite of New Brunswick. European colonists learned of this harvest practice from these native groups on their arrival in North America and collected fiddleheads as part of a subsistence diet (von Aderkas, 1984). In Vermont, we have seen a recent explosion of interest in buying and preparing fiddleheads with the rapid expansion of the local-foods movement. In the last three years, landowners have begun to post their lands with no-harvesting signs for the first time.

Little is known about the impact of fiddlehead harvesters on the demography or genetic structure of *Matteuccia struthiopteris*, as their activity is not regulated. However, it is known that the species' health can be significantly affected by the removal of young fronds. Bergerson and Lapointe (2001) conducted a five-year study in Québec, Canada, investigating the impacts of harvesting on *Matteuccia struthiopteris*. They found that harvesters should collect no more than half the croziers produced by a shoot, as removal of all shoots in a single year impaired carbohydrate accumulation and crozier production in subsequent years.

With the advent of molecular techniques, there has been substantial improvement in our understanding of the geographic distribution of genetic variation in plant populations. In a historical context, these distributional patterns are now commonly used to interpret the recent distributional history of species (Donoghue *et al.*, 2001; Hewitt, 2000). Analyses of these patterns make the basic assumption, an assumption that we will maintain, that populations near Pleistocene refugia, having remained established for the longest period of time, will display greater genetic diversity than more peripheral populations. This concept was first proposed in the stepping-stone model of population structure developed by Kimura and Weiss (1964). Building on this idea, dispersal models constructed by Ibrahim *et al.* (1996) and Hewitt (1996) predicted that as a species expands from its refugium the newly established populations contain less diversity than the parent population. The ideas of Kimura and Weiss (1964), Ibrahim *et al.* (1996), and Hewitt (1996) were confirmed in subsequent studies by Dufresne and Hebert (1997) and most of those reviewed by Barrington and Paris (2007).

On the other hand, genetic variation in populations relates to local evolutionary history (both selection and drift) and factors relating to population size (large central and small peripheral populations can evidence different patterns of genetic diversity). In northeastern North America for instance, the distribution of genetic diversity in *Cypripedium parviflorum* Salisb. appears to be the result of genetic drift related to differences in population size and not historical factors (Wallace and Case, 2000). Given the great differences in population size of

Matteuccia with elevation (very large in alluvial forests low in watersheds and small and isolated in wetlands at high elevations), history and recent evolution must both be considered.

Our interest in the historical biogeography of *Matteuccia struthiopteris* led us to assess genetic diversity of the fiddlehead fern at two scales. First, we used AFLP analysis to document the distribution of genetic diversity within an array of fiddlehead fern to assess variation within and among populations in Vermont. We expected one of two patterns to emerge: 1) concentration of variation in either large lowland or small highland populations implying recent drift or selection or 2) a regional pattern of decreasing diversity implying a historical cause. We also explored the pattern of variation in the downy versus smooth variants in these populations with similar expectations about pattern. Second, we set this local variation in the context of a less detailed view of the global genetic structure for the fiddlehead fern based on chloroplast and nuclear DNA sequences. In this study we sought to assess potential human impact on genetic diversity, as harvesting of these ferns is now intensive in the large populations in lower-elevation river valleys of the region.

Materials and Methods

Taxon sampling.—A total of 83 accessions representing four onocleaceous taxa was included in this study. All but three of these accessions were *Matteuccia struthiopteris.* Unless noted otherwise, at each site samples were collected as follows. Up to ten complete fronds were collected, each from a different shoot. In an effort to avoid collecting multiple ramets of single genets, each frond was taken at a minimum distance of 2 m from any other sample. The maximum distance was 5 m from any other sample. Downy individuals were deliberately included when encountered in a population, and each population was scored as downy abundant, rare, or absent. Material collected from each individual included a sample for DNA analysis stored in silica gel at −80 C and a dried voucher specimen, both from the same frond.

A total of 60 individuals of *Matteuccia struthiopteris* was collected across three watersheds in Vermont: the Passumpsic, Mettawee, and Winooski rivers (Fig. 1). The Passumpsic River is a tributary of the Connecticut River, located entirely within the Northeast Kingdom of Vermont. Three collections were made along the Passumpsic, the highest in elevation in Newark at 358 m, the middle in East Burke at 253 m, and the lowest in Barnet at 156 m (See Appendix 1 for accession data and voucher information).

The Mettawee River is the shortest of the three river systems from which plants were collected. One collection was made along the Mettawee River in Granville, NY at 173 m (Appendix 1).

The Winooski River is the largest of the three watersheds surveyed. The river drains an area of the northern section of the Green Mountains; it is a large tributary to Lake Champlain. Three collections were made along the river. The highest in elevation is in Cabot at 442 m, the next in Plainfield at 238 m, and the lowest in Richmond at 87 m (Appendix 1).

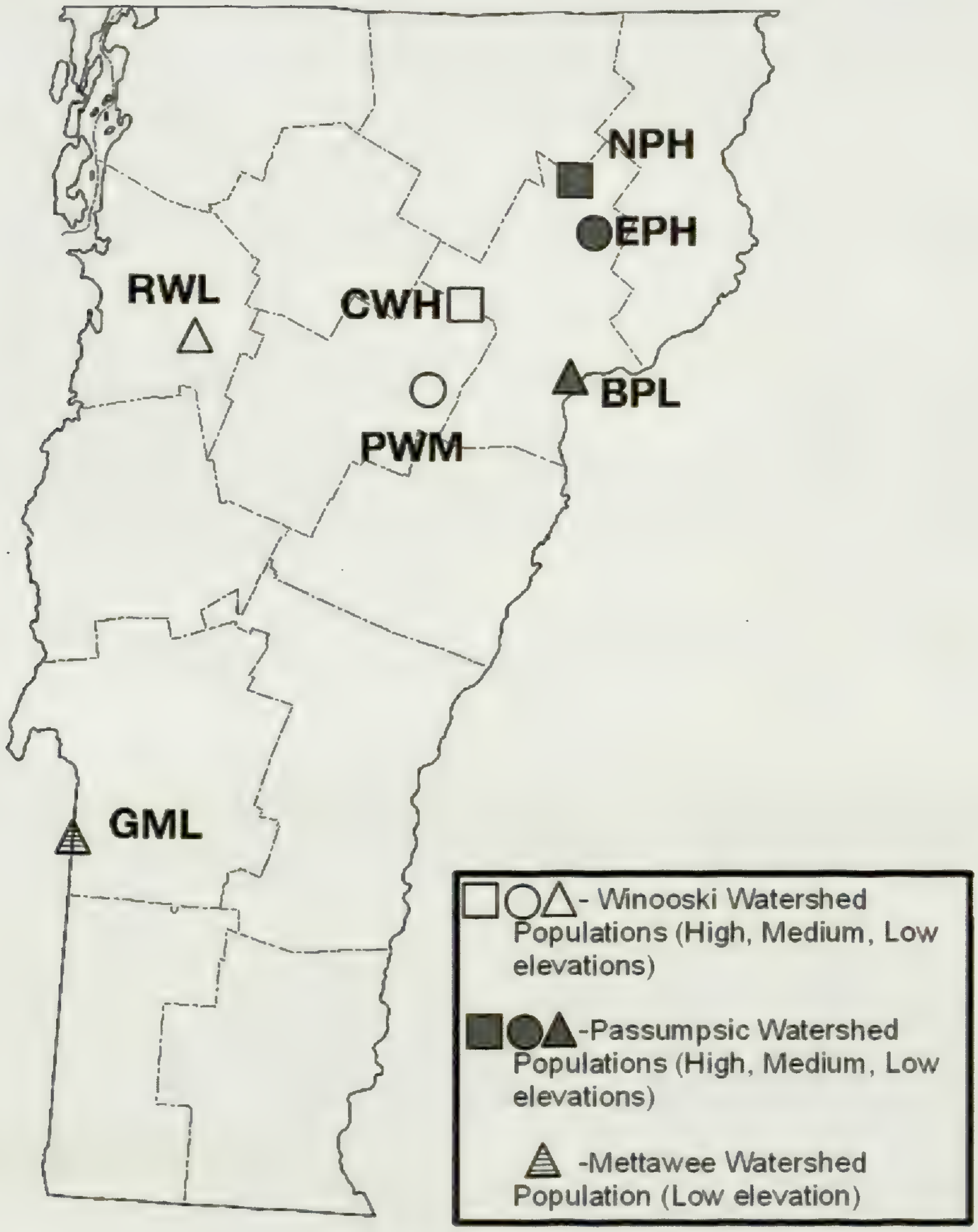

FIG. 1. Source of Vermont *Matteuccia struthiopteris* accessions for the AFLP study.

The remaining 23 accessions comprised two species of the *Matteuccia* segregate genus *Pentarhizidium* (*P. intermedium* (C.Chr.) Hayata and *P. orientale* (Hook.) Hayata), the out-group taxon *Onoclea sensibilis* L., and 20 additional *Matteuccia struthiopteris* accessions including 15 from the New World (Vermont, Maine, Quebec, New Brunswick, Michigan, British Columbia, and Alaska) and four from the Old World (China, Japan, and Sweden). Accessions from China and Sweden were provided by correspondents without vouchers or information on collection method (Appendix 1).

DNA extraction.—Total DNA was extracted from approximately 0.05 g of frond tissue following Dempster *et al.* (1999), which is based on the CTAB protocol of

Doyle and Doyle (1987). The following slight modifications were made in order to acquire samples of the high quality and concentration required for the AFLP technique. Homogenized frond material was allowed to incubate at 55°C for 24 hours in CTAB buffer. After the addition of isopropanol the nucleic acids were allowed to precipitate for one hour at −80°C. DNA was pelleted using centrifugation, then cleaned once in a 70% ethanol wash and once in a 95% ethanol wash. The pellets were dried via tube inversion or Speedvac (Genevac Inc, Gardiner, New York, USA). After the DNA was resuspended in 50 µL of ddH_2O the concentration was quantified using a NanoDrop1000 (Thermo Scientific, Waltham, Massachusetts, USA). Samples with concentrations lower than 100 ng/µL were discarded and re-extracted following Sigel (2008).

PCR amplification.—Screening of seven chloroplast markers and one nuclear marker for variability yielded two variable markers for sequencing, the highly labile cp marker psbA-trnH spacer (*psbA-trnH*, see Kress *et al.*, 2005) and the *PgiC* region including most of intron 15 and a portion of exon 16 (*PgiC* 15–16), labile in work with Dryopteridaceae in our lab (Sigel, 2008). *psbA-trnH* primers are those of Sang *et al.* (1997) with modification for use on ferns (psbA-trnHFor: 5′ GTTATGCATGAACGTAATGCTC 3′, psbA-trnHRev: 5′ CGCGCATGGTG-GATTCACAATCC 3′). The primers for *PgiC* 15–16 were modified for this study from those developed in our lab for use on *Polystichum* (Dryopteridaceae), following Ishikawa *et al.* (2002). They are PgiC15FM (5′ TTTGCTCCTCACATT-CAACA 3′), and PgiC16RM (5′ GTTGTCCATTAGTTCCAGGTTCCCC 3′). These regions were amplified by the polymerase chain reaction (PCR) method with all thermal cycling done using either a model TC-312 or T-3000 thermal cycler (Techne, Burlington, New Jersey, USA). Reactions were completed in 24 µL aliquots with the following reaction components: for psbA-TrnH: 100–200 ng of DNA, 0.1 µmol/L of each primer, 1× ExTaq Buffer (TaKaRa, Madison, Wisconsin, USA), 200 µmol/L of each dNTP, 200 µmol/L of $MgCl_2$, 200 µmol/L of BSA, and 0.625 U ExTaq polymerase (TaKaRa); for *PgiC* 15–16: 100–200 ng of DNA, 0.1 µmol/L of each primer, 1× ExTaq Buffer, 400 µmol/L of each dNTP, 400 µmol/L of BSA, and 0.625 U Ex Taq polymerase. The reaction conditions were as follows for *psbA-trnH*: initial denaturation was for 1 min at 95 C; followed by 30 cycles of 1 min at 95°C, 1 min at 50 C, and 4 min at 65 C; with a final extension of 72 C for 5 minutes. For *PgiC* 15–16: initial denaturation was for 3 min at 95 C; followed by three cycles of 1 min at 94 C, 1 min at 56 C, and 2 min at 72 C; followed by three cycles of 1 min at 94 C, 1 min at 53 C, and 2 min at 72 C; followed by 34 cycles of 45 sec at 94 C, 45 sec at 50 C, and 90 sec at 72 C; with a final extension of 72 C for 8 min. Before sequencing the PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, Ohio, USA).

Agarose gel electrophoresis and extraction.—Prior to sequencing, all amplified samples were visualized on 2% agarose gels with ethidium bromide (1.0 g agarose, 50 mL Tris Borate EDTA buffer, 2.5 µL ethidium bromide). If two or more bands were separated during electrophoresis, the band of the appropriate marker length was excised and the amplified DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, California, USA). Before

sequencing the extraction products were purified using ExoSAP-IT (USB Corp., Cleveland, Ohio, USA).

Sequencing.—Direct sequencing of both markers was undertaken using the ABI BigDye Terminator Cycle Sequence Ready Reaction Kit v. 31 (Perkin-Elmer/Applied Biosystems, Foster City, California, USA). The kit employs the following thermal-cycling protocol: initial denaturation was for 5 min at 80°C followed by 30 cycles of 10 sec at 96°C and five sec at 50°C with a final extension at 50°C for 4 min. An ABI Prism 3130x1 automated sequencer was used to resolve the sequencing products (Vermont Cancer Center DNA Analysis Facility, Burlington, Vermont USA).

Sequence alignment.—Raw forward and reverse sequences were assembled for each sample and ambiguous bases were corrected from inspection of the chromatograms; Sequencher v. 3.1.1 (Nishimura, 2000) and BioEdit v. 7.0.9 (Hall, 1999) were used for sequence editing and consensus-sequence construction. Consensus sequences were first aligned using ClustalX (Larkin *et al.*, 2007), then manually improved using MacClade v.4.08 (Maddison and Maddison, 2005).

Phylogenetic analysis.—Separate analyses were conducted for each data set that was generated (*psbA-trnH, PgiC* 15–16) and the two combined using Maximum Parsimony. For the analysis we used PAUP* version 4.0b10 (Swofford, 2001) with all characters treated as unordered with equal weights. A heuristic search was performed using 10,000 replicates and random taxon addition with ten trees held at the tree-bisection-reconnection (TBR) branch swapping step with each sequence addition. A maximum of ten trees was saved at each step, MulTrees option on, with ACCTRAN character-state optimization. Bootstrapping was performed for 1000 replicates using simple taxon addition, TBR branch swapping, and the MulTrees option on. For this analysis, we retained a single representative accession for sets of identical accessions (see Fig. 2 for details). We considered bootstrap percentages greater than 68 and 95 to be moderate and strong levels of clade support respectively following Driscoll and Barrington (2007). All trees were rooted using *Onoclea sensibilis* as the outgroup. We report only the combined analysis: separate analyses were congruent with but less resolved than the combined analysis.

AFLP protocol.—The AFLP protocol was adapted from Vos *et al.* (1995) with additional modifications as suggested by the Wolf Lab at Utah University (Wolf, 2000), the Gastony Lab at Indiana University (Nakazato *et al.*, 2006), previous studies in the Barrington Lab (Sigel, 2008), as well as modifications specific to this study. Several procedures were performed to minimize genotyping error in the final data. A previously typed sample was included in each round of amplification for comparison between different rounds. Ten percent of the samples were replicated between two and four times to assess the error within samples. Those samples with manifest errors in amplification, such as greatly reduced allele numbers, were discarded and re-amplified following Sigel (2008).

Restriction of the genomic DNA was performed using the restriction endonucleases *Eco*RI and *Mse*I (New England Biolabs, Ipswich, Massachusetts, USA). Ligation of the sticky end-adaptors (Invitrogen, Carlsbad, California, USA) to the restriction sites was also achieved in this initial reaction following the Wolf

(2000) protocol. Modifications from the Wolf Lab protocol (Wolf, 2000) are as follows: 20 μL of the enzyme master mix were used to avoid pipetting volumes under 0.4 μL. The master mix had the following components: 0.02 μL of *Mse*I, 0.05 μL of *Eco*RI, 0.025 μL of T4 Ligase, and 0.655 μL ddH_2O were used per 1 μL of total master-mix product. Each reaction volume totaled 11 μL, each containing 100 ng of DNA suspended in 5.5 μL ddH_2O. Samples were incubated for two hours at 37°C in a thermal cycler. In preparation for pre-selective amplification 3 μL of the restriction/ligation product were diluted with 24.5 μL TE 0.1 buffer.

In the first round of PCR, preselective amplification was achieved with primers that complement the *Eco*RI and *Mse*I adaptors plus one additional nucleotide i.e., *Mse*I+C (5′ GAT GAG TCC TGA GTA AC 3′) and *Eco*RI+A (5′ GAC TGC GTA CCA ATT CA 3′). Each preselective reaction totaled 25 μL, comprising the following: 3 μL diluted restriction/ligation product, 2.5 μL ExTaq Buffer (TaKaRa), 0.1 μL Ex Taq DNA polymerase (TaKaRa), 0.75 μL of 50mM $MgCl_2$, 1.2 μL of 2.5mM dNTPs, 16.45 μL ddH_2O, and 0.5 μL of a 10 μM solution of each of the primers. Reaction samples were initially denatured for 2 min at 72°C, followed by 30 cycles of: 94°C for 30 sec, 56°C for 30 sec and 72°C for 2 min, ending with 30 min at 6°C. Fifteen and a half μL of the preselective amplification product were diluted with 100 μL of TE 0.1 buffer. The quality of the undiluted preselective and restriction/ligation product was visualized on a 1.5% TBE-agarose gel.

In the second round of PCR, selective amplification was achieved using the *Mse*I+CAG and *Eco*RI+AAC (fluorophore NED [yellow] primers); each selective primer has an identical sequence to its corresponding preselective primer but with an additional two bases. The *Eco*RI primer is fluorescently tagged for visualization on the ABI 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Selective reactions were set up in 18 μL aliquots comprising the following: 3.6 μL of the diluted preselective DNA product, 1.8 μL Ex Taq Buffer, 1.8 μL Ex Taq DNA polymerase, 0.5 μL $MgCl_2$, 0.864 μL dNTPs, 1.44 μL of the 0.4mM *Mse*I selective primer (Invitrogen), 3.66 μL of the 0.08mM *Eco*RI selective primer (Invitrogen), 0.144 μL BSA, and 5.872 μL ddH_2O. Reactions were initially denatured at 94°C for 2 min, followed by 13 cycles of 94°C for 30 sec, 65°C for 30 sec (reduced by 0.7°C per cycle), 72°C for 2 min, and 24 cycles of 94°C for 30 min, 56°C for 30 sec, 72°C for 2 min, with a final hold at 72°C for 30 min. Selective amplification products were run on the 4-capillary ABI 3100-Avant Genetic Analyzer at the Vermont Cancer Center DNA Analysis Facility at the University of Vermont.

ALFP data scoring.—The most challenging aspects of AFLP analysis are data scoring and analysis (Bonin *et al.*, 2004; Bonin *et al.*, 2007; Meudt and Clarke, 2007; Pompanon *et al.*, 2005; Vekemans *et al.*, 2002; Whitlock *et al.*, 2008). In principle, the challenge to AFLP data scoring is producing a set of binary phenotypes (termed *loci*) for each individual from the presence/absence of the DNA fragments retrieved, while at the same time excluding experimental artifacts. We used the loci exclusion thresholds and phenotype exclusion thresholds outlined in Whitlock *et al.* (2008) to reduce the likelihood of scoring artifacts while maintaining a maximal number of informative markers. Appendix

B of Sigel (2008) provides a detailed account of AFLP analysis problems and solutions in our lab.

Initial AFLP fingerprints were determined with GeneMapper v. 3.7 (Applied Biosystems). Peak-height data for each individual were exported in tab-delineated form to an Excel worksheet. The top ten percent of the peak heights was trimmed to remove false flares in the recording instruments. The mean of the trimmed data was calculated for each locus across all individuals as well as for each individual across all its loci. These means were used as a basis for defining an array of candidate loci-exclusion and phenotype-calling thresholds. Using the trimmed mean rather than an arbitrary value of 100 relative fluorescence units (rfu) as suggested by GeneMapper (Applied Biosystems) is more accurate as it better represents the specific data set under study (Sigel, 2008). We generated 20 binary datasets with an array of loci-exclusion and phenotype-calling threshold combinations. The mismatch error rate (which represents the number of individuals with inconsistent band data in replicate amplifications) was calculated for each generated dataset. The optimum threshold was determined by selecting the generated dataset that maintained the maximum number of informative loci while retaining a mismatch error rate below five percent (Bonin *et al.*, 2004). Binary phenotype datasets generated using the identified optimum thresholds were used in the data analysis.

Data analysis and interpretation.—The binary dataset with the maximum number of informative loci with an error rate below five percent was used as a basis for data analyses. GENALEX version 6.2 (Peakall and Smouse, 2006) was used for all statistical analyses of the data. A pairwise, individual-by-individual genetic-distance matrix was generated. Each value in the matrix is a tally of differences between two genetic profiles (Peakall and Smouse, 2006, Appendix 1). As an initial probe of the data the genetic-distance matrix was used to do a principal-components analysis (PCA). We explored the PCA by visualizing individual plants on plots of pairs of principal components accounting for substantial variance in the data to search for clusters of individuals according to population, watershed, and elevation.

To assess patterns of genetic diversity across the seven Vermont populations and elevations the AFLP data were subjected to three calculations: the average genetic distance between individuals, the average heterozygosity, and the total number of loci present.

Results

Field observations.—In Vermont the downy trait was absent in the high-elevation population in the Winooski watershed and in both the high and middle populations in the Passumpsic watersheds. The trait was rare in the middle-elevation population in the Winooski watershed. Both watersheds had an abundance of downy individuals in the low-elevation population. The downy character trait was not scorable for individuals in the Mettawee population, as it was collected too late in the season. In the Maritimes, three low-elevation New Brunswick populations (the one near Campbellton New Brunswick, the one at the

intersection of Route 2 Canada and the Canaan River, and the one just west of Fredericton) included downy plants.

Sequence characteristics.—The aligned and concatenated sequence for *psbA-trnH* and *PgiC* 15–16 yielded a matrix of 798 characters, including five indels. *psbA-trnH* comprised 439 of these characters including two of the indels. Within *Matteuccia,* 59 characters (7.4%) were variable, of which 36 including four indels (4.5%) were parsimony informative. Twenty characters separated *Onoclea* (the outgroup) from the study group. In *PgiC,* direct sequencing yielded largely unambiguous sequences, presumably because most plants were homozygous for all nucleotides. An informative exception was our sequence for *Onoclea,* which contained an extensive region of double peaks at the 3′ end. The pattern of double peaks in this region was consistent with the interpretation that they were the result of the insertion of one nucleotide in one of the two homologs; for analysis we retained the allele that was identical to the rest of the study set.

Phylogenetic analysis.—The phylogenetic analysis conducted using Maximum Parsimony (MP) yielded one shortest tree consisting of 53 steps. The analysis (Fig. 2) retrieved a strongly supported clade (BS = 100) comprising the Asian endemics *Pentarhizidium intermedium* and *P. orientale* to be sister to a monophyletic (BS = 100) *M. struthiopteris.* Within our study species, Eurasian and American *M. struthiopteris* clades were moderately well supported (BS value for each is 86). Within each of the two *M. struthiopteris* clades all accessions were unresolved, with the exception of two of the accessions from eastern Canada, which were sister to each other (BS=100). An additional analysis, including the Alaska accession that was received while this contribution was in review, yielded similar results, but with the Old World clade bootstrap support reduced to 66% and a new clade comprising the Old World and Alaska retrieved with 65% bootstrap support.

Looking at individual characters for *Matteuccia struthiopteris*, New World and Eurasian accessions differed by three substitutions (0.3%), two of them synapomorphies for the Old World plants and one for the New World plants. With the addition of the Alaska accession, there was one synapomorphy unique to the Old World plants, one shared by the Old World and Alaska accessions, and one by the New World plants excluding the Alaska accession. The nearly complete lack of resolution within *M. struthiopteris* is due to absence of synapomorphies rather than character incongruence.

AFLP analysis.—AFLP analysis of the sixty *Matteuccia struthiopteris* samples using the *Mse*I+CAG *Eco*RI+AAG primer pair resulted in 254 distinguishable loci. A phenotype-calling threshold of 75% (92.51 rfu) and loci-exclusion threshold of 85% (104.85 rfu) were used to construct the binary data matrix from the raw peak-height data. This combination of thresholds applied to the raw data resulted in a 4.98% mismatch error rate between replicate samples.

The percent polymorphic loci across all populations was 86.22 while the percent polymorphic loci within the individual populations ranged from 33.36 in the Barnet population (middle elevation population in the Passumpsic watershed) to 61.81 in the Plainfield population (middle elevation population in the Winooski watershed, Table 1).

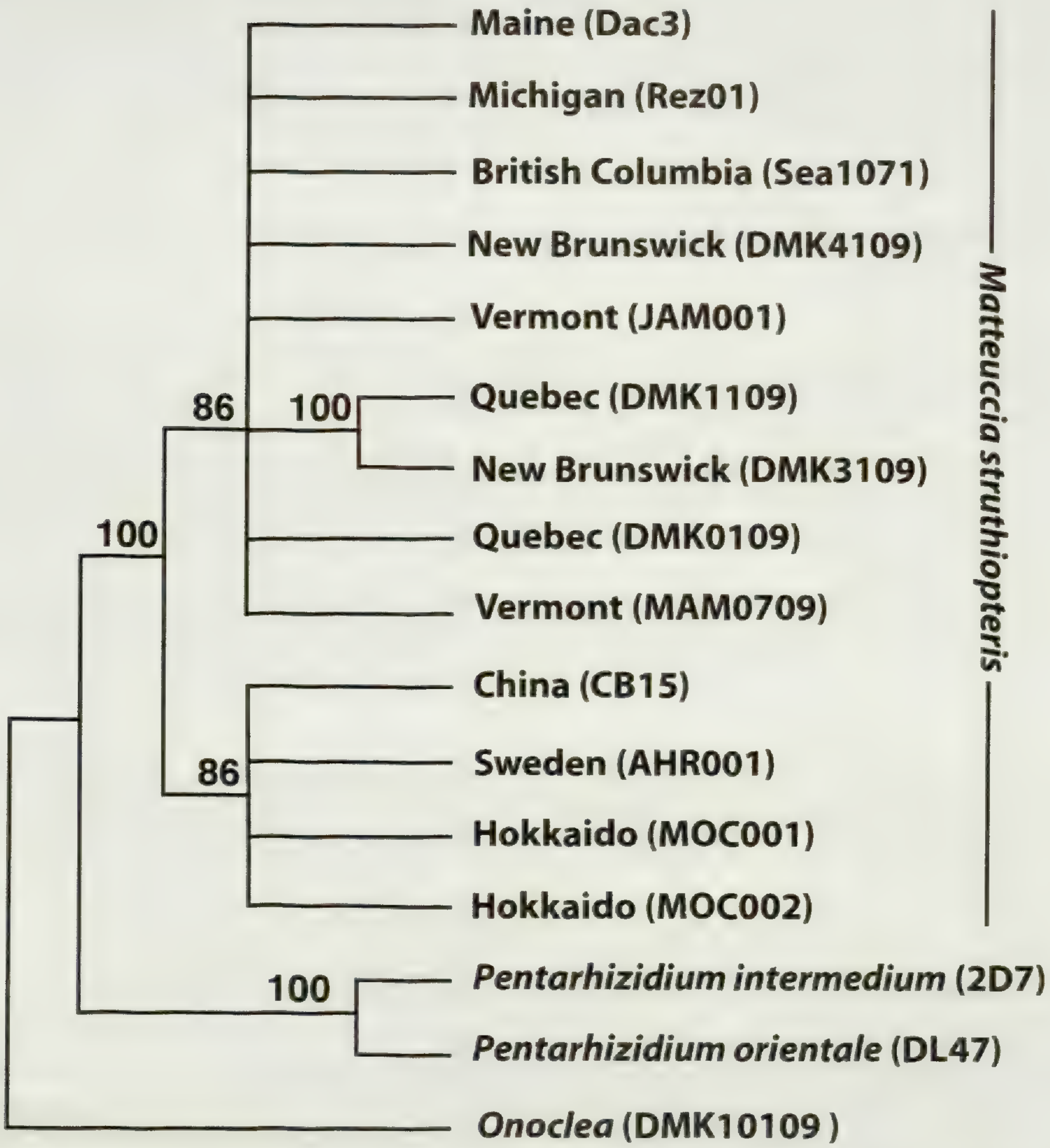

FIG. 2. Bootstrap analysis of *Matteuccia struthiopteris* and allies based on *psbA-trnH* and *PgiC* 15–16 combined, with *Onoclea sensibilis* as the outgroup. Numbers above common ancestors are bootstrap percentages. See methods for bootstrap analysis conditions. Identical sequences not included in this analysis were 1) DSB 2323, (same as Dac3, Rez01, Sea1071), 2) DMK 5109, 7109, 8109, and DMK9109 (same as DMK 4109 and JAM001), and 3) DMK2109 and 6109 (same as DMK 0109).

The principal-components analysis revealed substantial geographic clustering of genetically related individuals. The first three components retrieved from the analysis accounted for 75.52% of the variance; they represented 32.72%, 29.95%, and 12.85% of the total variance in the data respectively. Principal components 1 and 3 were most powerful in portraying the relationships among individuals and populations (Fig. 3). Most of the Passumpsic-watershed plants lay in a tight cluster; they were largely separated from the Winooski-watershed and Mettawee-watershed plants on principal component 1. In contrast the Winooski-watershed

TABLE 1. Genetic diversity calculations (number of loci, mean genetic distance, mean heterozygosity) for each of seven Vermont populations of *Matteuccia struthiopteris*. Rank is categorical order of each population; 1 is the most diverse and 7 the least diverse. Within watershed, the populations are listed from highest to lowest in elevation.

Watershed	Population	Number of Loci/ (Rank)	Mean Genetic Distance/ (Rank)	Mean Heterozygosity/ (Rank)
Winooski	Cabot (CWH)	139 (2)	50.944 (2)	0.152 (1)
	Plainfield (PWM)	160 (1)	55.222 (1)	0.145 (2)
	Richmond (RWL)	132 (3)	46.464 (3)	0.107 (5)
Passumpsic	Newark (NPH)	95 (6)	36.393 (6)	0.096 (6)
	East Burke (EPM)	108 (5)	41.238 (5)	0.112 (4)
	Barnet (BPL)	85 (7)	25.311 (7)	0.058 (7)
Mettawee	Granville, NY (GML)	126 (4)	44.444 (4)	0.127 (3)

plants were widespread on both components 1 and 3. Plants from the single Mettawee-watershed population were tightly clustered on both components 1 and 3; they were largely separated from the other two watersheds on component 3. Within the Passumpsic watershed, the high and middle populations (Newark and East Burke respectively) were tightly clustered on both components 1 and 3; the low-elevation population from this watershed included outliers that clustered with each of the other two watersheds.

The three approaches to assessing the distribution of genetic diversity revealed similar regional patterns (Table 1). The Winooski watershed was overall the most genetically diverse region according to all three calculations. In the number of loci and genetic distance calculation the Winooski watershed contained the three most diverse populations. Similarly, the mean heterozygosity data showed the Winooski watershed to contain two of the three most diverse populations. The Passumpsic watershed was the least diverse region according to all of the calculations. The number of loci as well as the genetic-distance metric revealed this watershed to contain the three least diverse populations, while the heterozygosity data indicates that two of the three least diverse populations lie in this watershed. The mean heterozygosity values for all populations were low to about average relative to other North Temperate ferns, ranging from 0.058 to 0.152 (compare, e.g., Kirkpatrick *et al.*, 1990; Suter *et al.*, 2000).

DISCUSSION

Phylogeny.—Our analysis of two highly labile DNA markers, one chloroplast and one nuclear, reveals *Matteuccia struthiopteris* to comprise a globally cohesive and minimally divergent system of populations. The relationships revealed by the phylogeny in this study are congruent with the relationships proposed by Gastony and Ungerer (1997). In both cases the clade containing *Pentarhizidium orientale* and *P. intermedium* is situated almost equally distant from *Onoclea sensibilis*

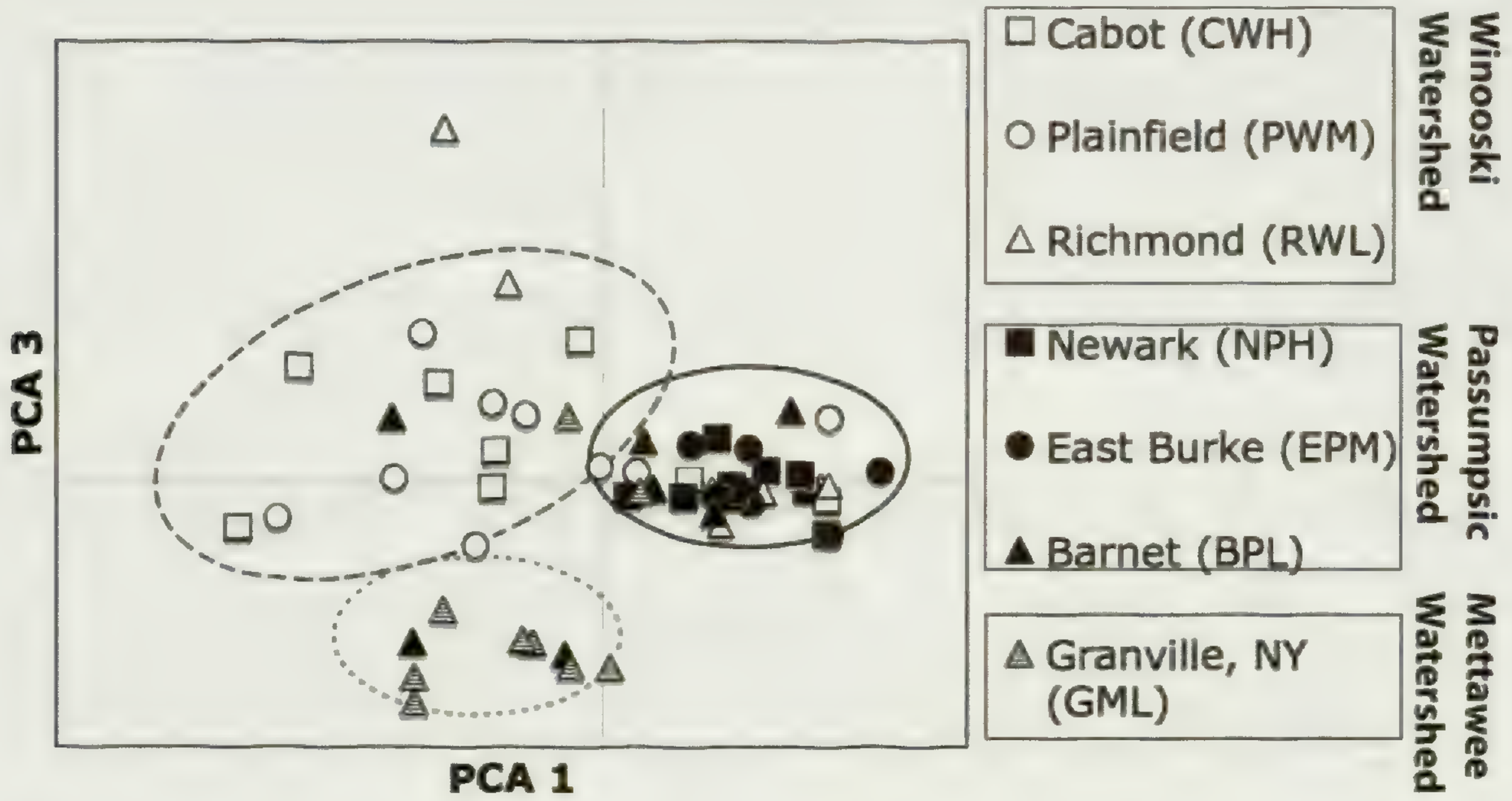

FIG. 3. PCA analysis of AFLP variation in *Matteuccia struthiopteris* by Vermont population and watershed. Shapes represent elevational category: square is high, circle is medium and triangle is low.

(Gastony and Ungerer: 49 steps, this study: 37 steps) and the clade containing *M. struthiopteris* (Gastony and Ungerer: 45 steps, this study: 40 steps).

Our recovery of a strongly supported and divergent clade comprising *Pentarhizidium orientale* and *P. intermedium*—corroborating the results of Gastony and Ungerer (1997)—reinforces the support for recognizing this pair in a genus separate from *Matteuccia*. Though Gastony and Ungerer's data suggest that *Onocleopsis hintonii* is better treated as a species of *Matteuccia sensu stricto*, we were unable to explore this possibility as we were unable to retrieve nuclear-DNA sequence data from this species.

AFLP analysis.—Analysis of the structure of genetic diversity in 60 *Matteuccia struthiopteris* samples revealed that the plants were differentiated between watersheds, rather than between elevations; we take this to suggest that historical biogeography rather than local recent events in Vermont populations accounts for the pattern we retrieved.

The large low-elevation population in the Winooski watershed was low in heterozygotes and lowest (within its watershed) in genetic diversity assessed from both number of loci and mean genetic distance. This population is located in Richmond, an area that receives high fiddlehead harvesting pressures (Maisonpierre, 2009). The low diversity in this population may be anthropogenic in nature. However, a relatively recent origin or expansion from a bottlenecked population is also possible.

Downy and smooth variants.—The geographic distribution of downy individuals presented a pattern at odds with the molecular-genetic signal in the sampled populations. Given the absence of molecular signal suggesting overall greater genetic diversity in the larger rivers, it appears that the downy variant may

be selected against at higher elevations. (Genetic drift seems a less likely explanation, since we would expect at least some high-elevation populations to be downy under a drift scenario). We are left with the working hypothesis that the morphological variant, unlike the molecular variation taken as a whole, has an ecological rather than a historical explanation.

Quaternary biogeography of Matteuccia struthiopteris.—The concentration of genetic diversity in the Winooski watershed (a geographic region), rather than at high elevations (i.e., peripherally) or low elevations (i.e., centrally), suggests that historical rather than environmental factors are driving the pattern of genetic diversity. The greater genetic diversity in the watershed draining westward into Lake Champlain suggests that it may be nearer to the location of populations that lay in a Pleistocene refugium. In this context, the relative decrease in genetic diversity and heterozygosity seen outside of the Champlain Valley may be a result of historical (post-glacial) expansion of the species from west to east across the northeast. A Mississippi Valley refugium for *Matteuccia struthiopteris* seems plausible, as refugia in the American South have been suggested for a set of North American species (Barrington and Paris, 2007; Davis 1983; Hewitt 2000, 2004; Willis and Hewitt, 2004).

In an attempt to gain insight into Holocene expansion in *Matteuccia struthiopteris*, we conducted a larger AFLP analysis (Koenemann, 2009). This larger analysis included *M. struthiopteris* populations from across northeastern North America and adjacent Québec and New Brunswick. However, while the results of this study did corroborate our suggestion that genetic diversity in the fiddlehead fern decreases as one moves from the south and west to the north and east, geographic patterning of the genetic diversity in populations was lost at this larger scale.

Conclusions.—*Matteuccia struthiopteris* is a globally cohesive species, characterized by minimal genetic variation, within which Eastern North American and Eurasion populations—the latter including the Alaskan population—form separable evolutionary lineages. The lower genetic diversity to the east in Vermont suggests expansion eastward in northeast North America. In the context of other studies, migration north and east from a Pleistocene refugium in the Mississippi Valley seems plausible as a working hypothesis.

ACKNOWLEDGMENTS

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Literature Cited

Barrington, D. S. and C. A. Paris. 2007. Refugia and migration in the Quaternary history of the New England flora. Rhodora 109:369–386.

Bergerson, M. E. and L. Lapointe. 2001. Impact of one year crozier removal on long-term frond production in *Matteuccia struthiopteris*. Canadian Journal of Plant Science 81:155–163.

Bonin, A., E. Bellemain, P. B. Eidensen, F. Pompanon, C. Brochmann and P. Taberlet. 2004. How to track and assess genotyping errors in population genetic studies. Molecular Ecology 13:3261–3273.

Bonin, A. D. Ehrich and S. Manel. 2007. Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. Molecular Ecology 16:3737–3758.

Davis, M. B. 1983. Quaternary History of Deciduous Forest of Eastern North America and Europe. Annals of the Missouri Botanical Garden 70:550–563.

Dempster, E. L., K. L. Pryor, D. Francis, J. E. Young and H. J. Rogers. 1999. Rapid DNA extraction from ferns for PCR-based analyses. Biotechniques 27:66–68.

Donoghue, M. J., C. D. Bell and J. Li. 2001. Phylogenetic patterns in Northern Hemisphere plant geography. International Journal of Plant Science 162:S41–S52.

Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh frond tissue. Phytochemical Bulletin 19:11–15.

Driscoll, H. E. and D. S. Barrington. 2007. Origin of Hawaiian *Polystichum* (Dryopteridaceae) in the context of a world phylogeny. Amer. J. Bot. 94:1413–1424.

Dufresne, F. and P. D. N. Hebert. 1997. Pleistocene glaciations and polyphyletic origins of polyploidy in an arctic cladoceran. Proceedings of the Royal Society of London 264:201–206.

Gastony, G. J. and M. C. Ungerer. 1997. Molecular systematics and a revised taxonomy of the onocleoid ferns (Dryopteridaceae: Onocleeae). American Journal of Botany 84:840–849.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41:95–98.

Hewitt, G. M. 1996. Some genetic consequences of ice ages, and their role, in divergence and speciation. Biological Journal of the Linnean Society 58:247–276.

Hewitt, G. M. 2000. The genetic legacy of the Quaternary ice ages. Nature 405:907–913.

Hewitt, G. M. 2004. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society B 359:183–195.

Ibrahim, K. M., R. A. Nichols and G. M. Hewitt. 1996. Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. Heredity 77:282–291.

Ishikawa, H., Y. Watano, K. Kano, M. Ito and S. Kurita. 2002. Development of primer sets for PCR amplification of the PgiC gene in ferns. Journal of Plant Research 115:65–70.

Kato, M. 1993. Biogeography of ferns: dispersal and vicariance. Journal of Biogeography 20:265–274.

Kimura, M. and H. Weiss. 1964. The stepping stone model of population structure and the decrease of genetic correlation with distance. Genetics 49:561–576.

Kirkpatrick, R. E. B., P. S. Soltis and D. E. Soltis. 1990. Mating system and distribution of genetic variation in *Gymnocarpium dryopteris* subsp. *disjunctum*. American Journal of Botany 77:1101–1110.

Koenemann, D. M. 2009. The Fiddlehead Fern (*Matteuccia struthiopteris*) in the Holocene of North America. Undergraduate Honors Thesis, University of Vermont, Burlington, Vermont, USA.

Kress, W. J., K. J. Wurdack, E. A. Zimmer, L. A. Weigt and D. H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences. 102:8369–8374.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948.

Lloyd, R. M. 1971. Systematics of the onocleoid ferns. University of California Publications in Botany 61:1–86.

Maddison, D. and W. Maddison. 2005. *MacClade: analysis of phylogeny and character evolution: version 4.03*. Sinauer Associates, Sunderland, Massachusetts, USA.

MAISONPIERRE, J. A. 2009. Impacts of harvesting practices on genetic diversity in Vermont's fiddlehead fern *Matteuccia struthiopteris* (L.) Todaro. Undergraduate Honors Thesis, University of Vermont, Burlington, Vermont, USA.

MEUDT, H. M. and A. C. CLARKE. 2006. Almost forgotten or latest practice? AFLP applications, analyses and advances. Trends in Plant Science 12:106–117.

MIYAZAWA, M., E. HORIUCHI and J. KAWATA. 2007. Components of the essential oil from *Matteuccia struthiopteris*. Journal of Oleo Science 56:457–461.

NAKAZATO, T., M. K. JUNG, E. A. HOUSWORTH, L. H. RIESEBERG and G. J. GASTONY. 2006. Genetic map-based analysis of genome structure in the homosporous fern *Ceratopteris richardii*. Genetics 173:1585–1597.

NISHIMURA, D. 2000. Sequencher 3.1.1. Biotech Software and Internet Report 1:24–30.

PEAKALL, R. and P. E. SMOUSE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:288–295.

POMPANON, F., A. BONIN, E. BELLEMAIN and P. TABERLET. 2005. Genotyping errors: causes, consequences, and solutions. Nature Reviews Genetics 6:847–859.

PRANGE, R. K. and P. VON ADERKAS. 1985. The biological flora of Canada. 6. *Matteuccia struthiopteris* (L.) Todaro, ostrich fern. Canadian Field Naturalist 99:517–532.

SANG, T., D. J. CRAWFORD and T. F. STUESSY. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). American Journal of Botany 84:1120–1136.

SIGEL, E. M. 2008. Polyphyly of polyploid species: an inquiry into the origin of *Dryopteris campyloptera* and *Dryopteris dilatata* (Dryopteridaceae). Master's Thesis, University of Vermont, Burlington, Vermont, USA.

SMITH, A. R. 1993. *Matteuccia*. In: Flora of North America Editorial Committee, eds 1993. *Flora of North America North of Mexico*. Vol. 2 Oxford University Press, New York and Oxford.

SUTER, M., J. J. SCHNELLER and J. C. VOGEL. 2000. Investigations into the genetic variation, population structure, and breeding systems of the fern *Asplenium trichomanes* subsp. *quadrivalens*. International Journal of Plant Sciences 161:233–244.

SWOFFORD, D. L. 2001. *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*. Sinauer Associates, Sunderland, Massachusetts, USA.

VEKEMANS, X., T. BEAUWENS, M. LEMAIRE and I. ROLDÁN-RUIZ. 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Molecular Ecology 11:139–151.

VON ADERKAS, P. 1984. Economic history of ostrich fern, *Matteuccia struthiopteris*, the edible fiddlehead. Economic Botany 38:14–23.

VOS, P., R. HOGERS, M. BLEEKER, M. REIJANS, T. Y. D. LEE, M. HORNES, A. FRIJTERS, J. POT, J. PELEMAN, M. KULPER and M. ZABEAU. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23:4407–4414.

WALLACE, L. and M. CASE. 2000. Contrasting allozyme diversity between northern and southern populations of *Cypripedium parviflorum* (Orchidaceae): Implications for Pleistocene refugia and taxonomic boundaries. Syst. Bot. 25:281–296.

WHITLOCK, R., H. HIPPERSON, M. MANNARELLI, K. BUTLIN and T. BURKE. 2008. An objective, rapid and reproducible method for scoring AFLP peak-height data that minimizes genotyping error. Molecular Ecology Resources 8:725–735.

WILLIS, K. J. and G. M. HEWITT. 2004. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions Royal Society London B 359:183–195.

WOLF, P. G. 2000. AFLP Protocol [online]. Available from http://bioweb.usu.edu/wolf/aflp_protocol.htm

APPENDIX 1. Accessions used in the study. Species numbers correspond to the following, 1: *Matteuccia struthiopteris*, 2: *Onoclea sensibilis*, 3: *Pentarhizidium intermedium*, 4: *Pentarhizidium orientale*. Vouchers and silica-dried material, where available, are at the University of Vermont, Burlington, VT 05405. The Genbank numbers marked with an asterisk are for plant JAM001.

Accessions Used at Location	Accession Number	Genbank Accession Numbers (*PgiC*/ *psbA-trnH*)	Population Location	Taxon	Watershed & Elevational category (AFLP)	Latitude	Longitude	Elevation (m)
1	DMK10109	HQ243688/ HQ243704	Burlington, VT, USA	2	NA	44.284 N	73.111 W	69 m
9	JAM001-JAM009	HQ243679/ HQ243695*	Cabot, VT, USA	1	Winooski – High	44.457° N	72.326° W	442 m
9	JAM011-JAM019	-	Plainfield, VT, USA	1	Winooski –Medium	44.285 N	72.415 W	238 m
8	JAM021-JAM028	-	Richmond, VT, USA	1	Winooski – Low	44.398 N	72.990 W	87 m
8	JAM031-JAM038	-	Newark, VT, USA	1	Passumpsic – High	44.702°N	72.005°W	358 m
10	JAM041-JAM050	-	East Burke, VT, USA	1	Passumpsic-Medium	44.599 N	71.949 W	253 m
7	JAM051-JAM057	-	Barnet, VT, USA	1	Passumpsic – Low	44.326 N	72.037 W	156 m
1	DSB2323	-	Winooski, VT, USA	1	NA	44.531°N	73.269°W	30 m
1	MAM0709	HQ243683/ HQ243699	Stowe, VT, USA	1	NA	44.474 N	72.221 W	235 m
1	Dac3	HQ243675/ HQ243691	Dacey Pond, ME, USA	1	NA	45.611°N	68.820°W	152 m
1	DMK9109	-	Lake George, ME, USA	1	NA	44.764°N	69.592°W	70 m
9	JAM061-JAM069	-	Granville, NY, USA	1	Mettawee – Low	43.444 N	73.282 W	178 m
1	Rez01	HQ243676/ HQ243692	Ann Arbor, MI, USA	1	NA	42.125°N	83.445°W	194 m

Appendix 1. Continued.

Accessions Used at Location	Accession Number	Genbank Accession Numbers (*PgiC*/ *psbA-trnH*)	Population Location	Taxon	Watershed & Elevational category (AFLP)	Latitude	Longitude	Elevation (m)
1	DMK0109	HQ243682/ HQ243698	Rivière Verte, QC, Canada	1	NA	48.042°N	69.302°W	30 m
1	DMK1109	HQ243680/ HQ243696	Rivière Saumon, QC, Canada	1	NA	48.697°N	67.931°W	65 m
1	DMK2109	–	Rivière Matane, QC, Canada	1	NA	48.735°N	67.495°W	33 m
1	DMK3109	HQ243681/ HQ243697	Campbelton, NB, Canada	1	NA	47.982°N	66.282°W	6 m
1	DMK4109	–	Redmondville, NB, Canada	1	NA	46.907°N	65.199°W	53 m
1	DMK5109	–	Moncton, NB, Canada	1	NA	46.315°N	64.624°W	15 m
1	DMK6109	–	Canon River Bank, NB, Canada	1	NA	45.969°N	65.196°W	29 m
1	DMK7109	–	Waterborough, NB, Canada	1	NA	45.871°N	66.007°W	51 m
1	DMK8109	–	Southampton, NB, Canada	1	NA	45.952°N	67.289°W	64 m
1	Sea1071	HQ243677/ HQ243693	Chilliwack, BC, Canada	1	NA	49.114°N	121.555°W	1012 m
1	MCS001	JF912402/ JF912403	Russian River Campground, Alaska	1	NA	60.484°N	149.973°W	300 m
1	MOC001	HQ243689/ HQ243705	Sapporo, Hokkaido, Japan	1	NA	43.067°N	141.350°E	25 m

APPENDIX 1. Continued.

Accessions Used at Location	Accession Number	Genbank Accession Numbers (*PgiC*/*psbA-trnH*)	Population Location	Taxon	Watershed & Elevational category (AFLP)	Latitude	Longitude	Elevation (m)
1	MOC002	HQ243690/ HQ243706	Sapporo, Hokkaido, Japan	1	NA	43.067°N	141.350°E	25 m
1	AHR001	HQ243685/ HQ243701	Västerås, Sweden	1	NA	59.611°N	16.551°E	17 m
1	CB15	HQ243684/ HQ243700	Mt. Changbaishan, Heilongjian Province, China	1	NA	NA	NA	NA
1	2D7	HQ243686/ HQ243702	Zhongdian, Yunnan Province, China	3	NA	NA	NA	NA
1	DL47	HQ243687/ HQ243703	Dali, Yunnan Province, China	4	NA	NA	NA	NA

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Archegonial Development and Oogenesis of the Fern *Plagiogyria euphlebia* and their Phylogenetic Significance

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ABSTRACT.—The cytological features of the cells taking part in archegonial development and oogenesis in the fern *Plagiogyria euphlebia* (Kunze) Mett. were described in detail by means of light and electronic microscopy. The archegonium develops from an initial cell, which contains dense cytoplasm in contrast to the somatic cells. Two divisions of the initial cell result in a tier of three cells. The middle of which finally develops into a neck canal cell, a ventral canal cell and an egg by two unequal divisions. During maturation, the egg cell becomes progressively isolated from the adjacent cells by forming a separation cavity, a casual wall and an egg envelope. Series sections show that a fertilization pore forms in the upper egg envelope. During maturation of the egg, the nucleus produces conspicuous evaginations. The phylogenetic relationship of the fern *P. euphlebia* is discussed according to the cytological features in oogenesis. The cytological features observed during oogenesis support the inclusion of Plagiogyriaceae among the tree-ferns as proposed from molecular analyses.

KEY WORDS.—Archegonial development, fern, oogenesis, phylogeny, *Plagiogyria euphlebia*

Recent investigations on oogenesis revealed that core-leptosporangiate ferns produce an obvious egg envelope and a fertilization pore in the mature egg (Cao *et al.*, 2009; Cao *et al.*, 2010a, b; Dai *et al.*, 2010); however, the mature egg of *Osmundaceae*, the basal-most member of leptosporangiate ferns (Smith *et al.*, 2006), does not possess a typical egg envelope and fertilization pore (Bao *et al.*, 2003; Cao *et al.*, 2012). Osmundaceae is considered a basal family within the leptosporangiate ferns with a long evolutionary history (Bell, 1986). In the bryophyte *Marchantia polymorpha* L., no extra membrane was observed around the mature egg (Zinsmeister and Carothers, 1974). It is concluded that the cytological features of the egg in oogenesis are closely related to the phylogenetic position. The sporophyte morphology supports the conclusion that the Plagiogyriaceae has a close relationship to Osmundaceae (Nayar, 1970; Mickel, 1974; Wu and Ching 1991). However, the relationships of Plagiogyriaceae to other ferns remain uncertain (Smith, 1995). Molecular data provide strong evidence for the inclusion of *Plagiogyria* within the tree ferns (Hasebe *et al.*, 1995; Pryer *et al.*, 2001; Pryer *et al.*, 2004; Smith *et al.*, 2006). In the present study, the oogenesis of the fern *Plagiogyria euphlebia* (Kunze) Mett. was studied and its phylogenetic similarity with respect to this character is discussed.

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Materials and Methods

Spores of *Plagiogyria euphlebia* were collected from plants in Wuyishan Nature Reserve of Jiangxi province, China. The spores were surface sterilized with 5% sodium hypochlorite solution for 3 min. After rinsing three times with distilled water, the spores were sown on a modified Knop's solution (0.8g $Ca(NO_3)_2 \cdot 4H_2O$; 0.2g KH_2PO_4; 0.2g KNO_3; 0.2g $MgSO_4 \cdot 7H_2O$, dissolved in 1 L distilled water), and solidified with 1.5% agar in culture dishes. These dishes were placed in an artificial climate chamber under conditions of 25°C in the light (18 h) and 20°C in the dark (6 h). After 8 to 9 weeks, archegonia had developed on the lower surface of the gametophytes just behind the growing apex.

Gametophytes bearing various archegonial stages were placed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer at room temperature for 6–12 h. The specimens were subsequently washed three times with the same buffer, postfixed in 2% aqueous osmium tetroxide for 2 h, rinsed three times in buffer and embedded in Spurr's resin (SPI-Chem, USA) via a graded acetone series. Specimens were thick sectioned for the presence of the archegonia and thin-sectioned with a diamond knife on an Ultracut-E ultramicrotome (Reichert-Jung, Germany). The thick sections were stained with Toluidine blue and observed using a light microscope. The thin sections were stained with uranyl acetate and lead citrate. All specimens were observed with H-600 electron microscope (Hitachi, Japan).

Results

The initial cell.—Archegonia of *Plagiogyria euphlebia* are usually produced on the lower surface of the gametophyte just behind the growing point (Fig. 1A). The cells coming to form archegonia (the initial cell, ic) can be identified by the cylindrical shape of the cell, which possesses more cytoplasm around the central placed nucleus in contrast to the somatic cells (Fig. 1B, C). The vacuoles in the initial cell are asymmetrically distributed. One or two large vacuoles are located in the lower part, and small vacuoles lie in the upper part of the cell (Fig. 1C). The chloroplasts in the initial cell, lacking well-developed lamellae and containing little starch, are usually smaller than those in the somatic cells (Fig. 1D). Mitochondria show little difference from those in the somatic cells. Crystals are frequently seen in plastids both in the archegonial initial and the somatic cells (Fig. 1D, asterisks). Subsequently, the initial cell divides into two by a periclinal division. The upper cell becomes the neck jacket initial (Fig. 1E, c1), which contains few vacuoles. The lower cell usually contains some large vacuoles at the basal part (Fig. 1E, c2). At this stage, there are well-developed plasmodesmata between the archegonial cells and the somatic cells (Fig. 1D, F).

The primary cell.—The lower cell forms a primary cell (pc) and a basal cell by a periclinal division (Fig. 1G). The primary cell is a square with almost equivalent height and width. The nucleus is larger and the chromatin becomes more dispersed than those in the adjacent cells (Fig. 1G). The plasmodesmata are well developed between the primary cell and the upper neck initial cell (Fig. 1H), but

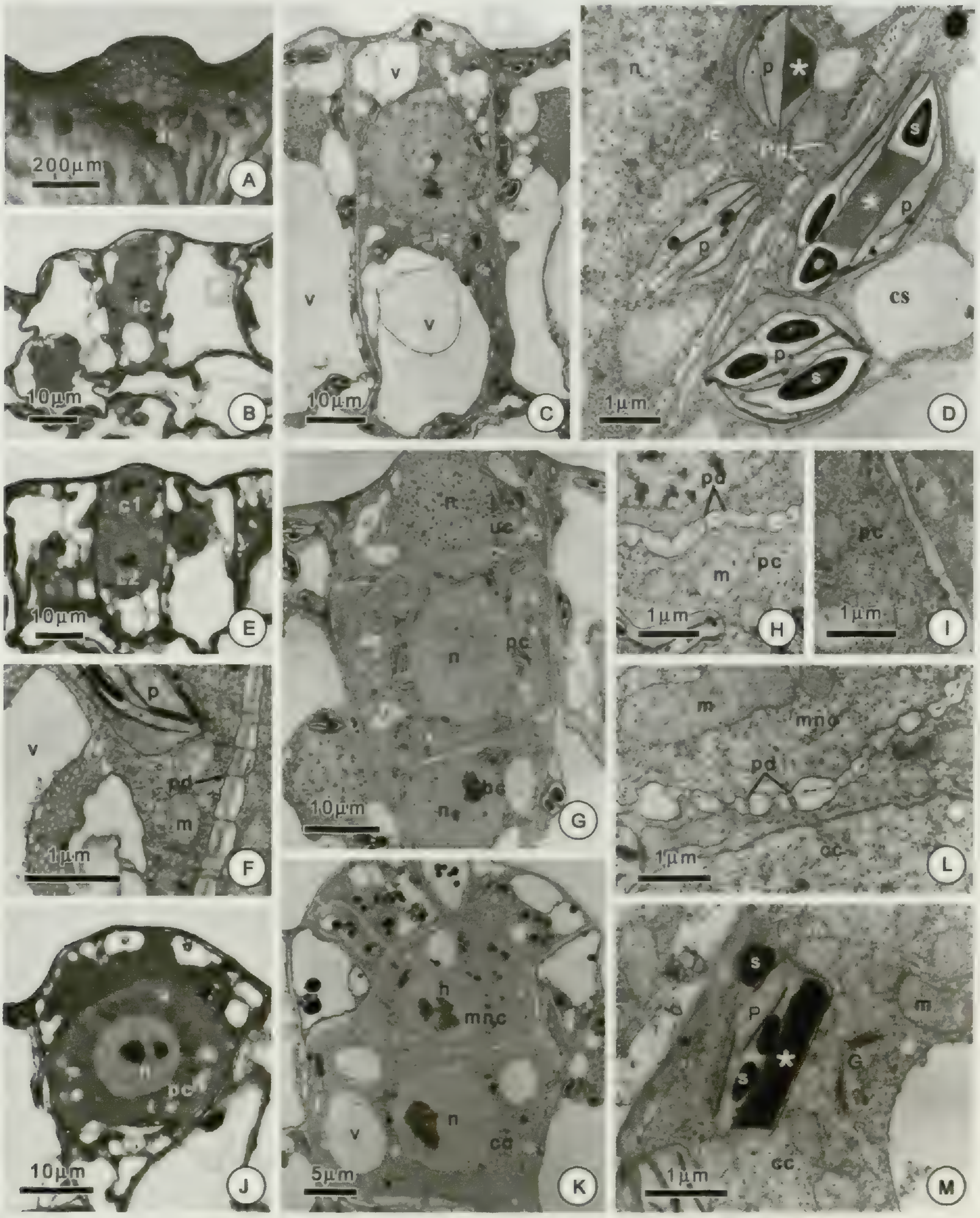

FIG. 1. Young archegonium of *Plagiogyria euphlebia* (**A**, under stereomicroscope; **B, E, J**, under LM; **The others**, under TEM). **A.** Archegonia (ar) are produced under the growing point of the prothallus; **B, C.** Initial cell (ic) possess a large nucleus and more cytoplasm around it. **D.** Plastids in the initial cell (ic) are obviously smaller and contain fewer starch grains than those in the somatic cells (cs). **E.** The initial cell divides into an upper cell (c1) and a basal cell (c2). **F.** Plasmodesmata are obvious between the basal cell and somatic cell. **G.** A tier of three cells are formed, which are the upper cell (uc), the primary cell (pc), and basal cell (bc). **H.** Plasmodesmata (pd) between the upper cell and the primary cell (pc). **I.** Plasmodesmata disappear between the primary cell and the somatic cell. **J.** The top of the primary cell (pc) bulges and the upper cell divides into four. **K.** The primary cell forms a central cell (cc) and a mononucleate neck canal cell (mnc) and by an unequal division; **M.** The central cell (cc) contains abundant organelles. G, Golgi bodies; m, mitochondrion; n, nucleus; p, plastid; pd, plasmodesmata; s, starch.

those are absent in the wall between the primary cell and the somatic cells (Fig. 1I). The organelles, including plastids and mitochondria, resemble those in previous stage. Soon, the pc enlarges and its upper surface protrudes upwards (Fig. 1J). Before division of the primary cell, the neck initial cell divides into a rosette of four cells by two anticlinal divisions (Fig. 1J).

The central cell.—The primary cell divides asymmetrically to form two cells. The cell towards the neck of the archegonium is a mononucleate neck canal cell (mnc) with little cytoplasm (Fig. 1K). The lower cell, obtaining more cytoplasm from its mother cell, is named as the central cell (cc) (Fig. 1K). A conspicuous feature of this stage and also at subsequent stages is that there are well-developed plasmodesmata between the neck canal cell and the central cell (Fig. 1L). Organelles in the central cell resemble those in the previous stage (Fig. 1M). Sometimes, a few large vacuoles can be seen in the cytoplasm (Fig. 1K).

The newly formed egg.—The central cell also divides asymmetrically to form a small ventral canal cell (vcc) and a large egg cell, which has most of the cytoplasm (Fig. 2A). Soon after the egg is formed, the nucleus of the mononucleate neck canal cell divides into two, without cell wall formation between the two nuclei, which resulting in a binucleate neck canal cell (ncc) (Fig. 2B). The newly formed egg and the neck and ventral canal cells are closely appressed to the archegonial jacket cells (Fig. 2B). Well-developed plasmodesmata connect the ventral canal cell and the neck canal cell (Fig. 2C), and also connect the egg and the ventral canal cell, but these are absent between the inner three cells and the jacket cells (Fig. 2D). The nucleus of the young egg is spherical and typical sections show it contains one or two nucleoli (Fig. 2A, B). The newly formed egg contains abundant vesicles in the cytoplasm (Fig. 2B). Plastids contain fewer starch grains and lamellae than previous stages (Fig. 2D). Organelles in the VCC and NCC (Fig. 2C) resemble those in the egg (Fig. 2D).

Egg maturation.—Egg maturation undergoes remarkable cytological changes including the formation of a separation cavity, an osmiophilic egg envelope, a fertilization pore and prominent nuclear evaginations. In the early stage of egg maturation, the most conspicuous feature in oogenesis is formation of separation cavity (sc). Serial sections show that the separation cavity initially begins to form around the periphery of the upper surface of the egg (Fig. 2E). The separation cavity expands centripetally and the connection region is correspondingly reduced. However, a pore region, which is about 2–3 μm, persistently connects the egg and the ventral canal cell (Fig. 2E, arrow; Fig. 2F, G). Well-developed plasmodesmata can be seen in the pore region (Fig. 2G). Sections stained with toluidine blue show that there is a deeply stained cell wall (Fig. 2E, arrowhead) between the egg and the ventral canal cell except in the pore region (Fig. 2E, arrow). This thickened cell wall is closely appressed to the ventral canal cell (Fig. 2F, arrowheads; Fig. 2G). In the lower part of the egg, a narrow separation cavity is formed between the plasmolemma and the cell wall (Fig. 2H). At this stage, the endoplasmic reticula and Golgi bodies become more observable than previous stage both in the egg and in the canal cells (Fig. 2G). Vesicles are mainly distributed in the upper part of the egg cytoplasm. The egg nucleus now becomes somewhat ellipsoid in shape with a depressed upper surface (Fig. 2E, F).

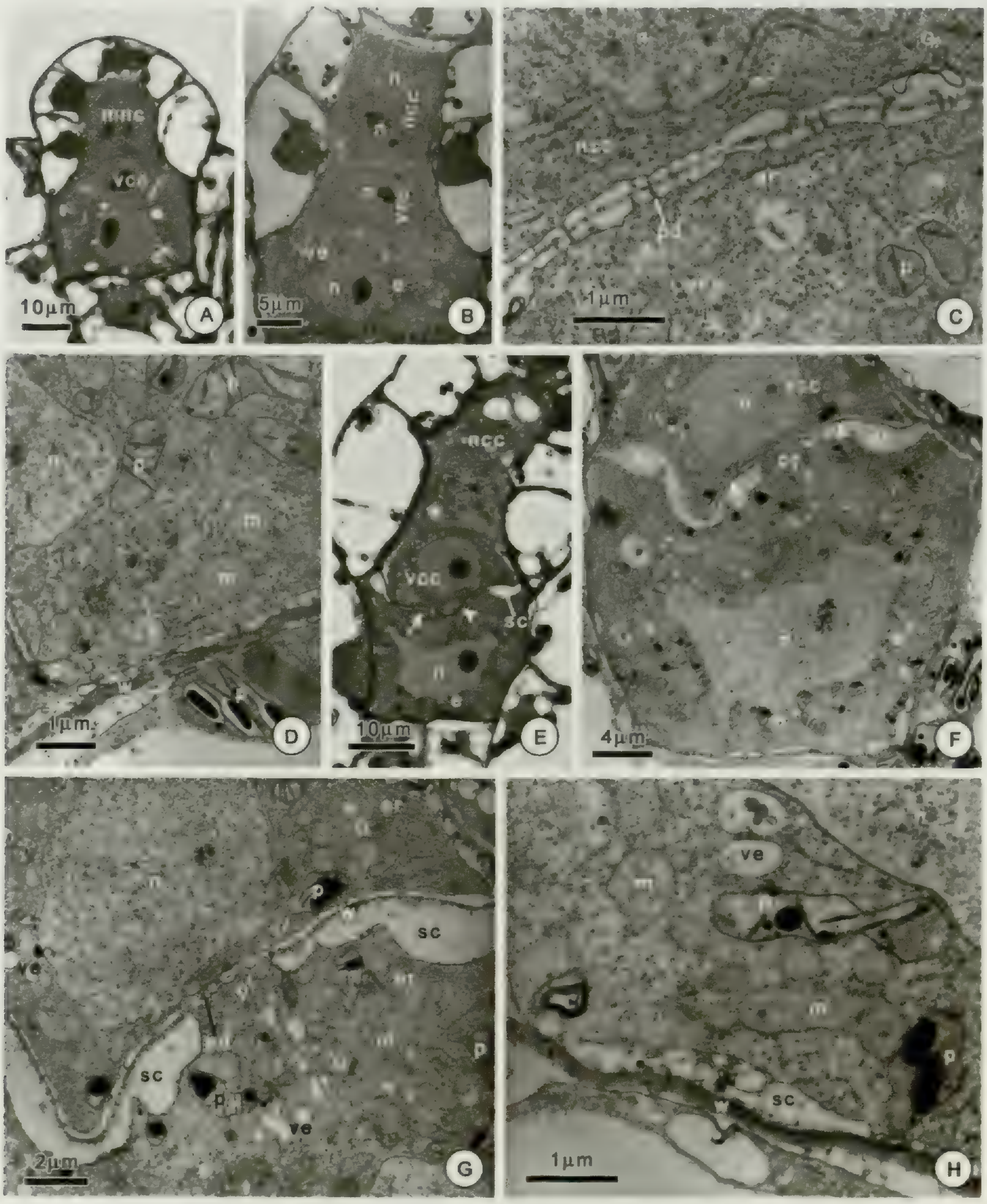

FIG. 2. Early stage of the egg development (**A**, **E**, under LM; **The others**, under TEM). **A.** An egg (e) and a ventral canal cell (vcc) is formed by an unequal division of the central cell; **B.** A binuclear elongated neck canal cell (ncc) is formed; **C.** Magnification of Fig. 2B showing the plasmodesmata between the vcc and the ncc; **D.** Part of the egg showing the organelles in the cytoplasm; **E.** Maturing egg stage, a separation cavity (sc) begins to form above the egg. A casual cell wall (arrowhead) between the egg and the vcc becomes obvious thickened. A pore region (arrow) connects the egg and vcc; **F.** The separation cavity (sc) forms around the egg. The casual cell wall (arrowheads) lies closely to the vcc. The pore region (pr) connects the egg and the vcc; **G.** Magnification of Fig. 2F showing the pore region (pr). **H.** The separation cavity (sc) in the lower part of the egg. G, Golgi bodies; m, mitochondrion; mnc, mononucleate neck canal cell; n, nucleus; p, plastid; pd, plasmodesmata; ve, vesicle; w, cell wall.

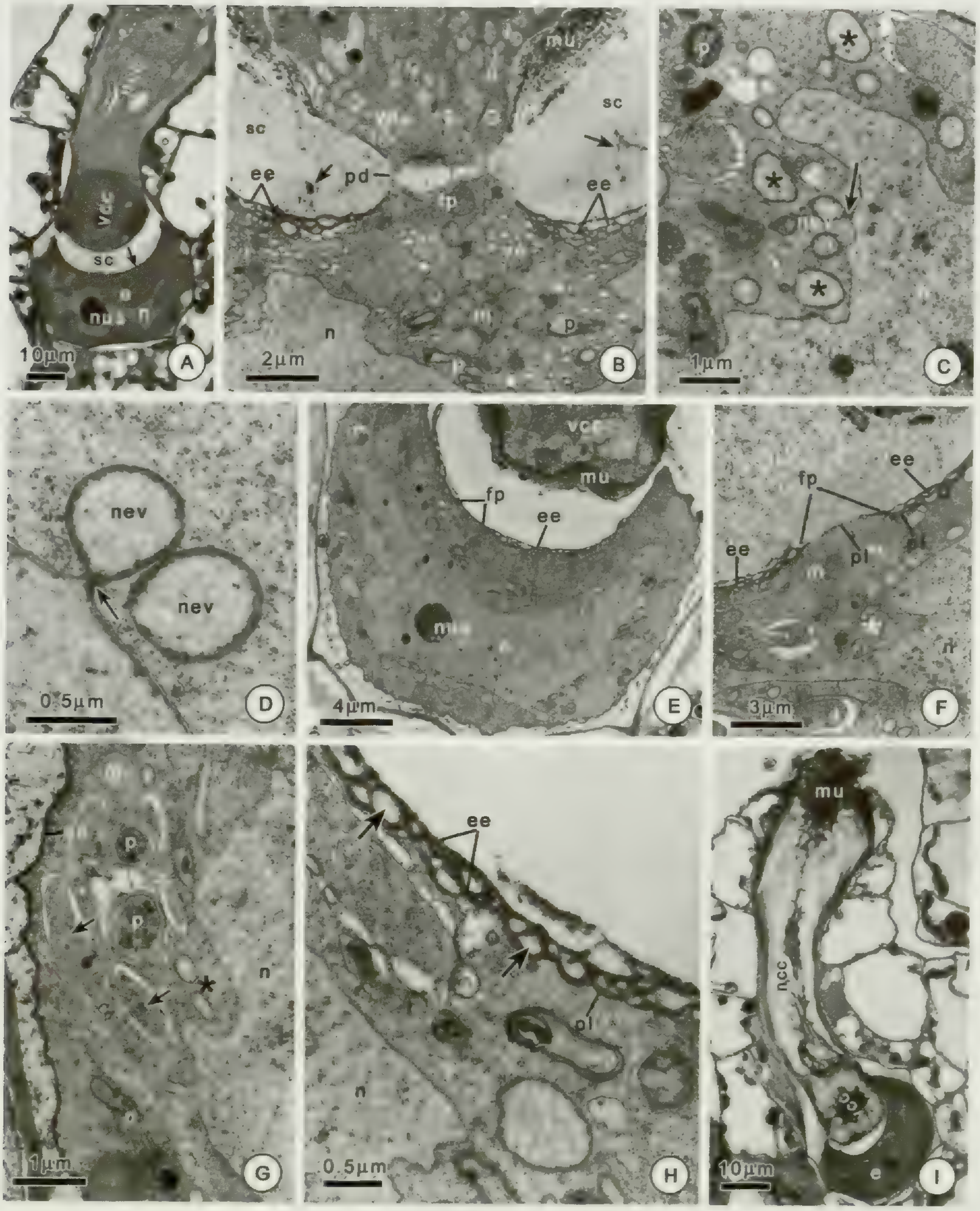

FIG. 3. Later stage of the egg development (**A**, **I**, under LM; **The others**, under TEM). **A.** An archegonium containing a maturing egg (e), a ventral canal cell (vcc) and a neck canal cell (ncc). The nucleus of the egg becomes highly irregular; **B.** The egg envelope (ee) covers the upper surface of the egg except in the connection region. Here a fertilization pore (fp) is formed; **C, D.** Nuclear evaginations (nev), some of which connected to the main body of the nucleus by a narrow isthmus (arrows); Some others are probably detached from the nucleus body (asterisks); **E.** The mature egg has a fertilization pore (fp). The vcc is dissociated from the egg. **F.** Another egg showing the fertilization pore (fp). Asterisk indicates the nuclear evaginations; **G.** Vacuolated cytoplasm (arrows) is often seen in the mature egg. Nuclear evaginations (asterisk) become ellipsoid; **H.** The egg envelope (ee) shows a reticular structure. Light-stained spaces

Subsequently, a special egg envelope is formed around the egg (Fig. 3A, arrow), which is accompanied by the disappearance of the cell wall between the egg and the ventral canal cell seen in previously stage (Fig. 3B). The upper egg envelope is much thicker and more conspicuous (Fig. 3B). The egg is still connected with the ventral canal cell in the pore region and no egg envelope is formed in this region (Fig. 3B), which leads to formation of a fertilization pore. In the separation cavity, amorphous materials are obvious above the egg envelope (Fig. 3B, arrows). The organelles in the egg cytoplasm differ greatly from those in the previous stage. Plastids degenerate further, but mitochondria are more prominent (Fig. 3B, C). In the ventral canal cell, vesicles and Golgi bodies increase, whilst mucilaginous materials accumulate around this cell (Fig. 3B, Mu).

At the middle stage of the egg development, the original ellipsoidal nucleus of the egg develops into an irregular cup-shape (Fig. 3A) and its surface produces numerous sac-like evaginations (Fig. 3C, D, nev). These are usually between 0.5–0.8 μm in diameter and remain connected to the main body of the nucleus via narrow isthmuses (Fig. 3C, D, arrow). Sacs similar to the nuclear evaginations, but without connection to the nucleus, appear in the cytoplasm of the egg (Fig. 3C, asterisks). The matrix of the evaginations resembles that of the main body of the nucleus (Fig. 3C, D). But the double membranes of the nuclear envelope cannot be recognized clearly (Fig. 3D).

The mature egg.—When the egg matures, the ventral canal cell is usually detached from the egg at the region of fertilization pore (Fig. 3E, F). The transverse diameter of the fertilization pore reaches 3 μm. No egg envelope covers this region. The plasmalemma covers the fertilization pore and the egg envelope lies outside of the plasmalemma on the remainder of the egg (Fig. 3F). The nucleus of the egg remains irregular in outline and evaginations can be seen here and there (Fig. E). However, most of these evaginations may have become ellipsoid in shape (Fig. 3F, G, asterisks). A most obvious change occurring in the cytoplasm of the egg is the appearance of the vacuolated organelles in the upper cytoplasm of the matured egg (Fig. 3G, arrows). Plastids, without any starch grains and lamellae, only can be identified by plastoglobuli (Fig. 3G). The egg envelope in the upper surface of the egg is especially obvious, which shows a reticular structure (Fig. 3H). The thickness of the upper egg envelope almost reaches 0.5 μm (Fig. 3H), but in the lower part the width of the egg envelope is only about 50–60 nm (Fig. 3G). As the egg matures completely, the canal cells degenerate. Most of the cytoplasm of the canal cells has decomposed into an amorphous mucilaginous material, which moves towards the opening of the archegonium when met with water (Fig. 3I).

←

(arrows) are seen in it; **I.** Mature archegonium with a mature egg and degenerated vcc and ncc. Mucilaginous material (mu) move towards the opening of the archegonium. G, Golgi bodies; m, mitochondrion; n, nucleus; nus, nucleolus; p, plastid; pd, plasmodesmata; pl, plasmalemma; sc, separation cavity; ve, vesicle.

Discussion

Early development of the archegonium.—The stages of archegonial development of *Plagiogyria euphlebia* is similar to that described for other derived ferns described previously (Bell and Mühlethaler, 1962a; Yang *et al.*, 2009; Dai *et al.*, 2010). However, the detailed ultrastructural features of the initial cell, the primary cell and central cell were lacking. The present investigation shows that the cells taking part in oogenesis usually possess much more cytoplasm. The plastids in these cells rarely contain starches, which is the typical feature of the meristematic cells. Furthermore, large polyhedral crystals in the plastids, previously reported in some angiosperms (Williams, 1974), are observed in fern cells for the first time. Vacuoles in the initial cell are distributed asymmetrically, which undoubtedly influence the polarity of the initial cell and finally result in three functionally different cells in an axial tier. The uppermost cell develops into the neck jacket cells of the archegonium. The middle cell finally develops into the egg and the two canal cells and the lowermost cell becomes a somatic cell and participates formation of the basal jacket cells in the later stage of the egg development.

The egg envelope and fertilization pore.—As in *Ceratopteris* (Cao *et al.*, 2009, 2010a) and *Adiantum* (Cao *et al.*, 2010b), *Plagiogyria euphlebia* also forms a prominent egg envelope and a fertilization pore. The egg envelope of the mature egg of *P. euphlebia* resembles those of *Pteridium aquilinum* (L.) Kuhn (Duckett and Bell, 1972), *Histiopteris incise* (Thunb.) J.Sm. (Bell, 1980), *Athyrium filix–femina* (L.) Roth (Fasciati *et al.*, 1994), *Dryopteris crassirhizoma* Nakai (Bao *et al.*, 2005) in structure, but differs somewhat from that of *Ceratopteris* and *Adiantum*, in which the egg envelope is composed of multilayered membranes (Cao *et al.*, 2008; Lopez-Smith and Renzaglia, 2008; Cao *et al.*, 2010a). In *Ceratopteris*, the egg envelope is believed to be formed by attachment of endoplasmic reticula (Cao *et al.*, 2008). However, endoplasmic reticula are rarely discovered in the maturing egg of *P. euphlebia*. The formation of the egg envelope may occur in another way. The osmiophilic amorphous materials in the separation cavity seem to be used to form the egg envelope on the outer surface of the egg plasmalemma. The structural difference in the egg envelope may have some significance in the classification of ferns. The ventral canal cell participates in the formation of the fertilization pore as suggested in *Ceratopteris* (Cao *et al.*, 2010a). The persistent connection of the egg and vcc in the pore region results in no deposition of the egg envelope on this region and finally forms a fertilization pore.

Nuclear behavior and evagination.—*Plagiogyria euphlebia* produces an irregular nucleus and obvious nuclear evaginations in the maturing egg, which resemble the derived ferns *Pteridium aquilinum* (Bell and Mülethaler, 1962b; Bell, 1972; Bell and Duckett, 1976; Bell, 1983), *Dryopteris filix-mas* (L.) Schott (Cave and Bell, 1975), *Histiopteris incisa* (Bell, 1980), *Dryopteris crassirhizoma* (Bao *et al.* , 2005) and *Adiantum* (Cao *et al.* , 2010b). In *Ceratopteris thalictroides* (L.) Brongn., the nucleus also becomes highly irregular, but it does not produce evaginations during oogenesis. The older fern lineage *Osmunda* possesses a regular nucleus (Bao *et al.*, 2003; Cao *et al.*, 2012). Therefore, the nuclear behavior and evaginations may have some significance in assessing the phylogenetic

affiliation of ferns. The derived ferns tend to produce more complicated evaginations.

Plasmodesmata diminishing and isolation of the egg.—The changes of the plasmodesmata are remarkable during oogenesis. The present investigation shows that the cells taking part in oogenesis, including the initial cell, the primary cell, the central cell and the egg cell, progressively lost the plasmodesmatal connections with their adjacent cells. For the egg, isolation is strengthened by forming a temporary cell wall between the egg and the vcc, and a permanent egg envelope around the egg. The biological significance of the isolation of the cells taking part in oogenesis most probably ensures the independent development of the sex cells. Bell and Duckett (1976) also indicated that the cells taking part in oogenesis in *Pteridium aquilinum* are progressively isolated from the remainder of the gametophyte.

The cytological features of oogenesis and their phylogenetic significance.—The family Plagiogyriaceae is usually considered to be basal based on morphology of the sporophytes. It was suggested that Plagiogyriaceae is closely related to the family Osmundaceae and may have evolved from a common ancestor (Nayar, 1970; Mickel, 1974). Molecular data do not support the conclusion that the Plagiogyriaceae is closely related to the Osmundaceae, but support the inclusion of the Plagiogyriaceae within the tree ferns (Hasebe *et al.*, 1995; Pryer *et al.*, 2001; Pryer *et al.*, 2004; Smith *et al.*, 2006). The present investigation shows that in *Plagiogyria euphlebia* the egg's cytological features, including the egg envelope and nuclear evaginations, are identical to the *Cibotium barometz* (L.) J.Sm. (Cao, unpublished data), which is a member of the tree ferns (Pryer *et al.*, 2001; Pryer *et al.*, 2004). *Plagiogyria euphlebia* and other polypodiaceous ferns usually possess a prominent egg envelope and fertilization pore in the mature egg (Cao *et al.*, 2009, 2010a, b; Dai *et al.*, 2010); however, *Osmunda* does not possess a typical egg envelope and fertilization pore (Bao *et al.*, 2003; Cao *et al.*, 2012). These observations support the inclusion of *Plagiogyria* among the tree ferns.

Acknowledgments

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Literature Cited

Bao, W. M., J. G. Cao and S. J. Dai. 2003. Ultrastructure of oogenesis in *Osmunda cinnamomea* var. *asiatica*. Acta Bot. Sin. 45:843–851.

Bao, W. M., Q. He, Q. X. Wang, G. W. Tian and J. G Cao. 2005. Ultrastructure of Oogenesis *in Dryopteris crassirhizoma* Nakai. J. Integr. Plant Biol. 47:201–213.

Bell, P. R. 1972. Nucleocytoplasmic interaction in the eggs of *Pteridium aquilinum* maturing in the presence of thiouracil. J. Cell Sci. 11:739–755.

Bell, P. R. 1980. Nucleocytoplasmic interaction during maturation of the egg of the fern *Histiopteris incisa* (Thunb.) J. Smith. Ann. Bot. 45:475–481.

Bell, P. R. 1983. Nuclear bodies in the maturing egg cell of a fern, *Pteridium aquilinum*. J. Cell Sci. 60:109–116.

Bell, P. R. 1986. Features of egg cells of living representatives of ancient families of ferns. Ann. Bot. 57:613–621.

Bell, P. R. and J. G. Duckett. 1976. Gametogenesis and fertilization in *Pteridium*. Bot. J. Linn. Soc. 73:47–78.

Bell, P. R. and K. Mühlethaler. 1962a. A membrane peculiar to the egg in the gametophyte of *Pteridium aquilinum*. Nature 195:198.

Bell, P. R. and K. Mühlethaler. 1962b. The fine structure of the cells taking part in oogenesis in *Pteridium aquilinum* (L.) Kuhn. J. Ultrastruct Res. 7:452–466.

Cao, J. G., X. F. Dai and Q. X. Wang. 2012. Cytological features of oogenesis and their evolutionary significance in the fern *Osmunda japonica*. Sex. Plant Reprod. DOI: 10.1007/s00497-011-0179-7.

Cao, J. G., Q. X. Wang and W. M. Bao. 2010a. Formation of the fertilization pore during oogenesis of the fern *Ceratopteris thalictroides*. J. Integr. Plant Biol. 52:518–527.

Cao, J. G., Q. X. Wang, X. L. Dai and J. G. Duckett. 2010b. Ultrastructural observations of oogenesis in the fern *Adiantum flabellulatum* L. (Adiantaceae). Amer. Fern J. 100:93–102.

Cao, J. G., N. Y. Yang and Q. X. Wang. 2008. Observations on the formation and ultrastructure of the egg membrane in the fern *Ceratopteris thalictroides* (L.) Brongn. Acta. Bot. Yunnan. 30:543–548.

Cao, J. G., Y. N. Yang and Q. X. Wang. 2009. Ultrastructure of the mature egg and fertilization in the fern *Ceratopteris thalictroides* (L.) Brongn. J. Integr. Plant Biol. 51:243–250.

Cave, C. F. and P. R. Bell P. R. 1975. Evidence for the association of acyl transferases with the production of nuclear evaginations in maturing eggs of the fern *Dryopteris filix-mas*. Histochemistry 44:57–65.

Dai, X. F., N. Jiang, J. G. Cao and Q. X. Wang. 2010. Primary Study on the Archegonium Formation and Oogenesis in the Fern *Phymatosorus hainanensis*. Bull. Bot. Res. 30:411–415.

Duckett, J. G. and P. R. Bell. 1972. Studies on fertilization in archegoniate plants II. Egg penetration in *Pteridium aquilinum* (L.) Kuhn. Cytobiologie 6:35–50.

Fasciati, R., J. Schneller, V. Jenni and U. P. Roos. 1994. Fertilization in the fern *Athyrium filix–femina* (Pterophyta): II. Ultrastructure. Crypt. Bot. 4:356–367.

Hasebe, M., P. G. Wolf, K. M. Pryer, J. R. Manhart, N. Murakami, E. H. Crane, C. H. Haufler and W. D. Hauk. 1995. Fern phylogeny based on *rbcL* nucleotide sequence. Amer. Fern J. 85:134–181.

Lopez-Smith, R. and K. Renzaglia. 2008. Sperm cell architecture, insemination, and fertilization in the model fern, Ceratopteris richardii. Sex. Plant Reprod. 21:153–167.

Mickel, J. T. 1974. Phelytic lines in the modern ferns. Ann. Missouri. Bot. Garden. 61:474–482.

Nayar, B. K. 1970. A phylogenetic classification of the homosporous ferns. Taxon 19:229–236.

Pryer, K. M., H. Schneider, A. R. Smith, R. Cranfill, P. G. Wolf, J. S. Hunt and S. D. Sipes. 2001. Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. Nature 409:618–622.

Pryer, K. M. and P. G. Wolf. 2004. Phylogeny and evolution of ferns (Monilophytes) with a focus on the early leptosporangiate divergenes. Amer J. Bot. 91:1582–1598.

Smith, A. R. 1995. Non-molecular phylogenetic hypotheses for ferns. Amer. Fern J. 85:104–122.

Smith, A. R., K. M. Pryer, E. Schuettpelt, P. Korall, H. Schneider and P. G. Wolf. 2006. A classification for extant ferns. Taxon 55:705–731.

Williams, E. 1974. Fine structure of vascular and epidermal plastids of the mature Maize Leaf. Protoplasma 79:395–400.

Wu, S. H. and R. C. Ching. 1991. Fern families and genera of China. Beijing, Science press.

Yang, N. Y., J. G. Cao and Q. X. Wang. 2009. Formation and Development of Archegonium in the fern *Ceratopteris thalictroides* (L.)Brongn. J. Wuhan Bot. Res. 27:569–573.

Zinsmeister, D. D. and Z. B. Carothers. 1974. The Fine Structure of Oogenesis in *Marchantia polymorpha*. Amer. J. Bot. 61:499–512.

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Morphological and Tissue Culture Studies of *Platycerium coronarium*, a Rare Ornamental Fern Species from Malaysia

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ABSTRACT.—The genus *Platycerium* consists of about 18 species, commonly found in tropical and subtropical forests. Among the different species, *Platycerium coronarium*, *P. platylobium*, *P. ridleyi* and *P. wallichi* are found in Peninsular Malaysia, and *P. coronarium* is considered the most attractive ornamentally. *Platycerium coronarium* is an epiphytic fern, bears a gigantic morphology and is native to tropical areas of South America, Africa, Southeast Asia, Australia and New Guinea. *Platycerium coronarium* nests on the upper branches of the tallest trees in the forest. Due to having a uniquely-shaped fronds, they are famous for ornamental purposes, where they can be found in gardens, especially in tropical regions. Detailed morphological studies of this species are lacking. In the present work, data are reported aiming at defining both the macro- and micro-morphological characteristics of intact and *in vitro P. coronarium*. Data from scanning electron microscopy (SEM) revealed similar ultrastructures of both types of leaves, i.e., the presence of multicellular trichomes on both the abaxial and adaxial surfaces. Sunken stomata were also detected on the abaxial surface of the leaves. In addition, tissue culture studies were done to obtain an efficient regeneration system as well as to serve as an approach for conservation. Successful regeneration of sporophytes from gametophyte explants were observed in MS medium supplemented with 1.0–1.5 mg/l GA_3 and 30 g/l sucrose, at pH 5.8 under 16 hours light and 8 hours dark.

KEY WORDS.—*Platycerium coronarium*, tissue culture, scanning electron microscopy (SEM), morphological studies, phytohormones

Platycerium (Polypodiaceae) consists of about 18 species, all of which are epiphytic and sometimes grow on rocks in tropical and subtropical forests. *Platycerium coronarium* (D. König ex O.F. Müll.) Desv., commonly known as staghorn fern, is an epiphyte that lives on large trees in most tropical forests of South-East Asia such as in Thailand, Myanmar, Philippines and Malaysia. The species is popular as an ornamental plant due to its uniquely shaped fronds. It is also valuable as a traditional medicinal plant to treat fever, irregular menstrual cycle and bile problems (Bidin, 1985). High demand for the limited number of species and forest destruction for development may lead to the extinction of this species (Porembski and Biedinger, 2001).

Few detailed morphological studies of *Platycerium coronarium* have been performed and limited tissue culture studies of *P. coronorium* had been carried out, such as those by Kwa et al. (1995a, 1995b, 1997). *In vitro* regeneration has

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TABLE 1. The effects of different concentrations of Naphthaleneacetic Acid (NAA) and 6-Benzylaminopurine (BAP) on the number of sporophyte leaves regenerated from gametophyte explants of *Platycerium coronarium*.

		Number of sporophyte leaves per explant		
NAA (mg/L)	BAP (mg/L)	Week 10	Week 20	Week 30
0.0	0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
0.1	0.1	3.25 ± 0.2	4.36 ± 0.2	5.25 ± 0.1
4.0	1.0	2.75 ± 0.5	4.65 ± 0.7	6.45 ± 0.3
0.1	1.0	0.00 ± 0.0	1.54 ± 0.6	2.32 ± 0.5
1.0	1.0	1.35 ± 0.6	3.65 ± 0.5	3.25 ± 0.4

Results: Mean ± S.E.

been recognized as a very useful technique for propagation and conservation of threatened plants. Gametophytic and sporophytic regeneration of scales of *Platycerium bifurcatum* (Cav.) C.Chr. has been reported (Ambro *et al.*, 1997) and regeneration of *P. coronarium* using leaf explants has also been achieved (Camloh *et al.*, 1994). To our knowledge, no successful protocol has been

FIG. 1. Habit of *Platycerium coronarium*.

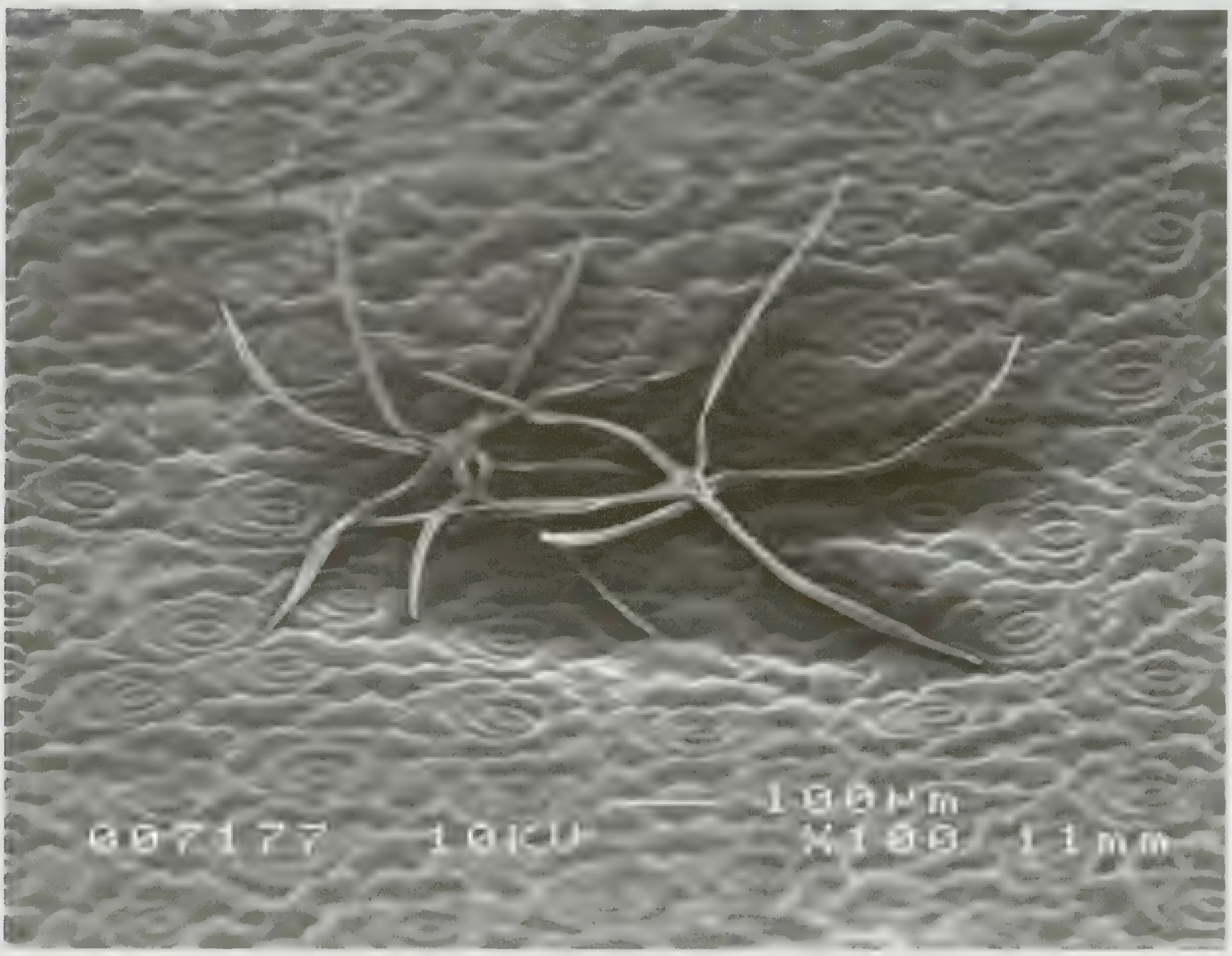

FIG. 2. Structure of trichome on the adaxial surface of *in vitro* leaf.

reported for shoot regeneration from *P. coronarium*, although recently different explants, such as spores (Aspiras, 2010), have been used.

In the present work, vegetative tissues (young sporophyte and gametophyte leaves) were used to regenerate *P. coronarium* utilizing the commonly used media (MS) (Murashige and Skoog, 1992), which has been used for regeneration of angiosperms (Taha and Tijan, 2002). The aim of the present investigation was to compare the macro- and micro-morphological characteristics of *P. coronarium* obtained both *in vivo* and *in vitro*. Also, an attempt was made to discover the most favorable sets of cultural and nutritional conditions for efficient *in vitro* regeneration, in order to establish an efficient regeneration system of *P. coronarium* and to ensure successful mass propagation of this species for conservation. Finally, the regenerated plants were compared with the intact plants to examine any variation that may have resulted from tissue culture protocols or due to different growth environments.

MATERIALS AND METHODS

Field studies and collection of specimens were conducted at Belum forest, Perak, Malaysia. For the tissue culture studies, sporophyte and gametophyte explants were used. Leaves sections (10 mm × 10 mm) from the young

FIG. 3. Structure of trichome on the abaxial surface of intact leaf.

sporophyte plants and spores were sterilized using a series of sodium hypochlorite and alcohol, and explants were cultured following standard methods (Taha, 1993). The medium used throughout this study was MS (Murashige and Skoog, 1962) basal medium, supplemented with various hormones such as Naphthaleneacetic Acid (NAA), 6-Benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D), Thidiazuron (TDZ) and etc. at different concentrations and combinations (see Table 1). The cultures were maintained at 25 ± 1°C under 16 hours light and 8 hours dark for at least 30 weeks.

The morphological study was carried out using light and scanning electron microscopes to observe both macro and micro-morphology of intact and *in vitro* leaf samples of *P. coronarium*. Intact leaves were obtained from plants grown under natural environment, while *in vitro* leaves were obtained from plants grown under aseptic condition in tissue culture system. Samples used for both observations were the sporophyte leaves. For micromorphological studies, sections of clean leaves (3 mm × 3 mm) were immersed in glutaraldehyde and Sorencen phosphate buffer solution at 1:1 ratio for 1 hour. They were then rinsed with 50% Sorencen phosphate buffer and soaked overnight in 2% osmium tetroxide (OsO_4) at 4°C for 14 hours. The samples were washed with sterile distilled water after thawing at room temperature and subsequently soaked for 15 minutes each in 13 steps of distilled water and

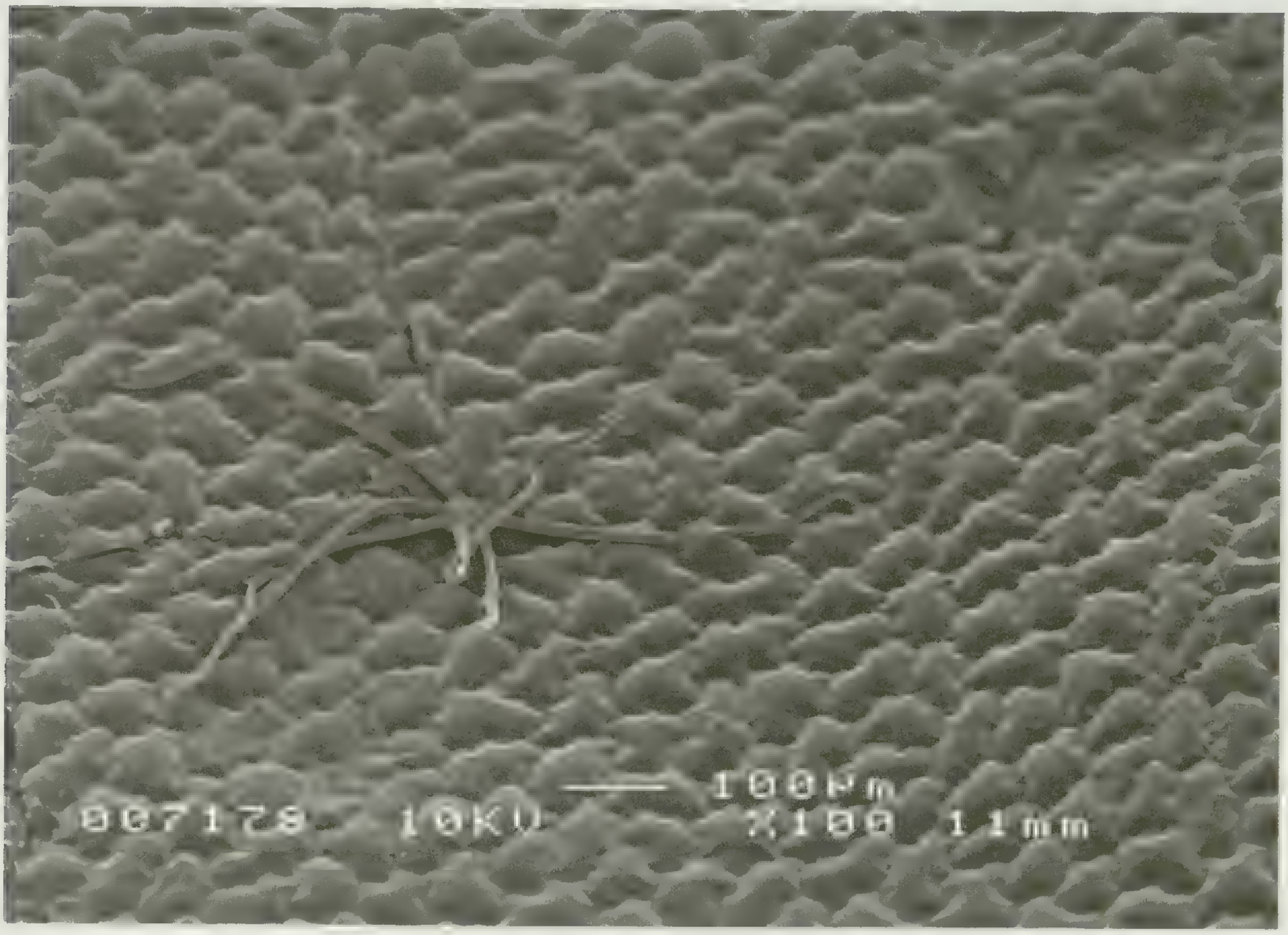

FIG. 4. Structure of trichome on the adaxial surface of intact leaf.

ascending concentration of diluted ethanol from 10% to 100%. This was followed by a hydration process using a series of 100% ethanol and acetone solutions in three different ratios (3:1, 1:1, 1:3). Samples were later soaked in 100% acetone for 20 minutes and this step was repeated four times.

The procedure continued with the drying process at critical point or Critical Point Drying (CPD) using a Polaron E 3000. Here, the samples were soaked in absolute acetone and inserted into the CPD E 3000 at low temperature (20 °C). The sample was subjected to 'flushing' and impregnation using aqueous CO_2 and by controlling the temperature. Finally, the samples were kept in a drying box before the mounting occurred to view the samples under Scanning Electron Microscopes (SEM).

RESULTS AND DISCUSSION

In naturally growing *Platycerium coronarium* the fertile and sterile leaves differ morphologically. As observed in this study, fertile leaves are pendulous (up to 200 cm long), branch dichotomously and arise from the axil of the sterile leaf. Sterile leaves are arranged in the upright position, to trap nutrients from organic matter and can reach up to 60 cm long. Dead sterile leaves remain in their upright positions to trap water and humus from the environment (Fig. 1).

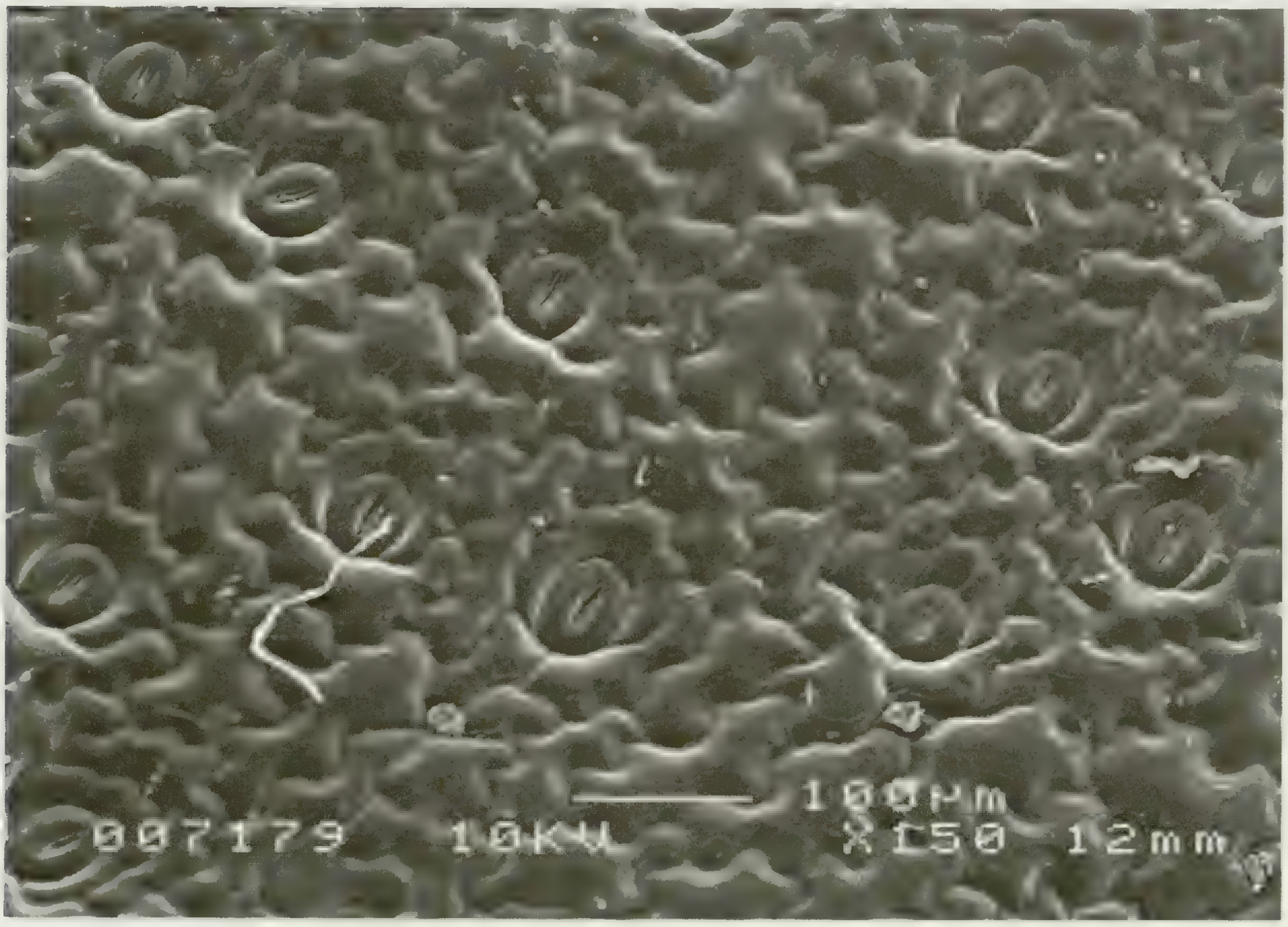

FIG. 5. Distribution of stomata on the abaxial surface of intact leaf.

Morphological observations were also made on sporophyte leaves of *Platycerium coronarium* regenerated in culture. Young sporophyte leaves have various shapes: round, cordate or lobed. The prothallus is thin, has a leaf-like structure and is photosynthetic and cordate. The size of the prothallus ranges from 0.05 to 0.3 mm. Rhizoids are present on the prothallus, functioning in the absorption of water for the gametophyte. Scanning electron microscopic observations of the prothallus showed that stomata and trichomes were absent. However, randomly distributed multicellular trichomes were observed on the abaxial surfaces of both *in vitro* and intact sporophyte leaves (Figs. 2–4). Anomocytic-type stomata, which are randomly distributed, were observed on both types of leaves, although more stomata were present on intact leaves compared to *in vitro* leaves (Figs. 5, 6, and 7). This may be due to the different environmental conditions of the species.

The most active part of the explant, being the sporophyte leaves, was identified when the explants were cultured on MS medium supplemented with 2 mg/l NAA and 1 mg/l BAP with a concentration of 30 g/l sucrose at pH 5.8 under 16 hours light and 8 hours dark compared to the other hormone combination. The formation of prothallus was observed after five weeks of culture, where the sizes ranged between 2.0–5.0 mm. MS medium supplemented with 0.1 mg/l NAA + 0.1 mg/l BAP induced production of 3.25 ± 0.2 sporophyte leaves after 10 weeks in culture using gametophyte explants (Table 1). MS medium supplemented with 1.0 mg/l NAA + 1.0 mg/l BAP

FIG. 6. Distribution of stomata on the abaxial surface of *in vitro* leaf.

resulted in production of the shoot-like structures after 10 weeks, with only 1.35 + 0.6 sporophyte leaves per explant. However, the number of leaves increased to 3.65 ± 0.5 and 3.25 ± 0.4 after 20 and 30 weeks, respectively. MS medium supplemented with 4.0 mg/l NAA and 1.0 mg/l BAP was the optimum media for regeneration of this shoot-like structure, which was observed after 20 to 30 weeks. The morphology of this shoot was globular and greenish in color. In the present study, the addition of 6-benzylaminopurine (BAP) alone to MS medium produced more shoots (1.35–6.45) but the formation of this shoot was slow compared to when MS medium without BAP was used.

TABLE 2. The effects of different concentrations of Gibberellic Acid (GA_3) on the number of sporophyte leaves regenerated from gametophyte explants of *Platycerium coronarium*.

	Number of sporophyte leaves per explant			
GA_3 (mg/L)	Week 5	Week 12	Week 23	Week 30
0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
0.1	0.00 ± 0.0	3.23 ± 0.2	9.25 ± 0.1	11.40 ± 0.4
1.0	0.00 ± 0.0	3.89 ± 0.7	20.45 ± 0.3	25.63 ± 0.6
1.5	0.00 ± 0.0	4.54 ± 0.6	18.32 ± 0.5	24.47 ± 0.8
2.0	1.23 ± 0.6	7.65 ± 0.5	10.25 ± 0.4	15.52 ± 0.2
3.0	0.00 ± 0.0	3.01 ± 0.5	8.75 ± 0.1	10.63 ± 0.3

Results: Mean ± S.E.

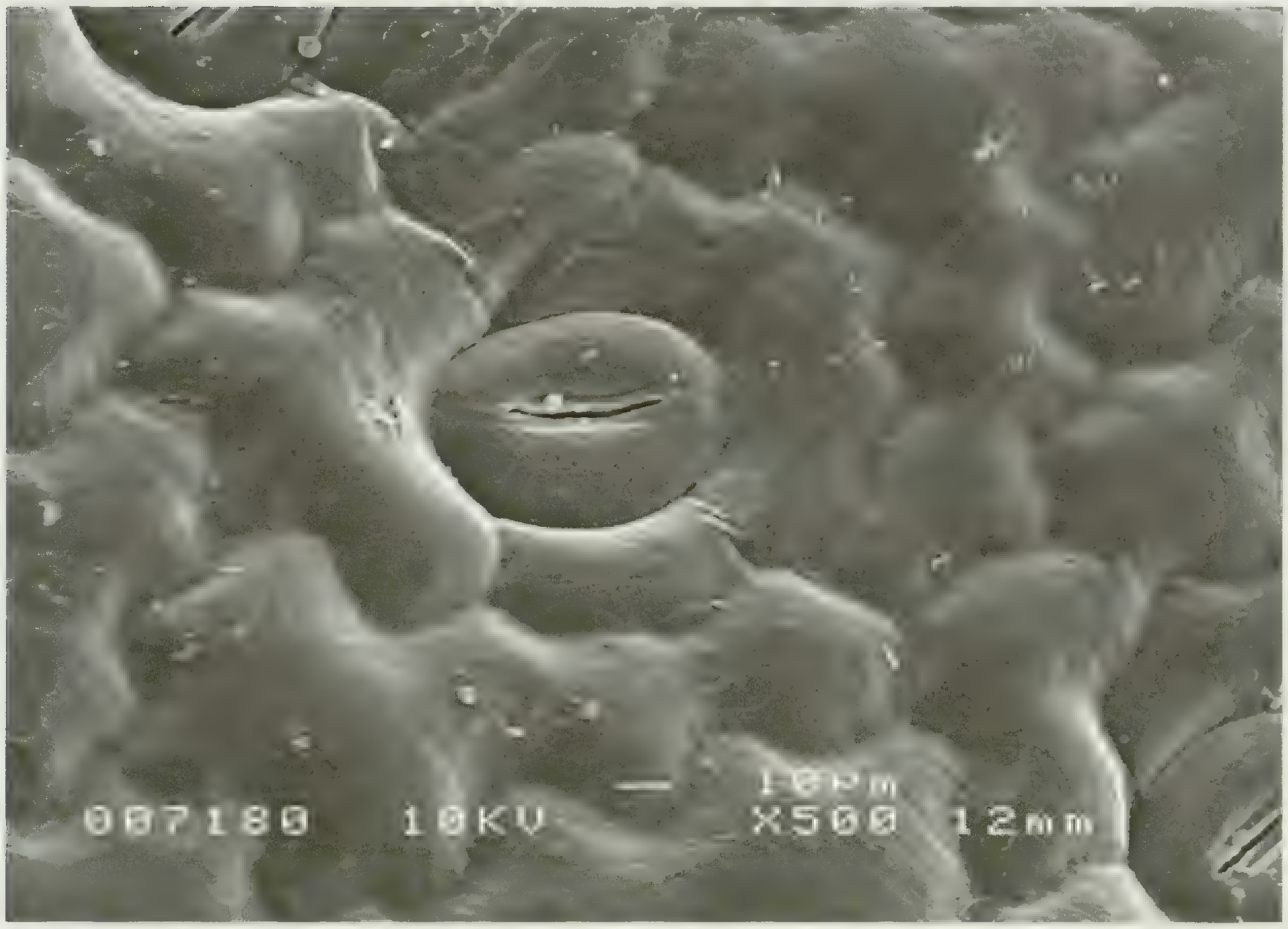

FIG. 7. Structure of stomata on the abaxial surface of intact leaf.

TABLE 3. The effects of various hormones : Indole-3-Acetic Acid (IAA), Kinetin, Thidiazuron (TDZ) and 2,4-Dichlorophenoxyacetic Acid (2,4-D) on the prothallus growth of *Platycerium coronarium*.

Hormones	Protallus growth (mm)		
	Week 1	Week 3	Week 6
0.1 IAA + 0.5 Kinetin	2.08 ± 0.5	2.38 ± 0.8	2.53 ± 0.3
0.5 IAA + 0.1 Kinetin	1.55 ± 0.1	2.09 ± 0.7	2.42 ± 0.2
3.0 IAA + 1.0 Kinetin	2.09 ± 0.8	2.56 ± 0.1	2.71 ± 0.1
1.0 IAA + 2.0 Kinetin	1.52 ± 0.4	2.05 ± 0.4	2.25 ± 0.5
0.1 2,4-D	1.26 ± 0.9	1.47 ± 0.9	0.00 ± 0.4
1.0 2,4-D	0.87 ± 0.7	0.98 ± 0.7	0.82 ± 0.6
1.5 2,4-D	1.08 ± 0.6	1.04 ± 0.6	0.00 ± 0.2
2.0 2,4-D	1.09 ± 0.3	1.09 ± 0.4	0.85 ± 0.1
3.0 2,4-D	1.06 ± 0.1	1.09 ± 0.7	0.96 ± 0.4
0.1 TDZ	1.35 ± 0.2	2.28 ± 0.9	2.58 ± 0.4
0.5 TDZ	1.08 ± 0.9	2.39 ± 0.8	2.59 ± 0.7
1.0 TDZ	1.59 ± 0.7	2.58 ± 0.5	2.77 ± 0.3
2.5 TDZ	1.0 ± 0.4	2.4 ± 0.4	2.63 ± 0.6

Results: Mean ± S.E.

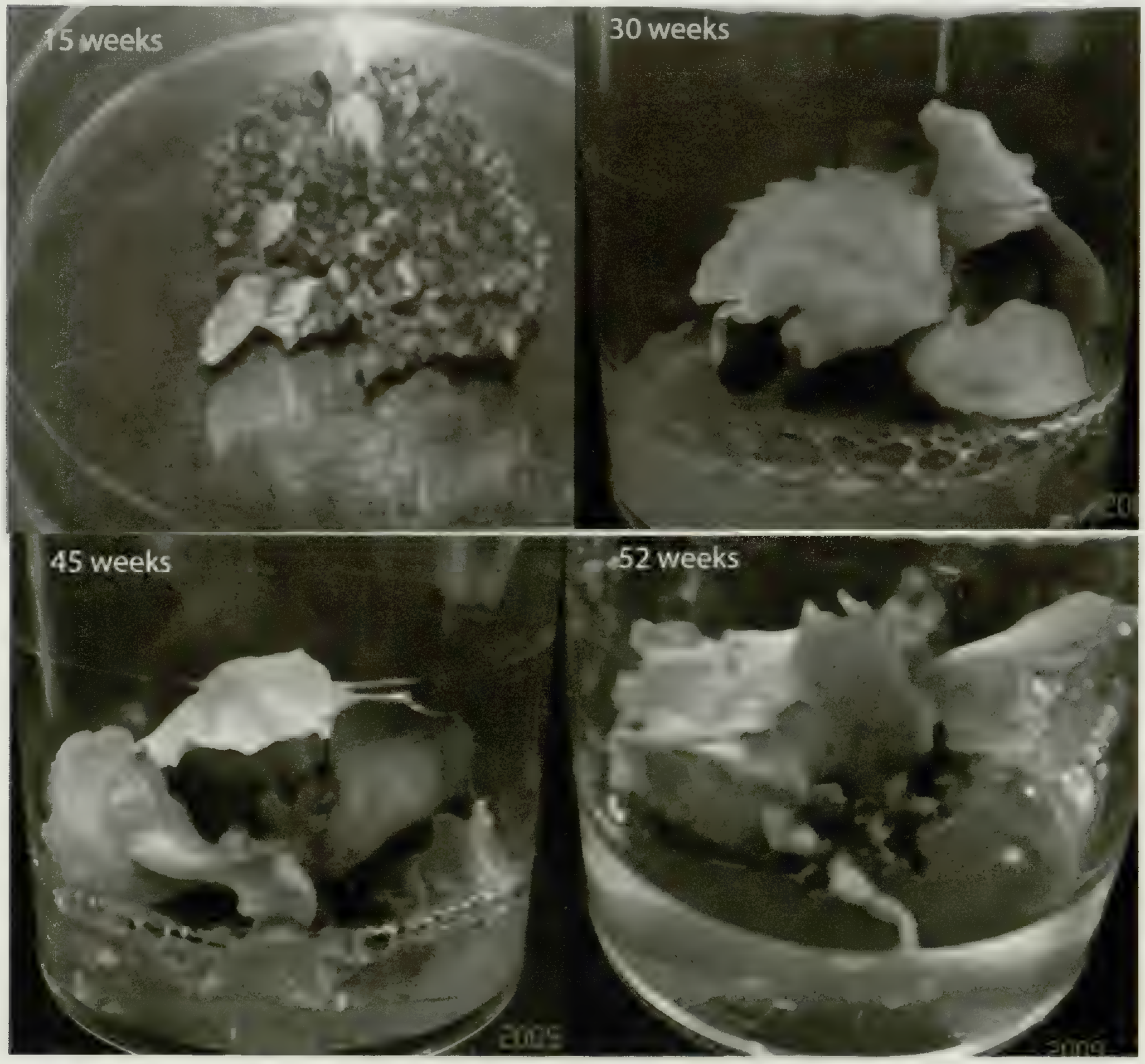

FIG. 8. Regeneration and propagation of *Platycerium coronarium* on MS medium supplemented with 1.0 mg/l Gibberellic Acid (GA_3) after 15, 30, 45 and 52 weeks.

Under favorable conditions, spores from gametophyte leaves will first develop into rhizoids and later mature to produce prothallus. In tissue culture systems, formation of rhizoid (root-like structure) from gametophyte leaves were obtained when the explants were cultured on MS medium supplemented with 0.1 mg/l NAA + 0.5 mg/l BAP and 1.5 mg/l NAA + 0.1 mg/l BAP after 10 weeks. However, the formation of the prothallus was very slow and no prothallus formed even after 20 weeks of culture when the above media and hormone combinations were utilized. Only after 30 weeks of culture, prothallus was formed. Thus, it can be concluded that formation of prothallus required a longer time compared to the rhizoids regeneration. In addition to NAA and BAP, Giberellic Acid (GA_3) was also tested using gametophytes as explants. The most responsive regeneration of sporophyte leaves was obtained in MS medium supplemented with 1.0–1.5 mg/l GA_3 (Table 2). Increasing concentrations of GA_3 (2.0–3.0 mg/l) generally lowered the number of

sporophyte leaves formed. GA_3 was more effective than NAA and BAP in the present study. Effects of Kinetin, IAA, 2,4-D and TDZ were also tested, and they were only effective in increasing the size of prothallus at a very low rates (Table 3). Figure 8 shows the development of *Platycerium coronarium* on MS medium supplemented with 1.0 mg/l GA_3 over the period of 15, 30, 45 to 52 weeks.

The present investigation indicates that regeneration and mass propagation of *Platycerium coronarium* is achievable and it can serve as an important tool for conservation of this rare and unique fern either *in vivo* after being acclimatized or in tissue culture systems as miniature plants. The present work differs from previous work, such as that of Aspiras (2010), where spores were used as explants, since we used vegetative tissues (gametophyte and sporophyte leaves) as explant sources. MS was utilized in the current work, which is normally used for seed plant regeneration. Hence, the current study has revealed that cultural and nutritional growth requirement of *Platycerium coronarium* can be met using this media. In the future, mass propagation of this fern species can be achieved using vegetative tissues and thus, reduces our dependence on spore germination for reproduction of this species.

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Literature Cited

Ambro, I., E. J. Dolin and M. Camloh. 1997. Gametophytic and sporophytic regeneration from scales of the fern *Platycerium bifurcatum* (Cav.) C. Chr. *In vitro*. Ann. Bot. 80:23.

Aspiras, R. A. 2010. Sporophyte and Gametophyte development of *Platycerium coronarium* (Koeing) Desv. and *P. gande* (Fee) C. Presl. (Polypodiaceae) through *in vitro* propagation. Saudi Journal of Biological Sciences 17:13–22.

Bhojwani, S. S. and M. K. Razdan. 1983. *Plant tissue culture: theory and practice developments in crops sciences* (5). Elsevier, Amsterdam.

Bidin, A. 1985. *Paku-pakis ubatan di semenanjung Malaysia*. Dewan Bahasa dan Pustaka, Kuala Lumpur.

Burr, R. W. 1976. Mass propagation of fern through tissue culture. Proc. Int. Plant Prop. Soc. 25:122.

Camloh, M., N. Gogala and J. Rode. 1994. Plant regeneration from leaf explants of the fern *Platycerium bifurcatum in vitro*. Sci. Hortic. 56:257–266.

Fernandez, B. 2003. New records for *Platycerium andinum* Baker in Peru. American Fern Journal 93:160–163.

Hoshizaki, B. J. 1970. Rhizome scales of *Platycerium*. American Fern Journal 60:144–160.

Hoshizaki, B. J. 1975. A staghorn fern (*Platycerium*) hybrid. American Fern Journal 65:99–101.

Kwa, S. H., Y. C. Wee, T. M. Lim and P. P. Kumar. 1995a. IAA- induced apogamy in *Platycerium coronarium* (Koenig) Desv. Gametophytes cultured *in vitro*. Plant cell Reports 14:598–602.

Kwa, S. H., Y. C. Wee, T. M. Lim and P. P. Kumar. 1995b. Establishment and physiological analyses of photoautotrophic callus cultures of the fern *Platycerium coronarium* (Koenig) Desv. under CO_2 enrichment. Journal of Experimental Botany 46:1535–1542.

KWA, S. H., Y. C. WEE, T. M. LIM and P. P. KUMAR. 1997. Morphogenetic plasticity of callus reinitiated from cell suspension cultures of the fern *Platycerium coronarium*. Plant Cell Tissue and Organ Culture, 86:37–44.

MURASHIGE, T. and F. SKOOG. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497.

TAHA, R. M. 1993. Tissue culture studies of citrus Hystrix D.C and *Severinia buxifolia* (Poir) Tenore. Asia Pacific J. of Molecular Biology and Biotechnology 1(1):36–42.

TAHA, R. M. and TIJAN, M.. 2002. An *in vitro* production and field transfer protocol for *Solanum melongena* L. plants. South African Journal of Botany 68:447–450.

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Pityrogramma opalescens (Pteridaceae), a New Species from Cerro del Torrá, Colombia

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ABSTRACT.—A new species, *Pityrogramma opalescens*, is described; it is known only from Cerro del Torrá, an isolated mountain peak in the Cordillera Occidental of Colombia, which is a region of high diversity and endemism. It is most similar to *Pityrogramma lehmannii*, and the two species differ from all other *Pityrogramma* species by having creeping rhizomes, rhizome scales with turgid cells, an elongate, proximally 1-pinnate lamina, with sessile segments supplied by multiple veins that emerge from the rachis rather than from a single main vein, and farina on the abaxial side of the lamina that is borne at the apex of short hairs. It differs from *P. lehmannii* by more numerous segments that are narrower (3.5–4.0 mm wide vs. 10.0–17.0 mm wide), with more acutely rounded apices, and dull-yellow rather than white farina abaxially.

KEY WORDS.—Taenitidoid, *Pityrogramma lehmannii*, Cerro del Torrá, Colombia

While identifying specimens of Andean ferns, a new species was encountered that had been collected in the Cordillera Occidental of Colombia. It appears most similar to *Pityrogramma lehmannii* (Hieron.) R. M. Tryon, an infrequently collected species known from northern Ecuador and Colombia.

Pityrogramma is a primarily neotropical genus of Pteridaceae naturalized in many tropical regions of the Old World (Tryon *et al.*, 1990). It was most recently revised by Tryon (1962) who recognized 14 species; however, subsequent authors have considered it slightly larger, with Tryon and Tryon (1982) recognizing 16 species, and Mickel and Smith (2004) recognizing 17 species. Domin (1928, 1929, 1941) made critical early taxonomic contributions, but his 1929 monograph inflated the number of infra-specific taxa and confounded the nomenclature by typifying names on incomplete specimens of cultivated material that lack precise locality data.

Tryon *et al.* (1990) treated *Pityrogramma* as part of Pteridaceae subfam. Taenitidoideae (the "taenitids"), a group of about 15 genera defined by having exindusiate sporangia spread along the veins and spores with a prominent equatorial flange. *Pityrogramma* is distinguished from other taenitid genera by having rhizomes that bear scales and not bristles, laminae that are usually 2–4-pinnate and often abaxially farinose, and spores with 1–4 accessory ridges parallel to the equatorial flange and coarse markings on the distal face (Tryon *et al.*, 1990; Yatskievych *et al.*, 1990).

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Although not all genera have been sampled, Taenitidoideae have been found to be monophyletic, with the inclusion of *Cosentinia* (Nakazato and Gastony, 2003; Sánchez-Baracaldo, 2004a; Prado *et al.*, 2007; Schuettpelz *et al.*, 2007), which had previously been placed among cheilanthoid ferns by Tryon *et al.* (1990) and Zimmer (1991), or affiliated with other primarily neotropical taenitids by Pichi Sermolli (1985) in his Hemionitidaceae. Several genera of the Taenitidoideae by comparison, including *Anogramma*, *Eriosorus*, and *Jamesonia*, have been revealed to have problems in their circumscription (Nakazato and Gastony, 2003; Sánchez-Baracaldo, 2004a, 2004b; Prado *et al.*, 2007; Schuettpelz *et al.*, 2006). Nakazato and Gastony (2003) demonstrated the polyphyly of *Anogramma* sensu Tryon (1962), and commented that studies with a broader sampling of *Pityrogramma* were necessary to determine whether species unrelated to *Anogramma* s.s. should be considered congeneric with *Pityrogramma* or treated as a coordinate independent genus. This close relation of some *Anogramma* species to *Pityrogramma* was anticipated (Domin, 1929b; Tryon, 1962; Tryon and Tryon, 1982). Nonetheless, *Pityrogramma* sensu Tryon *et al.* (1990) has been recovered as monophyletic in each of these phylogenetic studies, although sampling has been limited.

Pityrogramma opalescens Sundue ***sp. nov.*** TYPE.—COLOMBIA, Depto. Chocó, Municipio San José del Palmar, Cerro del Torrá, 4°46′N, 76°29′W, abajo del helipuerto en un antiguo alug, y a la orilla del bosque, vereda de Río Negro, 1700 m, 25 Aug 1988, *J. E. Ramos, P. A. Silverstone, L. H. Ramos 1523* (Holotype: CUVC-n.v., Isotypes: COL-n.v., NY!; photos of CUVC and COL at NY). **Figs. 1–3.**

Ex affinitate *P. lehmannii* Hieron., sed laminis cum pluribus ((30)60–70) segmentis (vs. cum paucioribus (20–25) segmentis in *P. lehmannii*), segmentis

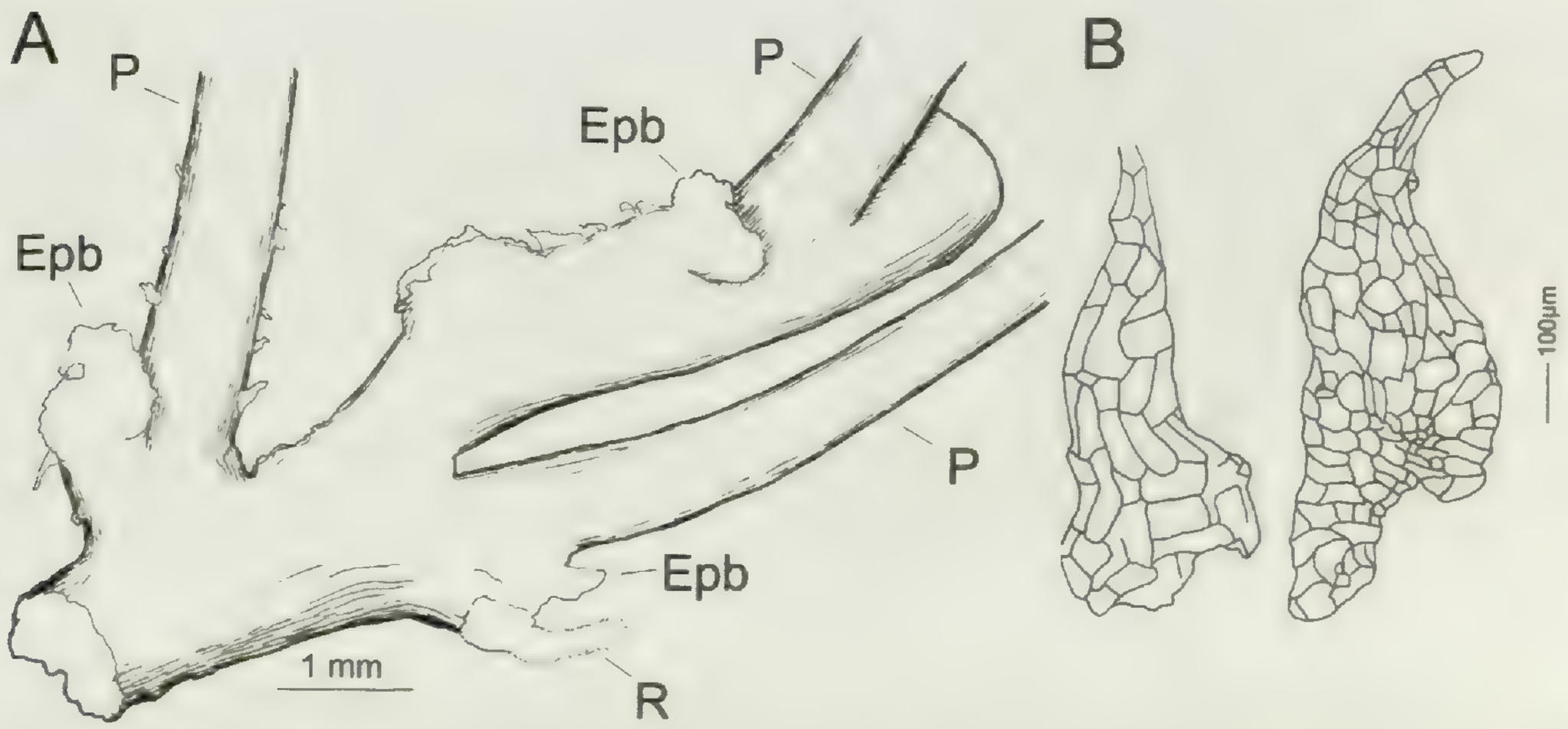

FIG. 1. *Pityrogramma opalescens* (based on the isotype, NY). A. Rhizome depicting position of epipetiolar branch buds, Epb – epipetiolar branch bud, P – petiole, R – root. B. detail of rhizome scales.

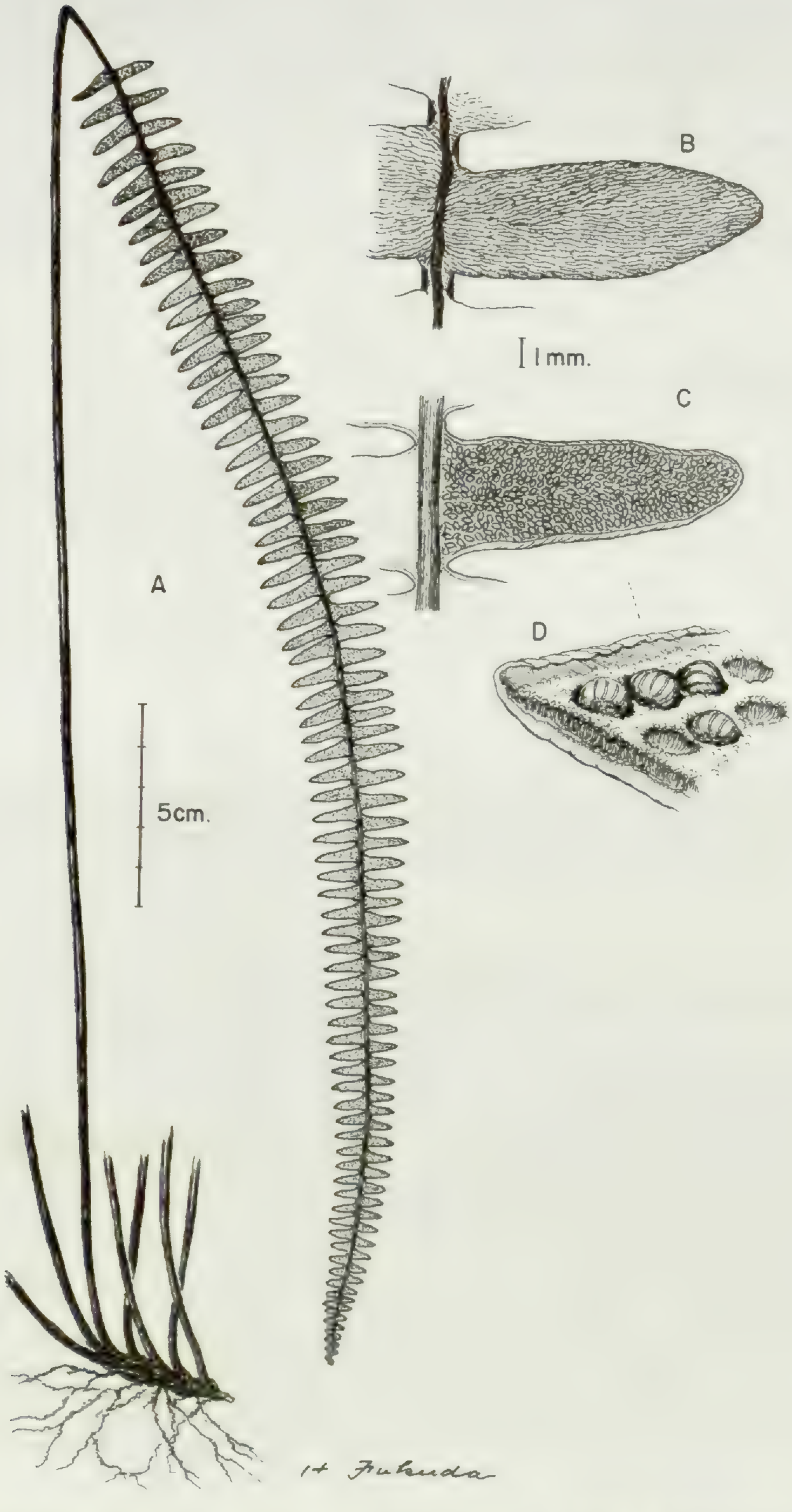

FIG. 2. *Pityrogramma opalescens* (based on the isotype, NY). A. habit. B. adaxial pinna. C. abaxial pinna. D. detail of sporangia and farinose indument.

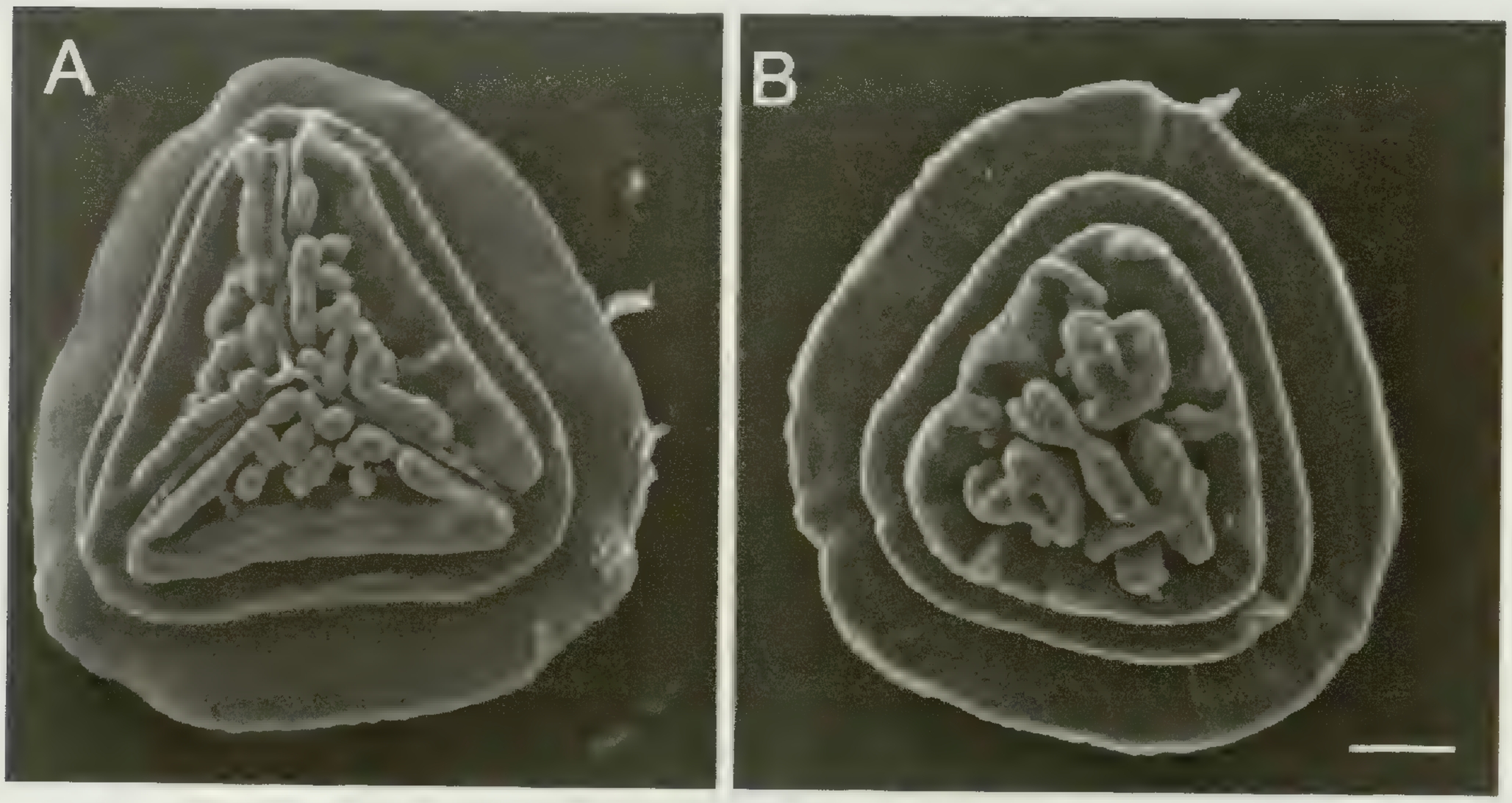

FIG. 3. Spores of *Pityrogramma opalescens* (based on the isotype, NY). Scale bar = 10 μm. A. proximal face. B. distal face.

3.5–4.0 mm latis (vs. segmentis latioribus, 10.0–17.0 mm latis, in *P. lehmannii*), et farina sordido-aurea (vs. farina alba in *P. lehmannii*) differt.

Plants terrestrial; roots inserted radially, densely provided with golden-brown root hairs; rhizomes (Fig. 1A) 1.0–2.5 mm diam., radially symmetrical, short-creeping, light brown, sparsely scaly, the rhizome surface easily seen between scales, the scales (Fig. 1B) 0.5–1.2 × 0.3–0.5 mm, basifixed, narrowly triangular, spreading, golden-brown, the cells somewhat turgid, lustrous, translucent, the margin entire, the apex acute; fronds erect, 35–65 cm long; petioles 20–35 cm long, 1.0–2.5 mm diam., with two vascular bundles, castaneous, lustrous, proximally and medially terete, distally with a shallow adaxial groove, essentially glabrous with a few scattered scales proximally, the scales similar to those of the rhizome, the petiole base provided with an epipetiolar bud, the buds bearing a tuft of scales and usually associated with 1–3 roots; rachises glabrous, abaxially prominent and similar to the petioles, adaxially the castaneous tissue of the rachis visible proximally, buried in the lamina tissue distally; laminae (Fig. 2A) 12–28 × (1)2.0–2.5 cm, linear, even sided, the base truncate, the apex attenuate, proximally 1-pinnate, distally 1-pinnatisect, with (30)60–70 pairs of segments, the segments (Fig. 2B) (5)10–12 × 3.5–4.0 mm, narrowly triangular to nearly oblong, widest at the base, the base sessile, the apex narrowly rounded, the margins entire, the abaxial lamina surface (Figs. 2C, D) densely and evenly puberulent, the hairs ca. 0.1 mm long, light brown, erect, apparently secretory and the apices provided with a dull-yellow farina, the farina forming a confluent layer near the height of the sporangial capsules, the adaxial lamina surface (Fig. 2B) glabrous, striate, whitish on weathered leaves; veins free, forking, each lamina segment with 5–7 veins, these emerging directly from the rachis without a prominent costa or

main vein; sporangia spread along veins on the abaxial lamina surface; spores (Figs. 3A, B) 61–63 μm wide, brown, frequently refracting light and reflecting blue and purple hues, trilete, with a broad and prominent equatorial flange, 10 μm wide, that can be easily observed through the dissecting microscope at 60× magnification.

ETYMOLOGY.—The name "opalescens" is derived from the iridescent mineral opal, which the spores of *Pityrogramma opalescens* resemble when they reflect light.

DISTRIBUTION.—Known only from the type collection made at 1700 m in very wet cloud forest on Cerro del Torrá, in Chocó, Colombia. This isolated mountain peak (summit 2770–2800 m) in the western cordillera (Cordillera Occidental) is one of four peaks in the Cordillera de San Miguel, which is separated from the Serranía de los Paraguas (a branch of the Cordillera Occidental) by a valley of about 600 m elevation (Silverstone-Sopkin and Ramos-Perez, 1995). It lies within the Chocó floristic region, one of the wettest and most species-rich areas in the world for plants (Gentry, 1986). Cerro del Torrá and the Serranía de los Paraguas are particularly rich in rare and narrowly restricted species (Silverstone-Sopkin and Graham 1986; Davidse and Clarke, 1996; Taylor, 1997; Struwe, 2003; Pedraza 2008).

With the description of *P. opalescens,* 11 species of *Pityrogramma* are recorded from Colombia (compiled from Tryon, 1962; Lellinger, 1977; Tryon and Stolze, 1990). Most of these are broadly distributed, but *P. opalescens*, and *P. dukei* Lellinger are both known only from Depmartmento Chocó.

MORPHOLOGY.—Spore morphology of *P. opalescens* (Figs. 3A, B) is similar to that of other species of *Pityrogramma*, as evidenced a broad equatorial flange, a second ridge that is parallel to it, and coarse markings on the face, which A. F. Tryon called "hieroglyphic marks" (Tryon and Lugardon, 1991). The equatorial flange in *P. opalescens*, however, is unparalleled in size, measuring 10 μm, large enough to be seen through the dissecting microscope at 60×. The spores of *P. opalescens* are also conspicuously iridescent, reflecting blue and purple hues when illuminated by a halogen bulb and observed with a dissecting microscope. Iridescence can be observed to a lesser degree in other species of *Pityrogramma* (e.g., *P. ebenea* (L.) Proctor) as well, and may also occur elsewhere in the family among genera with similar spores, but to my knowledge this has not been recorded in descriptive literature (Domin, 1928, 1929, 1941; Tryon, 1962; Tryon *et al.*, 1990). Iridescence has been documented from the megaspores of some species of *Selaginella*, where it is caused by colloidal crystal configuration of the exine in the spore wall (Hemsley *et al.*, 1994, 1998).

Epipetiolar buds of ferns are shoot initials that develop from the base of petiole. They develop into new stem tissue without a resting phase and without detaching from the primary plant body (Moran, 2004). These were termed epipetiolar rhizomes by Lellinger (2002). Wardlaw's (1952) "detached meristems" differ from epipetiolar buds by being induced by damage to the

plant and by lacking a regular position. Bulbils, a type of asexual reproduction in ferns, also differ by generally detaching from the primary plant or becoming active after senescence of the leaf that bears them. The epipetiolar buds on *P. opalescens* appear as scaly protuberances at the base of the petioles. Roots can be seen emerging from most of these buds (Fig. 2A), suggesting that they are actively growing, not dormant. Epipetiolar buds are well documented from several families of ferns, including Hymenophyllaceae, Gleicheniaceae, and Dennstaedtiaceae (Bower, 1923; Troop and Mickel, 1968; Bierhorst 1969, 1974; Imaichi, 1980, 1985). To my knowledge, within the Pteridaceae they have been previously reported only from *Pteris wallichiana* J. Agardh (Chandra and Nayar, 1970). They are not mentioned within descriptions of the family in general taxonomic references for ferns (e.g., Tryon and Tryon, 1982; Tryon *et al.*, 1990).

DISCUSSION.—Despite being known from only a single gathering, the type collection of *P. opalescens* includes ample material, with all parts of the plant well represented in each duplicate. Photos of the isotypes were generously provided to me by Philip Silverstone-Sopkin (CUVC) before they were distributed. Based on my own searches and those conducted for me by colleagues, I do not believe that any other collections of *P. opalescens* reside in herbaria that harbor strong collections of Colombian ferns available to me at this time: CUCV, GH, MO, NY, UC, and US. The type locality of *P. opalescens* is remote and difficult to access. Consequently, I choose to describe the plant based on the material at hand in order to draw further attention to this remote, under-collected, and species-rich area.

The NY sheet of the type collection was originally distributed as "*Pityrogramma sp.*" and most likely corresponds to the collection identified as such in the phytogeographic study of Cerro del Torrá conducted by Silverstone-Sopkin and Ramos-Pérez (1995). This species was probably not described until now because unlike most collections from the study, the duplicates of *Ramos et al. 1523* were not widely distributed.

Superficially, *Pityrogramma opalescens* bears a strong resemblance to *Jamesonia verticalis* Kunze, so much so that the isotype at NY was previously misidentified as that species. The two share elongate even-sided laminae with short undivided segments and castaneous axes. Both species grow sympatrically at Cerro del Torrá. *Pityrogramma opalescens*, however, clearly does not belong to *Jamesonia* or the closely allied *Eriosorus* because *P. opalescens* has rhizomes with scales rather than bristles, veins that end before the lamina margins, and farina present on the abaxial lamina surface. By comparison, *Eriosorus* and *Jamesonia* have rhizome scales with bristles instead of scales, veins that end at the lamina margins, and non-farinose surfaces. *Jamesonia verticalis* itself can be further distinguished by its puberulent rachis.

Pityrogramma opalescens is probably most closely related to *P. lehmannii* (Hieron.) R. M. Tryon, an infrequently collected species known from 1200–2400 m, northern Ecuador and southern Colombia. The two share creeping rhizomes, with scales that have turgid cells, an elongate lamina, with sessile

undivided segments supplied by several veins that emerge from the rachis rather than from a single main vein, and farina on the abaxial side of the lamina that is borne at the apex of short hairs. *Pityrogramma lehmannii*, however, differs by having fewer segments (20–25) that are nearly as long as they are wide (12–17 × 10–17 mm), with more broadly rounded apices, and white rather than dull-yellow farina abaxially. Based on the material that I have seen, *Barclay 9463* (GH), *Lehmann 8944* (GH, isotype), *Madison 883* (GH), *P. lehmanii* does not have epipetiolar buds or iridescent spores. In other respects, however, the spores are similar and have a very wide equatorial flange like that of *P. opalescens* (Tryon and Tryon, 1982).

The characters that unite these two species and distinguish them from other species of *Pityrogramma* are unique within the genus. Other species of *Pityrogramma* generally have erect rhizomes with scales that have flat cells, deltate or lanceolate laminae that are more finely dissected, and stipitate segments that are served by a main vein. If they are farinose, the farina is sub-sessile on the abaxial lamina surface borne on surficial glands rather than clearly at the apex of short hairs (0.1mm long). When describing *P. lehmannii* (as *Gymnogramme lehmannii*), Hieronymus (1905) thought it distinct enough from its congeners in *Gymnogramme* to warrant a new section, *Isgnogramme*. Christensen maintained *P. lehmannii* in *Gymnogramme* when he adopted *Pityrogramma* in his 1934 supplement of Index Filicum. Tryon (1962) made the transfer into *Pityrogramma*, but noted the morphological discrepancy, as did Yatskievych *et al.* (1990).

These observations raise doubts as to the placement of both *P. opalescens* and *P. lehmannii* in *Pityrogramma*, but no alternate placement seems more appropriate. One possible alternate placement to consider for these plants is *Pterozonium*, but that would result in equal morphological discrepancy. *Pterozonium* is a morphologically diverse genus, and some species are similar to *P. opalescens* and *P. lehmannii* in having creeping rhizomes provided with scales that have turgid cells, lamina segments with rounded apices and that do not have a main vein, and farina on the abaxial lamina surface that is borne at the apex of short hairs. However, the stipitate segments, tuberculate or regulate spore surfaces that lack "hieroglyphic marks" (Tryon and Lugardon, 1991), and peculiar geography of *Pterozonium* argue against it as the correct home for *P. opalescens* and *P. lehmannii*. Consequently, they are maintained in *Pityrogramma* pending further study.

Given their unique suite of characters, *P. lehmannii* and *P. opalescens* should be prioritized in future phylogenetic studies aimed at unraveling the evolution of the taenitid ferns.

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Literature Cited

Bierhorst, D. W. 1969. On *Stromatopteris* and its ill-defined organs. Amer. J. Bot. 56:160–174.

Bierhorst, D. W. 1974. Variable expression of the appendicular status of the megaphyll in extant ferns with particular reference to the Hymenophyllaceae. Ann. Miss. Bot. Gard. 61:408–426.

Bower, F. O. 1923. *The Ferns: (Filicales): treated comparatively with a view to their natural classification*, Vol. 1. Cambridge University Press.

Chandra, S. and B. K. Nayar. 1970. Some aspects of the morphology of the rhizome of *Pteris wallichiana*. Proc. Indian Acad. Sc. 71:79–85.

Christensen, C. 1934. *Index Filicum Supplementum Terium Pro Annnis 1917–1933*. Hafniae apud H. Hagerup, Copenhagen.

Davidse, G. and L. G. Clark. 1996. Two new species of *Neurolepis* (Poaceae: Bambuseae) from Colombia. Novon 6:150–156.

Domin, K. 1928. Generis *Pityrogramma* (link) species ac sectiones in clavem analyticam dipositae. Spisy Přír. Fak. Karlovy Univ. 88:6.

Domin, K. 1929. The hybrids and garden forms of the genus *Pityrogramma* (Link). Rozpr II. Tř. České Akademie 38:3–80.

Domin, K. 1929b. Letter to Carl Christensen. Februrary 2nd 1929. Archives of the Alice and Rolla Tryon Pteridophyte Library. Pringle Herbarium. The University of Vermont.

Domin, K. 1941. Additamentum primum ad filicum Americae tropicae australis et mediae insularis cognitionem. Genus *Pityrogramma* (Link.) Dom. Věstn. Ceské Společn. Nauk. Tř Mat.-Přír. [Mém. Soc. Sc. Bohême, Cl. Sci.]. 15:1–8.

Gentry, A. 1986. Species richness and floristic composition of Chocó region plant communities. Caldasia 15:71–91.

Hemsley, A. R., M. E. Collinson, W. L. Kovach, B. Vincent and T. Williams. 1994. The role of self-assembly in biological systems: evidence from iridescent colloidal sporopollenin in. *Selaginella* megaspore walls. Phil. Trans. Roy. Soc. London B. 345:163–173.

Hemsley, A. R., B. Vincent, M. Collinson and P. C. Griffiths. 1998. Simulated self-assembly of spore exines. Ann. Bot. 82:105–109.

Hieronymus, G. 1905. Plantae Lehmannianae, pteridophyta. Bot. Jahrb. Syst. 34:417–582.

Imaichi, R. 1980. Developmental studies on the leaf and the extra-axillary bud of *Histiopteris incisa*. Bot. Mag. Tokyo. 93:25–38.

Imaichi, R. 1985. On the extra-axillary buds of *Histiopteris* and *Hypolepis* collected in Bolivia. Bull. Fac. Agr., Tamagawa Univ. 25:23–33.

Lellinger, D. B. 1977. Nomenclatural and taxonomic notes of the pteridophytes of Costa Rica, Panama, and Colombia. Proc. Biol. Soc. Wash. 89:703–732.

Lellinger, D. B. 2002. A modern multilingual glossary for taxonomic pteridology. Pteridologia 3:1–263.

Mickel, J. T. and A. R. Smith. 2004. The Pteridophytes of Mexico. Mem. New York Bot. Gard. 88:1–1054.

Moran, R. C. 2004. A Natural History of Ferns. Timber Press, Portland.

Nakazato, T. and G. J. Gastony. 2003. Molecular phylogenetics of *Anogramma* species and related genera (Pteridaceae: Taenitidoideae). Syst. Bot. 28:490–502.

Pedraza, P. 2008. Three new species of *Disterigma* (Ericaceae: Vaccinieae) from western Colombia, with comments on morphological terminology. Brittonia 60:1–10.

Pichi Sermolli, R. E. G. 1985. The fern genus *Cosetinia* Todaro. Webbia 39:179–189.

Prado, J., C. Del Nero Rodrigues, A. Salatino and M. L. F. Salatino. 2007. Phylogenetic Relationships among Pteridaceae, including Brazilian Species, Inferred from *rbcL* Sequences. Taxon. 56:355–368.

SÁNCHEZ-BARACALDO, P. 2004a. Phylogenetic relationships of the subfamily Taenitidoideae, Pteridaceae. Amer. Fern J., 94:126–142.

SÁNCHEZ-BARACALDO, P. 2004b. Pylogenetics and biogeography of the neotropical fern genera *Jamesonia* and *Eriosorus* (Pteridaceae). Amer. J. Bot. 91:274–284.

SCHUETTPELZ, E., H. SCHNEIDER, L. HUIET, M. D. WINDHAM and K. M. PRYER. 2007. A molecular phylogeny of the fern family Pteridaceae: assessing overall relationships and the affinities of previously unsampled genera. Molecular Phylogenetics and Evolution 44:1172–1185.

SILVERSTONE-SOPKIN, P. A. and S. A. GRAHAM. 1986. Alzateaceae, a plant family new to Colombia. Brittonia 38:340–343.

SILVERSTONE-SOPKIN, P. and J. E. RAMOS-PÉREZ. 1995. Floristic exploration and phytogeography of the Cerro del Torrá, Chocó, Colombia: 169–186 In *Biodiversity* and *Conservation of Neotropical Montain Forests, Proceedings of a Symposium, New York Botanical Garden, 21–26 June 1993*, Churchill, S. P., Balslev, H., Forero, E. and Luteyn, J., eds, The New York Botanical Garden, New York.

STRUWE, L. 2003. Two new winged species of *Symbolanthus* (Gentianaceae: Helieae) from Colombia. Novon 13:133–140.

TAYLOR, C. M. 1997. New species of *Palicourea* and *Psychotria* (Rubiaceae: Psychotrieae) from northwestern Colombia. Novon 7:191–200.

TROOP, J. E. and J. T. MICKEL. 1968. Petiolar shoots in the dennstaedtioid and related ferns. Amer. Fern J. 58:54–70.

TRYON, A. F. and B. LUGARDON. 1991. *Spores of the Pteridophyta: surface, wall structure, and diversity based on electron microscope studies*. Springer-Verlag, New York.

TRYON, R. 1962. Taxonomic fern notes. II. *Pityrogramma* (including *Trismeria*) and *Anogramma*. Contr. Gray Herb. 189:52–76.

TRYON, R. M. and R. G. STOLZE. 1989. Pteridophyta of Peru, part II, 13. Pteridaceae–15. Dennstaedtiaceae. Fieldiana, Bot. 22:1–128.

TRYON, R. M. and A. F. TRYON. 1982. *Ferns and Allied Plants with Special Reference to Tropical America*. Springer-Verlag, New York.

TRYON, R. M., A. F. TRYON and K. U. KRAMER. 1990. *Pteridaceae. In: The Families and Genera of Vascular Plants. Vol. 1. Pteridophytes and Gymnosperms*. Kramer, K. U., Green P. S., eds, Springer-Verlag, Berlin, pp. 230–256.

WARDLAW, C. W. 1952. *Morphogenesis in plants*. John Wiley and Sons, New York.

YATSKIEVYCH, G., M. D. WINDHAM and E. WOLLENWEBE. 1990. A reconsideration of the genus *Pityrogramma* (Adiantaceae) in western North America. Amer. Fern J. 80:9–17.

ZIMMER, B. 1991. Remarks on the Greek cheilanthoid ferns. Botanika Chronika 10:211–237.

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New Species of *Elaphoglossum* Schott ex J.Sm. (Dryopteridaceae) from Brazil

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Abstract.—Two new species of *Elaphoglossum* (Dryopteridaceae) are described and illustrated: *Elaphoglossum bradeanum* Melo & Salino and *Elaphoglossum commissurale* Melo & Salino, both endemic to Minas Gerais state (Brazil).

Key Words.—Taxonomy, ferns, *Elaphoglossum*, Dryopteridaceae, Minas Gerais, Brazil

Elaphoglossum is a large fern genus with over 600 species, with about three-fourths of them occurring in tropical America (Mickel and Smith, 2004). There are approximately 84 species in Brazil. Most are limited to rain and mountainous forests in the southeastern and southern regions of Brazil. However, they can be found in several formations, such as semi-deciduous and deciduous forests and in savanna areas like the Brazilian Cerrado. The genus is difficult taxonomically, mainly due to morphological variation of vegetative characters. This fact, combined with the lack of recent studies in Brazil, has led to misinterpretation of the circumscription of many species. In this paper two new species are described as a partial result of a taxonomic study of *Elaphoglossum* from Minas Gerais state.

Elaphoglossum bradeanum Melo & Salino, ***sp. nov.*** TYPE.—Brazil. **Minas Gerais**: Santa Maria do Salto, Distrito de Talismã, Fazenda Duas Barras, 16° 24′ 16″ S, 40° 03′ 27″ W,750–850m, 10 Oct 2003, *A. Salino 9248 et al.* (holotype, BHCB; isotype, NY). **Fig. 1A–C**

Elaphoglossum bradeanum E. iguapensi Brade affine, sed squamis rhizomatis margine integra et frondibus apice basique cuneatis differt.

Plants epiphytic and terrestrial; *rhizome* short creeping, 10 mm in diameter, densely scaly, scales oblong-lanceolate with acuminate and crispate apices, entire, blackish-brown, opaque, 3–5 × 1.4–7 mm; phyllopodia 16–27 mm long; *sterile leaves* 2.5–3.5 mm apart, 60–107 cm long; petioles 29–45 cm × 2–3.4 mm, sulcate, castaneous, with scales at the base oblong-lanceolate, 3–5 × 1.8–2.1 mm, appressed, ascending, sparsely ciliate, light brown, other scales elliptical, ciliate; *blades* oblong-linear, subchartaceous, apically and basally cuneate, the margins glabrous, 38–62 × 6–8 cm; *veins* obscure, free, simple and furcate, ca. 1 mm apart, set at 90° to costae, *hydathodes* lacking; blades on abaxial surface with minute, brown, stellate scales; costae sulcate until ½ the sterile leaf length, with scales oblong-lanceolate, 1–1.2 mm long, light

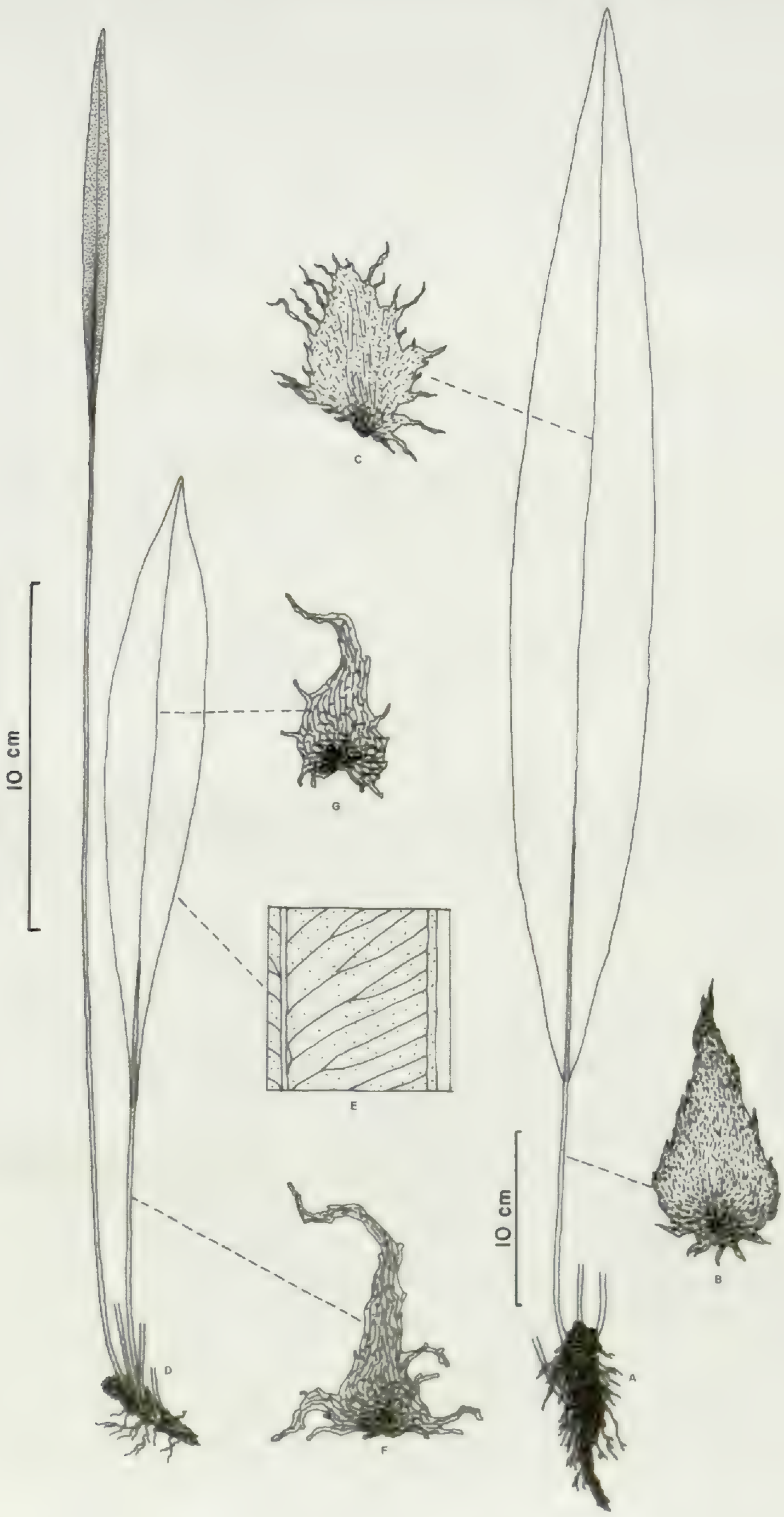

FIG. 1. A–C. *Elaphoglossum bradeanum* (*Salino 9248*, BHCB). A. Habit. B. Scales of petiole. C. Scales of abaxial surface of costa. D–G. *Elaphoglossum commissurale* (*Salino 9496,* BHCB). D. Habit. E. Venation showing the commissural vein parallel to the margin. F. Scales of petiole. G. Scales of abaxial surface of costa.

castaneous, margins erose and long ciliate, sometimes amorphous; *fertile leaves* unknown.

ADDITIONAL SPECIMENS EXAMINED.—BRAZIL. **Minas Gerais:** Santa Maria do Salto, Distrito de Talismã, Fazenda Duas Barras, 16° 24′ 16″ S, 40° 03′ 27″ W, 09 Mar 2004, *A. Salino 9520 et al.* (BHCB); idem, 24 Feb 2005, *A. Salino 10063 et al.* (BHCB).

Elaphoglossum bradeanum is most closely related to *E. iguapense* Brade (Brazil), but it is distinguished by the blackish-brown rhizome scales with entire margins (vs. castaneous scales with ciliate margins), sterile leaves ca. 100 cm long, and blade oblong-lanceolate with base and apex cuneate. *Elaphoglossum iguapense* has short sterile leaves (ca. 50 cm long), and blade lanceolate with base long decurrent and acuminate apex. *Elaphoglossum bradeanum* is endemic to Minas Gerais state near the border with the Bahia state. It is known only from the type locality, where it grows in the Atlantic rainforest at 750–850 m. We named this species in honor of Alexander Curt Brade, a botanist who contributed to the knowledge of this genus in Brazil in the last century.

Elaphoglossum commissurale Melo & Salino, ***sp. nov.*** TYPE.—BRAZIL. **Minas Gerais**: Santa Maria do Salto, Distrito de Talismã, Fazenda Duas Barras, 16° 23′ 54″ S, 40° 3′ 39″ W, 725m, 08 Mar 2004, *A. Salino 9496 et al.* (holotype: BHCB). **Fig. 1D–G**

Elaphoglossum pteropo C. Chr. et *E. ovalifolio* (Fée) Chirst affine, sed frondibus sterilibus nervo commisurali praeditis differt.

Plants terrestrial; *rhizome* creeping, 1.7–1.8 mm in diameter, densely scaly, scales lanceolate with acuminate and crispate apex, entire to ciliate at the base, light brown, 1.7–2.7 × 0.6–0.7 mm; *phyllopodia* 4–6 mm long; *sterile leaves* 3–3.7 mm apart, 10–20 cm long; *petioles* 2–4 cm × 0.6–0.75 mm, sulcate, yellowish, with scales lanceolate, 1.5–2.6 × 0.4–0.7 mm, ascending, entire to sparsely ciliate at the base, castaneous, point of attachment blackish; other scales stellate to amorphous; *blades* oblong-lanceolate, subchartaceous, base long cuneate (decurrent), apex acute to cuneate, margins glabrous, 8–16 × 0.9–2 cm; veins evident on abaxial surface, simple and furcate, ca. 1.5 mm apart, uniting to a marginal commissural vein, set at 50–55° to costae; *hydathodes* lacking; costae abaxially and adaxially with scales irregularly divided to amorphous, 0.2–1 mm long, castaneous; *fertile leaves* one time as long as the sterile ones, 16.5–30 cm long; *petioles* 10–19 cm × 0.5–0.7 mm; *blades* linear, 5–10 × 0.8–1 cm, base long cuneate, decurrent, apex cuneate, margins glabrous; costae with scales similar in shape to those of the sterile leaves; *intersporangial scales* absent.

Elaphoglossum commissurale is characterized by the commissural vein of the sterile fronds, and by the scales of the rhizome with a collapsed rounded cells in the base and apex. This species is most related to *E. pteropus* C. Chr. and *E. ovalifolium* (Fée) Christ. It is distinguished from *E. ovalifolium* which

has an oblong-elliptic blade, and lanceolate and blackish scales on the petiole and costae, whereas *Elaphoglossum commissurale* has a oblong-lanceolate blade, and castaneous scales on the petiole and costa abaxially. It is distinguished from *E. pteropus* with its rhizome scales lanceolate with margins entire to sparsely ciliate and veins ending free at the margin of the blade, whereas in *Elaphoglossum commissurale* the rhizome scales are densely ciliate at the base, and the veins unite to a marginal commissural vein. This species is endemic to Minas Gerais state near the border with Bahia state. It is known only from the type locality, where it grows in the Atlantic rainforest at 700–800 m. *Elaphoglossum commissurale* is named for its commissural vein.

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LITERATURE CITED

MICKEL, J. T. and SMITH, A. R. 2004. *The Pteridophytes of Mexico*. Memoirs of the New York Botanical Garden 88:1–1055.

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The Genus *Hymenasplenium* (Aspleniaceae) in Cuba, Including New Combinations for the Neotropical Species

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Abstract.—Morphological, cytological and taxonomical analyses of *Asplenium delitescens* and *A. laetum* in Cuba resulted in the recognition of both species belonging to the genus *Hymenasplenium* Hayata. The combinations of the Neotropical species to this genus are presented. Some remarks on gametophyte morphology of *H. delitescens* are included.

Key Words.—*Hymenasplenium*, Cuba, new combinations

Hymenasplenium was briefly described in Japanese by Hayata (1927), citing *Asplenium unilaterale* Lam. (from Tropical Africa, Southeastern Asia, Japan, Polynesia north to Hawaii, Iwatsuki, 1975), as the type species, based on the differences in the stelar morphology of rhizome. The stelar morphology of *H. unilaterale* (Lam.) Hayata and its allied species was studied by Iwatsuki and Kato (1975). They proposed that the dorsiventral stele anatomy of this group might have evolved from the radial dictyostele typical of *Asplenium* by elongation of the rhizome, probably as an adaptation to the petrophytic habitat. However, they considered it inappropriate to separate *Hymenasplenium* based exclusively on this feature. For that reason, Iwatsuki (1975) proposed to treat *Hymenasplenium* as a section within *Asplenium*.

Murakami (1992) studied the stelar structure of Neotropical species of sect. *Hymenasplenium* and concluded the basic stelar structure was identical to the Old World Tropic species. Later, in the monograph for this group in the Neotropics, Murakami and Moran (1993) followed the criteria of Iwatsuki (1975) of treating *Hymenasplenium* as a section of *Asplenium*.

The first molecular analysis conducted for the Neotropical species of sect. *Hymenasplenium* revealed that this group is a distinct and coherent clade that is sister to the rest of *Asplenium* (Murakami and Schaal, 1994). Subsequently, Murakami (1995) published a review of the studies on *Hymenasplenium*. In the presented molecular phylogenetic tree all paleo- and neotropical species formed a monophyletic group, which lead Murakami (1995) to propose again the recognition of *Hymenasplenium* as a genus, although without doing the formal combination of the species names. These studies did not include West Indian samples. Succeeding works on molecular data confirmed the

monophyletic status of *Hymenasplenium* (Murakami *et al.*, 1999; Gastony and Johnson, 2001; Schneider *et al.*, 2004). Schneider *et al.* (2004) also pointed out that no hybrids have been found between *Hymenasplenium* and *Asplenium* species, although hybrids are otherwise common in Aspleniaceae.

The genus *Hymenasplenium* is defined by the unusual dorsiventral vascular system in long creeping rhizomes, swollen petiole bases and a chromosome number of x=39, 38, which differ from the radially symmetrical steles, non swollen petiole bases and chromosome number of x=36 typical of *Asplenium* (Murakami and Moran, 1993).

In the taxonomic treatment of Aspleniaceae for the "Flora de la República de Cuba", we recognized only *Asplenium* L. and *Schaffneria* Fée ex T. Moore (Sánchez and Regalado, 2003). We recorded two Cuban species of sect. *Hymenasplenium*, *Asplenium delitescens* (Maxon) L. D. Gómez and *A. laetum* Sw. We decided to treat both species within *Asplenium* because we could not reliably verify the chromosome numbers of both species. The only cytological data so far available for *Asplenium laetum* was a report of a sexual diploid cytotype from Jamaica with 2n=72 (Walker, 1966), which corresponds with chromosome numbers found in *Asplenium* (Murakami and Moran, 1993). While *Asplenium laetum* is a rather common rupestral species growing along streams in evergreen mountain rain forests (Fig. 1), *A. delitescens* has not been collected in the Cuban territory since 1914. Even though it was described from Cuba (Maxon, 1908), no specimens were deposited in Cuban herbaria. We could only study the pictures of type specimens (US, NY) and two specimens at Stockholm (S), encompassing the total amount of known Cuban collections.

In 2004, we rediscovered a population of *Asplenium delitescens* in Eastern Cuba, giving us the opportunity to examine the chromosome number and the viability of the spores. We here present the results of our morphological, palynological, anatomical and cytological analyses of these species and their taxonomic position in regard to *Hymenasplenium* and some remarks on the

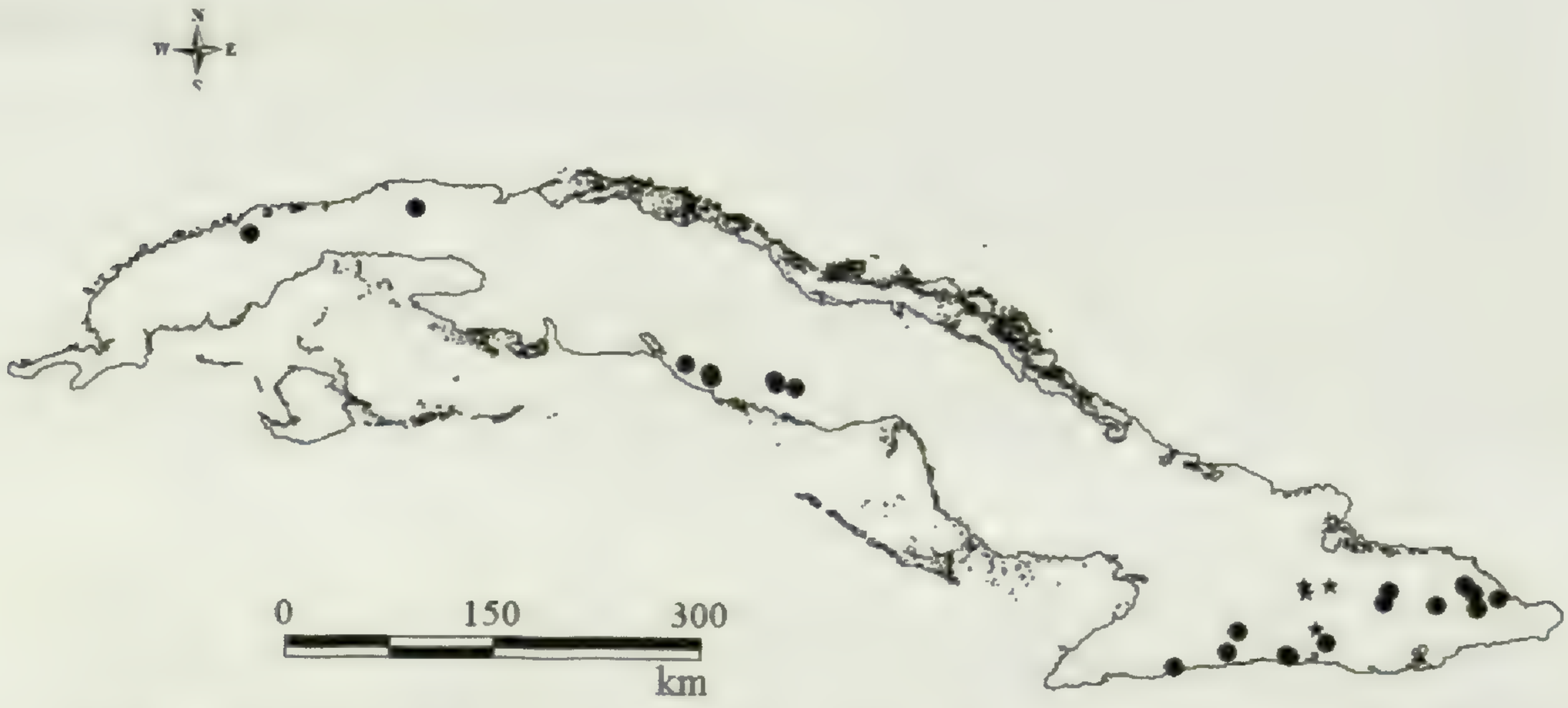


FIG. 1. Distribution of *Asplenium delitescens* (★) and *Asplenium laetum* (●) in Cuba.

gametophyte morphology of *A. delitescens*. The new combinations of Neotropical species to *Hymenasplenium* are also included.

Materials and Methods

The first specimen of *Asplenium delitescens* was collected in San Luis region in Eastern Cuba, by Pollard and Palmer in 1902, and formed the basis of the description of this species (Maxon 1908). It was also collected twice by Ekman, in Canapú, Sierra of Nipe, in woods over limestone in 1914. In November 2004, we found six individuals in the stream of La Caridad, behind the American's house in Sierra of Nipe, protected area "Pinares de Mayarí", growing terrestrially over a concave stone with abundant soil and humus in an evergreen forest near an intermittent stream at 341 m above sea level. One of those individuals (*Regalado et al. 42361* BSC, HAC, HAJB) was fragmented in three specimens deposited in HAC, HAJB, and BSC.

Specimens examined.—***Asplenium delitescens***: Cuba. **Holguín:** Oriente, Sierra de Nipe, ad cañon flumin. Canapú in rupibus calcar., 18.VIII.1914, *Ekman 2533* (S); Oriente, prope Bayate, Cayo del Rey, in cañon ad flum. Canapú, 6.IX.1914, *Ekman 2760* (S); Pinares de Mayarí, Sierra de Nipe, stream near La Caridad, near to Casa del Americano, 18.XI.2004, *Regalado et al. 42361* (BSC, HAC, HAJB). Honduras. Toledo District, on hill top in high ridge, Branch Hills, 8.I.1945, *Gentle 5139* (MO). Mexico. **Chiapas:** Marqués de Comillas, 6 km southeast from Ejido to Benemerito de las Américas, 9.X.1984, *Martínez 8136* (NY). **Oaxaca:** Dtto. Tuxtepec and 4 km west of Rt 175. ca. 100 m, 30.VII.1971. *Mickel 5784* (NY). ***Asplenium laetum:*** Cuba. **Pinar del Río:** Mil Cumbres (Chavarría Ranch), path from Mil Cumbres to La Altura, 29.III.2004, *Bécquer 81192, 81195* (HAJB). **La Habana:** Tapaste, San Rafael Hills, Callejón del matadero, 11.VI.1914, *León & Ekman 4294*, (HAC). **Cienfuegos:** San Blas, Santa Clara Prov., 16.III.1929, *J. G. Jack 7093* (AJBC). **Sancti Spiritus:** Escambray, head of the J. González stream, Cudina over limestone, *Caluff & Shelton s.n.* (BSC); Topes de Collantes, head of Caburní River, IV.1985, *Caluff 1370* (BSC); Escambray, Cudina, head of Canas River, south exposition, I.1986, *Caluff 2000, 2001* (BSC); Banao Hills, Tetas de Juana, summit, moist forest over the cliff, northern slope, 24.IX.1999, *Sánchez et al. 3025* (HAJB); Banao Hills, stream near Teta de Juana, IV.1994, *Caluff & Shelton3642* (BSC); Banao Hills, Plátanos glen, in a dry dale, 5.1995, *Caluff & Shelton4156* (BSC); Banao Hills, Cayajaná stream, V.1995, *Caluff & Shelton 4212* (BSC); Banao Hills, stream near the old way from Caja de Agua to Teta de Juana, 10.V.2000, *Regalado et al.42420* (HAC); Banao Hills, Plátanos glen, in a dry dale, 10.V.2000, *Regalado 90164, 90165* (MACB). **Guantánamo:** prope villam Monte Verde dictam, Cuba Orientali, I.1859, *Wright 1026* (HAC); Santo Domingo, Sabaneta, Hibrahím Sánchez Hills, in a stream, IV.1988, *Caluff & Motito 2704* (BSC); Viento Frío, stream between Vía Mulata and Barbudo River, IV.1988, *Caluff & Motito 2734* (BSC); Sierra del Purial, Viento Frío, banks of Barbudo River, IV.1992, *Caluff & Shelton3136* (BSC); Viento Frío, stream behind the farmhouse, IV.1992, *Caluff*

& Shelton3168, 3223 (BSC); Cuba Mountain slope, directly south of Jagüey, Yateras, Oriente; 420–510 m alt. on ground in rocky forest, rare, 24.IV.1907, *Maxon 4158* (S); Bayate, XII.1928, *Hioram Hno. 6990* (HAC); Bayate, XII.1923, *Hioram Hno. 9322* (HAC); Bayate, Mountains of Guantánamo (Oriente), XII.1923, *Hioram Hno. 16180* (HAC); Banks of Jaguaní River, between Cocalito and Los Lirios rivers, 100–160 m., 15.II.2004, *Caluff 42385* (HAC); Vázquez, stream between the coffee depulpery and Piloto River (National Park Alejandro de Humboldt), 1.IV.1999, *Sánchez & del Risco 77905, 77909, 77913, 77914, 77915* (HAJB); Yunque de Baracoa, ascension and summit (National Park Alejandro de Humboldt), 22.I.2002, *Sánchez et al. 79402, 79445* (HAJB); Yunque de Baracoa, slopes (National Park Alejandro de Humboldt), 23.I.2002, *Sánchez et al. 79495* (HAJB); Yunque de Baracoa, over limestone, 12.1985, *Caluff 1816* (BSC). **Holguín:** La Melba, III.1972, *Cárdenas s.n.* (HAC); Oriente, Sierra de Nipe, prope flumin Canapú, ad rupibus calcar., 1.VIII.1914, *Ekman 2531* (S); Oriente, Bayate, Cayo del Rey, in the cañon flumin Canapú, ad rup. calcar., 6.IX.1914, *Ekman 2749a* (S); La Melba, Palmares Stream over ultramaphic rocks, I.2001, *Sánchez et al. 78652, 78695* (HAJB); National Park Alejandro de Humboldt, Arroyo Bueno, La Melba, Facistor Stream (tributary of Jaguaní River), 5.II.2001, *Sánchez et al. 78844* (HAJB). **Santiago de Cuba:** Loma del Gato, Sierra Maestra, 1923, *Clemente Hno. 757* (S); Loma del Gato, 850 m, VIII.1923, *Clemente Hno. 784* (HAC); Ravin d'Arménie, roches Loma del Gato, 800 m, 6.1924, *Clemente Hno. 1194* (HAC); III Frente, El Julio, La Pimienta, Pozo Prieto, II.1991, *Caluff & Shelton3022* (BSC); S. Maestra, south of Turquino Edge Cardero Hill 2600 feet alt., 31.VII.1935, *Roig et al. 6612* (HAC); Sierra del Cobre, Loma San Juan, VII.1928, *Hioram Hno. 7127* (HAC); Loma San Juan, XII.1923, *Hioram Hno. 9321* (HAC); Sierra Maestra, edge of Sierra Maestra between Alcarraza and Punta de Lanza, IV.1971, *Bisse & Lippold 19634* (HAJB). **Prov. unknown:** Eastern Cuba (Lesquereux Collection), I.1859, *Wright 1086* (HAC).

Digital specimen images at the Herbarium Berolinense (B) (Röpert 2000) were consulted.

SAMPLES FOR STUDYING EPIDERMIS, INDUSIA AND STIPE CROSS SECTIONS.—***Asplenium delitescens:*** Regalado et al. 42361 (HAC), *Ekman 2533, 2760* (S), *Mickel 5784, Martínez 8136* (NY), *Gentle 5139* (MO); ***A. laetum:*** *Caluff 42385* (HAC), *Sánchez & del Risco 77905, 77909, 77915, Sánchez et al. 79402, Bécquer 81192, 81195* (HAJB).

SAMPLES FOR STUDYING SPORES (In bold specimens studied at Scanning Electron Microscope (SEM)).—***Asplenium delitescens:*** *Regalado et al. 42361* (HAC), *Ekman* ***2760*** (S); ***A. laetum:*** *Wright 1026, 1086, Regalado et al. 42420* (HAC), *Sánchez et al. 79402, 79495, Sánchez & del Risco 77905,* ***77909****, Bécquer 81192, 81195* (HAJB), *Regalado 90164, 90165* (MACB), *Maxon* ***4158*** (S).

Cytology.—For the cytological analysis, immature sporangia of *A. delitescens* were fixed in the field with glacial acetic acid/ethanol (1:3) and conserved at 4 °C until the processing for observing chromosomes in meiosis. The samples were gathered in Pinares de Mayarí, Sierra de Nipe, stream of La

Caridad, near American's house, 18 November 2004, 14:00 hours and 17 October 2005, 15:35 hours.

Sporangia were washed in 95% ethanol for 20 minutes, stained with Wittman's Hematoxylin for 12–24 hours, washed in glacial acetic acid for 5 minutes and mounted in Hoyer's mounting medium (Soriguer *et al.*, 1993).

Micromorphology.—Samples for studies of epidermis, indusia, and spores are listed after the examined specimens. Surfaces of spores were studied in dry samples from herbarium specimens fixed on stubs with double-sided tape and coated with gold palladium (Au/Pd, c. 20 nm) and examined using a SEM Jeol JSM 25 S-11 in the Laboratory of Palynology of the Swedish Museum of Natural History and a SEM Hitachi S-3000-N at the Real Jardín Botánico de Madrid. Values of stomata and spore length (major equatorial diameter) are reported as the average of 30 measures per sample; sizes are expressed as minimum, mean and maximum length. Terminology proposed by Punt *et al.* (1994) was followed for descriptions.

Anatomy of stipe cross sections.—Stipes were distally cut, about 2 mm below the basal pair of pinnae. Materials used for sectioning are cited after the examined specimens. They were fixed in formalin-acetic acid-alcohol solution and free-hand cross sections were made with razor blades. Petioles were cleared in 3% NaOCl solution for 3 minutes and were washed in distilled water for 2–3 minutes. Sections were stained with toluidine blue and mounted in permanent slides. For section descriptions, the terminology of Lin and De Vol (1977) was followed.

Spore sowing and gametophyte development.—Spores for cultures were taken from a sporophyte of *A. delitescens* (*Regalado et al. 42361*HAC). Spores were sown on mineral agar (Dyer, 1979) in Petri dishes (6 cm diameter) for the study of germination percentage and first stages of prothallial development. The cultures were kept in a growth chamber at 20°C at 12 hours of illumination with fluorescent tubes (28 $\mu Em^{-2}s^{-1}$)/12 hours dark overnight. These cultures were maintained with enough humidity to ensure sexual contact among the gametophytes. Gametophytes were stained with chloral hydrate acetocarmine (Edwards and Miller, 1972) and mounted in water for the morphological study. The classification of Nayar and Kaur (1968) for spore germination and the categorization of gametophyte type of development from Nayar and Kaur (1971) were followed.

Results

External morphology.—*Asplenium delitescens* and *A. laetum* share several morphological characters as having creeping rhizomes, swollen petiolar bases, 1-pinnate herbaceous laminae with scattered filiform scales over the rachises; veins 1–2 forked, sori occasionally diplazioid (Table 1). They differ in following characters: *Asplenium delitescens* has dull greenish brown petioles, lanceolate pinnae, and deltate, abruptly reduced laminae ending in a deltate apical portion, whereas *A. laetum* has lustrous dark brown petioles, trapezoid

TABLE 1. Morphological characters of *Asplenium delitescens* and *A. laetum*.

Morphological characters		*A. delitescens*	*A. laetum*
Rhizome	shape	creeping	short creeping
	diameter	−4 mm	2.5–3.5 mm
	color	reddish brown	reddish brown
	shape	linear-lanceolate	linear-lanceolate
	scales size (length × width)	0.5–2.5 × 0.4–2 mm	1.7–3.5 × 0.1–0.5 mm
Leaves	position	distant from each other	near to each other
	length	22–65 cm	13.6–55.3 cm
Petiole	color	dull greenish brown or stramineous	lustrous dark brown
	length	9–32 cm	3–18.8 cm
	diameter	1.5–2 mm	0.6–1.5 mm
Blade	degree of division	pinnate	pinnate
	shape	ovate to deltoid	lanceolate to oblong-lanceolate
	apex	attenuate, pinnatifid	acuminate, entire, often broken
	base	truncate, sometimes obtuse	mostly obtuse to truncate
	size (length × width)	13–33 × 12–28 cm	10.3–36.5 × 3.5–8.5 cm
	texture	herbaceous	herbaceous
Rachis	color	greenish	brown
	indumentum	brownish to blackish filiform scales and scarce pluricellular hairs	brownish to blackish filiform scales
Pinnae	shape	lanceolate	trapezoid dimidiate
	orientation with respect to rachis	ascending	patent
	apex	acute to attenuate	acute or obtuse
	petiolule	petiolulate	shortly petiolulate
	margin	denticulate	biserrate or crenate-serrate
	size (length × width)	7–12 × 1–2 cm	2–4.6 × 0.6–1.8 cm
	number of pairs	6–11	10–23
Venation	veins	2-forked	1-2-forked
Sori	shape	linear	linear
	number of pairs	5–8	1–8
	length	4–10 mm	2–7 mm
	position	medial	supramedial
Indusium	color	light brown	light brown
	margin	entire	entire
	width	0.3–0.6 mm	0.3–0.5 mm

dimidiate pinnae, and lanceolate or oblong-lanceolate, gradually reduced laminae with fragile pinnatifid apical portions (Fig. 2).

Micromorphology.—Both species have similar patterns in structure of scales, epidermis, indusia and spores. Typical features of Aspleniaceae are clathrate petiole scales with glandular tips when young. These are reddish brown, filiform linear-lanceolate in both species, but have entire margins in *A. laetum* and some marginal projections ending in glands in *A. delitescens* (Fig. 3). Epidermal cells have undulate anticlinal walls in each surface, and stomata are mainly basipolocytic. Anticlinal walls of the subsidiary cells are straight in *A. laetum* while they are slightly sinuous in *A. delitescens*. Stomata length is

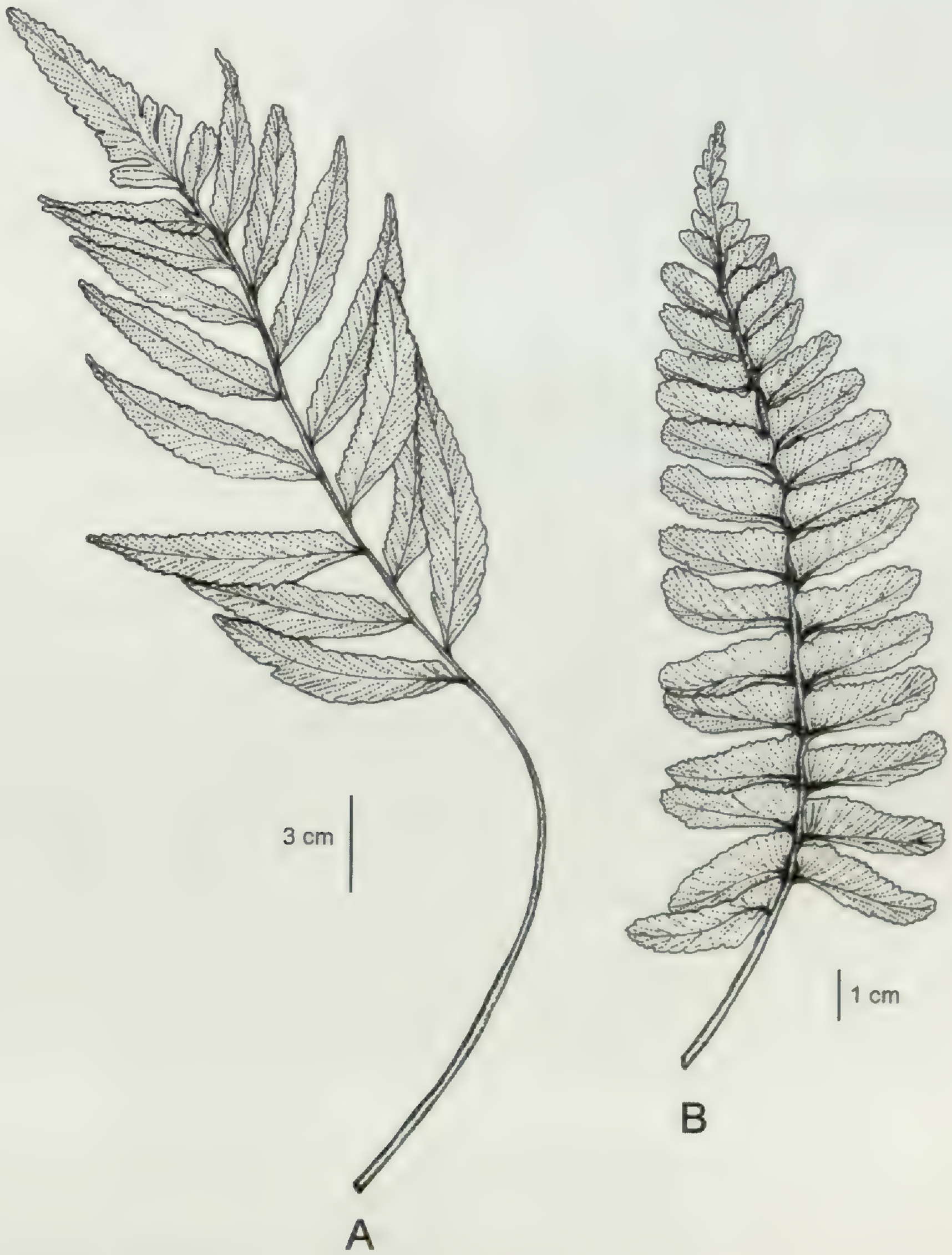
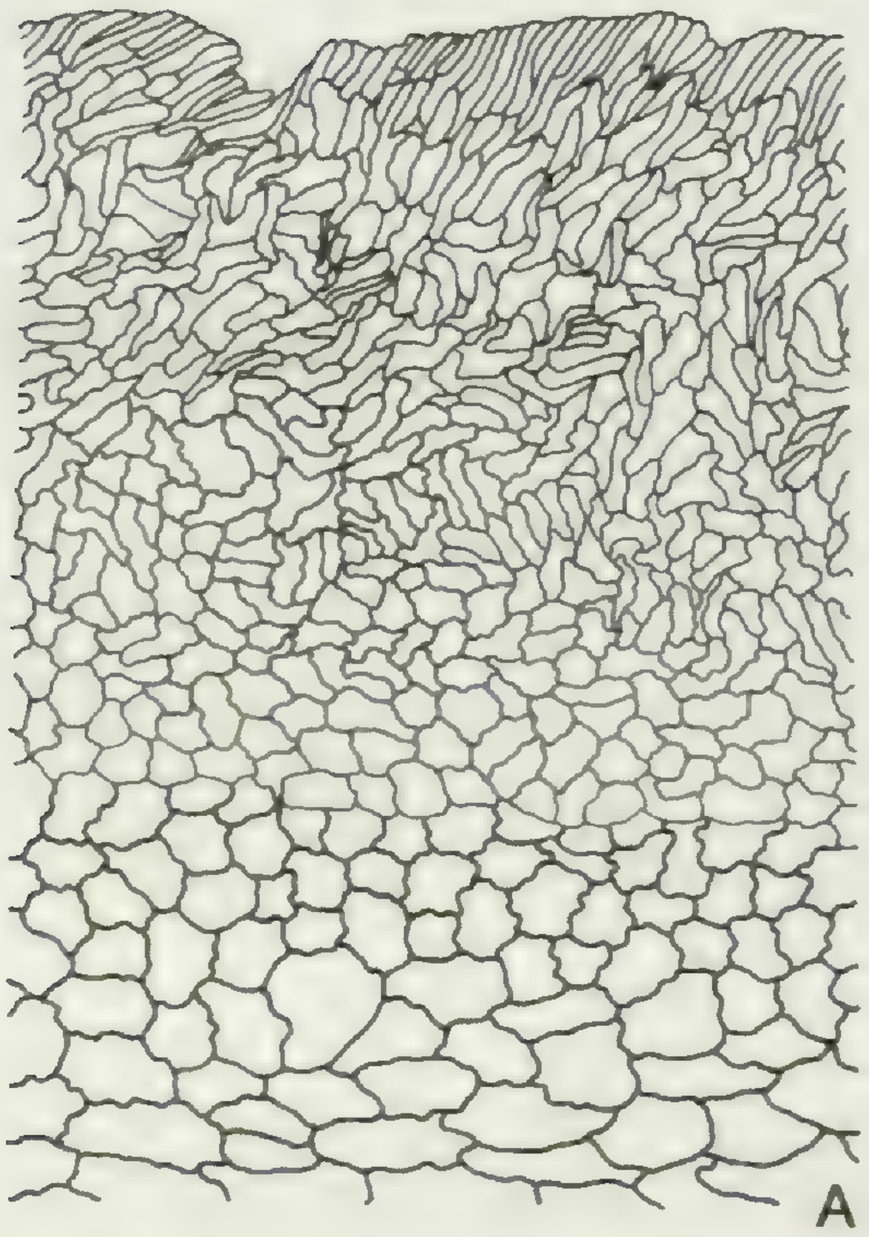


FIG. 2. Silhouettes of leaves of *Asplenium delitescens* (A) and *A. laetum* (B).

FIG. 3. Stem scales, cell structure. A. *Asplenium laetum* (78844, HAJB). B. *A. delitescens* (41361, HAC).



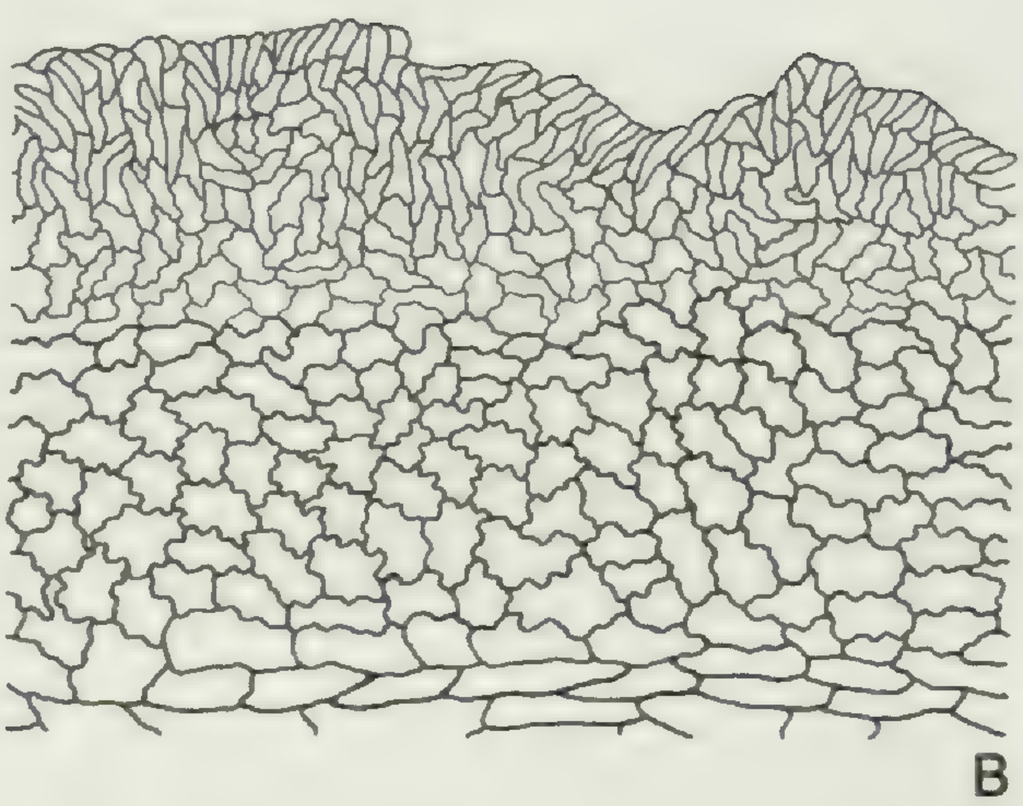


FIG. 4. Indusium. A. *Asplenium laetum* (79402, HAJB). B. *A. delitescens* (42361, HAC).

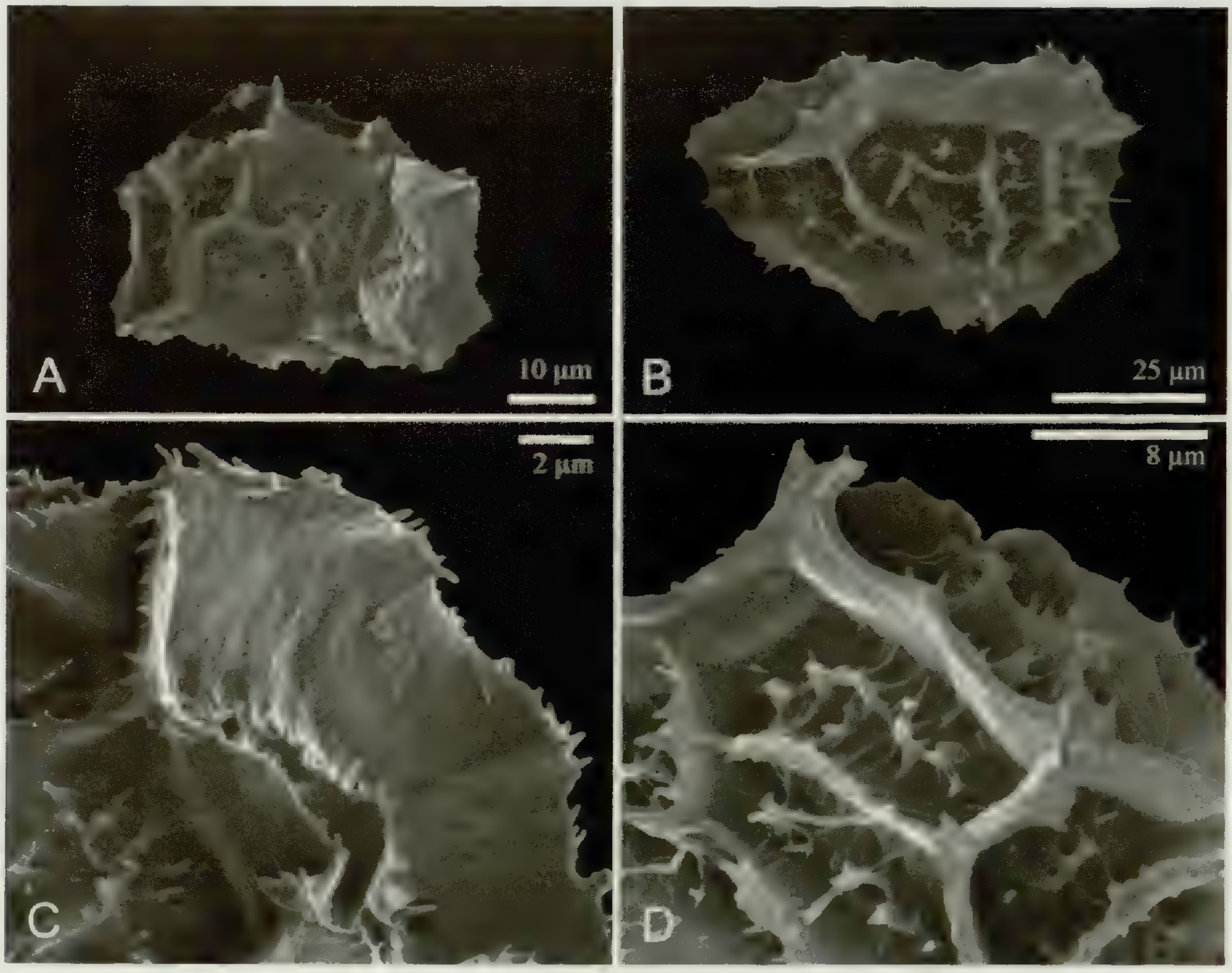


FIG. 5. Scanning electron micrographs of spores. A, C. *Asplenium delitescens* (2760, S). B, D. *A. laetum* (77909, HAJB).

(70–) 96.4 (–130) µm in *A. laetum* and (75–) 85.8 (–90) µm in *A. delitescens*. Abaxial surface is smooth in both species.

Indusia are structured into two different zones. The distal zone is composed of rectangular cells with slender anticlinal walls, irregularly situated. Proximal cells are rather radial symmetric and they are arranged with their major axis parallel to the indusial margin (Fig. 4). Upper indusium surface is also smooth in these species.

The spores of both species have an echinolophate perispore, with slim folds forming regular lacunae in *A. delitescens* and irregular ones in *A. laetum*. The surface of the lacunae is rugulate to microechinate (Fig. 5). Mean values of spore length measured along the major equatorial diameter were (30–) 34 (–35) µm in *A. delitescens* and (30–) 35.7 (–45) in *A. laetum*. Most of the examined samples of *A. laetum* presented abortive sporangia and spores.

Stipe anatomical characters.—Stipes of both species have oval transverse sections with a deep adaxial groove (Fig. 6). From periphery to center, stipe sections have a monostratified epidermal layer, with uniform polygonal isodiametric cells. The epidermis is covered with a thin and smooth cuticle. The ground tissue consists of several regularly thin-walled cells of parenchyma. A sclerenchyma zone of 4–5 cell layers is found in the abaxial cortex, near

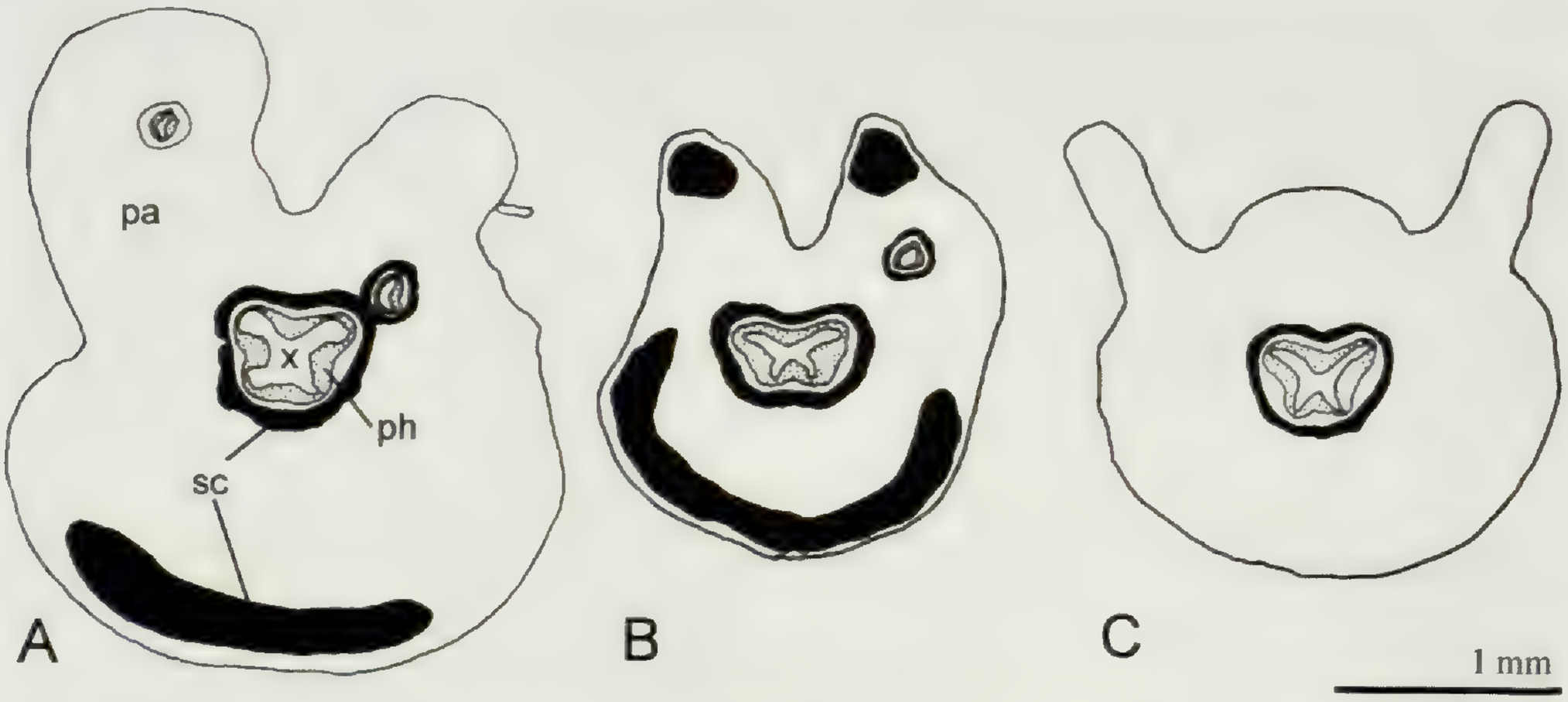


FIG. 6. Stipe cross sections of A. *Asplenium delitescens* (42361, HAC). B. *Asplenium laetum* (81195, HAJB). C. *Asplenium abscissum* (Caluff 42387, HAC). pa: parenchyma. sc: sclerenchyma. ph: phloem. x: xylem.

to the epidermis. Sclerenchyma also forms a ring that surrounds the central vascular bundle. One to two small bundles can be found in the adaxial groove sides. These bundles are enclosed by a single-layered endodermis. An internal X shaped xylem strand (curved-shaped xylem strand in smallest bundles) is surrounded by phloem (Fig. 6) and parenchyma.

Cytology.—Meiosis in *Asplenium delitescens* was studied in four spore mother cells, from a plant fixed on 17 October 2005 and showed 78 regular pairs (Fig. 7), being tetraploid, with basic chromosome number of 39.

Spore germination and gametophyte development of Asplenium delitescens.—Spore germination rate was 20%, which can be considered a low percentage. One reason for this fact could be that spores were somewhat

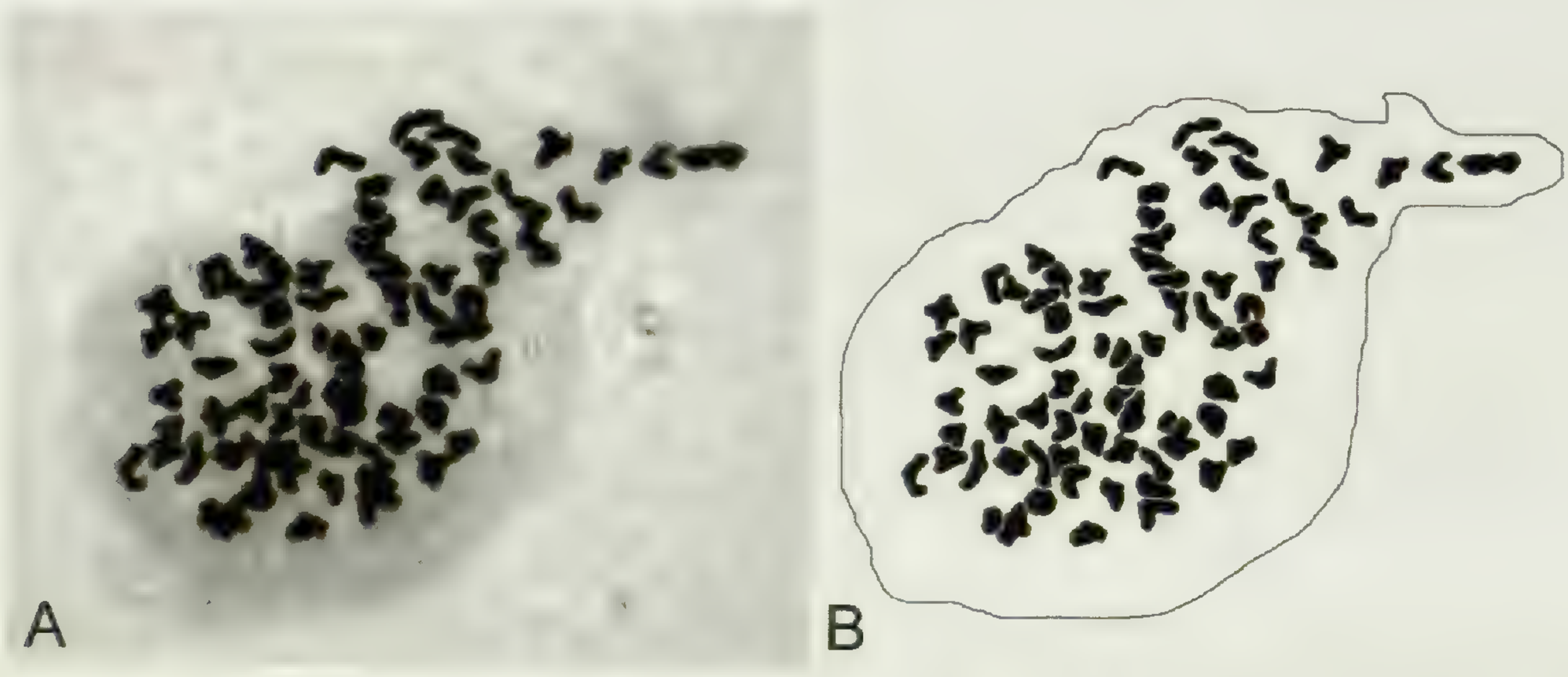


FIG. 7. Chromosomes in meiosis of *A. delitescens* (42361, HAC). A. Picture of spore mother cell (1000 X). B. Explanatory diagram. Some of the bivalents are shown partially because of superimposition.

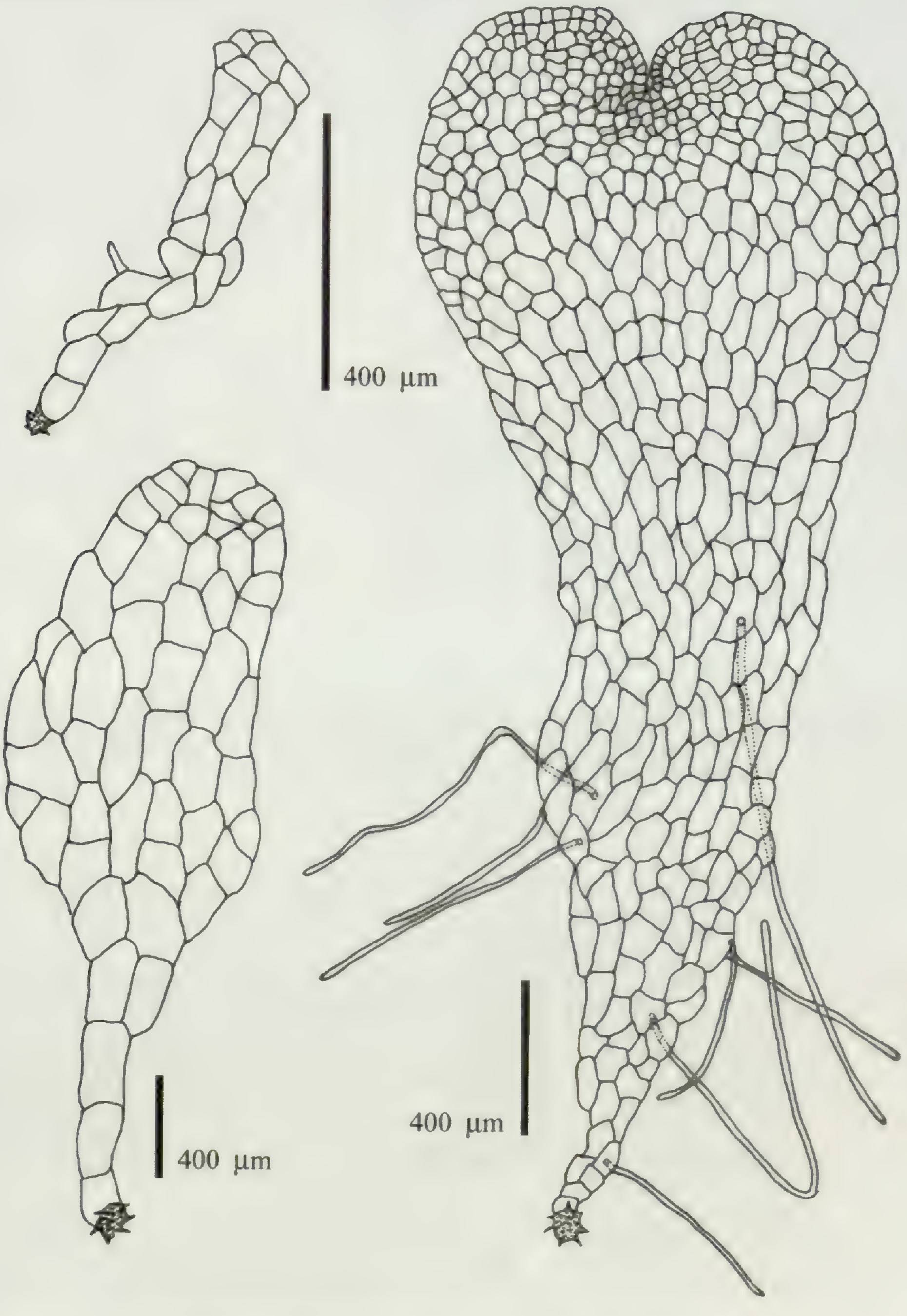


FIG. 8. First stages of gametophyte morphology of *A. delitescens* (42361, HAC).

immature at time of harvest. Spore germination follows the *Vittaria* type and the gametophyte pursues the *Adiantum* type of development, according to the classification of Nayar and Kaur (1968, 1971). A cuneiform submarginal cell originates the meristem; the bidimensional phase starts with the longitudinal

division of a subapical cell in a filament of two or three cells. Mature prothalli have the typical cordate shape, a few longer than broad in first stages, and gametangia of both sexes occur mixed in the central stripe (Fig. 8). Gametophyte morphology of *H. delitescens*, observed from lab cultures reveals the typical cordate-shaped gametophytes, characteristic of terrestrial species, according to Farrar *et al.* (2008). Although both antheridia and archegonia of normal aspect were formed in gametophytes of *A. delitescens*, no sporophytes were produced in cultures after two years from spore sowing.

Discussion

External morphology and stipe anatomy.—Characters shared by *Asplenium delitescens* and *A. laetum* are creeping (always short creeping in *A. laetum*) rhizomes, swollen petiole bases, and occasionally diplazioid sori, which are also defining characteristics of the genus *Hymenasplenium* (Iwatsuki, 1975). Other characters that relate these species to *Hymenasplenium* are the adaxially shallowly grooved rachises and costae, which were first observed in Asiatic species by Iwatsuki (1975). Murakami and Moran (1993) used this character in a comparison between the Neotropical species *A. delitescens* and *A. abscissum* Willd. These species have very similar laminar architecture and pinnae shape and can be confused if the rhizomes are absent. *Asplenium abscissum*, as other *Asplenium* species, has erect rhizomes and rounded cross sections of stipes, rachises and costae even in dried specimens, with adaxial wings of parenchyma, while *A. delitescens* and *A. laetum* have adaxially grooved, not winged, stipes, rachises and costae (Fig. 6).

Spores and chromosome number.—A common spore ornamentation pattern (Murakami and Moran, 1993) was observed in both species. Most of the Cuban specimens of *Asplenium laetum* are sterile hybrids producing a high percentage of aborted spores. From 12 examined samples, only three specimens *Maxon 4158* (S) and *Sánchez et al. 79402, 79495* (HAJB) show some well-formed spores. Murakami and Moran (1993) cited 16 specimens from Belize, Costa Rica, Venezuela, Colombia, Ecuador, Peru and Bolivia as presumable hybrids, having intermediate morphology between *Asplenium delitescens* and *A. laetum*, most of them with aborted spores. However, 10 Cuban specimens (*Wright 1026* (G, K, L, MO, NY, S, UC) and *Wright 1086* (BM, G, K)) were cited by Murakami and Moran (1993) as *Asplenium laetum*, not under the hybrid section of their monograph. Our examined samples of *Wright 1026, 1086* (HAC) had collapsed sporangia and malformed spores. Since all these specimens probably belong to the same populations, this fact suggests that normal and hybrid specimens can be found living together.

From 24 described species of the genus/sect. *Hymenasplenium*, 67% have been checked for their chromosome number: 50% of them have x=39, 13% show x=36 (*A. laetum, A. triquetrum* N. Murak. & R. Moran and *H. costarisorum* N. Murak. & X. Cheng), 4% possess x=38 (*H. subnormale* Copel.) and 33% remain unchecked. This unchecked percentage belongs mainly to Neotropical species. Our chromosome counts of x=39 for *Asplenium*

delitescens agree with the reported number for *Asplenium repandulum* Kunze (Smith and Mickel, 1977, rectified by Murakami, 1995 as *A. riparium* Liebm.), *Hymenasplenium cataractarum* (Rosenst.) N. Murak., *H. hondoense* (N. Murak. & Hatanaka) T. Nakaike and *H. obliquissimum* Hayata (Mitui *et al.*, 1989), *H cardiophyllum* (Hance) T. Nakaike (Kato *et al.* 1990), *A. excisum* C. Presl, *H. apogamum* (N. Murak. & Hatanaka) T. Nakaike, *H. cheilosorum* (Kunze ex Mett.) Tagawa, *H. latipinnum* N. Murak. & X. Cheng, *H. laterepens* N. Murak. & X. Cheng and *H. obscurum* (Blume) Tagawa (Cheng and Murakami, 1998). The reports of a sexual diploid cytotype of *Asplenium laetum* from Jamaica (John Crow Mountains) in two specimens with 2n=72 and n=36 (Walker, 1966) and a tetraploid 2n=144, n=36 for *A. triquetrum* in Misiones, Argentina (Guillén and Daviña, 2005), are divergent from the characteristic n=39 usually found in the genus/sect. *Hymenasplenium* and would speak in favor for keeping *Asplenium*. Cheng & Murakami (1998), also reported sexual diploids (n=36) and tetraploids (n=72) of *H. costarisorum* from southwestern China. However, regarding the position of *H. costarisorum* and *A. laetum* in the molecular phylogeny obtained from *rbcL* sequences by Murakami (1995), the chromosome number x=36 can not be considered a plesiomorphic state of this character in *Hymenasplenium*, but could be a reversion or a convergence from x=39 to x=36, as it was interpreted by Cheng and Murakami (1998).

Murakami and Moran (1993) stated that *Asplenium laetum* is the most common and widely distributed species in the New World, ranging from Mexico to northern Argentina. They identified some variation correlated with geography in continental Neotropical territories, in characters such as pinnae apex and pinnae margins, and affirmed that future studies could reveal that *A. laetum* consists of several species. In Cuba, *Asplenium laetum* could hybridize with other species of the genus *Asplenium*, which seems possible because they share the same chromosome base number, considering the report of n=36 for Jamaican specimens (Walker, 1966) or with *Asplenium delitescens*, although different chromosome base numbers could result in a lower probability of hybridism. However, there is no morphological evidence of intermediate characters in Cuban examined specimens of *A. laetum* that permit identification of probable parental species. Putative hybrids of *A. laetum* occur equally frequently in the three distinct ranges in Cuba (Fig. 1). Distances among the ranges and with other islands of the Greater Antilles or the continental areas of Central America are not a barrier to the high dispersal capacity of fern spores (Tryon, 1970, 1979); therefore any continental or Greater Antilles species has the same opportunity of being one of the parental species of these hybrids. In addition, phylogenies of *rbcL* sequences by Murakami and Schaal (1994) and Murakami *et al.* (1999) only reflect the maternal line, and samples may not been checked for putative hybridism. A cytological and nuclear phylogenetic study, covering the whole distribution area of *A. laetum*, is needed to validate an expected different position of this species, based on a hypothesis of hybrid origin. These analyses including more species would allow also us to

understand whether the n=36 reported for some *Hymenasplenium* species is a reversion or a convergence from x=39 to x=36.

Taxonomical Section.—In this work we agree with previous studies (Murakami, 1995; Murakami *et al.*, 1999; Schneider *et al.*, 2004 and Smith *et al.*, 2006) to recognize *Hymenasplenium* at a generic rank. The formal combination to *Hymenasplenium* of all 11 Neotropical species is presented below.

Hymenasplenium laetum (Sw.) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium laetum* Sw., Syn. Fil.: 79: 271. 1806. TYPE. — [icon] Schkuhr, 24: 103. Kl. Linn. Pfl.-Syst. t. 70. 1806–1809 (neotype: [designated by Proctor 1985: 371]). = *Asplenium salicifolium* var. *krugii* H. Christ, Bot. Jahrb. Syst. 24: 103. 1897. TYPE.—CUBA. **Guantánamo**: "ad Jagüey", 500 m, "In silvestr.", VIII.1889, *Eggers 4927* (lectotype: [designated by Sánchez and Regalado 2003: 32] P [photo]).

DISTRIBUTION.—Mexico, Continental Tropical America, Greater and Lesser Antilles, Trinidad, Tobago, Tropical Africa and Madagascar.

Hymenasplenium delitescens (Maxon) L. Regalado & C. Prada, **comb. nov.** ≡ *Diplazium delitescens* Maxon, Contr. U.S. Natl. Herb. 10: 497, t. 56, f. 1. 1908. ≡ *Asplenium delitescens* (Maxon) L. D. Gómez, Brenesia 8: 52. 1976. TYPE.—CUBA. **Santiago:** "Vicinity of San Luis", 15–18.II.1902, *Pollard & Palmer 348* (holotype: US 403261 [photo]; isotypes: MO 1875901 [photo], NY 127304 [photo]).

DISTRIBUTION.—Mexico, Continental Tropical America and Cuba.

Hymenasplenium hoffmannii (Hieron.) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium hoffmannii* Hieron., Hedwigia 60: 258. 1919. TYPE.—COSTA RICA. **Aguacate:** VIII.1857, *Hoffmann 836* (holotype: B [photo]; isotype: NY 149237 [photo]).

DISTRIBUTION (as reported by Murakami and Moran, 1993).—Mexico, Guatemala, Honduras, El Salvador, Nicaragua, Costa Rica, Panamá, Colombia, Venezuela and Trinidad.

Hymenasplenium obtusifolium (L.) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium obtusifolium* L. Sp. Pl. 1080. 1753. TYPE.—Petiver, Pter. Amer. 117, t.2, fig 14. [Plumier, Traité Foug. Amér. t.67.1705] (erroneously cited as f. 4 in the original description). Based on a plant from Morne de la Calebasse, Martinique.

DISTRIBUTION (as reported by Murakami and Moran, 1993).—Panamá, Jamaica, Puerto Rico, Lesser Antilles, Colombia, Venezuela and Trinidad.

Hymenasplenium ortegae (N. Murak. & R. C. Moran) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium ortegae* N. Murak. & R. C. Moran, Ann. Missouri Bot. Gard. 80: 23, fig. 11 b, c. 1993. TYPE.—VENEZUELA. **Paéz**: Apure, Campamento de Corpo Andes, along Río Arauca at Colombian border, near Torunos. 200 m. (656.2 ft.), 29.VI.1983, *van der Werff & González 4599* (holotype: MO n.v.; isotype: NY 149265 [photo]).

DISTRIBUTION (as reported by Murakami and Moran, 1993).—Venezuela, Colombia, Ecuador, Peru and Brazil.

Hymenasplenium purpurascens (Mett. ex Kuhn) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium purpurascens* Mett. ex Kuhn, Linnaea 36: 102. 1869. TYPE.—ECUADOR. **Chimborazo:** "ad pedem montis Chimborazo", *Spruce 5697* (holotype: B [photo]).

DISTRIBUTION (as reported by Murakami and Moran, 1993).—Ecuador.

Hymenasplenium repandulum (Kunze) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium repandulum* Kunze, Linnaea 9: 65. 1834. TYPE.—PERU. **Huánuco**: Pampayaco, in sylvis montosis ad arborum truncos, VII.1829, *Poepigg s. n.* (holotype: B n.v.; isotype: NY (a pinna) 149290 [photo]).

DISTRIBUTION (as reported by Murakami and Moran, 1993).—Ecuador and Peru.

Hymeasplenium riparium (Liebm.) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium riparium* Liebm. Mexic. Bregn. (seors. 92): 244. 1849. ≡ *Asplenium obtusifolium* var. *riparium* (Liebm.) Domin, Pterid. Isl. Dominica, Rozpr. Král. Ceské Spolecn. Nauk. Tr. Mat.- Prir., Nov. Rad. 2: 175. 1929. TYPE.—MEXICO. **Veracruz**: Hacienda de Jovo, *Liebmann s. n.* [Pl. Mex. 310] (lectotype [designated by Smith 1981: 51]: C n.v.).

DISTRIBUTION (as reported by Mickel and Smith, 2004).—Mexico, Guatemala, Belize, Honduras, Nicaragua, Costa Rica, Panama, Colombia, Venezuela and Ecuador.

Hymenasplenium triquetrum (N. Murak. & R. C. Moran) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium triquetrum* N. Murak. & R. C. Moran, Ann. Missouri Bot. Gard. 80: 31, fig. 8 b, c. 1993. TYPE.—BOLIVIA. La Paz: Prov. **Nor-Yungas:** Polo-Polo bei Coroico [Im tiefen Schatten des Hochwaldes oder auf nassem Boden], 1100 m, 10.XI. 1912, *Buchtien 625* (holotype: MO n.v.; isotypes: NY [photo], BM, K, Z n.v.).

DISTRIBUTION (as reported by Murakami and Moran, 1993).—Bolivia and Brazil.

Hymenasplenium volubile (N. Murak. & R. C. Moran) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium volubile* N. Murak. & R. C. Moran, Ann. Missouri

Bot. Gard. 80: 32, fig. 13 e. 1993. TYPE.—ECUADOR. **Cotopaxi**: Quevedo-Latacunga road, km 46 from Quevedo, NE exposed slopes with rainforest, 600 m, 0°55′S, 79°11′W, 4.IV.1973, *Holm-Nielsen et al. 2905* (holotype: 2914385 MO [photo], isotypes: AAU, F, UC n.v.).

DISTRIBUTION (as reported by Adams, 1995).—Costa Rica, Panama, W Colombia, W Ecuador.

Hymenasplenium basiscopicum (R. C. Moran & M. A. Sundue) L. Regalado & C. Prada, **comb. nov.** ≡ *Asplenium basiscopicum* R. C. Moran & M. A. Sundue, Brittonia 56: 124. 2004. TYPE.—BOLIVIA. **Santa Cruz**: Prov. Ichilo, Parque Nacional Amboró, steep slopes above and 1 km S of Río Saguayo, 750 m, 17°41′S, 63°44′W, 20.I.1988, *M. Nee 36020* (holotype: NY [photo], isotypes: LPB, MO n.v.).

DISTRIBUTION (as reported by Moran & Sundue, 2004).—Bolivia.

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LITERATURE CITED

ADAMS, D. B. 1995. *Asplenium*. *In:* G. Davidse, et al. (ed.). Flora Mesoamericana, Psilotaceae a Salviniaceae, 1:290–324, Universidad Nacional Autónoma de México, México.

CHENG, X. and N. MURAKAMI. 1998. Cytotaxonomic study of Genus *Hymenasplenium* (Aspleniaceae) in Xishuangbanna, Southwestern China. J. Plant Research 111:495–500.

DYER, A. F. 1979. The culture of fern gametophytes for experimental investigation. *In:* Dyer, A. F. (ed.) *The experimental biology of ferns*, 253–305, London Academic Press.

EDWARDS, M. E. and J. H. MILLER. 1972. Growth regulation by ethylene in fern gametophytes. III: Inhibition of spore germination. Amer. J. Bot. 59:458–465.

FARRAR, D. R., C. DASSLER, J. E. WATKINS JR. and C. SKELETON. 2008. Gametophyte Ecology. *In:* Ranker, T. A. and C. H. Haufler (eds). *Biology and Evolution of ferns and Lycophytes*, 222–256, Cambridge University Press.

GASTONY, G. J. and W. P. JOHNSON. 2001. Phylogenetics placements of *Loxoscaphe thecifera* (Aspleniaceae) and *Actinopteris radiata* (Pteridaceae) based on analyses of *rbcL* nucleotide sequences. Amer. Fern J. 91:197–213.

GUILLÉN, R. H. and DAVIÑA, J. R. 2005. Estudios cromosómicos en especies de *Asplenium* (Aspleniaceae) de la Argentina. Darwiniana 43:44–51.

HAYATA, B. 1927. On the systematic importance of the stelar system in the Filicales, 1. Bot. Mag. Tokyo 41:697–718.

IWATSUKI, K. 1975. Taxonomic studies of Pteridophyta X. Acta Phytotax. Geobot. 27:39–54.

IWATSUKI, K. and M. KATO. 1975. Stelar structure of *Asplenium unilaterale* and its allied species. Kalikasan 4:165–174.

KATO, M., NAKATO, N. AKIYAMA, S. and K. IWATSUKI. 1990. The systematic position of *Asplenium cardiophyllum* (Aspleniaceae) Bot. Mag. Tokyo 103:461–468.

LIN, B. L. and C. E. DE VOL. 1977. The use of stipes characters in fern taxonomy. I. Taiwania 22:91–99.

MAXON, W. R. 1908. Studies of Tropical American Ferns. No. 1. *Diplazium delitescens*. Contr. U.S. Nat. Herb. 10:497.

MICKEL, J. T. and A. R. SMITH. 2004. *The pteridophytes of Mexico*. Mem. New York Bot. Gard. 88.

MITUI, K., MURAKAMI, N. and K. IWATSUKI. 1989. Chromosomes and systematics of *Asplenium* sect. *Hymenasplenium* (Aspleniaceae). Amer. J. Bot. 76(11):1689–1697.

MORAN, R. C. and M. A. SUNDUE. 2004. *Asplenium* (sect. *Hymenasplenium*) *basiscopicum*, a new species from Bolivia. Brittonia 56:124–127.

MURAKAMI, N. 1992. Stelar structure of *Asplenium obtusifolium* and Its Allied Species in the New World Tropics, with Comparison to the Asian Members of *Asplenium* sect. *Hymenasplenium*. Bot. Mag. Tokyo 105:135–147.

MURAKAMI, N. 1995. Systematics and Evolutionary Biology of the Fern Genus *Hymenasplenium* (Aspleniaceae). J. Plant Res. 108:257–268.

MURAKAMI, N. and R. C. MORAN. 1993. Monograph of the Neotropical species of *Asplenium* sect. *Hymenasplenium* (Aspleniaceae). Ann. Missouri Bot. Gard. 80(1):1–38.

MURAKAMI, N. and B. A. SCHAAL. 1994. Chloroplast DNA variation and the phylogeny of *Asplenium* sect. *Hymenasplenium* (Aspleniaceae) in the New World tropics. J. Plant Research 107:245–251.

MURAKAMI, N., NOGAMI, S. WATANABA, M. and K. IWATSUKI. 1999. Phylogeny of Aspleniaceae inferred from rbcL nucleotide sequences. Amer. Fern J. 89(4):232–243.

NAYAR, B. K. and S. KAUR. 1968. Spore germination in homosporous ferns. Journal of Palynology 4:1–14.

NAYAR, B. K. and S. KAUR. 1971. Gametophytes of homosporous ferns. Botanical Review 37(3):296–396.

PROCTOR, G. R. 1985. *Ferns of Jamaica*. Br. Mus. (Nat. Hist.), London.

PUNT, W., BLACKMORE, S., NILSSON, S. and A. LE THOMAS. 1994. *Glossary of Pollen and Spore Terminology*. LPP Contrib, Ser. No. 1, LPP Found., Utrech.

RÖPERT, D. (ed.) 2000- (continuously updated), Digital specimen images at the Herbarium Berolinense- Published on the Internet http://ww2.bgbm.org/herbarium/ (Barcode: B 20 0053513 / ImageId: 263518) [accessed 11-Apr-11].

RÖPERT, D. (ed.) 2000- (continuously updated), Digital specimen images at the Herbarium Berolinense- Published on the Internet http://ww2.bgbm.org/herbarium/ (Barcode: B 20 0016097 / ImageId: 263074) [accessed 11-Apr-11].

SÁNCHEZ, C. and L. REGALADO. 2003. Aspleniaceae. Flora de la República de Cuba. 8(1):1–65, Koeltz Scientific Books, Königstein.

SCHNEIDER, H., RUSSELL, S. J., COX, C. J., BAKKER, F., HENDERSON, S., RUMSEY, F., BARRETT, J., GIBBY, M. and G. C. VOGEL. 2004. Chloroplast Phylogeny of Asplenioid Ferns based on *rbcL* and *trnL-F* Spacer Sequences (Polypodiidae, Aspleniaceae) and its Implications for Biogeography. Systematic Botany 29(2):260–274.

SMITH, A. R. and J. T. MICKEL. 1977. Chromosome counts for Mexican ferns. Brittonia 29:391–398.

SMITH, A. R., PRYER, K. M., SCHUETTPELZ, E., KORALL, P., SCHNEIDER, H. and P. G. WOLF. 2006. A classification for extant ferns. Taxon 55:705–731.

SORIGUER, R. C., FONDOS, P., MARTíNEZ, T., GARCÍA, E., A. LAZO and A.. 1993. Utilización de las colecciones de epidermis vegetales, sus posibles aplicaciones y problemas de conservación. International Symposium and First Congress on preservation and conservation of Natural History Collections. 1:113–118.

TRYON, R. M. 1970. Developmental and evolution of fern floras of oceanic islands. – Biotropica 2:76–84.

TRYON, R. M. 1979. Biogeography of the Antillean Fern Flora. In: Bramwell, D. (ed.). *Plants and Islands*, 55–68, London Academic Press.

WALKER, T. G. 1966. A cytotaxonomic survey of the Pteridophytes of Jamaica. – Trans. Roy. Soc. Edin. 66:169–237.

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Studies on the Genus *Bolbitis* (Dryopteridaceae) from Vietnam and Laos

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ABSTRACT.—The genus *Bolbitis* from Vietnam and Laos is revised; 14 species and two varieties are recognized. A new species, *Bolbitis lanceolata* S. K. Wu & J. Y. Xiang is described and illustrated. *Bolbitis scandens* W. M. Chu ex Ching et C. H. Wang and *B. yunnanensis* Ching ex Ching et C. H. Wang are two new records in both Vietnam and Laos. *Bolbitis hekouensis* Ching and *B. latipinna* Ching are treated as synonyms for the first time.

KEY WORDS.—Bolbitoid ferns, Dryopteridaceae, Vietnam, Laos, taxonomic review

The genus *Bolbitis* was established by Schott in 1834, to include species with a creeping rhizome and anastomosing veins. Simultaneously Schott established *Egenolfia* to describe the species with free veins. The first monograph of the genus *Egenolfia* was completed by Ching (1931), in which nine species and one variety, including two species from Vietnam were recognized. In the Flore Générale de I'Indo-Chine, C. Christensen and Tardieu-Blot (1941) described 12 species of *Bolbitis* and *Egenolfia*. Iwatsuki (1959) revised the Japanese species and treated *Egenolfia* as a synonym of *Bolbitis*. In a broader study on *Bolbitis*, Hennipman (1977) accepted Iwatsuki's generic delimitation, completed a taxonomic review for the genus *Bolbitis*, recognized 44 species, among which 12 species and three varieties were distributed in the Indo-China peninsula. Recently, Phan (1998) recorded 14 species (varieties) of *Bolbitis* and two species of *Egenolfia* from Vietnam.

Moran *et al.* (2010a) studied the phylogeny of the bolbitidoid ferns using two non-coding chloroplast spacers: *trnL-trnF* and *rps4-trnS* with samples from 57 species. They found that traditionally recognized *Bolbitis* was resolved as polyphyletic, with the Neotropical species sister to *Elaphoglossum*, separate from the other species of *Bolbitis*. Moran *et al.* (2010b) recognized the Neotropical clade as a new genus, *Mickelia*, including 10 species and one hybrid, leaving *Bolbitis* as a pantropical genus of about 50 species.

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From 2004 to 2008, five botanical expeditions were conducted by the authors in both Vietnam and Laos. Nearly 10,000 specimens were collected during these expeditions, with nearly 250 of them belonging to *Bolbitis*. A taxonomic revision of *Bolbitis* is made accordingly, based on field observations and herbarium study. Based on these studies, 14 species and two varieties from Vietnam and Laos are recognized, including one new species, two new records and two new combinations as follows.

The species reported in our study different from those of Phan (1998) in several ways. *Bolbitis annamensis* Tardieu-Blot & C. Chr. has been subsumed in *B. heteroclita* (Presl) Ching, *B. prolifera* (Fée) C. Chr. & Tard. has been subsumed in *B. virens* (Hook. & Grev.) Schott, and *Egenolfia asplenifolia* Fée has been subsumed in *Bolbitis appendiculata* (Willd.) K. Iwats. as Hennipman (1977) suggested. *Bolbitis cadieri* (C. Chr.) Ching, which was suggested by Hennipman (1977) as a possible hybrid, has not been seen in our fieldwork. *Bolbitis crispatula* (Copel.) Ching var. *copelandii* (Ching) Hennipman is accepted as *B. copelandii* Ching ex C. Chr. & Tardieu. Two new records are here reported, *Bolbitis scandens* W. M. Chu ex Ching et C. H. Wang and *B. yunnanensis* Ching ex Ching et C. H. Wang. *Bolbitis semicordata* (Bak.) Ching is a species distributed in South India, we have not seen it and doubt the validity of the record. *Egenolfia sinensis* (Bak.) Maxon should be *Bolbitis sinensis* (Baker) K. Iwats. *Bolbitis prolifera* (Fée) C. Chr. & Tard. has been subsumed in *B. virens* as Hennipman suggested.

Key to the species of *Bolbitis* in Vietnam and Laos

1. Veins free
   2. Perisopre reticulate; lateral pinnae subentire or crenate, apex obtuse or acute
      3. Stipe and rachis subglabrous; base of pinnae asymmetrical; fertile pinnae ovate or oblong . . . . . ***B. appendiculata***
      3. Stipe and rachis densely scaly; base of pinnae symmetrical; fertile pinnae moniliform . . . . . ***B. hookeriana***
   2. Perispore cristate-undulate; lateral pinnae pinnatifid, apex acuminate
      4. Stipe scaly at the base, rachis and costa very sparsely scaly; lateral sterile pinnae less than 20 pairs; pinnae subentire or shallowly lobed . . . . . ***B. sinensis***
      4. Stipe, rachis and costa underneath densely scaly; lateral sterile pinnae more than 30 pairs; pinnae lobed more than halfway towards the costa. . . . . ***B. tonkinensis***
1. Veins more or less anastomosing
   5. Areoles with free included veinlets
      6. Veins forming a costal areole, otherwise free; terminal segment similar to the lateral ones
         7. Fertile pinnae more or less pteridoid, sporangia inserted along the margin only . . . . . ***B. copelandii***
         7. Fertile pinnae acrostichoid, sporangia inserted all over the lower surface. . . ***B. crispatula***
      6. Veins forming 2 to more areoles
         8. Terminal segment similar to the lateral ones. . . . . ***B. yunnanensis***
         8. Terminal segment different from the lateral ones
            9. Fronds in 2 series on rhizome, terminal segment ± conform to the pinnae. . . . . ***B. heteroclita***
            9. Fronds in 3 series on rhizome, terminal segment triangular. . . . . ***B. christensenii***
   5. Areoles lacking free included veinlets
      10. Rhizome high climbing, up to 2 m. . . . . ***B. scandens***
      10. Rhizome terrestrial or epiphyte, short-creeping

11. Lamina hard, coriaceous, lustrous when dried, lateral veins regular, obvious on both sides . . . . . ***B. viriens***
11. Lamina herbaceous to chartaceous, matte, lateral veins obscure
    12. Dried lamina purplish. . . . . ***B. scalpturata***
    12. Dried lamina greenish
        13. Margin of pinnae usually shallowly lobed, rounded or rounded cuneate at the base, terminal segment acute or short-flagelloid. . . . . ***B. subcordata***
        13. Margin of pinnae entire, narrowly cuneate at the base, terminal segment similar to the lateral ones. . . . . ***B. lanceolata***

Bolbitis appendiculata (Willd.) K. Iwats. Acta Phytotax. Geobot. 18: 48. 1959; *Acrostichum appendiculatum* Willd. Sp. Pl. 5: 114. 1810. *Gymnogramma auriculata* Kaulf., Enum. Fil. 79, 1824, non Bl. 1828. *Polybotrya appendiculata* J. Smith, Hook. J. Bot. 4: 150, 1841. *Lacaussadea appendiculata* Gaudich., Voy. Bonite Bot. pl. 119. 1852. *Egenolfia appendiculata* J. Smith, Ferns Br. For., 111, fig. 1866. TYPE.—INDIA. *Klein 912*, without precise locality (B).

Acrostichum asplenifolium Bory in Bélanger, Voy. Ind. Or. Bot. 2: 21, pl. 3. 1833. *Polybotrya asplenifolia* Presl, Tent. Pterid. 231, 1836. *Polybotrya appendiculata* (Willd.) J. Smith var. *asplenifolia* Bedd., Handb. Ferns Br. India: 424. fig. 255. 1883. *Egenolfia asplenifolia* Fée, Genres Polyp.: 358. 1852. *B. asplenifolia* Iwatsuki, Acta Phytotax. Geobot. 18: 49. 1959. TYPE.—INDIA. *Bélanger s.n.*, South India, Madura, Dendigal, 1831 (P).

Egenolfia crenata Ching & Chiu, Acta Phytotax. Sin. 21:212. 1983. TYPE.—CHINA. **Yunnan:** Jinping, *Sino-USSR Yunnan Exped. 892* (Holotype: PE; isotype: KUN).

SPECIMENS EXAMINED.—LAOS. **Bolikhamsai Province:** Kham Keuate District, Thong Pei Village, Phou Koma, 18°18′104″N, 109°09′28″E, 600–650 m, Nov. 3, 2007, *Wu, Liu, et al. 219* (IEBR, KUN). **Champasak Province:** Parson District, Nang long Village, Dasta Waterfall, 1100–1200 m, Dec. 17, 2008, *Wu, Gong, et al. 2124* (GH, KUN, MO, TMRC). **Vientiane Province:** Vang Vieng District, Na Mouang Village, 18°53′293″ N, 102° 25′183″ E, 230–300 m, Nov. 14, 2007, *Wu, Liu, et al. 392* (KUN, TMRC). VIETNAM. **Đắk Nông Province:** Dak Gloong District, Dak P'Iao Commune, Ta Dung Nature Reserve, approximately 18°52′18″ N, 108°0l′37″ E, 1200–1400 m, Nov. 13, 2006, *Wu, Phan, et al. 1525* (GH, IEBR, KUN, MO). **Hà Giang Province:** Yen Minh District. Du Gia Mun., about 2 km to SW Giang Tru C Village, approximately 22°56′51″ N, 105°10′24″ E, 900–1200 m, Nov. 29, 2004, *Wu, Phan, et al. 788* (GH, IEBR, KUN, MO); Yen Minh District, Du Gia Mun., about 2 km to Sw Giang Tru C Village, 22°56′51″ N, 105°10′24″ E, 1000–1200 m, Nov. 30, 2003, *Wu, Phan, et al. 832* (IEBR, KUN, MO). **Kon Tum Province:** Kon Plong District, Po E Mun., Violac Village, 14°45′ 16″ N, 108°30′ 41″E, 900–1000 m, Nov. 22, 2003, *Wu, Phan, et al. 188* (GH, IEBR, KUN, MO). **Quảung Bình Province:** Bo Trach

District, Hung Trach Mun., Phong Nha-Ke Bang National Park, ca. 650 m, between km 51–56 of west branch of Ho Chi Minh road, approximately 17°27′30″ N 106°23′06″ E, Dec. 07, 2004, *Wu, Phan, et al. 929* (GH, IEBR, KUN, MO); Bo Trach District, Tan Trach Mun., ca. 750 m, km 37 of the road No. 565, approx. 17°24′24″ N, 106°13′08″ E, Dec. 13, 2004, *Wu, Phan, et al. 1118* (GH, IEBR, KUN, MO).

DISTRIBUTION.—Japan (Ryukus), China (South), India, Thailand, Cambodia, Laos, Vietnam, Malaysia. Very common in South Asia.

ECOLOGY.—In dense forest, usually along the stream on muddy rocks, 250–1400 m.

Bolbitis christensenii (Ching) Ching in C. Chr. Ind. Fil., Suppl. 3. 47. 1934. *Campium christensenii* Ching, Bull. Fan Mem. Inst.. Biol., Bot. Ser. 2: 214, pl. 31. 1931. TYPE.—CHINA. Kweichow, Puding, *Esquirol 2672* (K?).

Bolbitis hekouensis Ching, Acta Phytotax. Sin. 21: 212. 1983. TYPE.—CHINA. **Yunnan:** Hekou, *S. K. Wu 4056* (PE).

SPECIMENS EXAMINED.—LAOS. **Bolikhamsai Province:** Kham Keuate District, Phou Kom, Thong Pei Village, 18°18′104″ N, 109°28′00″ E, 600–650 m, Nov. 3, 2007, *Wu, Liu, et al. 199* (KUN, TMRC). VIETNAM. **Hà Giang Province:** Yen Menh District., Du Gia Mun., about 1 km to SW Giang Tru C Village, 22°56′51″ N, 105°10′24″ E, 500–850 m, Nov. 28, 2004, *Wu, Phan, et al. 763* (IEBR, KUN).

DISTRIBUTION.—China (Yunnan), Thailand, Vietnam. New record for Laos.

ECOLOGY.—In primary and secondary evergreen broad-leaved forest or scrub in limestone area.

Bolbitis copelandii Ching ex C. Chr. & Tardieu. Notul. Syst. (Paris) 7: 101. 1938. *Bolbitis crispatula* (Copel.) Ching var. *copelandii* (Ching) Hennipm., Leid. Bot. Ser. 2: 159. f. 40: 1, 42. 1977. TYPE.—CAMBODIA. Angkor, *H. M. Smith 302* (Holotype: BM, isotype: MICH, US).

SPECIMENS EXAMINED.—LAOS. **Attapeu Province:** Xaysetha District, Dachen Village, Dec. 9, 2008, *Wu, Gong, et al. 1989* (GH, KUN, MO, TMRC). **Khammouane Province:** Hinboun District, Naxin Village, Tad Nam Sanam Waterfalls, 18°14′00″ N, 104°42′234″ E, Nov. 8, 2007, *Wu, Liu, et al. 319* (GH, KUN, MO, TMRC); Gnommalate District, Houay Jat Mountain, Keovilay Village, 17°40′154″ N, 105°10′908″ E, Oct. 28, 2007, *Wu, Liu, et al. 52* (GH, KUN, MO, TMRC). **Saravane Province:** Tateng District, Panentay Village, Dec. 3, 2008, *Wu, Gong, et al. 1808* (GH, KUN, MO, TMRC); Tateng District, Songtia Village, TormaHore Waterfall, Dec. 4, 2008, *Wu, Gong, et al. 1860* (GH, KUN, MO, TMRC); Lao gham District, Nasia Village, Dec. 14, 2008, *Wu, Gong, et al.*

2055 (GH, KUN, MO, TMRC). **Vientiane Province:** Vang Vieng District, Na Khoum Village, 18°52′465″ N, 102°24′384″ E, Nov. 13, 2007, *Wu, Liu, et al. 361* (GH, KUN, MO, TMRC). VIETNAM. **Đồng Nai Province:** Tan Phu District, Nam Cat Tien Commune, Cat Tien National Park, 11°26′57″ N, 107°21′41″ E, Nov. 17, 2006, *Wu, Phan, et al. 1642* (GH, IEBR, KUN, MO).

DISTRIBUTION.—Thailand, Laos, Vietnam. A common species, especially in South Laos.

ECOLOGY.—In primary and secondary evergreen forest, 200–800 m.

DISCUSSION.—This species was treated as a variety of *Bolbitis crispatula* by Hennipman (1977). Tagawa & Iwatsuki (1988) treated it at the rank of species and pointed out that *B. crispatula* has narrow fertile fronds, 2–5 mm wide, covered almost entirely with sporangia, while *B. copelandii* have wider fertile fronds, 4–12 mm, bearing the sporangia in a broad band near the margins often curving along the lobes and dispersing inwards below the sinus, and with a broad sterile portion on both sides of the costae.

Bolbitis crispatula (Copel.) Ching in C. Chr. Ind. Fil. Suppl. 3: 47. 1934; *Acrostichum crispatulum* (Wall.) C.B. Clarke, Trans. Linn. Soc. Bot. 1: 580, pl. 84, fig. 2B, 2D, 1880. excl. var. nom. illeg., non Fée 1852. TYPE.— BANGLADESH. Kumaon, *Wallich 24* p.p. (Blinkworth *leg.*), (Holotype: K, herb. Wallich; isotype: K, MICH.).

SPECIMEN EXAMINED.—LAOS. **Sekong Province:** Tateng District,Songtia Village, Torma Hore Waterfall, Dec. 4, 2008, *Wu, Gong, et al. 1860* (GH, KUN, MO, TMRC).

DISTRIBUTION.—India, Thailand and Vietnam. New record for Laos.

ECOLOGY.—In tropical semi-deciduous forest, 500–600 m.

DISCUSSION.—*Bolbitis crispatula* is most similar to *B. copelandii* Ching ex C. Chr.; see discussion under *B. copelandii.*

Bolbitis heteroclita (C. Presl) Ching ex C. Chr. Ind. Fil., Suppl. 3: 48. 1934. *Acrostichum heterocilitum* C. Presl, Rel. Haenk. 1: 15. pl. 2:.2. 1825. *Leptochilus heterocilita* (C. Presl) C. Chr. Ind. Fil. 385. 1906. TYPE.— INDONESIA. **West Java:** Mt. Salak, Tjikoja, *Zollinger 1441*, (Holotype: P; isotype: BM, G).

Bolbitis annamensis Tardieu & C. Chr., Not. Syst. 7:100. 1938. TYPE.— VIETNAM. Annam, Thanh Tan, 100–200m, *Cadière 149* (Holotype: BM; isotype: P).

Bolbitis confertifolia W. M. Chu ex Ching et C. H. Wang, Acta Phytotax. Sin. 21: 211. 1983. TYPE.—CHINA. **Yunnan:** Xishuangbanna, *Yunnan Complex Exped. 1851.* (PE).

SPECIMENS EXAMINED.—LAOS. **Vientiane Province:** Vang Vieng District, Na Khoum Village. 18°52′ 465″ N, 102° 24′ 354″ E, 250 m. Nov. 13, 2007, *Wu, Liu, et al. 355* (GH, KUN, MO, TMRC). VIETNAM. **Hà Giang Province**: Yen Minh District, Du Gia Mun., about 2 km to SW of Giang Tru C Village, approximately 22°56′51″ N, 105°10′24″ E, 900–1200 m, Nov. 29, 2004, *Wu, Phan, et al. 769* (GH, IEBR, KUN, MO). **Quảng Bình Province:** Bo Trach District, Hung Trach Mun., Phong Nha-Ke Bang National Park, ca. 650 m, between km 51 and 56 of west branch of Ho Chi Minh road, approximately 17°27′30″ N, 106°23′06″ E, Dec. 7, 2004, *Wu, Phan, et al. 894* (KUN); Bo Trach District, Xuan Trach Mun., Phong Nha-Ke Bang National Park, arounda Deo pass, ca. 460 m, about 25 km to the north of Khe Gat. along Ho Chi Minh road. approximately 17°39′24″ N, 106°05′66″ E, Dec. 8, 2004, *Wu, Phan, et al. 937* (GH, IEBR, KUN, MO).

DISTRIBUTION.—China, North India, Burma, Thailand, Malaysia to New Guinea. New record for Laos and Vietnam.

ECOLOGY.—In disturbed primary and secondary broad-leaved lowland and submontane forests, and it prefers very moist habitats, usually growing along the stream, 150–1200 m.

DISCUSSION.—Tagawa and Iwatsuki (1988) reported that "This species does not form colonies like *Bolbitis subcordata* of Japan"; however, we do find large colonies of *B. heteroclita* in Vietnam. In some colonies, particularly in very moist habitats, vegetative propagation is common, and almost no fertile fronds can be found.

Bolbitis hookeriana K. Iwats. in Acta Phytotax. Geobot. 18: 59. 1959. *Polybotrya vivipara* Buch.-Ham. Fl.: t. 107. 1825. *Egenolfia vivipara* (Buch.-Ham.) C. Chr. Ind. Fil., Suppl. 111: 102. 1934. *Bolbitis appendiculata* ssp. *vivipara* var. *vivipara* (Hook.) Hennipman *in* Blumea 18: 147. 1970. TYPE.—INDIA. *Wallich 29 p.p.* (Hamilton leg), Assam, Goalpara, 1808 (Holotype: K, isotype: K).

SPECIMENS EXAMINED.—LAOS. **Khammouane Province:** Nhommalat District, Tha Thote Village, Oct. 27, 2007, *Wu, Liu, et al. 16* (GH, KUN, MO, TMRC). VIETNAM. **Đồng Nai Province:** Tan Phu District, Nam Cat Tien Commune, Cat Tien National Park, approximately 11°26′57″ N, 107°21′41″ E, ca.115 m, Nov. 17, 2007, *Wu, Phan, et al. 1644* (GH, IEBR, KUN, MO).

DISTRIBUTION.—North India, Thailand. New record for Laos and Vietnam.

ECOLOGY.—In broad-leaved dense forest, usually along the stream on muddy rocks, 150–1000 m.

Bolbitis lanceolata S. K. Wu & J. Y. Xiang, ***sp. nov.*** TYPE.—LAOS. **Khammoun Province:** Nakai District, Nakai Village, Phou Ar. (Dan Feuang), 17°43′217″ N, 105°07′663″ E, in evergreen forest, on limestone rocks, 650–750 m, Oct. 31, 2007, *Wu SG, Liu ED, Xiang JY, Somsanith B, Onevilay S 121* (KUN, MO, TMRC). **Fig. 1: A–C, Fig. 2**.

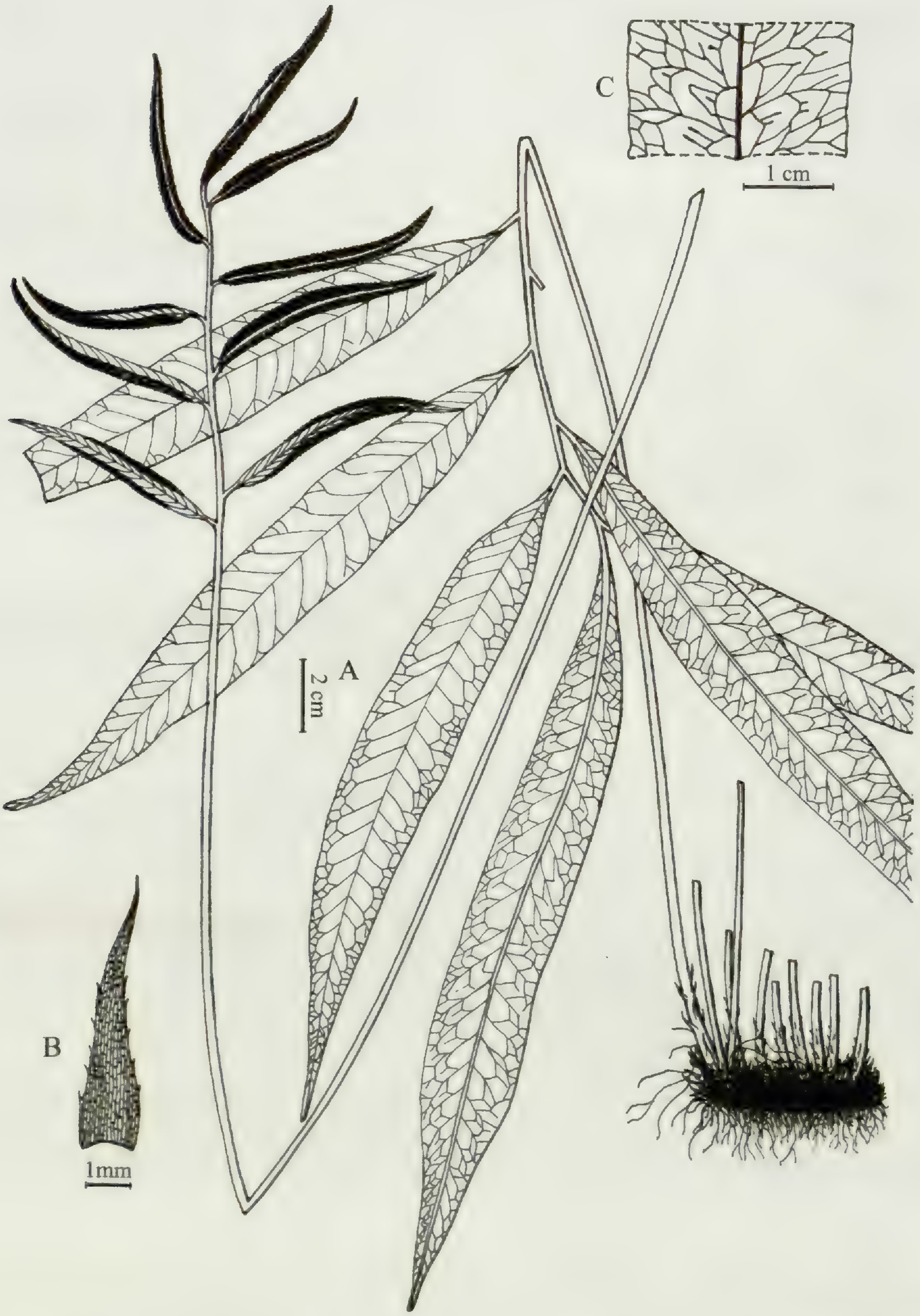

FIG. 1. *Bolbitis lanceolata* S. K. Wu & J. Y. Xiang. A) habitat; B) Rhizome Scale. C) Part of sterile pinna. Drawn from *Wu, Liu, et al. 336* (KUN).

FIG. 2. Scanning electron micrograph of a spore of *Bolbitis lanceolata*. Taken from *Wu, Liu, et al. 121* (KUN).

Bolbitidi scalptulatae (Fée) Ching similis, sed frondibus 3–4 seriebus, approximatis, pinnis sterilibus angustioribus 15–20 × 2–2.5 cm, pinnis lateralibus 3–5-jugis, lanceolatis, basi acuminatis, margine integris, dilute chartaceis, in sicco viridulis non rubescentibus, pinna terminali ad apicem sine bulbilo differt.

Terrestrial. Rhizome short-creeping, up to c. 10 cm long, 5–10 mm thick, with 3 or 4 rows of leaves. Rhizome scales subclathrate, oblanceolate, 2–3 × 0.5–1 mm, entire or irregularly laciniate at margin, brown, clathrate. Roots ventral, irregularly arranged. Leaves rather close together. Fronds dimorphic, 40–70 cm long, stipe stramineous, 25–30 cm long, grooved on the adaxial side, with scales at the base, gradually becoming glabrous towards the apex. Sterile lamina ca. 25–30 cm long, 20–25 cm wide, pinnate, 3–5 pairs of pinnae, pinnae alternate, lanceolate, 15–20 × 2–2.5 cm, apex caudate, base acuminate, margin entire, short-stalked, patent or ascending. Lamina papyraceous, green when dried, glabrous, terminal segment similar to the lateral ones, without bulbil on the adaxial surface. Veins raised abaxially, immersed adaxially, main lateral veins 4–6 mm apart, with two or three rows of areoles between two adjacent lateral veins, with one or two included veinlets in each areole. Fertile lamina ca. 40–70 × 3–8 cm, petiole 25–40 cm, lateral pinnae 3–4 pairs, linear-lanceolate, 2.5–7 × 0.3–0.7 cm, stalked 1–2 mm. Sporangia distributed evenly

over the lower surface. Spores monolete, spheroidal, undulate with gross ridges.

SPECIMENS EXAMINED.—LAOS. **Khammouane Province:** Hinboun District, Khounkham Village, Houay Muang (Phou Hai), 18°13′469″N, 104 °32′671″ E, in broad-leaved evergreen forest, limestone rocks, 450–500 m, Nov. 9, 2007, *Wu, Liu, et al. 336* (Holotype: KUN, isotype: TMRC).

Bolbitis lanceolata is most similar to *Bolbitis scalpturata* (Fée) Ching, but differs by having 3 to 4 rows of leaves close together; pinnae of sterile lamina narrower, 15–20 × 2–2.5 cm, lateral pinnae 3–5 pairs, pinnae base cuneate, entire at margin, thin-chartaceous, greenish when dry, without bulbil at the apex of the terminal pinnate. In contrast, *Bolbitis sculpturata* has 2 rows of spaced leaves, pinnae of sterile lamina wider, 15–20 × 3–4 cm, lateral pinnae 5–7 pairs, pinnae base acuminate pinnae margin more or less serrate-crenate, herbaceous to subcoriaceous, purplish or purplish-brown when dry, with or without bulbil at the apex of the terminal pinnate.

ECOLOGY.—This species is endemic to Khammoun Province of Laos, where it is epipetric under the broad-leaved evergreen forest on limestone mountain.

Bolbitis scalpturata (Fée) Ching in C. Chr. Ind. Fil. Suppl. 3: 50 1934. *Heteroneuron scalpturatum* Fée, Hist. Acrost. 95, pl. 56. 1845. *Acrostichum scalpturatum* Kunze, Bot. Zeit. 103, 1848. Mettenius, Fil. Lips. 21, 1856. *Leptochiilus scalpturatus* C. Chr., Ind. Fil. 387, 1906. *Campium scalpturatum* Copel., Philip. J. Sc. 37: 383, f. 35. 1928. LECTOTYPE.— (Chosen by Hennipman, 1977). PHILIPPINES. Manila, xi-1836, *Gaudichaud s.n.* (Holotype: P ; isotype: B, BM, P.).

SPECIMENS EXAMINED.—LAOS. **Salavane Province:** Lao Gham District, Phu Sa Sat protected area, Nasia Village, 750–800 m, Dec. 14, 2008, *Wu, Gong, et al.2057* (GH, KUN, MO, TMRC). VIETNAM. **Đắk Nông Province:** Dak Gloong District, Ta Dung, Dak. P'lao Commune, National Reserve, 11°52′18″ N, 108°01′37″ E, 1200–1400 m, Nov. 13, 2006, *Wu, Phan, et al. 1526* (GH, IEBR, KUN, MO).

DISTRIBUTION.—Burma, Malaysia, Thailand, Laos, Vietnam.

ECOLOGY.—Epipetric on granite, in primary evergreen tropical forest.

Bolbitis scandens W. M. Chu ex Ching & C. H. Wang, Phytotax. Sin. 21: 213. 1983. TYPE.— CHINA. **Yunnan:** Lu Chun, *W. M. Chu et al. 6733* (Holotype: PYU ; isotype: PE)

SPECIMENS EXAMINED.—LAOS. **Attapeu Province:** Xaysetha District, Dakcheng Village, 150–200 m, Dec. 7, 2008, *Wu, Gong, et al. 1929* (KUN, TMRC). Vientiane Province, Vieng District, Na Khoum Village, 18°52′465″ N, 102°24′354″ E, Nov. 13, 2007, *Wu, Liu, et al. 355* (KUN, MO, TMRC, GH).

DISTRIBUTION.—China (South Yunnan). New record for Laos.

ECOLOGY.—under broad–leaved evergreen forest, along small streams, 1500–2000 m.

DISCUSSION.—This species is closely related to *Bolbitis heteroclita*, differing by its hemiepiphytic habit, having a rhizome that climbs high upon tree trunks. Its leaf texture is chartaceous, thicker than the herbaceous lamina of *B. heteroclita*, and its terminal pinnae never obviously prolonged like *B. heteroclita*.

Bolbitis sinensis (Baker) K. Iwats. Acta Phytotax. Geobot. 18: 49. 1959. *Acrostichum sinense* Baker in Kew. Bull. 14. 1906. *Polybotrya sinensis* C. Chr. Ind. Fil., Suppl. 1: 57. 1913. *Egenolfia sinensis* (Baker) Maxon, Proc. Biol. Soc. Wash. 36: 173. 1923. *Campium sinense* C. Chr., Contr. U.S. Nat. Herb. 26: 292, 1931. TYPE.—CHINA. **Yunnan:** Szemao, *Henry 12494*, (Holotype: K; isotype: B, BM, US).

Egenolfia bipinnatifida J. Sm., Hist. Fil. 132. 1875. TYPE.—MYANMAR. **Tanintharyi Region:** Dawna Range near Moulmein, *Parish 60* (K).

SPECIMENS EXAMINED.—LAOS. **Champasak Province:** Parson District, Nanglong Village, Dasta Waterfall, Dec. 17, 2008, *Wu, Gong, et al. 2124B* (GH, KUN, MO, TMRC). **Vientiane Province:** Khamkeut District. Puang Pu tao Village, 550–650 m, Nov. 6, 2007, *Wu, Liu, et al. 302* (KUN). **Xiangkhouang Province:** Kham District, Tha Village, 1300m, Dec. 21, 2008, *Wu, Gong, et al. 2200* (GH, KUN, MO, TMRC). VIETNAM. **Đắk Nông Province:**, Dak Gloong distict, Dak P'lao Commune, Ta Dung Nature Reserve, approximately 11°52′ 18″ N, 108°01′37″ E, 1200–1400 m, Nov. 12, 2006, *Wu, Phan, et al. 1543* (GH, IEBR, KUN, MO); approximately 11°52′18″ N, 108°01′ 37″ E, 1100–1250 m, Nov. 13, 2006, *Wu, Phan, et al. 1587* (GH, IEBR, KUN, MO). **Kon Tum Province:** Sa Thay District, Sa Nhon Mun., west of Sa Nhon Forest Protection Station, 14°27′ 43″ N, 107°45′ 52″ E, between 800–1000 m, Nov. 17, 2003, *Wu, Phan, et al. 085* (GH, IEBR, KUN, MO); Sa Son Mun., Bar Gok villiage, 14°26′ 29″ N, 107°42′ 44″ E, 800–1000 m, Dec. 8, 2004, *Wu, Phan, et al. 937* (GH, IEBR, KUN, MO). **Lâm Đắng Province:** Da Lat city, Ta Nung Commune, Tran Le agriculture farm, approximately 11°56′08″ N, 108°22′47″ E, alt. 1300–1360m, Nov. 8, 2006, *Wu, Phan, et al. 1468* (GH, IEBR, KUN, MO).

DISTRIBUTION.—China (Yunnan), India, Burma, Cambodia, Thailand, Indonesia. New record for Laos and Vietnam.

ECOLOGY.—In primary tropical broad-leaved evergreen forest.

Bolbitis subcordata (Copel.) Ching in C. Chr. Ind. Fil. Suppl. 3: 50. 1934. *Campium subcordataum* Copel., Philipp. J. Sci. 37: 369, fig. 23, pl. 16. 1928. TYPE.—CHINA. Hainan, *McClure C.C.C. 9436* (Holotype: P?; isotype: BISH, BM, C, MO, P).

SPECIMENS EXAMINED.—VIETNAM. **Đắk Nông Province:**, Dak Gloong District, Dak P'Lao Commune, Ta Dung Nature Reserve, 11°52′18″ N, 108°01′37″ E, Nov. 13, 2006, *Wu, Phan, et al. 1544* (GH, KUN, MO, TMRC).

DISTRIBUTION.—Japan, China (Hainan), Thailand, Laos. New to Vietnam.

ECOLOGY.—In primary evergreen tropical forest, 1200–1400 m.

Bolbitis tonkinensis (C. Chr. ex Ching) K. Iwats., Acta Phytotax. Geobot. 18: 49. 1959. *Egenolfia tonkinensis* C. Chr. ex Ching, Bull. Fan Mem. Inst. Biol. Bot. Ser. 2:306 1931. TYPE.—VIETNAM. Tonkin, Lang-son Herb. *École Sup. Agric. & Sylvic. Hanoi 3396* (Colani leg.), (Holotype: BM; isotype: BM, P, PE, UC, US).

SPECIMENS EXAMINED.—LAOS. **Champasak Province:** Parson District, Nanglong Village, Dasta Waterfall, *Wu, Gong, et al. 2124B* (GH, KUN, MO, TMRC).

DISTRIBUTION.—China (South Yunnan), Thailand, Cambodia, Vietnam. New record for Laos.

ECOLOGY.—In primary broad-leaved evergreen forest along stream, 1120–1200 m.

Bolbitis virens (Hook. & Grev.) Schott, Gen. Fil.: ad t. 14. 1834. *Acrostichum virens* [Wall., Cat. No. 1033, nom. nud. 1829.] Hooker & Grev., Ic. Fil. 221. 1831. *Campium virens* Presl., Tent. Pterid. 239, 1836. *Cyrtogonium virens* J. Smith, Hook. J. Bot. 4: 154. 1841. *Heteroneuron virens* Fèe. Hist. Acrost. 93. 1845. *Poecilopteris virens* Moore. Ind. Fil. XX. *Gymnopteris virens* Keyserling. Polyp. & Cyath. Herb. Bungeani 33. 1873. *Leptochilus virens* C. Chr. Ind. Fil. 388. 1906. TYPE.—MYANMAR. Tovag, *Wallich 1033* (K).

Bolbitis latipinna Ching, Acta Phytotax. Sin. 21: 213. 1983. TYPE.—CHINA. **Yunnan:** Xishuangbanna, Sep. 1936, *C. W. Wang 78807* (PE).

a. Central pinnae of fertile leaves index >15, 8–20 × 0.2–0.9 cm..... ..... **var. *virens***

b. Central pinnae of fertile leaves index 3–8, 4–11.5 × 0.8–2 cm..... ..... **var. *compacta***

Bolbitis virens var. ***virens***

SPECIMENS EXAMINED.—LAOS. **Salavan Province:** Phu Sa Sat Protect area, Dec. 14, 2008, *Wu, Gong, et al. 2057* (GH, KUN, MO, TMRC). **Champasak Province:** Pak Song District, Lak Sao Village, Yuang Waterfall, Dec. 16, 2008, *Wu, Gong, et al. 2069* (GH, KUN, MO, TMRC). **Vientaine Province:** Van Vieng District, Pha Tang Village, 19 04′180″ N, 102 24′433″ E, Nov. 15, 2007, *Wu, Liu, et al. 420* (GH, KUN, MO, TMRC). VIETNAM. **Kon Tum Province:** Sa Thay District, Sa Son Mun., Bar Gok Village, 14 26′29″ N,107 42′44″ E, Nov. 15, 2003, *Wu, Phan, et al. 35* (GH, IEBR, KUN, MO).

DISTRIBUTION.—China (Southern Yunnan), Bangladesh, India, Burma, Thailand. New record for Laos and Vietnam.

ECOLOGY.—In primary tropical evergreen broad-leaved forest, 1200–1400 m.

DISCUSSION.—A very distinct and locally common species. It is similar to *Bolbitis costata* and *B. subcrenata*. It differs from *B. costata* by its greenish dried leaves whereas the latter has purplish dried leaves. It differs from *B. subcrenata* by there being two or more free veins in costal areoles in *B. virens*, while there are only one or two free veins in costal areoles of *B. subcrenata*, and its "venation pattern reminiscent of that of the meniscioid ferns" (Hennipman, 1977).

Bolbitis virens var. ***compacta*** Hennipm. Blumea 18: 149. 1970. TYPE.—THAILAND. Peninsular Thailand, Nakhon Sri Thammarat, Trang, Khao Chong, 600–1100 m, *Tagawa et al. T. 6802*, (Holotype: L; isotype: India, KYO).

SPECIMENS EXAMINED.—LAOS. **Bolikhamsai Province:** Khamkeut District, Nam Gha Nay Mountain, Thong Pai Village, ca. 550 m, Nov. 4, 2007, *Wu, Liu, et al. 227* (KUN, TMRC). VIETNAM. **Kon Tum Province:** Kon Plon District, Po E Mun., Violac Village, 4°15′16″ N, 108°30′41″ E, 900–1000 m, Nov. 22, 2003, *Wu, Phan, et al. 195* (KUN). **Quảng Bình Province:** Phong Nha –Ke Bang National Park, 17°27′30″ N, 106°23′06″E, ca. 650 m, Dec. 07, 2004, *Wu, Phan, et al. 909* (GH, IEBR, KUN, MO); 17°39′24″ N, 106°05′66″ E, ca. 460 m, Dec. 08, 2004, Dec. 08, 2004, *Wu, Phan, et al. 950* (GH, IEBR, KUN, MO). Bo Trach District, Hung Trach Mun., top of U Bo pass, 17°28′ 36″ N, 106°22′39″E, 800–900 m, Nov.15, 2003, *Wu, Phan, et al. 1047* (GH, IEBR, KUN, MO).

DISTRIBUTION.—Malaysia, Thailand, New record for Laos and Vietnam.

ECOLOGY.—In primary and secondary evergreen broad-leaved forest or scrub in limestone area near stream.

DISCUSSION.—Differs from var. *virens* by shorter and broader fertile pinnae (ca. 5–8 × 1–2 cm).

Bolbitis yunnanensis Ching ex Ching & C. H. Wang, Acta Phytotax. Sin. 21: 214. 1983. *Bolbitis subcordata* auct non Copel. X. Cheng in C. Y. Wu (ed.), Fl. Yunn. 21: 222. 2005. TYPE.—CHINA. **Yunnan:** Xishuangbanna, Mar. 1956. *R.C.Ching, s.n.* (PE).

SPECIMENS EXAMINED.—LAOS. **Champasak Province:** Parson District, Nanglong Village, 1100–1200 m, Dec. 17, 2008, *Wu, Gong, et al. 2138* (KUN). VIETNAM. Cat Tien National Park, approximately 11°26′57″ N, 107°21′41″ E, ca. 115 m. Nov. 17, 2006, *Wu, Phan, et al. 1643* (GH, IEBR, KUN, MO).

DISTRIBUTION.—China (South Yunnan). New record for both Vietnam and Laos.

ECOLOGY.—In disturbed primary and secondary broad-leaved lowland forest on old alluvial black soil with superficial volcanic rocks.

DISCUSSION.—This species is very close to *Bolbitis virens*, but differs from it in having broad fertile pinnae, 5–8 × 0.8–1.2 cm, sterile pinnate 3–5 pairs, entire

or crenate at margin. This species was placed in synonymy under *B. subcordata* (Copel.) Ching in the Flora Yunnanica (Wu, 2005), but it is obvious that lamina of *B. yunnanensis* is imparipinnate while laminae of *B. subcordata* have a pinnate apices. Morphological study of type specimens, Dong (2005) reduced *Bolbitis yunnanensis* as synonym of *B. hainanensis* Ching & Wang. We however have not seen the type of *B. hainanensis*, and maintain the name here until further study can be undertaken.

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Literature Cited

Cheng, X. 2005. Bobitidaceae, Pp. 212–225, *in* Wu, C. Y., ed. *Flora Yunnanica*. Science Press, Beijing.

Ching, R. C. 1931. On the genus *Egenolfia* Schott. Bull. Fan Mem. Inst. Biol. Ser 2:297–317.

Hennipman, E. 1977. *A Monograph of the Fern Genus Bolbitis* (Lomariopsidaceae), Leid. Bot. Ser. No 2:1–330, Leiden University Press.

Ho, P. H. 1991. *An Illustrated Flora of Vietnam*. Mekong Printing, Montréal.

Iwatsuki, K. 1959. Taxonomic studies of Pteridophyta IV. Emendation of *Bolbitis*, with special reference to the Far Eastern species. Acta Phytotax. Geobot. 18:44–59.

Moran, R. C., P. H. Labiak and M. Sundue. 2010a. Phylogeny and character evolution of the bolbitidoid ferns (Dryopteridaceae). Int. J. Plant Sc. 171:547–559.

Moran, R. C., P. H. Labiak and M. Sundue. 2010b. Synopsis of *Mickelia*, a newly recognized genus of bolbitidoid ferns (Dryopteridaceae). Brittonia 62:337–356.

Tagawa, M. and K. Iwatsuki. 1988. *Bolbitis*. Pp. 310–322, in: Smitinand, T. and Larsen, K., eds. *Flora of Thailand. Part 3. Blechnaceae to Athyriaceae*, 297–480, 1988. Chutima press, Bangkok.

Tardieu-Blot, M. and C. Christensen. 1940. Flore Générale de l'Indo-chine. *7*:423–438.

Wu, S. H. and C. H. Wang. 1999. Bolbitidaceae, Pp. 104–124, *in* Wu, C. Y., ed. Flora of Reipublicae Popularis Sinicae. Science Press, Beijing.

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Systematic Studies of *Polystichum* (Dryopteridaceae) in Japan (I): *P. fibrillosopaleaceum* var. *marginale* is a Diploid Hybrid between *P. fibrillosopaleaceum* and *P. igaense*

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Abstract.—Morphological, cytological, and isozyme studies of three plants of *Polystichum fibrillosopaleaceum* var. *marginale* (Dryopteridaceae) from Gotenba, at the foot of Mt. Fuji, Shizuoka Prefecture, Japan, and comparative observations of their lamina, scales, sori, spores, and perispore ornamentation, revealed them to be intermediate between *P. fibrillosopaleaceum* and *P. igaense*. Cytologically, the meiotic chromosome number of n=c. 38II + 6I (2x) and the malformed spores of the three plants are characteristic of hybrid sterility. The electrophoretic polymorphisms of four isozymes (Pgi, Pgm-1, Pgm-2, and Skdh) were examined in the three plants and in 22 individuals of the putative parental species, *P. fibrillosopaleaceum* (11) and *P. igaense* (11). The heterozygous genetic patterns (Pgi-ab, Pgm-1ab, Pgm-2ab, and Skdh-ab) fixed in the putative hybrids indicate that they have a combination of the Pgi-b, Pgm-1b, Pgm-2a, and Skdh-a alleles of *P. igaense* and Pgi-a, Pgm-1a, Pgm-2b, and Skdh-b alleles of *P. fibrillosopaleaceum*. The morphological, electrophoretic features and chromosome numbers confirm the three plants to be diploid hybrids of *P. fibrillosopaleaceum* and *P. igaense*. We therefore conclude that *P. fibrillosopaleaceum* var. *marginale* is in fact a diploid hybrid and thus should not be given the variety status.

Key Words.—*Polystichum*, hybrid, diploid, variety, electrophoretic polymorphisms

Polystichum (Dryopteridaceae) is a genus estimated to contain about 200 to more than 300 species, occurring in mountainous, warm temperature areas, especially in eastern to southeastern Asia (Kramer and Green, 1990; Iwatsuki, 1992; Kung *et al.*, 2001, Little and Barrington 2003). Hybridization and polyploidization have played important roles in species diversification and reticulation in *Polystichum*, reflected in the high frequency of tetraploid species and hybrids (Daigobo, 1972; Vida and Reichstein, 1975; Wagner, 1973; Wagner, 1979; Barrington, 1985, 1986, 1990; Soltis and Soltis, 1987). Systematic studies have been undertaken to resolve the evolutionary and taxonomic questions regarding *Polystichum* in Europe and America during the

past half century (Manton, 1950; Wagner, 1973: Wagner, 1979; Barrington, 1986, 1990; Little and Barrington, 2003), and in Asia recently (Zhang and Kung, 1996; Lu *et al.*, 2007; Li *et al.*, 2008).

Based on morphological characters, 32 species and about 40 interspecific hybrids involving 13 putative parental species are recognized in Japan (Iwatsuki, 1992). It is generally difficult to confirm the taxonomy of closely related species or discriminate between them using only a single analytical method because the distribution of character states can overlap. It is therefore necessary to combine morphological, cytological, and electrophoretic analyses to determine the evolutionary relationships among the species in Japan.

Among the 13 Japanese putative parental species classified in sect. *Metapolystichum* (Daigobo 1972), six are diploid species (Takamiya 1996), for which systematic studies are few. It is important to clarify the identity of the diploid species before we can understand the origins of the hybrids and the polyploid species, and to resolve the history of speciation, hybridization and polyploidization in Japanese *Polystichum*. This study focused on the diploid species *P. igaense* Tagawa and *P. fibrillosopaleaceum* (Kodama) Tagawa, which are believed to be involved in the origin of more than 10 hybrids (Iwatsuki, 1992). *Polystichum igaense*, endemic to Japan, occurs in mountain forests on Honshu, Shikoku, and Kyushu. *Polystichum fibrillosopaleaceum*, a well-known Japanese endemic species, is widely distributed on the Pacific Ocean side of Japan from Honshu and Shikoku to Kyushu in forests around lowland villages, overlapping locally with *P. igaense*.

Plants from Shizuoka Prefecture, Honshu Island, Japan have been segregated as *Polystichum fibrillosopaleaceum* var. *marginale* Seriz. With only a few individuals found in the wild, it is endangered, classified as grade IA (CR) in the Red Data Book of the Japanese Environmental Agency (2007). *Polystichum fibrillosopaleaceum* var. *marginale* has morphological features similar to those of *P. fibrillosopaleaceum*, such as nearly entire, twisted scales, deltoid outline of pinnules, and conspicuous fibrillose scales on the undersurface of the pinnules, but other characters, such as shorter stipes, untwisted pinna and rachis scales, and marginal sori, are similar to the features of *P. igaense* (Serizawa, 1971). Serizawa (1971) considered temporarily that it might be a hybrid between *P. igaense* and *P. fibrillosopaleaceum*. The meiotic chromosome number of *P. fibrillosopaleaceum* var. *marginale* is reported to be n = 41 II (2x), the same as that of *P. fibrillosopaleaceum* (Daigobo, 1973; Shimura and Ooishi, 1980). Those early studies support the proposition that *P. fibrillosopaleaceum* var. *marginale* is probably a variety of *P. fibrillosopaleaceum*, although irregular sterile spores are sometimes produced (Shimura, 1975), and Nakaike (1992) elevated it to species as *P. shizuokaense* Nakaike. Lack of detailed studies of *P. fibrillosopaleaceum* var. *marginale*, however, has hindered further classification of this taxon.

In the present study, three individuals were collected in the area where *P. fibrillosopaleaceum* and *P. igaense* occur in sympatry; they were tentatively identified as *P. fibrillosopaleaceum* var. *marginale* on the basis of their morphological features. To understand the nature and evolutionary origin of *P.*

TABLE 1. Materials used in morphology, cytology and electrophoresis.

Taxa	Locality & voucher no.	Chomosome no. (n, 2n)	Electro. examined samples
P. fibrillosopaleaceum (2x)	Asahi, Yokohama, Kanagawa Pref. (40215, 06100105)	2n=c.82	2
	Midori, Yokohama, Kanagawa Pref. (40039, 40050)		2
	Fujisawa, Kanagawa Pref. (40219, 40221, 40223)		3
	Gotenba, Shizuoka Pref. (40013, 40021, 40036; 40230, 40242, 40253)		3
	Kannan City, Shizuoka Pref. (05060727, 05060729)		2
	Misato, Gunma, Gunma Pref. (40513, 40514, 40515)		1
Hybrid (2x) (*P. fibrillosopaleaceum* var. *marginale*)	Gotenba, Shizuoka Pref. (40238) (40018, 40020)	n=c.41	3
P. igeanse (2x)	Gotenba, Shizuoka, Pref. (40006, 40009, 40022, 40033, 40038; 40227, 40228, 40234, 40245, 06100302)	2n=82	5
	Susono, Shizuoka Pref. (40260, 40261, 05060519, 05060520, 05060531, 06100344)		6

fibrillosopaleaceum var. *marginale*, morphological, cytological and electrophoretic analyses were carried out to compare these three plants with *P. fibrillosopaleaceum* and *P. igaense*.

MATERIALS AND METHODS

Thirty herbarium specimens of *Polystichum* from Japan, deposited in the herbaria of Botanical Garden, University of Tokyo (TI), the Department of Botany, Kyoto University (KYO), and the Japanese National Museum of Nature and Science (TNS), were used for morphological observations. Three samples collected from Gotenba, Shizuoka Prefecture, which had previously been presumed to be *P. fibrillosopaleaceum* var. *marginale*, were examined and compared morphologically with *P. fibrillosopaleaceum* and *P. igaense*. The habitat of the three taxa overlapped in a very narrow area (about 5×5 m^2) in an evergreen forest (cedar) in Gotenba.

For cytological and electrophoretic analyses, the three samples of *P. fibrillosopaleaceum* var. *marginale* from Gotenba, Shizuoka Prefecture, 11 samples of *P. fibrillosopaleaceum* and 11 samples of *P. igaense* were collected in Shizuoka and Kanagawa Prefectures (Table 1). The voucher specimens for these analyses are deposited in the herbarium, Department of Biological Science, Faculty of Life and Environmental Science, Shimane University, Japan.

Spores were obtained from fertile leaves. Spore form and spore number per sporangium (s/s) were observed under a light microscope. The spores obtained from the type specimen of *P. fibrillosopaleaceum* var. *marginale* (Serizawa 6180 = TNS VS-573649) and the three samples collected from Godenba (40018, 40020, and 40238) were compared. To observe perispore ornamentation, dried spores were coated with gold under vacuum with no pretreatment (E-102, Pt-Pd, 3 minutes, 100 Å) and observed by scanning electron microscopy (SEM; S-800 Hitachi). To examine spore germination, spores were gathered from mature sporangia at the stage just before opening to avoid contamination, sown on standard 1/10 MS medium, and cultured at 20–25°C under uninterrupted fluorescent light of about 800–1000 lux.

For cytological analysis, root tips were pretreated with 0.002 M 8-hydroxyquinoline solution for 5 hrs at 20 °C, fixed in an acetic acid–alcohol (1:3) solution for more than 15 min, macerated in a mixed solution of 1 N hydrochloric acid (HCl): 45% acetic acid (3:1) for 2.5 min at 58 °C, and stained with 2% aceto–orcein. The root tissue (about 1–2 mm of root tip) was then cut and squashed for somatic observation (Lin *et al.*, 1990). Fresh pinnae of fertile leaves with sori were fixed in an acetic acid–alcohol (1:3) solution (more than 15 minutes) and then stored at 4–10°C for meiotic observation.

Flow cytometric analysis was used to confirm the ploidy level of those samples for which cytological materials could not be obtained. Approximately 1cm^2 fresh leaf tissue was chopped with a razor, and subsequent methods followed Ebihara *et al.* (2005).

For electrophoretic analysis, fresh leaf material (100 mg) from each sample was ground in 0.5 ml of Tris-HCL extraction buffer pH 7.5 (Soltis *et al.*, 1983). The slurry was centrifuged and the supernatant was subjected to electrophoresis on 10% polyacrylamide gels. Five isozyme systems were examined: aspartate aminotransferase (Aat), leucine aminopeptidase (Lap), phosphoglucomutase (Pgm), phosphoglucose isomerase (Pgi), and shikimate dehydrogenase (Skdh). All isozymes migrated anodally. When the isozymes were encoded by more than one locus, the most anodally migrating isozyme was designated 1, the next 2, and so on.

Results

Morphology.—The three samples from Godenba were observed morphologically and identified to be *P. fibrillosopaleaceum* var. *marginale* based on the characters such as nearly entire and twisted scales, and submarginal sori as described by Serizawa (1971). The average size and shape of the leaves of the samples (40018, 40020, and 40238) showed morphological features intermediate between those of *P. fibrillosopaleaceum* and *P. igaense* (Figs 1–2; Table 2). The stipes of the three plants were 15.4 (± 5.1) cm long with dense scales. The lanceolate lamina was 54.8 (± 9.7) cm long and 16.6 (± 2.1) cm wide, longer than that of both species. The scales on the lower portion of the stipes were slightly tortuous, and the margins of the scales were more or less denticulate with sparse projections more similar to those of *P. igaense*, which

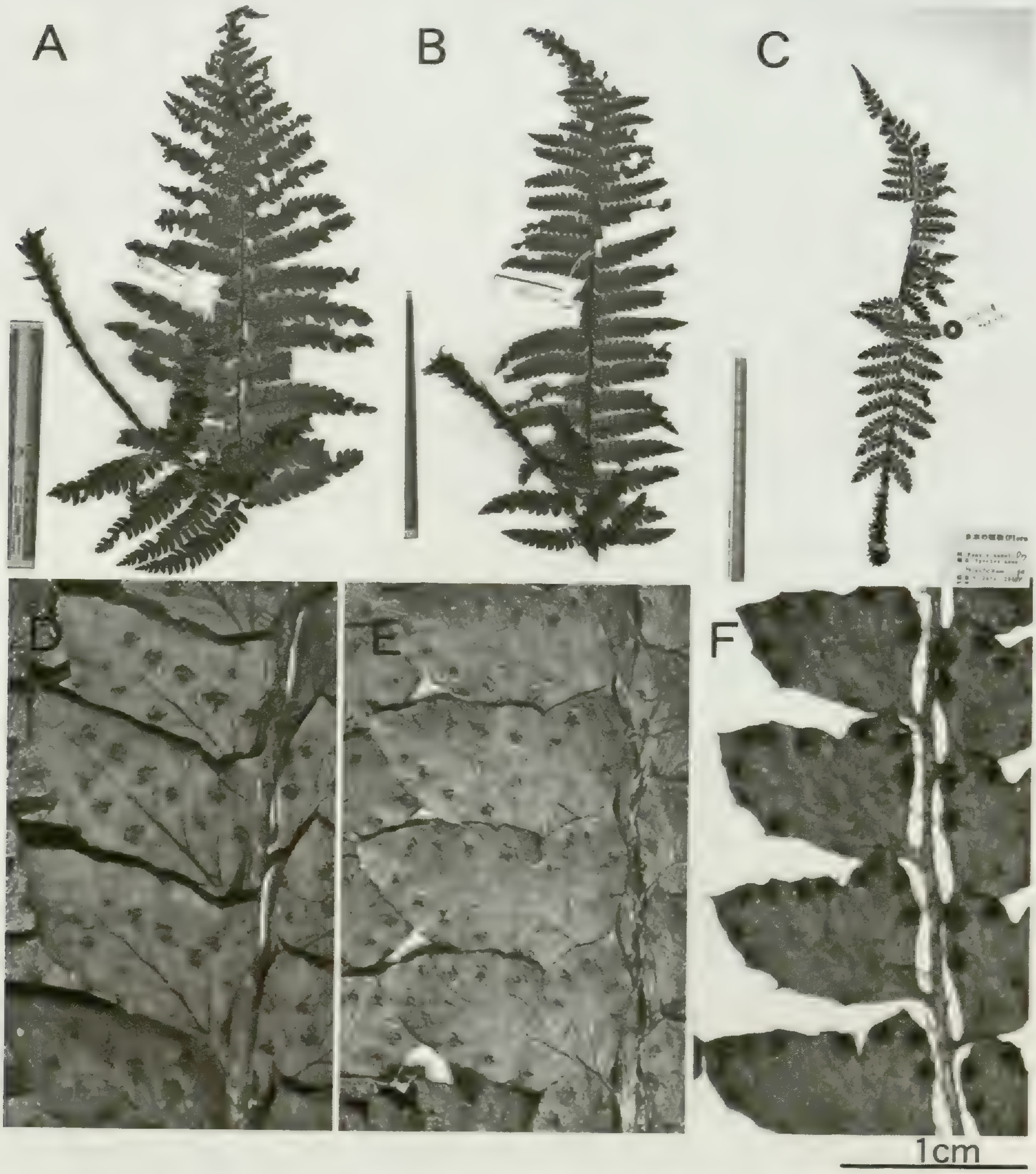

FIG. 1. Leaf photographs. A, D: *P. fibrillosopaleaceum*; B, E: *P. fibrillosopaleaceum* var. *marginale*; C, F: *P. igaense*.

has scales with projection margins, slightly tortuous when dried (the scales of *P. fibrillosopaleaceum* have entire margins and are strongly twisted). The sori were marginal or submarginal on the pinnules, like those of *P. igaense*.

The spores of *P. fibrillosopaleaceum* and *P. igaense* are normally 64 s/s and monolete, with distinct features in the perispore. Those of *P. fibrillosopaleaceum* are reticulate echinate, whereas those of *P. igaense* are fenestrate cristate (Fig. 3). The spores of the three samples were highly irregular in size (30–60 μm) and number (32 s/s, c. 48 s/s, 64 s/s, c. 112 s/s), and were malformed,

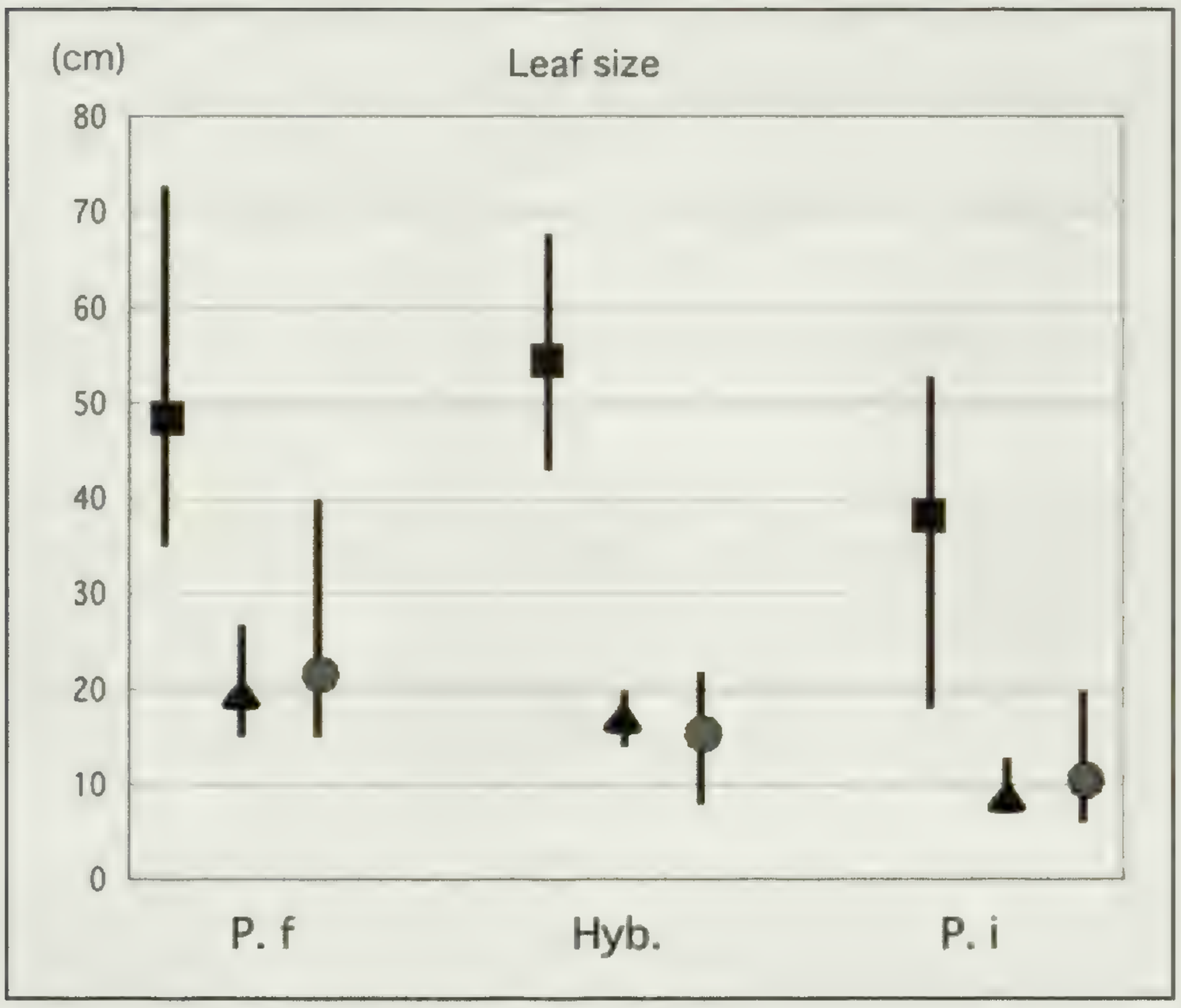

FIG. 2. Leaf size data. Squares show frond lengths; triangles show the widths of fronds; circles show the lengths of stipes.

and of three different types: reticulate echinate (like *P. fibrillosopaleaceum*), fenestrate cristate (like *P. igaense*), and cristate with coalescent echinate. These types were observed either in single sporangia or in different sporangia of a single plant (Fig. 3B, D). Three herbarium specimens of *P. fibrillosopaleaceum* var. *marginale* including a holotype specimen (Serizawa 6180 = TNS VS-573649) were studied for comparison. The spores obtained from the holotype specimen (Fig. 4) and specimens 40018, 40020, and 40238 were also irregular, and with variable perispores.

TABLE 2. Morphological characters in the putative hybrid and in its putative parent species *P. fibrillosopaleaceum* and *P. igaense*. **P.f**: *P. fibrillosopaleaceum*; **Hyb.**: the putative hybrid; **P.i**: *P. igaense*.

Taxa	P.f	Hyb.	P.i
Characters			
Leaf shape (cm)			
Laminae length	48.4±12.5	54.8±9.7	37.3±9.1
Laminae width	19.1±3.4	16.6±2.1	8.7±1.4
Stipes	21.7±7.0	15.4±5.1	10.5±2.9
Scales (length, cm)	tortuous, entire (1.45±0.29)	a little tortuous, sparsely projects (1.37±0.40)	a little tortuous when dry, projects (0.87±0.16)
Sori	medial	marginal or submarginal	marginal
Spore	reticulate echinate	irregular	cristate

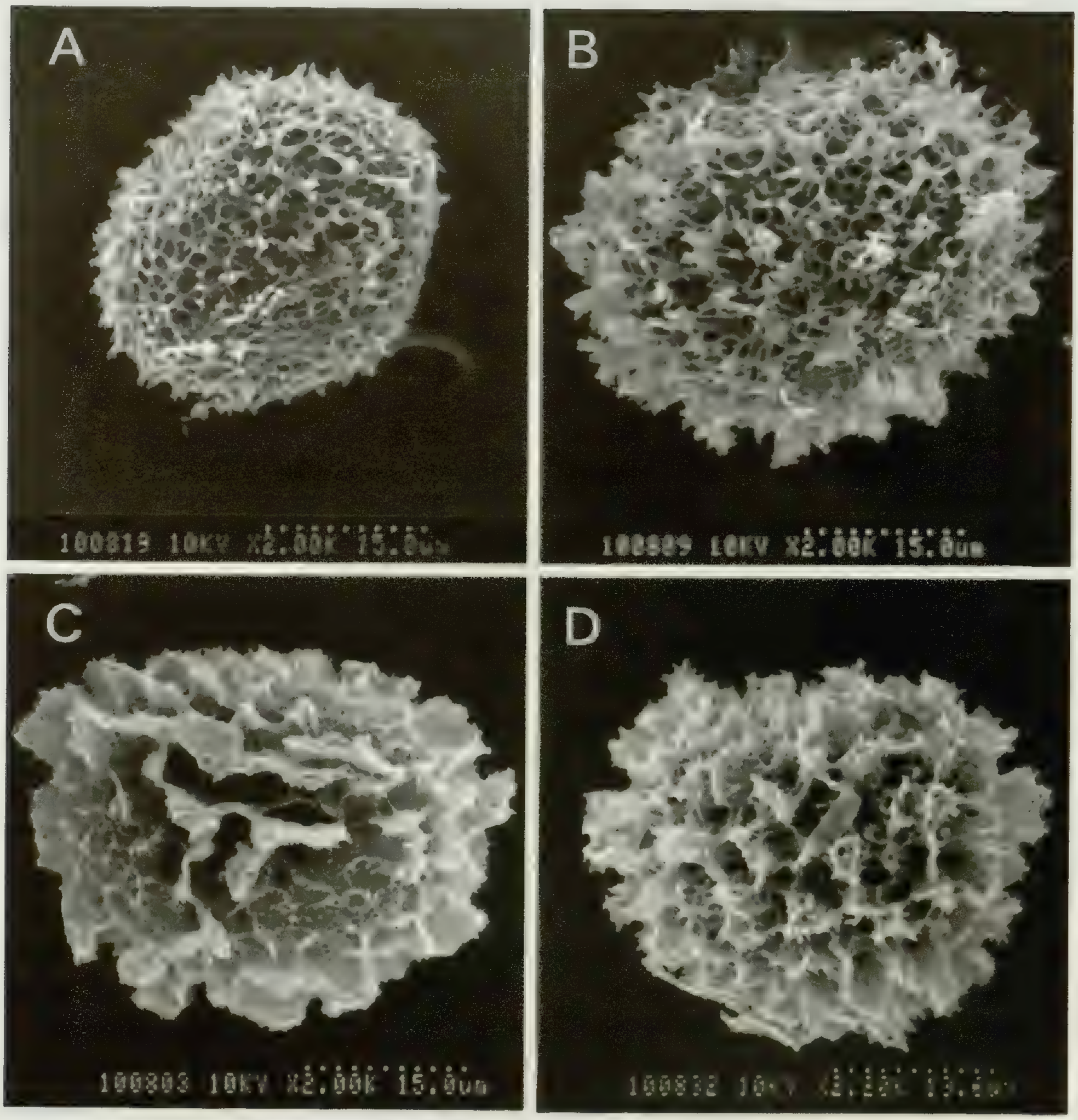

FIG. 3. SEM images of spore morphology. A: *P. fibrillosopaleaceum*; C: *P. igaense*; B, D: *P. fibrillosopaleaceum* var. *marginale*.

The spore viability of an individual sample (40238) was tested. Only about 1% of the spores germinated and grew to prothalli within 3–4 months of sowing, whereas about 75% of the control samples (viable spores) from a plant of *P. fibrillosopaleaceum* germinated in 1 month. The prothalli of 40238 were irregular or cordate in shape, and there were several archegonia (which soon withered) in the cushion zone, but no antheridia or young sporophytes were observed before the prothalli died.

Cytological evidence and polyploidy.—The chromosome numbers, meiotic behavior and polyploidy of *P. fibrillosopaleaceum*, *P. igaense*, and the three samples of *P. fibrillosopaleaceum* var. *marginale* were examined (Table 1). Both *P. fibrillosopaleaceum* and *P. igaense* were diploid with 2n = c. 82 chromosomes (Fig. 5A–D), consistent with previous studies (Mitui, 1965, 1966, 1968; Kurita, 1966; Daigobo, 1973). Because the suitable cytological

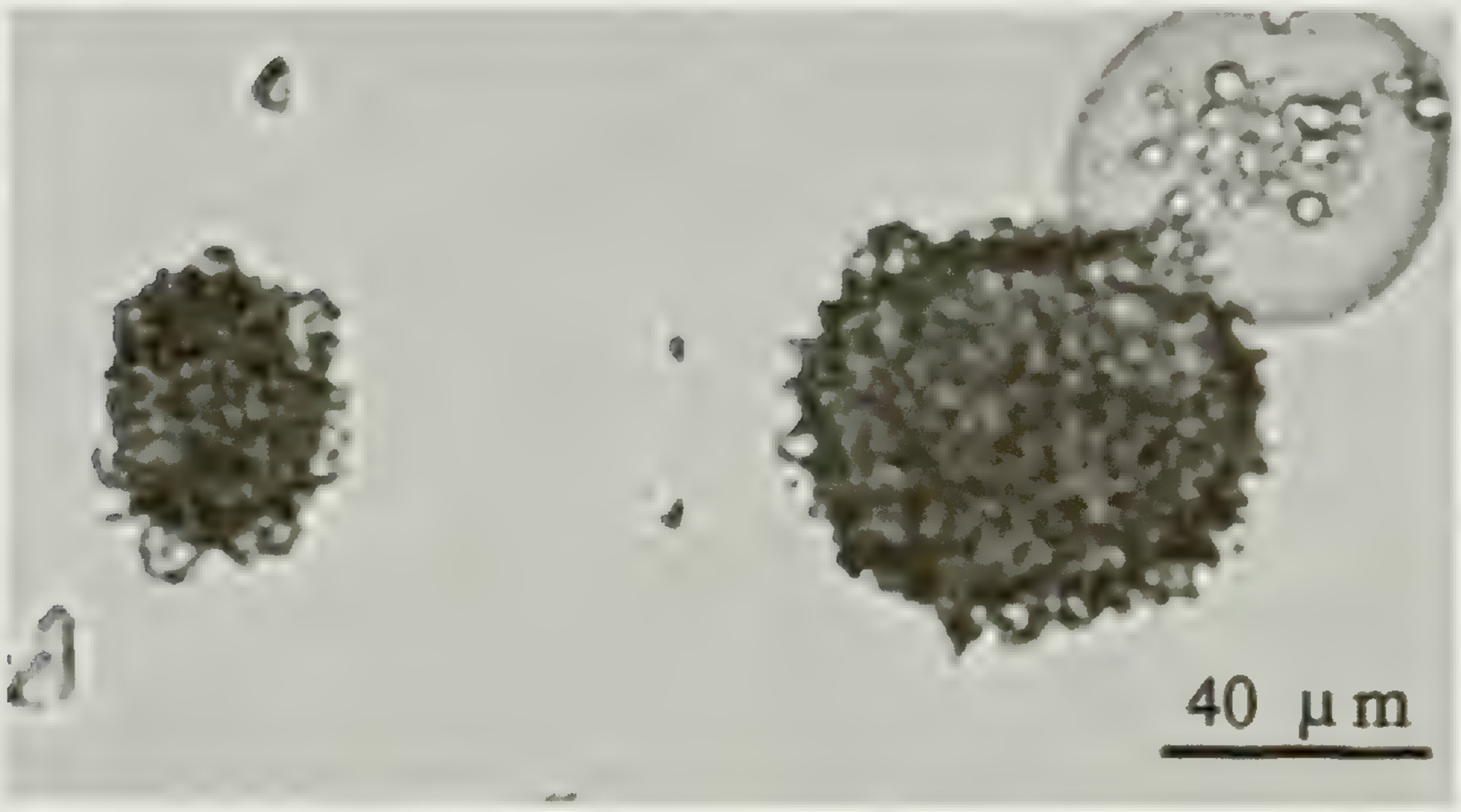

FIG. 4. Spores of *P. fibrillosopaleaceum* var. *marginale* (holotype specimen 6180).

materials could not be obtained in field for two of the three *P. fibrillosopaleaceum* var. *marginale* samples (40018 and 40020), the both samples were examined by flow-cytometric analysis, and were shown to be diploid by flow-cytometric analysis. The meiotic behavior of the other sample (40238) was observed. Occasional irregular meiotic division, such as a chromosome bridge at anaphase II, and chromosomal fragments at metaphase I and telophase II were observed (Fig. 6A–C). However, a few normal-looking meiotic mother cells, with approximately 41 II bivalent chromosomes, were observed in the same plant (Fig. 5E–F), and it was presumed that such sporocytes could produce normal tetrads. Various sporangia (with 16 sporocytes) were observed in this individual, which ranged from almost normal (64 s/s) to extremely irregular (c. 48 s/s, 32 s/s, and c. 112 s/s). The variability of the sporangia suggests genetic instability during meiosis.

Isozyme analysis.—Seven loci were clearly interpretable: Aat, Lap, Pgi, Pgm-1, Pgm-2, Pgm-3, and Skdh. Aat, Lap, and Pgm-3 were monomorphic and subsequently omitted from the analysis. *Polystichum fibrillosopaleaceum* and *P. igaense* have different alleles at the loci encoding Pgi, Pgm-1, Pgm-2, and Skdh. None of the genetic markers present in all 11 samples of *P. igaense* (Pgi-b, Pgm-1b, Pgm-2a, and Skdh-a) were found in *P. fibrillosopaleaceum*. Conversely, the alleles Pgi-a, Pgm-1a, Pgm-2b, and Skdh-b were detected in all 11 individuals of *P. fibrillosopaleaceum*but absent in the samples of *P. igaense*. The three putative hybrids showed the same heterozygous 4-locus genotypes, Pgm-1ab, Pgm-2ab, Pgi-ab, and Skdh-ab, a combination of alleles from both *P. fibrillosopaleaceum* and *P. igaense* (Fig. 7).

DISCUSSION

In general, intermediate morphological characters and irregular, sterile spores suggest hybridity in ferns. All morphological characters of the three *P. fibrillosopaleaceum* var. *marginale* samples examined here showed a combi-

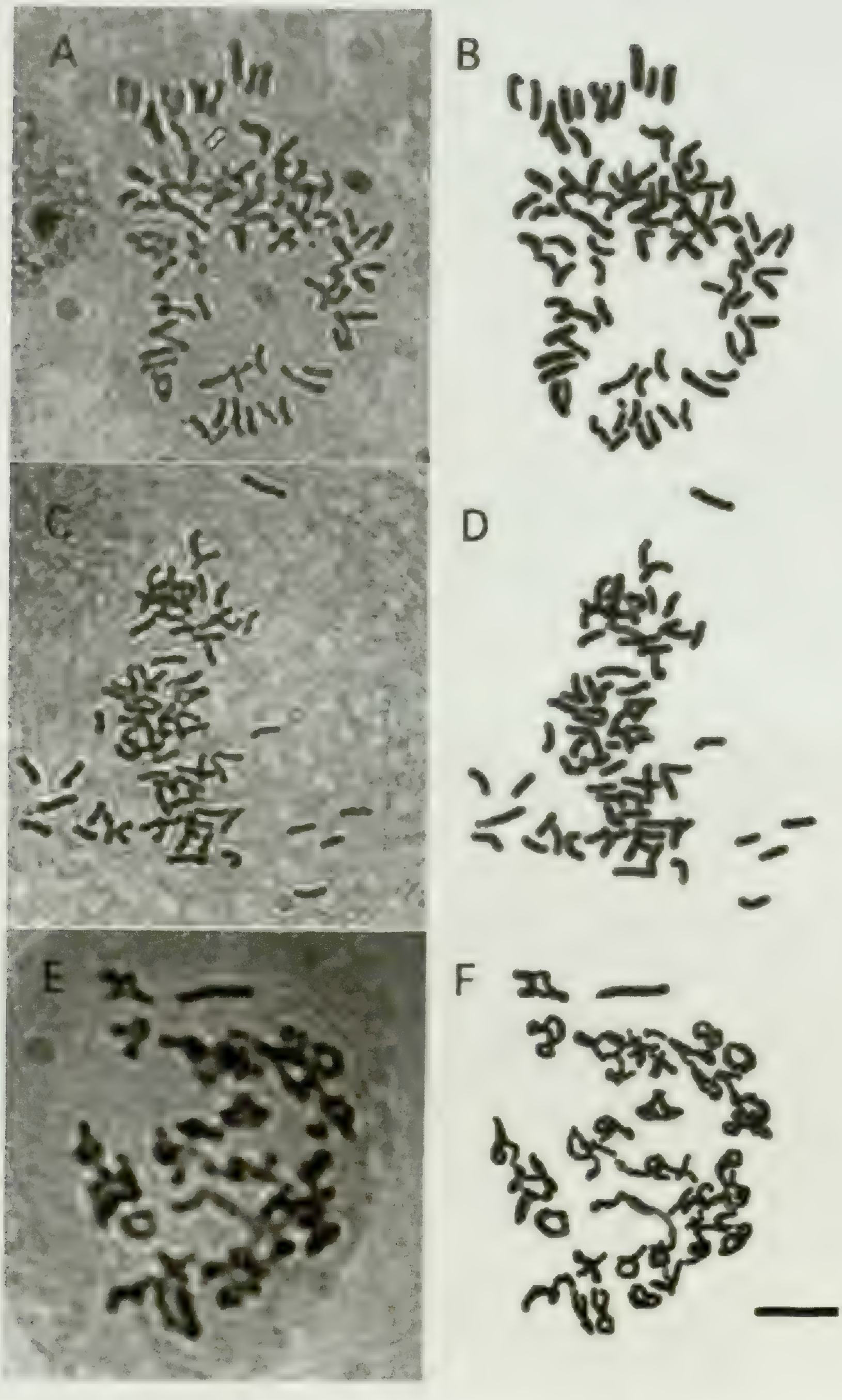

FIG. 5. Photographs of chromosomes. A, B: *P. fibrillosopaleaceum*, 2n = 82 (2x); C, D: *P. igaense*, 2n = 82 (2x); E, F: *P. fibrillosopaleaceum* var. *marginale*, n = c. 41 (2x). (bar = 10 μm).

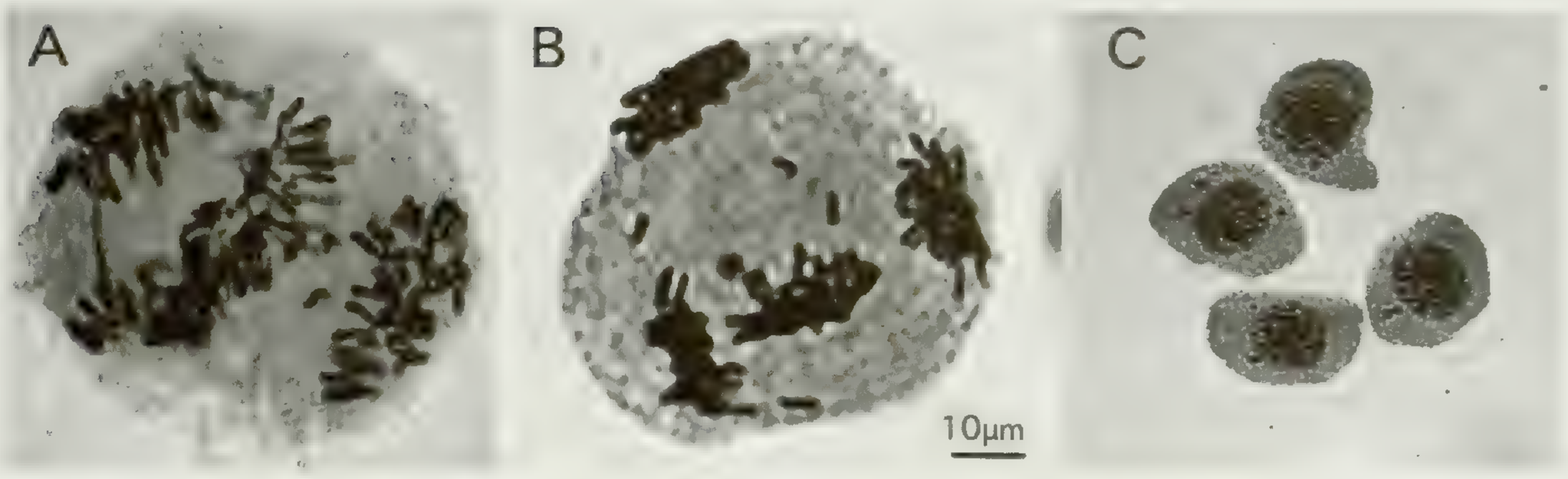

FIG. 6. Photographs of tetrads of *P. fibrillosopaleaceum* var. *marginale*. A: showing a bridge in spore mother cell at meiotic anaphase II; B: showing some fragments or univalent chromosomes in spore mother cell at meiotic telophase II; C: tetrad with chromosome fragments.

nation of the features of *P. fibrillosopaleaceum* and *P. igaense*, or similarity with the form of *P. fibrillosopaleaceum* or *P. igaense*. Chromosome numbers and flow-cytometric data showed both the putative hybrids and their putative parents to be diploid (n=41 and 2n=82). The presence of one to several univalent chromosomes or fragments at metaphase I or telophase II and anaphase II suggested the lack of full homology in the pairing of homologous chromosomes. There appears to be an association between lack of chromosome pairing and irregular spores and reduced spore germination in these plants. The electrophoretic evidence demonstrates that the genomes of the putative hybrids are a combination of contributions from the diploid progenitors, *P. fibrillosopaleaceum* and *P. igaense*. All results obtained in this study support our hypothesis that the three samples of *P. fibrillosopaleaceum* var. *marginale* are diploid hybrids derived from the disparate diploid species *P. fibrillosopaleaceum* and *P. igaense*.

In the dataset for DNA barcoding of Japanese pteridophytes (Ebihara *et al.*, 2010), *P. fibrillosopaleaceum* var. *marginale* showed almost identical chloroplast r*bcL* sequences as *P. igaense*. This suggests *P. igaense* is probably the maternal parent species of *P. fibrillosopaleaceum* var. *marginale*. These data further support the idea that *P. fibrillosopaleaceum* var. *marginale* could be of hybrid origin.

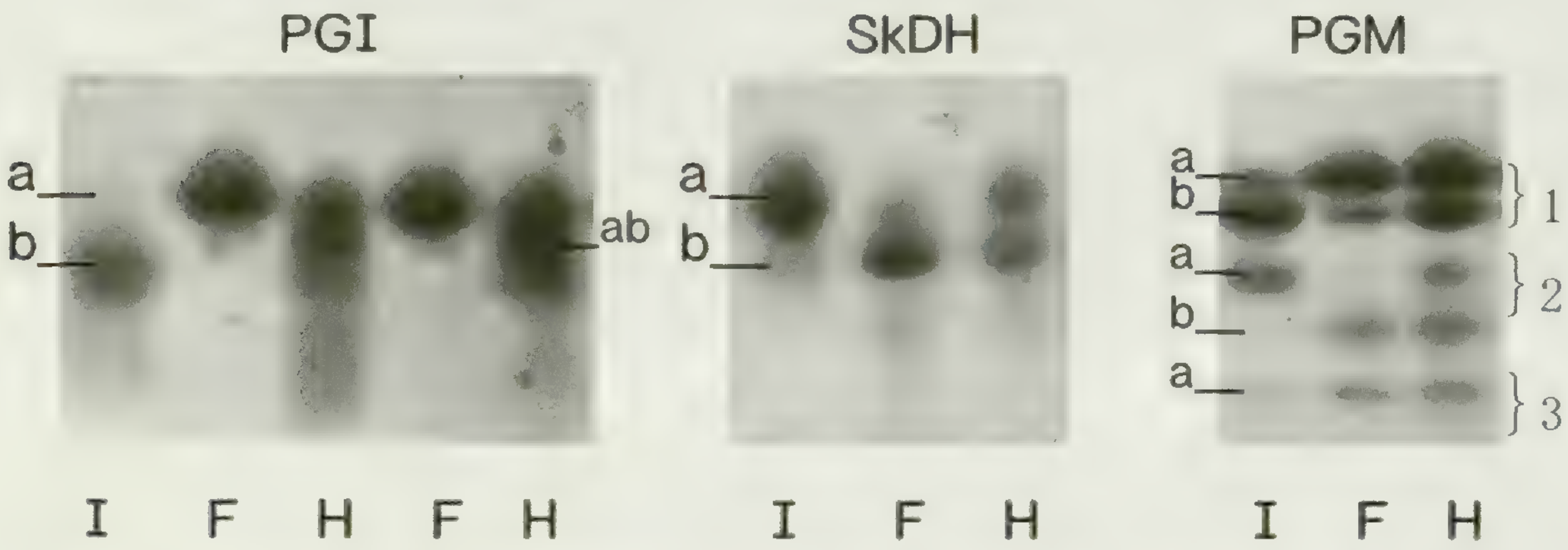

FIG. 7. Photographs showing electrophoretic band patterns of Pgi, Skdh, and Pgm. (I: *P. igaense*; F: *P. fibrillosopaleaceum*; H: Putative hybrid (*P. fibrillosopaleaceum* var. *marginale*).

Although abnormal spores were observed on some specimens, Serizawa (1971) considered *P. fibrillosopaleaceum* var. *marginale* to be a variety of *P. fibrillosopaleaceum* rather than a hybrid, because Daigobo (personal communication) found that its meiosis was normal with a complement of 41 II chromosomes. Daigobo (1974) and Shimura (1975, 1980) also reported a normal complement of 41 II chromosomes, but Shimura (1980) noted irregular spores and a germination rate of only 2~7.5% in *P. fibrillosopaleaceum* var. *marginale*.

A similar phenomenon was also observed in the hybrid between *P. concinnum* and *P. speciosissimum*, which showed virtually complete chromosomal pairing in some cells, even though the progenitor species are strongly differentiated morphologically (Barrington, 1990). Barrington (1990) reported that in such situations, chromosome pairing may be under less stringent genetic control in *Polystichum* than in other fern groups, thus allowing the relatively high frequencies of homologous and homoeologous pairing as observed in *Polystichum* hybrids. Thus it is very difficult to morphologically distinguish the hybrids of closely allied species of *Polystichum*, because of their continuous morphological variation. *Polystichum fibrillosopaleaceum* is considered to be involved in several hybrids as a putative diploid parent (Iwatsuki, 1992) and as the ancestor of various polyploid species (Lin *et al.*, unpublished data). Therefore it can be concluded that *P. fibrillosopaleaceum* plays an important role in the hybridization, polyploidization, and reticulate evolution of Japanese *Polystichum*.

Taxnomic treatment:

Polystichum* x *shizuokaense Nakaike, pro sp.—*Polystichum shizuokaense* Nakaike, New Fl. Jap. Pterid. 842 (1992) —*Polystichum fibrillosopaleaceum* (Kodama) Tagawa var. *marginale* Seriz., J. Geobot. 19: 104 (1971).

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LITERATURE CITED

BARRINGTON, D. S. 1985. The morphology and origin of a new *Polystichum* hybrid from Costa Rica. Syst. Bot. 10:199–204.

BARRINGTON, D. S. 1986. The morphology and cytology of *Polystichum* x *potteri* hybr. Nov. (=*P. acrostichoides* x *P. braunii*). Rhodora 88:297–31.

BARRINGTON, D. S. 1990. Hybridization and allopolyploidy in Central American *Polystichum*: Cytological and isozyme documentation. Annals of the Missouri Botanical Garden 77:297–305.

DAIGOBO, S. 1972. Taxonomical Studies on the fern Genus *Polystichum* in Japan, Ryukyu, and Taiwan. Sci. Rep. Tokyo Kyoiku Daigaku. 15:57–80.

DAIGOBO, S. 1973. Chromosome numbers of the fern genus *Polystichum* (1). J. Jap. Bot. 48:337–344.

DAIGOBO, S. 1974. Chromosome numbers of the fern genus *Polystichum* (2). J. Jap. Bot. 49:371–378.

EBIHARA, A., H. ISHIKAWA, S. MATSUMOTO, S.-J. LIN, K. IWATSUKI, M. TAKAMIYA, Y. WATANO and M. ITO. 2005. Nuclear DNA, chloroplast DNA and ploidy analysis clarified biological complexity of the *Vandenboschia radicans* Complex (Hymenophyllaceae) in Japan and adjacent areas. Amer. J. Bot. 92:1535–1547.

EBIHARA, A., J. H. NITTA and M. ITO. 2010. Molecular Species Identification with Rich Floristic Sampling: DNA Barcoding the Pteridophyte Flora of Japan. PLoS Hubs. 5(12):e15136.

IWATSUKI, K. 1992. Ferns and Fern Allies of Japan. Heibonsha Publishers, Tokyo. 311 pp. 165–176.

JAPANESE ENVIRONMENTAL AGENCY. 2007. Threatened Wildlife of Japan, Plant I, Vascular Plants, Red Data Book. Japan Wildlife Research Center, Tokyo.

KRAMER, K. U. and P. S. GREEN, eds. 1990. I. Pteridophytes and Gymnosperms *In:* K. Kubitzki, *The families and genera of vascular plants.* Springer-Verlag. Berlin.

KUNG, H.-S., W.-M. CHU, Z.-R. HE and L.-B. ZHANG. 2001. Polystichum. *In* C.-Y. Wu (ed), *Flora Reipublicae Popularis Sinicae. vol. 5(2).* Kung, H.-S. Science Press, Beijing. pp. 1–246.

KURITA, S. 1966. Chromosome numbers of some Japanese ferns (5). J. Jpn. Bot. 41:82–84.

LI, C. X., S. G. LU and D. S. BARRINGTON. 2008. Phylogeny of Chinese Polystichum (Dryopteridaceae) based on chloroplast DNA sequence data. J. Plant Res. 121:19–26.

LIN, S.-J., M. KATO and K. IWATSUKI. 1990. Sporogenesis, reproductive mode, and cytotaxonomy of some species of *Sphenomeris*, *Lindsaea*, and *Tapeinidium* (Lindsaeaceae). Amer. Fern J. 80:97–109.

LITTLE, D. P. and D. S. BARRINGTON. 2003. Major evolutionary events in the origin and diversification of the fern genus *Polystichum* (Dryopteridaceae). Amer. J. Bot. 90(3):508–514.

LU, J. M., D. S. BARRINGTON and D. Z. LI. 2007. Molecular phylogeny of the polystichoid ferns in Asia based on *rbcL* sequences Sys. Bot. 32(1):26–33.

MANTON, I. 1950. Problems of Cytology and Evolution in the Pteridophyta University Press, Cambridge.

MITUI, K. 1965. Chromosome studies on Japanese ferns (1). J. Jpn. Bot. 40:117–124.

MITUI, K. 1966. Chromosome studies on Japanese ferns (2). J. Jpn. Bot. 41:60–64.

MITUI, K. 1968. Chromosomes and speciation in ferns. Sci. Rep. Tokyo Kyoiku Daigaku. Sec. B, 13:285–333.

NAKAIKE, T. 1992. *New Flora of Japan: Pteridophyta.* Shibundo, Tokyo. pp. 842.

SERIZAWA, S. 1971. Two new varieties of Japanese ferns. J. Geobot. 19:103–105.

SHIMURA, Y. 1975. A study of spores in some ferns belonging to *Metapolystichum* in Japan (III). Bull. Fac. Edu. Shizuoka Univ. 26:35–53.

SHIMURA, Y. and M. OOISHI. 1980. A study on the chromosome numbers of some taxa of Japanese *Polystichum*. J. Phytogeogr. and Taxon 28:24–27.

SOLTIS, D., C. HAUFLER, D. DARROW and G. GASTONY. 1983. Starch gel electrophoresis of ferns: A complication of grinding buffers, gel and electrode buffers, and staining schedules. Amer. Fern J. 73:9–27.

SOLTIS, P. and D. SOLTIS. 1987. Electrophoretic and morphological confirmation of interspecific hybridization between *Polystichum kruckebergii* and *P. munitum*. Amer. Fern J. 77:42–49.

TAKAMIYA, M. 1996. Index to Chromosomes of Japanese Pteridophyta (1910–1996). Japan Pteridological Society, Tokyo.

VIDA, G. and T. REICHSTEIN. 1975. Taxonomic problems in the fern genus *Polystichum* caused by hybridization *In* S. M. Walters, (editors). *European Floristic and Taxonomic Studies.* E. W. Classey Faringdon, England. pp. 126–135.

WAGNER, D. 1979. Systematics of *Polystichum* in western North America and north of Mexico. Pterodologia 1:1–64.

WAGNER, W. 1973. Reticulation of holly ferns (*Polystichum*) in the western United States and adjacent Canada. Amer. Fern J. 63:99–115.

ZHANG, L.-B. and H.-S. KUNG. 1996. Taxonomic study on the fern genus *Polystichum* sect. Metapolystichum Tagawa from China (II). Acta Phytotaxonomica Sinica 34:68–76.

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Cytotaxonomic Study of 12 Species in the Polypodiaceae from Southern China

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ABSTRACT.—Chromosome numbers and reproductive biologies of 12 fern species from eight genera in the Polypodiaceae, *(Colysis, Lepidogrammitis, Lepisorus, Microsorium, Neolepisorus, Phymatopteris, Phymatosorus,* and *Pyrrosia)* from the southern region of China (Yunnan, Guangxi and Hainan) were investigated. The base number is $x=36$ in these genera of Polypodiaceae. The chromosome numbers for four species: $2n=72$ ($2x$) in *Lepidogrammitis drymoglossoides*, $2n=72$ ($2x$) in *Neolepisorus ovatus*, $2n=72$ ($2x$) in *Phymatopteris rhynchophylla*, and $2n=72$ ($2x$) in *Phymatosorus hainanensis* are here reported for the first time. Four records: $2n=72$ ($2x$) in *Colysis hemionitidea*, $2n=72$ ($2x$) in *Lepisorus thunbergianus*, $2n=108$ ($3x$) in *Phymatopteris crenatopinnata*, and $2n=108$ ($3x$) in *Phymatosorus cuspidatus* are new cytotypes. *Lepisorus thunbergianus* $2n=72$ ($2x$) has the base number of $x=36$, diverging from those cited in previous reports ($x=25$, 38, 50, and 51). The reproductive type in *P. crenatopinnata* and *P. cuspidatus* is apogamous, whereas in the other species it is of the sexual reproductive type.

KEY WORDS.—China, Chromosome number, Cytotaxonomy, Polypodiaceae, Reproductive mode

The Polypodiaceae is one of the most diverse groups of extant ferns (Schneider *et al.*, 2004). They are not only a lineage of derived ferns that has a high number of species, but they also display a vast range of morphological variation (Kreier *et al.*, 2008). Ching believed that China (especially SW China and the Himalayas) was the center of origin of Polypodiaceae, with about half the number of the total species of Polypodiaceae occurring there (Ching, 1979; Lin, 2000).

Cytological and reproductive studies may provide very useful information for phylogenetic and evolutionary studies of various groups of ferns. Data on chromosome numbers, base numbers, karyotypes, aneuploids, polyploidy levels, and reproductive modes are useful for understanding of the origin,

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evolution and speciation of the ferns (Manton, 1950; Wang, 1984; Wu, 1984; Wang *et al.*, 2007). Data on chromosomes are also important for discriminating species and species complexes.

Previous cytological studies have recorded chromosome numbers in less than 60 species of Polypodiaceae in South China (Yunnan, Zhejiang, Hainan, and Fujian) (Wang, 1984; Weng, 1990; Kato *et al.*, 1992; Cheng, 1992; Kato and Nakato, 1999; Nakato *et al.*, 1995; Lin *et al.*, 1996, 2002). This number accounts for only 23% of the Chinese Polypodiaceous ferns. Data on chromosomes of some genera/species in Polypodiaceae have been reported, such as $2n=72$ ($2x$) in *Lepidogrammitis rostrata*, *Phymatopteris oxyloba* and *Lepisorus macrosphaerus*; $2n=108$ ($3x$) in *Phymatopteris trisecta*; and $2n=70$ ($2x$) in *Lepisorus bicolor* and so on (Wang *et al.*, 2007; Wang and Lu, 2008; Lu and Wang, 2008).

Cytological research on these ferns has been severely lacking, because of difficulties in manipulating root tips due to their small size, inaccessibility of the species, and the large number of chromosomes. Cytological studies of the southern China Polypodiaceous ferns can contribute to our understanding of the origin and evolution of the Polypodiaceae. Accordingly, we chose 12 species in eight genera of Polypodiaceae as materials, following the alphabet order. Chromosome counts had already been performed for eight of the species used in this study: $n=36$ in *Colysis elliptica* (Tsai and Shieh, 1983; Weng, 1990), $2n=144$ ($4x$) in *C. hemionitidea* (Mehra, 1961), $2n=72$ ($2x$) in *C. pothifolia* (Tsai and Shieh, 1983; Kato *et al.*, 1992), $2n=50$, 51, 75, 76, 100, 101, 102 and 103 in *Lepisorus thunbergianus* (Takamiya, 1996), $n=36$, 37 in *Microsorium fortunei* (Roy and Holttum, 1965; Mitui, 1973), $2n=72$ ($2x$) in *Phymatopteris crenatopinnata* (Panigrahi and Patnaik, 1961), $2n=74$ ($2x$) in *Phymatosorus cuspidatus* (Kato and Nakato, 1999), and $2n=72$ ($2x$) in *Pyrrosia lingua* (Takei, 1969, 1983a). The aim of this study is to provide new cytological information on the classification and phylogenetics of Polypodiaceous ferns.

Materials and Methods

Living plants (Table 1, Fig. 1) were collected from the field in Yunnan, Guangxi and Hainan provinces, southern China, and cultivated in the lab. Voucher specimens were deposited in the Herbarium of Yunnan University (PYU).

For the examination of mitotic chromosomes, root tips were pretreated in 0.002 mol/L 8-hydroxyquinoline solution for 3–6h before being fixed in Carnoy's solution (95% ethanol: glacial acetic acid=3:1) for 12–24 h; then they were hydrolyzed in 1 mol/L HCl at 60 C for 10–15 min. After washing 3–4 times to eliminate residual hydrochloric acid, materials were stained in 2% aceto-orcein and squashed by the usual method (Manton, 1950). The "second squashed" method (Wang and Zhang, 1981) was used when chromosomes occurred in different planes. Mitotic cells were examined and photographed by using an Olympus BX51-DP70 photomicroscope. More than 30 chromosome micrographs were observed in each individual.

In the higher leptosporangiate ferns, there are two reproductive types: the sexual reproductive type with 64 spores in a sporangium, and the apogamous

TABLE 1. Taxa studied of Polypodiaceae, their collection information and chromosome data.

Taxon	Chromosome number	Spore N.*	Locality	Elevation (m)	Voucher	Figs
Colysis elliptica (Thunb.) Ching	2n=72	64	Mulun, Guangxi	350	XC Deng *et al.* 31786 (PYU)	2A, 3A
Colysis hemionitidea (Wall. ex Mett.) C. Presl	2n=72	64	Guilin, Guangxi	210	RX Wang *et al.* 017 (PYU)	2B, 3B
Colysis pothifolia (D. Don.) C. Presl	2n=72	64	Guilin, Guangxi	280	RX Wang *et al.* 010 (PYU)	2C, 3C
Lepidogrammitis drymoglossoides (Bak.) Ching	2n=72	64	Bama, Guangxi	380	RX Wang *et al.* 015 (PYU)	2D, 3D
Lepisorus thunbergianus (Kaulf.) Ching	2n=72	64	Huanjiang, Guangxi	450	XC Deng *et al.* 31758 (PYU)	2E, 3E
Microsorium fortunei (T. Moore) Ching	2n=72	64	Guilin, Guangxi	180	XC Deng *et al.* 31790 (PYU)	2F, 3F
Neolepisorus ovatus (Bedd.) Ching	2n=72	64	Guilin, Guangxi	160	RX Wang *et al.* 014 (PYU)	2G, 3G
Phymatopteris crenatopinnata (C. B. Clarke) Pichi-Serm.	2n=108	32	Weixi, Yunnan	2300	RX Wang *et al.* 009 (PYU)	2H, 3H
Phymatopteris rhynchophylla (Hook.) Pichi-Serm.	2n=72	64	Pingbian, Yunnan	1700	RX Wang *et al.* 008 (PYU)	2I, 3I
Phymatosorus cuspidatus (D. Don.) Pichi-Serm.	2n=108	32	Bama, Guangxi	520	RX Wang *et al.* 016 (PYU)	2J, 3J
Phymatosorus hainanensis (Noot.) S. G. Lu	2n=72	64	Mt. Wuzhishan, Hainan	250	SG Lu 4001 (PYU)	2K, 3K
Pyrrosia lingua (Thunb.) Farwell	2n=72	64	Pingbian, Yunnan	1600	XC Deng *et al.* 31797 (PYU)	2L, 3L

* Number of spores per sporangium.

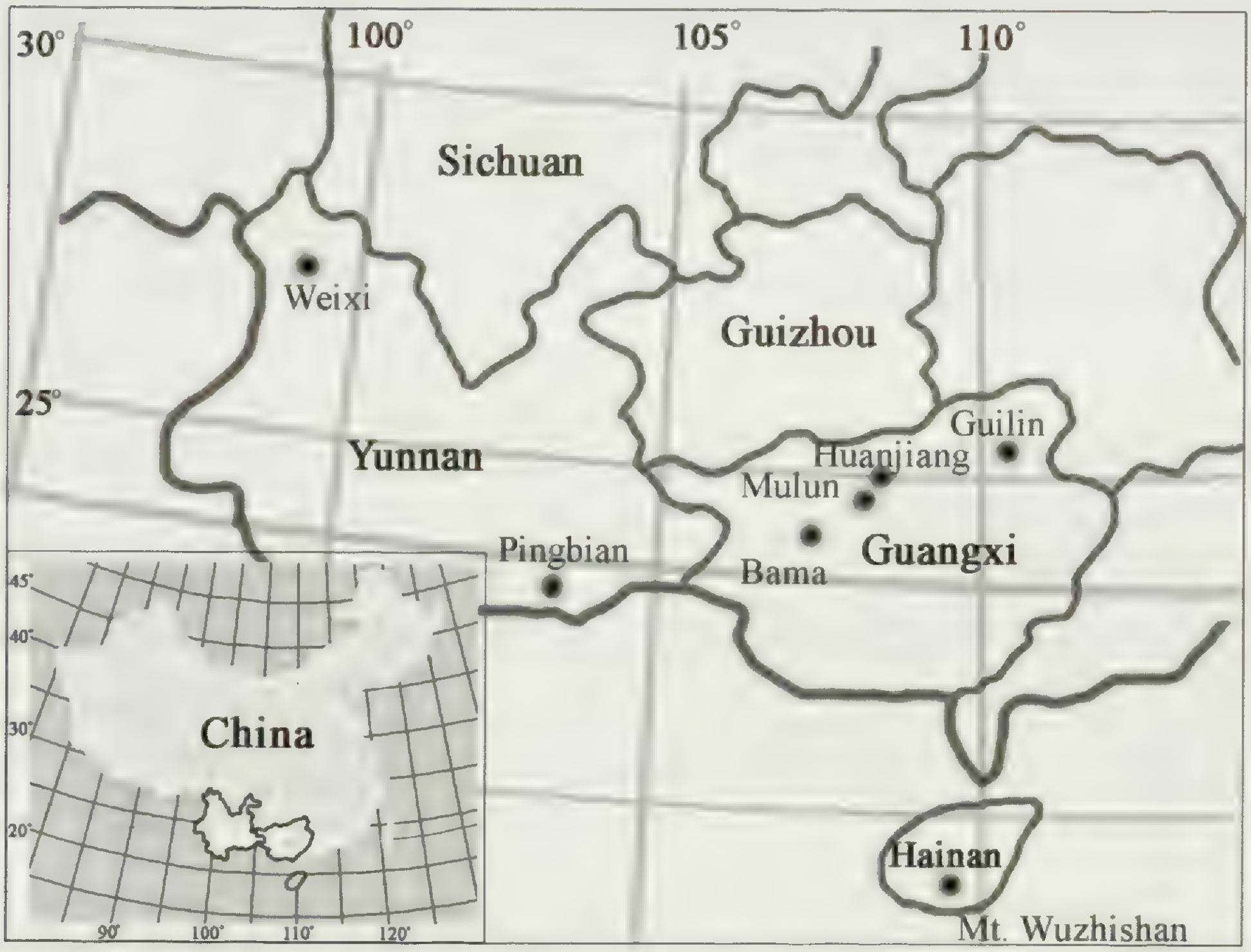

FIG. 1. Distribution map showing localities of the seven collection sites.

type with 32 spores (Manton, 1950; Lovis, 1977; Walker, 1979). To determine the reproductive type, the number of spores per sporangium was counted. At least five unopened sporangia were counted in each individual.

RESULTS AND DISCUSSION

The chromosome photographs of root tip materials of 12 species in eight genera of Polypodiaceae are shown in Figures 2 and 3. The chromosome numbers for four species are newly reported for China: $2n=72$ ($2x$) in *Lepidogrammitis drymoglossoides*, $2n=72$ ($2x$) in *Neolepisorus ovatus*, $2n=72$ ($2x$) in *Phymatopteris rhynchophylla*, and $2n=72$ ($2x$) in *Phymatosorus hainanensis*. Four records are new cytotypes: $2n=72$ ($2x$) in *Colysis hemionitidea*, $2n=72$ ($2x$) in *Lepisorus thunbergianus*, $2n=108$ ($3x$) in *Phymatopteris crenatopinnata*, and $2n=108$ ($3x$) in *Phymatosorus cuspidatus*. *Lepisorus thunbergianus* $2n=72$ ($2x$) has the base number $x=36$, which is different from the previous reports $x=25$, 38, 50, and 51. We confirmed the counts of $2n=72$ ($2x$) in *Colysis elliptica*, $2n=72$ ($2x$) in *C. pothifolia*, $2n=72$ ($2x$) in *Microsorium fortunei*, and $2n=72$ ($2x$) in *Pyrrosia lingua*.

The reproductive type in *P. cuspidatus* and *P. crenatopinnata* was apogamous, whereas in the other species it was of the sexual reproductive type. The chromosome number and reproductive mode for each species are described and discussed below.

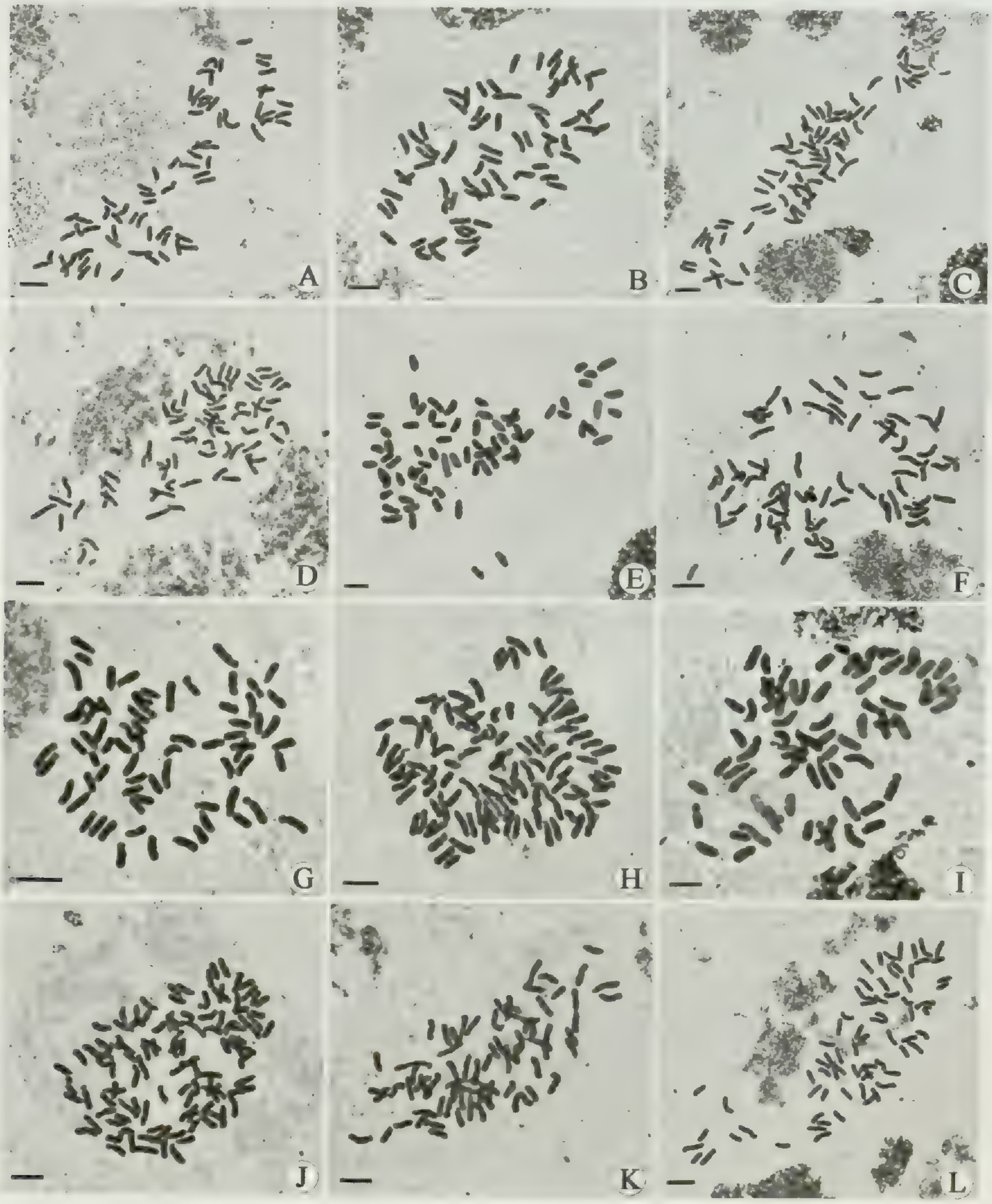

FIG. 2. Photographs of somatic chromosomes at metaphase A. *Colysis elliptica*, 2*n*=72. B. *C. hemionitidea*, 2*n*=72. C. *C. pothifolia*, 2*n*=72. D. *Lepidogrammitis drymoglossoides*, 2*n*=72. E. *Lepisorus thunbergianus*, 2*n*=72. F. *Microsorium fortunei*, 2*n*=72. G. *Neolepisorus ovatus*, 2*n*=72. H. *Phymatopteris crenatopinnata*, 2*n*=108. I. *P. rhynchophylla*, 2*n*=72. J. *Phymatosorus cuspidatus*, 2*n*=108. K. *P. hainanensis*, 2*n*=72. L. *Pyrrosia lingua*, 2*n*=72 (*scale bars*=5μm).

***Colysis elliptica* (Thunb.) Ching**

This species is known from across India, Vietnam, Japan, and southern China. The material from Mulun, Guangxi counted in this study had 72 chromosomes in the mitotic cells (Fig. 2A, 3A). The species is a diploid, with 64 spores per sporangium.

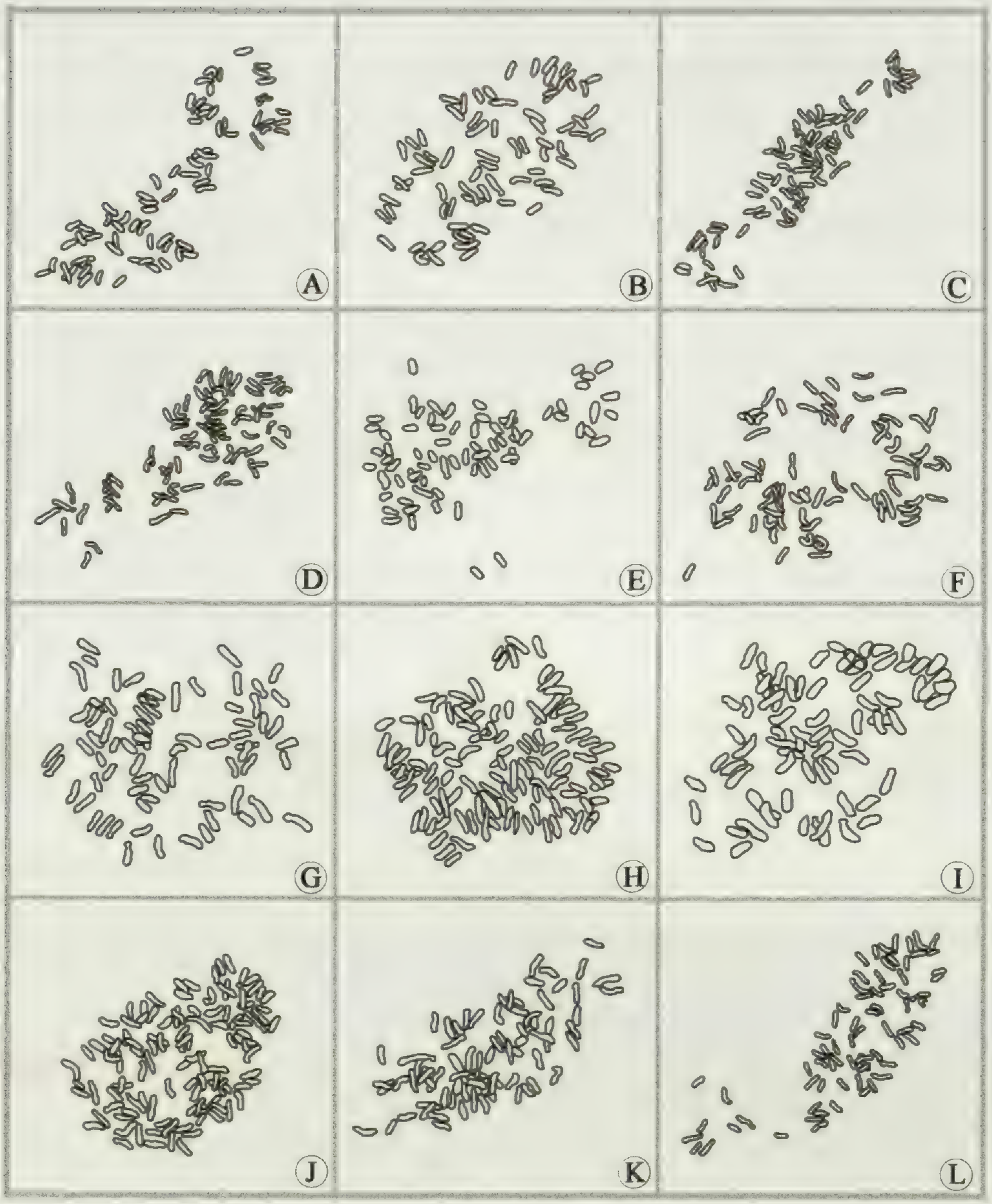

FIG. 3. Explanatory drawings to Fig. 2. A. *Colysis elliptica*, 2*n*=72. B. *C. hemionitidea*, 2*n*=72. C. *C. pothifolia*, 2*n*=72. D. *Lepidogrammitis drymoglossoides*, 2*n*=72. E. *Lepisorus thunbergianus*, 2*n*=72. F. *Microsorium fortunei*, 2*n*=72. G. *Neolepisorus ovatus*, 2*n*=72. H. *Phymatopteris crenatopinnata*, 2*n*=108. I. *P. rhynchophylla*, 2*n*=72. J. *Phymatosorus cuspidatus*, 2*n*=108. K. *P. hainanensis*, 2*n*=72. L. *Pyrrosia lingua*, 2*n*=72.

Tsai and Shieh (1983) reported the material from Taiwan, China had 36 chromosomes in the meiotic cells. Weng (1990) suggested the material from Fujian, China also had 36 chromosomes in the meiotic cells, and 64 spores per sporangium, which suggests it is a diploid. Kato *et al.* (1992) believed the material from Dali, Yunnan, had 72 chromosomes in the mitotic cells. The

materials from five Japanese populations had 36 chromosomes in the meiotic cells (Mitui, 1966, 1968; Kurita, 1968; Takei, 1983b), and 64 spores per sporangium, which suggested that these species are sexual diploids. These results indicated stability of chromosome numbers in the different populations in China and Japan.

The record made by Löve *et al.* (1977) showed that different chromosome numbers occur in six species of the genus *Colysis*, indicating that diploid ($2n=72$), triploid ($2n=108$), tetraploid ($2n=144$), and hexaploid ($2n=216$) counts exist in this genus.

***C. hemionitidea* (Wall. ex Mett.) C. Presl**

This species is widespread in tropical and subtropical Asia. The material from Guilin, Guangxi counted in this study had 72 chromosomes in the mitotic cells (Fig. 2B, 3B). Sixty-four spores per sporangium were found, suggesting it is a sexual diploid. This record is a new cytotype.

Mehra (1961) shown the material from Mt. Himalaya had 144 chromosomes in the mitotic cells, and 64 spores per sporangium, suggesting it is a sexual tetraploid.

***C. pothifolia* (D. Don.) C. Presl**

This species is widespread in tropical and subtropical Asia. The material from Guilin, Guangxi counted in this study had 72 chromosomes in the mitotic cells (Fig. 2C, 3C). We suggest this species is a sexual diploid based on the observation of 64 spores per sporangium.

Tsai and Shieh (1983) found that the material from Taiwan, China had 36 chromosomes in the meiotic cells. Kato *et al.* (1992) collected material from Dali, Yunnan and Lin *et al.* (2002) collected materials from Fujian, each of which had 72 chromosomes in the mitotic cells. All materials above had 64 spores per sporangium, suggesting that these are sexual diploids.

***Lepidogrammitis drymoglossoides* (Bak.) Ching**

This species is endemic to China, distributed widely in the southern region. The material from Bama, Guangxi counted in this study had 72 chromosomes in the mitotic cells (Fig. 2D, 3D). Sixty-four spores were present in each sporangium, suggesting that the species is a sexual diploid. This chromosome count is reported for the first time.

***Lepisorus thunbergianus* (Kaulf.) Ching**

This species is known from across southern China, Japan, and the Philippines. The material from Huanjiang, Guangxi in this study was found to have $2n=72$ chromosomes in the mitotic cells (Fig. 2E, 3E). The species is a sexual diploid, with 64 spores per sporangium. This is a new cytotype.

Lin *et al.* (2002) reported that the material from Fujian had $2n=100$ chromosomes in the mitotic cells, and suggested it was a sexual tetraploid.

Lepisorus thunbergianus is a cytologically complicated species, its chromosome numbers have been variously reported as $n=25$, 50, $2n=50$, 51, 75, 76, 100, 101, 102 and 103, including examples of polyploidy and aneuploidy from Japan (Takamiya, 1996). It has been shown that diploids, triploids, tetraploids, and polyploids exist in *L. thunbergianus* (Takamiya, 1996).

***Microsorium fortunei* (T. Moore) Ching**

This species is known from across southern China, Burma, Bhutan, and Vietnam. Material from Guilin, Guangxi in this study had 72 chromosomes in

the mitotic cells (Fig. 2F, 3F). Sixty-four spores were present in each sporangium, suggesting it is a sexual diploid.

Roy and Holttum (1965) reported *M. fortunei* from southern China had 36 chromosomes in the meiotic cells, suggesting the same results as this paper. In contrast, Mitui (1973) suggested the material from Japan had 37 chromosomes in the meiotic cells, which may be the result of aneuploidy.

***Neolepisorus ovatus* (Bedd.) Ching**

This species is widespread in southern regions of China and India. The material from Guilin, Guangxi counted in this study had 72 chromosomes in the mitotic cells (Fig. 2G, 3G). There were 64 spores per sporangium. The results suggest it should be considered a sexual diploid species. This chromosome count is reported for the first time.

***Phymatopteris crenatopinnata* (C. B. Clarke) Pichi-Serm.**

This species occurs in southern China and also in NE India. The material from Weixi, Yunnan, had 108 chromosomes in the mitotic cells (Fig. 2H, 3H). We counted 32 spores per sporangium. The results suggest it is apogamous with $2n=108$ in this study. This is a new cytotype.

Panigrahi and Patnaik (1961) reported the material from eastern India had 72 chromosomes in the mitotic cells, and 64 spores per sporangium, suggesting it is a sexual diploid.

***P. rhynchophylla* (Hook.) Pichi-Serm.**

This species is widespread in tropical and subtropical Asia. The material we collected from Pingbian, Yunnan, had 72 chromosomes in the mitotic cells (Fig. 2I, 3I). There were 64 spores per sporangium, suggesting it is a sexual diploid. This chromosome count is reported for the first time.

***Phymatosorus cuspidatus* (D. Don.) Pichi-Serm.**

This species is known from across southern China, India, Vietnam, Nepal, and Thailand. The material from Bama, Guangxi counted in this study had 108 chromosomes in the mitotic cells (Fig. 2J, 3J). Thirty-two spores were present per sporangium. The results indicate it is an apogamous triploid with $2n=108$ and it represents a new cytotype.

Kato and Nakato (1999) reported the material from Hainan had 74 chromosomes in the mitotic cells, and 64 spores per sporangium, suggesting it is a sexual diploid.

***P. hainanensis* (Noot.) S. G. Lu**

This species is found in Hainan, China and also in India and Vietnam. The material we collected from Mt. Wuzhishan, Hainan, had 72 chromosomes in the mitotic cells (Fig. 2K, 3K). We counted 64 spores per sporangium, suggesting it is a sexual diploid. This chromosome count is reported for the first time.

***Pyrrosia lingua* (Thunb.) Farwell**

This species is known from across southern China, India, Vietnam, and Japan. The material from Pingbian, Yunnan counted in this study had 72 chromosomes in the mitotic cells (Fig. 2L, 3L). The species is a sexual diploid, with 64 spores per sporangium.

Takei (1969, 1983a) believed the material from Japan also had 72 chromosomes in the mitotic cells. Our result suggests that *Pyrrosia lingua* is in a stable state.

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LITERATURE CITED

CHENG, X. 1992. Chromosome numbers in some Hengduan Mountains species of *Phymatopsis* J. Sm. (Polypodiaceae). In Bhardwaja, T. N. and C. B. Gena. *Perspectives in Pteridophytes*. Today and Tomorrow's Printers and Publishers, New Delhi.

CHING, R. C. 1979. The Himalaya as a center of concentration for the south-eastern Asiatic polypodiaceous ferns. Acta Bot. Yunnan. 1:23–31.

KATO, M. and N. NAKATO. 1999. A cytotaxonomic study of Hainan (S. China) pteridophytes, with notes on polyploidy and apogamy of Chinese species, Pp. 1–19. In: Zhang, X. C. and K. H. Shing, eds. *Ching Memorial Volume*. China Forestry Publishing House, Beijing.

KATO, M., N. NAKATO and X. CHENG. 1992. A cytotaxonomic study of ferns of Yunnan, southwestern China. Bot. Mag. (Tokyo) 105:105–124.

KREIER, H. P., X. C. ZHANG, H. MUTH and H. SCHNEIDER. 2008. The microsoroid ferns: Inferring the relationships of a highly diverse lineage of Paleotropical epiphytic ferns (Polypodiaceae, Polypodiopsida). Mol. Phylogenet. Evol. 48:1155–1167.

KURITA, S. 1968. Cytotaxonomy of Colysis. Ann. Rep. Foreign Students'Coll. Chiba Univ. 3:47–51.

LIN, S. J., K. IWATSUKI and M. KATO. 1996. Cytotaxonomic study of ferns from China I. Species of Yunnan. J. Jpn. Bot. 71:214–222.

LIN, S. J., H. R. ZHANG, K. IWATSUKI and H. S. LU. 2002. Cytotaxonomic study of ferns from China II. Species of Fujian. J. Jpn. Bot. 77:129–138.

LIN, Y. X. 2000. Flora Reipublicae Popularis Sinicae. Science Press, Beijing, 6(2):7–266.

LÖVE, A., D. LÖVE and R. E. G. PICHI SERMOLLI. 1977. Cytotaxonomical atlas of the Pteridophyta. A. R. Gantner Verlag, Vaduz.

LOVIS, J. D. 1977. Evolutionary patterns and processes in ferns. Adva. Bot. Res. 4:229–451.

LU, S. G. and R. X. WANG. 2008. A Cytotaxonomic Study of *Drymotaenium miyoshianum* Makino. J. Trop. Subtrop. Bot. 16(2):173–175.

MANTON, I. 1950. Problems of cytology and evolution in the Pteridopyhta. Cambridge University Press, London.

MEHRA, P. N. 1961. Chromosome numbers in Himalayan ferns. Res. Bull. Panjab. Univ., N.S. 12:139–169.

MITUI, K. 1966. Chromosome studies on Japanese ferns (2). J. Jpn. Bot. 41:60–64.

MITUI, K. 1968. Chromosome and speciation in fern. Sci. Rep. Tokyo Kyoiku Daigaku, Sec. B 13:285–333.

MITUI, K. 1973. A cytological survey on the pteridophytes of the Bonin Islands. J. Jpn. Bot. 48:247–254.

NAKATO, N., M. KATO and B. D. LIU. 1995. A cytotaxonomic study of some ferns from Jiangsu and Zhejiang provinces, China. J. Jpn. Bot. 70:194–204.

PANIGRAHI, G. and S. N. PATNAIK. 1961. Cytology of some genera of Polypodiaceae in eastern India. Nature 191:1207–1208.

ROY, S. K. and R. E. HOLTTUM. 1965. Cytological observation on ferns from South China. Amer. Fern J. 55:154–158.

SCHNEIDER, H., A. R. SMITH, R. CRANFILL, T. J. HILDEBRAND, C. H. HAUFLER and T. A. RANKER. 2004. Unraveling the phylogeny of polygrammoid ferns (Polypodiaceae and Grammitidaceae): exploring aspects of the diversification of epiphytic plants. Molecular Phylogenetics and Evolution 31:1041–1063.

TAKAMIYA, M. 1996. *Index to Chromosomes of Japanese Pteridophyta (1910–1996).* Japan Pteridological Society, Tokyo.

TAKEI, M. 1969. Karyological studies in Polypodiaceae I. Karyotypes of a few species of the genus *Lemmaphyllum* and *Pyrrosia* in Japan. Bot. Mag. (Tokyo) 82:482–487.

TAKEI, M. 1983a. Karyological and karyosystematical studies on Polypodiaceae in Japan (III). Res. Bull. Fac. Educ. Oita Univ. (Nat. Sci.) 6(4):17–46.

TAKEI, M. 1983b. Karyological and karyosystematical studies on Polypodiaceae in Japan (IV). Res. Bull. Fac. Educ. Oita Univ. (Nat. Sci.) 6(5):5–43.

TSAI, J. L. and W. C. SHIEH. 1983. A cytotaxonomic survey of the pteridophytes in Taiwan. J. Sci. Eng. 20:137–158.

WALKER, T. G. 1979. The cytogenetics of ferns, Pp. 87–132. *In*: Dyer, A. F., ed. *The Experimental Biology of Ferns.* Academic Press, London.

WANG, R. X. and S. G. LU. 2008. A cytotaxonomic study of 10 species of the Polypodiaceae from Yunnan, China. J. Syst. Evol. 46:499–504.

WANG, R. X., S. G. LU and X. C. DENG. 2007. Cytotaxonomic studies of the Chinese pteridophytes: A review. Acta Phytotax. Sin. 45:98–111.

WANG, Z. R. and Z. X. ZHANG. 1981. Cytological observation on some Chinese ferns. Acta Bot. Sin. 23:26–34.

WANG, Z. R. 1984. The cytogenetical approach to the investigation of fern systematics and evolution. Chin. Bull. Bot. 2:26–34.

WENG, R. F. 1990. Cytological observations of some Chinese fern. Acta Phytotax. Sin. 28:27–33.

WU, Z. H. 1984. A survey of cytology in Pteridophyta. Guihaia 4:1–8.

American Fern Journal 101(4):317–318 (2011)

Shorter Notes

Herbivory on *Pecluma pectinatiformis* (L.) Price (Polypodiopsida) by Caterpillars of *Argyrosticta* Hübner (Lepidoptera) – a Possible Case of Mimicry?—Field work in a mixed humid forest with *Araucaria*, at the beginning of the austral Fall season (April), in the Northeastern region of the State of Rio Grande do Sul, Brazil (Princesa dos Campos, ca. 28° 56′W and 50° 28′S, ca. 800 m alt.), revealed herbivory by caterpillars on fronds of *Pecluma pectinatiformis* (L.) Price (Polypodiaceae). This fern occurs in forested areas as an epiphyte, occasionally on rocks or on the ground, in Brazil (South and Southearn regions), Paraguay and Argentina. Spore morphology, together with sporophyte description, illustrations, data on the ecology and distribution of this species in the State of Rio Grande do Sul were presented by Lorscheitter *et al.* (Palaentographica, 270: 57–60. 2005).

On some plants damage to the laminar tissue was observed (Fig. 1), and on closer examination the herbivorous caterpillars were found. The caterpillars were overlooked on a first casual observation due to their coloration pattern, which, to some extent, resembled a fertile pinna (abaxially) of the *Pecluma* plant. The pattern consists of a reddish line with black longitudinal portions in the middle of the dorsal surface (such as a central "midrib"), presenting on each side a sequence of white and yellow segments forming a striped line, somewhat interrupted by dark semi-circular spots (curved stripe) close to each yellow segment at the inner side. This produces a visual pattern that mimicks a fertile segment of the fern with its yellowish mature sori before the release of

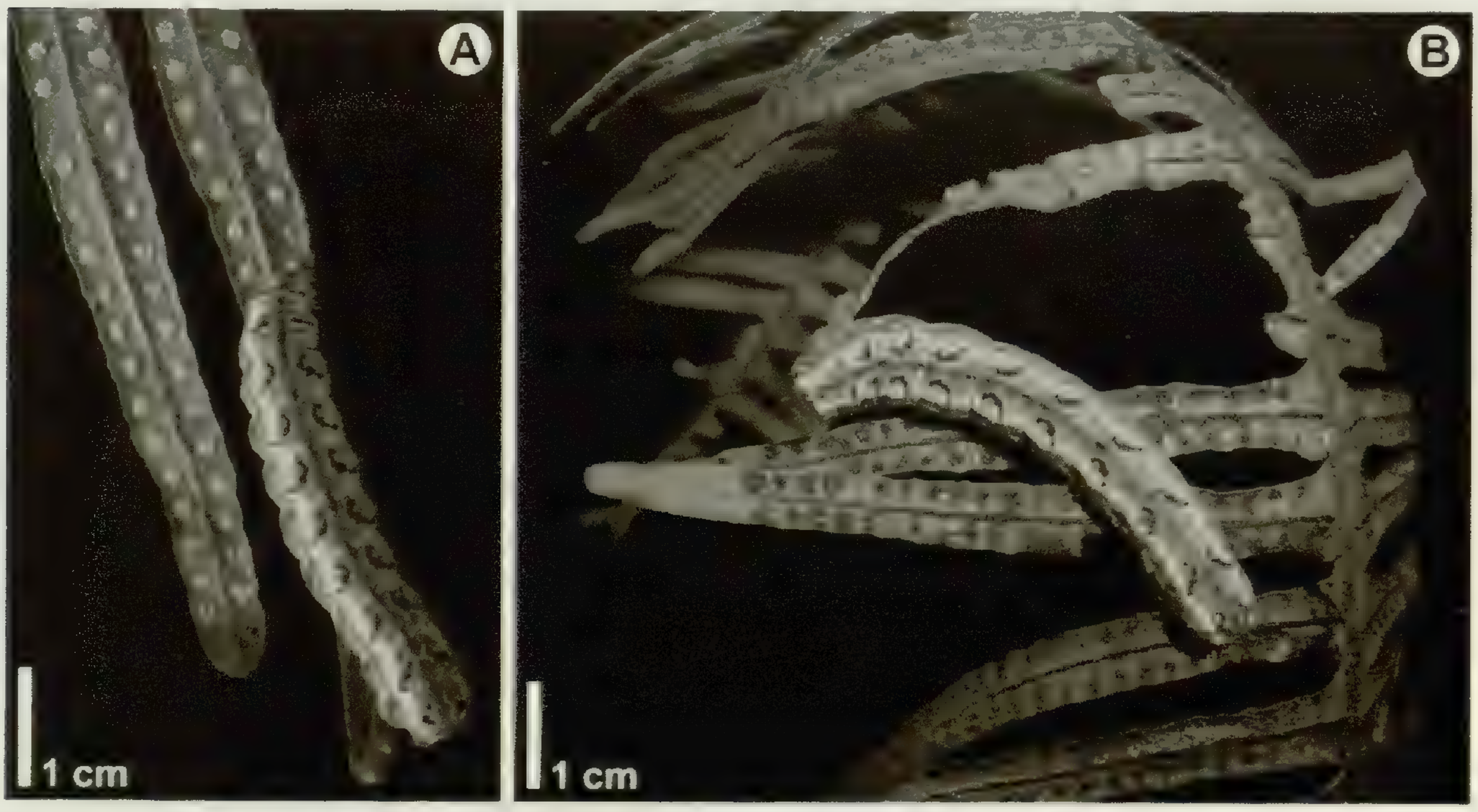

Fig. 1. *Argyrosticta* herbivory on *Pecluma pectinatiformis*. A. Detail of the *Argyrosticta* caterpillar. B. Section of the frond with tissue damage (color images available from corresponding author).

the spores. It is also possible that the dark semicircular marks simulate the incisions between adjacent pinnae, when the animal is over the reddish-brown frond rachis.

The images in Fig. 1 were taken after disturbing the caterpillar, but the animal tends to stretch while feeding, thus having a width similar to that of the pinnae. Feces produced by the caterpillar dissolved in water and observed using an optical microscope, revealed the presence of spores morphologically corresponding to those of host plant. On a isolated plant, the development of one of these caterpillars was followed for 14 days (no molting or changes in the color pattern were observed) up to the pupa stage (in the humic soil substrate), and hatching after another 28 days as an adult moth. Entomologist Alfred Moser, classified the insect as a *Noctuidae*, genus *Argyrosticta* Hübner (1821) [cf. *A. meres* (Druce, 1903) under investigation]. All stages were documented through digital images. A voucher has been deposited at the ICN Herbarium in Porto Alegre (ICN 167777).

A rapid survey of 32 herbarium sheets of this species revealed 15 with damages to the laminar tissue which could be attributed to herbivory. However, attempts to find more carterpillars in the field, on different occasions, were unsuccessful. There are still only few records of this kind of interaction with neotropical ferns, while this subject opens interesting questions and chalenges for future research (see Mehltreter *in* Mehltreter *et al.*, *Fern Ecology*, Cambridge University Press, p. 232–235. 2010).

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